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# GENOME SIZE VARIATION OF *LOTUS PEREGRINUS* AT EVOLUTION CANYON I MICROSITE, LOWER NAHAL OREN, MT. CARMEL, ISRAEL

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On the basis of previous studies showing a positive correlation between number of copies of retrotransposons and geographical environment, we hypothesized that different ecogeographical conditions on opposite slopes of Evolution Canyon I could cause intraspecific variation in plant genome size. To test this hypothesis, we chose *Lotus peregrinus* L. (annual, self-pollinator) as the first candidate because of its biological contrast to the previously studied carob tree (long-lived, cross-pollinator). Absolute nuclear DNA content of 60 genotypes of *L. peregrinus* was estimated by PI flow cytometry, with tomato (*Lycopersicon esculentum* cv. Stupicke) as internal reference standard. The mean 2C-value in *L. peregrinus* was 2.546 pg, ranging from 2.39 pg to 2.71 pg. The mean 2C-value was higher in plants from the south-facing slope (2.549 pg) than from the north-facing slope (2.544 pg), but we were not able to show significant interslope differences in genome size.

**Key words:** *Lotus peregrinus*, bird's-foot trefoil, Evolution Canyon, flow cytometry, PI staining, genome size, nuclear DNA content, intraspecific variation, species plasticity.

## INTRODUCTION

Genome size is a highly important factor in plant biodiversity, especially in plant biology branches such as physiology, systematics, evolutionary biology, and ecology (Ohri, 1998, 2005). Since there is much debate over the correct terminology used to describe genome size, we follow the proposal recently published by Greilhuber et al. (2005). The term 'holoploid genome' refers to the whole chromosome complement with chromosome number *n*, irrespective of the degree of generative polyploidy. Consequently, the term 'C-value' can be used for 'holoploid genome size.' The term 'monoploid genome' should refer to one chromosome set of an organism, and the number of chromosomes should be expressed by the base number *x*. Therefore, 'monoploid genome size' can be abbreviated as 'Cx-value.' In connection with quantitative data the terms 'C-value' and 'Cx-value' should always be given with the prefix number indicating the DNA level (1C, 2C, 4C). The terms 'genome' and 'genome size' should be used more generally to describe terms in relation to *n* or *x* chromosomes, especially in titles and introductory or concluding phrases with further specification.

Genome size has been regarded as a species-specific constant (Greilhuber, 1998), and genome size variation of similar species has been called the C-value paradox (Thomas, 1971). Most recently, the C-value paradox has been addressed by Cavalier-Smith (2005), who states that the vast amounts of non-genic DNA in many eukaryotes are the necessary outcome of novel cell structures imposing novel selective forces – genomes and cell architecture coevolve. However, many subsequent studies have reported intraspecific and intrapopulation genome size variability (Ohri, 1998). Relationships between genome size and other biological characteristics (e.g., speed of growth, cell cycle, meiosis duration, life cycle) have been reported (e.g., Bennett, 1972; Price, 1988). Ecogeographically correlated intraspecific genome size variability has been documented for many plant species including *Aegilops squarrosa* (Furuta et al., 1975), *Fraxinus americana* (Black and Beckmann, 1983), *Poa annua* (Grime, 1983), *Gibasis venustula* (Kenton, 1984), *Bulbine bulbosa* (Watson, 1987), various species of *Eleusine*

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(Hiremath and Salimath, 1991), *Milium effusum* (Bennett and Bennett, 1992), *Dactylis glomerata* (Reeves et al., 1998), *Zea mays* ssp. *mays* (Poggio et al., 1998), *Crepis pulchra* (Dimitrova and Greilhuber, 2000), *Eleocharis uniglumis* ssp. *sterneri* (Bureš et al., 2002) and *Ceratonia siliqua* (Bureš et al., 2004). Ecogeographical and evolutionary implications of intraspecific genome size variability were discussed by Grime and Mowforth (1982), Bennett (1987) and Knight et al. (2005).

A long-term research project in Evolution Canyon I (EC I) was begun at the Institute of Evolution, University of Haifa, Israel, in 1991. Its main objective is to evaluate the importance of different evolutionary forces on local biodiversity differentiation (Nevo, 1995, 1997, 2001). With regard to genome size, local differentiation in *Hordeum spontaneum* (Kalendar et al., 2000) and in carob tree *Ceratonia siliqua* (Bureš et al., 2004) has been studied at EC I so far. In *H. spontaneum*, significantly larger amounts of retrotransposon BARE–1 were found in plants from the drier, African-savanna-like south-facing slope (SFS) than from the wetter, cooler, Mediterranean garrigue-covered north-facing slope (NFS). This interslope difference was interpreted as the result of significantly higher BARE–1 activity at the drier 'African' SFS than at the wetter 'European' NFS (Kalendar et al., 2000). The expected relationship between BARE–1 activity and genome size was based on the positive correlation between the two parameters, but the higher genome size in plants from the SFS than from the NFS was not statistically significant (Kalendar et al., 2000). In *C. siliqua*, genomes in adult trees were significantly larger in plants from the SFS than from the NFS (Bureš at al., 2004). The results obtained so far at EC I may indicate an interslope trend toward increased genome size along the aridity stress gradient. Here we report a study of interslope variability in genome size in the annual, mostly self-pollinating bird's-foot trefoil, *Lotus peregrinus* L., whose biological and ecological properties differ from those of the carob tree. We posited that the genome would be larger at the SFS than at the NFS in this species.

## MATERIALS AND METHODS

### MICROSITE

The EC I microsite, located in the lower part of Nahal Oren, Mt. Carmel, Israel (32°43'N; 34°58'E), is a Plio Pleistocene canyon (Nevo, 1995). The opposite slopes, running east-west, are only 100 m apart at the bottom and 400 m at the top. The valley bottