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Genome size microscale divergence of Cyclamen persicum in Evolution Canyon, Israel

Research Article

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Abstract: Using DAPI flow cytometry, we examined genome size divergence of the Persian violet, *Cyclamen persicum* (Primulaceae) (2n=48) on close opposite slopes of Evolution Canyon (EC), Mt. Carmel, Israel. The range of genome size variation detected among measured cyclamens was 6.41% in relation to the smallest measured DNA content. Our data on *C. persicum* at EC showed that local variability in the 2C-value exists. Significantly less DNA was recorded in plants growing in one station of the African savannah-like south-facing slope (AS) but not in the remaining two stations of the same slope. We were not able to reject the null hypothesis that there are no significant interslope differences in the genome size between the temperate European garrigue-like north-facing slope (ES) and the drier AS. In spite of the nonsignificant interslope trend for the higher genome size in *C. persicum*, the data-fusion (meta-analysis) test using correlations between C-values in *C. persicum*, and earlier studied carob tree (*Ceratonia siliqua*), trifoil (*Lotus peregrinus*) and a beetle (*Oryzaephilus surinamensis*) and their distribution along the aridity gradient indicates a positive relationship between drought and genome size at the microsite.

Keywords: Cyclamen persicum • 2C-value • DNA • Flow cytometry • Genome size • Evolution Canyon

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1. Introduction

The Evolution Canyon (EC) project involved the evaluation of the relative importance of different evolutionary forces and outcomes on local biodiversity differentiation at the microsite, EC, lower Nahal Oren, Mt. Carmel, Israel (see reviews in [1-3]). In regard to genome size in the carob trees (Ceratonia siliqua), significantly more DNA was recorded in adult trees growing on the more illuminated, warmer, drier, and microclimatically more fluctuating south-facing slope (referred to as African savannah-like and abbreviated AS in the text) than in trees on the nearby opposite, less illuminated, cooler, and more humid European garrigue-like north-facing slope (abbreviated ES in the text) [4], in spite of an interslope distance of only 100 m at the canyon bottom and 400 m at the top. In wild barley, Hordeum spontaneum, it was proposed

that larger genomes, due to retrotransposon activity, are adapted to aridity stress locally at EC [5] as well as regionally in Israel [6].

To check the generality of local interslope divergence in genome size, we chose to test the Persian violet, *Cyclamen persicum* (family Primulaceae). This species is a geophyte to be contrasted with the previously studied species at EC, *H. spontaneum*, which is fast-growing and self-pollinating, and the slow-growing perennial tree, *Ceratonia siliqua*.

2. Experimental procedures

2.1. Microsite

The microsite named Evolution Canyon (EC), located at lower Nahal Oren, Mt. Carmel, Israel (32°43'N; 34°58'E), is a Plio-Pleistocene canyon [1]. The oppo-

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18]. So far, the reported number of chromosomes in

site slopes, which run in an east-west direction, are separated by only 100 m at the bottom and 400 m at the top. The African savannah-like south-facing slope (AS) is covered by an open park forest of evergreen Ceratonia siliqua-Pistacia lentiscus with dominant savannah-like grassland. In contrast, the European garigue-like north-facing slope (ES) is covered by a dense Mediterranean maquis of evergreen Quercus calliprinos and deciduous Pistacia palaestina [7]. Geology (Upper Cenomanian Limestone: [8]), regional Mediterranean climate (mean annual rainfall ca. 600 mm, potential evaporation 1,700 mm, and mean August and January temperatures 28°C and 13°C, respectively: [9]), and pedology (terra rossa: [10]) are the same on both slopes. Due to differences in geographic orientation in the Northern Hemisphere, on average, the AS receives more radiation per year than the ES having the same inclination [11]. Indeed, the AS of EC is significantly more illuminated, warmer, drier, and microclimatically, spatiotemporally, more fluctuating than the opposite temperate ES [12]. The microclimate differences are reflected in strong local biodiversity differences. The AS is, on average, richer in terrestrial species and displays higher genetic diversity than the ES, which is richer in aquatic-dependent taxa [1-3]. To elaborate on the interslope comparison, three collection stations were established on each slope. They were located at the upper (elevation 120 m asl.; AS1 and ES7, respectively), middle (elevation 90 m asl.; AS2 and ES6, respectively), and lower sections (elevation 60 m asl.; AS3 and ES5, respectively). The order of stations according to the decrease in water availability due to interslope differences in evaporation rates and to water runoff is as follows: ES5>ES6>ES7>AS3>AS2>AS1 [12]. Environmental factors (water availability, humidity, temperature, and solar radiation) are possible determinants of the species richness in stations and slopes (Pavlíček & Nevo, submitted). We chose to correlate 2C-value (= nuclear content, 2C-value divided by a ploidy level = genome size) and water availability at EC because drought was identified as a possible environmental factor influencing genome size at the microsite [4,5].

2.2. Model species

Cyclamen persicum Mill. (the Persian violet or cyclamen, plant family: Primulaceae) is a perennial herb with large tubers (up to 15 cm in diameter); its flowers appear between December and early May and is distributed in the east Mediterranean from east Greece through west and south Asia Minor up to Israel, Lebanon, west Syria, and Jordan; it is present also on Crete, Cyprus, Karpathos, Rhodes, and Tunisia [13-

the wild forms of *C. persicum* has been consistently 2n = 48 [19]. It has smaller chromosomes (approx. 2 µm long: Figure 1) compared to other species of the subgenus Eucosme (C. graecum, C. rohlfsianum), while species of other Cyclamen subgenera (i.e., Psilanthum, Gyrophoebe, and Cyclamen) have larger chromosomes as well as different basic chromosome numbers [19-23]. In EC, lower Nahal Oren, Mt. Carmel, the Persian violet grows on both slopes, but it is more abundant on the ES [7]. On the ES, it grows under trees and bushes, and also in openings between them, among dense herbal vegetation. However, on the AS they grow in the shade of trees, bushes, inside rock crevices, and in shade under boulders, but not in open areas exposed to direct sun radiation. Flowering can take place in the absence of leaves or with fully developed foliage (Pavlíček, pers. obs.). The flowers are self-incompatible and their morphology indicate that they were buzz-pollinated [24] but it is speculated that specialized pollinators have dissapeared or lost their attraction to the flowers [25]. The study of pollination in two wild populations of C. persicum in Israel showed that the most frequent pollinators during springtime were various unspecialized species of thrips (Thysanoptera) and hoverflies (Syrphidae), and, rarely, a large Anthophora species [25]. In the winter-flowering populations, the most common visitor was a small primitive moth, Micropteris elegans (Micropterigidae, Lepidoptera) [25]. The limited dispersal capacity of Cyclamen arises from the dependence on ant dispersal for a small numbers of large seeds [26].

2.3. Collection of samples

Sixty tuber samples, ten samples from each station, were collected on January 28, 2004, at EC. Since the preliminary results showed promising interslope patterns, we collected, 15 additional specimens on February 15, 2004, to increase stastical power. The distance between collected specimens was 10 or more meters on both collection dates. Each tuber sample was represented by a piece of about 3x2 cm size that was cut off from the tuber, kept at low temperature, and delivered to the Department of Botany, Masaryk University, in Brno (Czech Republic). The measurements took place immediately upon the delivery of samples. While collecting tuber samples, we also measured the diameter of each tuber and counted the number of flowers and leaves on the source plant.

2.4. Preparation of chromosomal spreads

Root tips of original plants were transferred to ice water for 24-28 h to accumulate metaphases and then fixed in 3:1 (v/v) 100% ethanol : acetic acid. Chromosome spreads were prepared according to the technique [27] with modifications [28]. Three plants from each slope were analyzed. The chromosomes were stained by AT-specific fluorochrome Hoechst 33258 [29]. Slides were examined on a Leica DMR microscope using a digital camera (Olympus C3030).

2.5. Measurement of genome size

A ploidy analyzer (PA-1, Partec GmbH, Münster, Germany) equipped with an HBO-100 mercury arc lamp was used for the estimation of relative DNA content. Sample preparations were carried out in a two-step procedure [30, 31] in the Laboratory of Flow Cytometry, Department of Botany, Masaryk University, Brno, Czech Republic. One particular individual of Glycine max 'Polanka' (with 2C = 2.50 DNA pg content : [32]) was used as an internal standard in all measurements. Samples from a measured individual (approx. 100 mg of tuber tissue) and a reference standard (0.5 cm² of leaf blade) were chopped with a new razor blade for about 20 s in a Petri dish containing 0.5 ml of ice-cold Otto I buffer (4.2 g citric acid monohydrate + 1 ml 0.5% Tween 20 adjusted to 200 ml and filtered through 0.22 µm filter); upon completion, another 0.5 ml Otto I buffer was added. The solution was then filtered through nylon cloth (50 µm mesh size). For DNA staining, 2 ml of Otto II buffer (0.4 M disodium hydrogenphosphate dodekahydrate) including DAPI (4',6diamidino-2-phenylindole; 4 µg/ml final concentration) was used. DAPI is known to form fluorescent complexes with natural double-stranded DNA, showing fluorescence specificity preferentially for AT-bases, and it is more accurate in the estimation of small infraspecific differences of DNA content than other substances, for example, ethidium bromide. Each sample was measured three times on different days and the average value was included in the statistical analysis. Calibrated relative DNA content R_{sample} was calculated using the following formula:

$$R_{sample} = \frac{\sum_{i=1...3} \left(\frac{1}{CV_{sample}i} \times \frac{1}{CV_{reference}i} \times \frac{F_DAPI_{sample}i}{F_DAPI_{reference}i}\right)}{\sum_{i=1...3} \left(\frac{1}{CV_{sample}i} \times \frac{1}{CV_{reference}i}\right)}$$

where CV is the variance coefficient and F_DAPI is the relative fluorescence channel (mean of peak) for samples and references in the first, second, and third measurement (i = 1...3), *i.e.*, each measurement of relative DNA content: $R_{sample}i = F_DAPI_{sample}i / F_DA-PI_{reference}i$ is included in the calculation of the average with weight equal to $1/CV_{sample}i \times 1/CV_{reference}i$. During measurements, samples from the AS and ES of Evolution Canyon were measured in an alternating sequence.

2.6. Statistical analysis

We used the statistical program "Statistica for Windows" version 5.0 (StatSoft. Inc.) for most parametric and non-parametric tests. The calculation of the difference between two means is based on the standard normal distribution (see distribution tables at : http:// classweb.gmu.edu/ralexan3/SYST683/Resources/ stat-tables.html). We correlated the ranks representing species 2C-value against ranks representing water availability (and attached other environmental factors to it) at the same stations at EC. To test the overall significance of the multiple correlations we used the powerful Fit data-fusion (meta-analysis) test [33] based on p values for each independent test in the following form:

$$Fit = -2\sum_{i=1}^{\kappa} \ln(p_i)$$

that approximatelly follows χ^2_{2k} distribution.

3. Results

3.1. Verification of chromosome number

We checked the chromosome number in three plants from each slope and all of them were found to be 2n = 48, as expected (Figure 1).

3.2. Inter- and intraslope differences in genome size of *Cyclamen*

The range of genome size variation detected among measured cyclamens was 6.41% in relation to the smallest measured DNA content. The order of stations according to the increase in their mean genome size was: AS3<ES6<ES5<AS2 <AS1<ES7. The mean relative genome size was higher on the ES than on the AS (Table 1). No deviation from the normality of the data on the AS or the ES was detected by the visual examination of histograms or by Shapiro-Wilkes W test (ES: W = 0.971, p < 0.507; SFS: W = 0.980, p < 0.813). Mean variance was slightly higher on the ES than on the AS (see standard deviation (SD) in Table 1), but not significantly different (Levene's test, F = 0.22,

p = 0.54). Therefore, the results of both normality and variance tests enabled the use of parametric statistics. The t-test for independent samples showed nearly sig-



Figure 1. Metaphase chromosomes of Cyclamen persicum Mull. (2n=48) stained with Hoechst 33258. Bar represents 10 μm.

nificant interslope differences (t-value = -1.857, df = 73, p < 0.067). ANOVA showed the same interslope result as expected, but there was signficant difference in the interstation comparison (SS effect = 0.007, df = 5, F = 3.92, p = 0.003). The most important role in the pattern of the interstation comparison was taken by AS3 because the only significant pairwise comparisons were AS3 x ES5 (t-test, t-value=-3.215, p = 0.004), AS3 x ES6 (t-test, t-value=-2.64, p = 0.014), AS3 x ES7 (t-test, t-value=-4.25, p = 0.0003), AS1 x AS3 (t-test, t-value=-3.43, *p* = 0.003) and AS2 x AS3 (t-test, t-value=-4.17, p = 0.0004). If we removed AS3 from our analysis then nearly significant interslope differences disappeared (AS mean = 1.285, ES mean = 1.285, t-value = 0.075, df = 60, p = 0.94) and the ANOVA interstation comparison became non-signficant (SS effect = 0.0007, df = 4, F = 0.523, p = 0.72).

Also, at the AS there seems to be an upslope trend of increasing genome size from the bottom to top (Table 1). However, at the ES the largest amount of DNA per station was recorded at ES7, and the smallest were recorded at ES6.

3.3. Correlation analysis

Results show no significant correlations present between relative genome sizes of cyclamen and the tu-

Station/ Slope	Sample size (N)	Mean relative genome size + SD	Median	Mean number of leaves	Mean tuber diameter (cm)
AS1	10	1.287±0.02	1.286	11.50	5.08
AS2	12	1.284±0.01	1.285	19.30	5.70
AS3	13	1.262±0.02	1.264	18.00	6.31
ES5	13	1.283±0.02	1.284	18.08	5.48
ES6	14	1.282±0.02	1.280	15.29	6.40
ES7	13	1.290±0.02	1.288	16.92	6.32
AS	35	1.277 ± 0.02	1.276	16.57	5.75
ES	40	1.285 ± 0.02	1.284	16.73	6.07
EC	75	1.281 ± 0.02	1.283	16.65	5.91

 Table 1. Mean relative genome size in tubers of C. persicum at "Evolution Canyon", organized by slopes and stations, Mt. Carmel, Israel.

AS1 – south-facing slope, station 1; AS2 – south-facing slope, station 2; AS3 – south-facing slope, station 3; ES5 – north-facing slope, station 5; ES6 - north-facing slope, station 6; ES7 - north-facing slope, station 7; AS – "African" south-facing slope; ES – "European" north-facing slope; EC – Evolution Canyon, lower Nahal Oren, Mt. Carmel, Israel.

ber diameter (see Table 1 for mean values per station) and the number of flowers and leaves (see Table 1 for mean values per station). However, the last two parameters, the tuber diameter, and the number of flowers and leaves, were significantly positively correlated (Spearman R = 0.651, $p < 1.10^{-5}$) as expected.

We used powerful Fisher's Fit data-fusion test [33] to analyse the significance of multiple correlations between genome size and ranking of stations according to their level of drought (higher level of drought - higher water deficit due to increased evaporation and higher water runoff) in species in which the interslope differences in 2C-values were studied at EC (Table 2). The test on four diploid species (C. persicum, beetle Oryzaephilus surinamensis, carob tree Ceratonia siliqua, and trefoil Lotus peregrinus) showed that a possibility to disproved the null hypohesis, assuming no interslope differences in genome size, is nearly significant (Fit = 14.56, df = 8, p = 0.068) in spite of the individual significance only in Ceratonia siligua (Table 2). The result of the test could be considered as significant since we expected a priori the higher genome size at AS than ES, and this allowed us to release the significance threshold to $\alpha = 0.10$ instead of $\alpha = 0.05$.

4. Discussion

4.1. Interslope divergence in genome size

Our results in *Cyclamen persicum* support the idea that variability in the genome size is distributed *nonrandomly* at the microsite EC. Significant interslope differences were shown in a carob tree, *Ceratonia*

Station	Rank*	Oryzaephilus surinamensis ¹	Cyclamen persicum	Ceratonia siliqua ²	Lotus peregrinus ³
AS1	6		1.287±0.02	0.572±0.003	2.553±0.05
AS2	5	0.508±0.01	1.284±0.01	0.576±0.003	2.541 ± 0.06
AS3	4		1.262±0.02	0.572±0.004	2.552±0.06
AS		0.508	1.278	0.573	2.549
ES5	1	0.504±0.01	1.283±0.02	0.565±0.005	2.542±0.05
ES6	2	0.506±0.01	1.282±0.02	0.567±0.003	2.536±0.06
ES7	3	0.505±0.01	1.290±0.02	0.570 ± 0.005	2.553±0.06
ES		0.505	1.285	0.567	2.544
R		0.800	0.314	0.899	0.406
pi		0.20	0.54	0.01	0.42
ln(pi)		-1.609	-0.609	-4.206	-0.857

 Table
 2. Mean relative genome size in the four studied species at Evolution Canyon according to slopes and stations.

 AS1 - south-facing slope, station 1; AS2 - south-facing slope, station 2; AS3 - south-facing slope, station 3; ES5 - north-facing slope, station 5; ES6 - north-facing slope, station 6; ES7 - north-facing slope, station 7; AS - African savannah-like south-facing slope; ES - European garrigue-like north-facing slope; 'Pavlíček et al. (submitted), 2[4], 3[36], pi - significance of the Spearman correlation, Rank - ranking of a stations according to the increasing drought, R - Spearman rank correlation.

siliqua [4] and between females of O. surinamensis (Sharaf et al., in prep.), and the non-significant interslope differences in wild barley, Hordeum spontaneum [5] and Lotus peregrinus [36]. However, in comparison with the carob tree, the pattern of differences in genome size in C. persicum is different at EC. In the carob study, genome size was significantly higher on the AS than on the ES; all three AS stations showed higher genome sizes than the opposite ES stations, and the two pairwise opposite stations (AS2-ES6, AS3-ES5) were significantly different [4]. In our case, only AS3 was significantly different from the rest of stations and if AS3 was excluded from the analysis, regarded as the outlier, then the interslope differences virtually disappeared. Notably, the Spearman correlation between 2C-values per station and rank of stations according to their experienced drought is positive in C. persicum as has been found in other species studied so far. This positive relationship suggests that we interpret significantly lower 2Cvalues at AS3 as exceptional and not reflecting the general interslope pattern. This finding is unlikely to be a technical artifact, since samples from AS and ES were measured in an alternate sequence between specimens from different stations, homogenized in the same solutions; measurements were done by the same flow cytometry. Since the measurements of genome size were done on the samples from the heterogeneous microsite, there is a possibility that some unknown and unexpected environmental factor induced a local variation in DNA contents in a similar way as described in *Helianthus annuus* [34]. There is no obvious geographic barrier between AS3 and AS2, separated by only a few tens of meters, which might explain the isolation of these two sub-populations one

from another. The only difference we could observe was that *C. persicum* at AS3 grows in the shade of trees and bushes, whereas in the other AS stations it grows more in the cracks of rock and in the shade made by boulders. The distance between AS3 and ES5 is about 100 m at the valley bottom in a dry summer creek. The bottom valley could serve as a barrier to seed dispersal by ants but probably could not prevent pollinators from flying one slope to another. However, more investigation is needed to determine if there might be some unusual pollination pattern at AS3 in comparison with other stations at EC.

4.2. Correlations

We were not able to prove that genome size in Cyclamen significantly correlates with the morphological properties of studied plants (number of leaves and flowers, diameter of bulbs) or with environment (drought stress, temperature, humidty, and plant density) or that significant interslope differences exist. However, the Fit data-fusion test on Spearman correlations between 2C-values in four species (C. persicum, the carob tree Ceratonia siliqua, trifoil, Lotus peregrinus and the sawthoothed grain beetle, Oryzaephilus surinamensis) studied so far at EC and the ranking of stations according to their increasing aridity indicate a possibility that genome increases with the level of drought at EC, in line with earlier predictions [4, 5, 35, 37, 38]. The test results could be regarded as significant if the significance threshold released to $\alpha = 0.10$ instead of $\alpha = 0.05$, but the possible generalization of the test results is limited because only four species were included and, for the most part, it was C. siliqua and O. surinamensis that contributed mostly to the test outcome. However, all four tested species showed the same interslope trend in correlation between genome size and ranking of stations according to increasing drought, i.e., higher genome size at the AS than the ES. Notably, the trend of higher genome size at the AS rather than on the ES was claimed also in H. spontaneum in EC, but no raw data were published [5]. This conclusion is in line with suggestions that increased tolerance to drought (and to another stresses such a frost) is associated with larger genome size in plants [4, 5, 35, 37, 38]. It should be noted that other ecogeographically correlated intraspecific variability in genome size was documented apart from Ceratonia siliqua [4]; for example, between genome size and altitude at the local geographic scale in Zea mays [39] and Dactylis glomerata [40, 41]. On the global geographical scale larger genome size variability was documented in relict parts of species distribution range of Festuca pallens while marginal parts were invaded by the smaller genomes [42].

4.3. Selection for genome size in Cyclamen?

Unfortunately, we do not know the underlying molecular factor(s) explaining genome size variability in *C. persicum* at EC. Transposition controlled by a differential drought (environmental) selection was the mechanism connected to the observed interslope differences in the amount of DNA in wild barley, *Hordeum spontaneum*, at EC [6]. However, other known mechanisms might play a role in our study as well, such as (a) a differential transposition of somatically active transposable elements, which have been described in, so far, both invertebrates and vertebrates [43, 44] or (b) the presence of heritable changes involving DNA within a single generation that have been observed in the annual flax [45]. We cannot exclude from our consideration the possibility of environmentally induced changes of genome size, as these changes have been observed during cell culturing [46-48]. Also, observed genome size changes may have been associated (not necessarily directly selected for) with changes in cell volume and/or in karyoplasmatic ratio. For example, in some plants, selection for maximum growth can lead to modulation of DNA content [49].

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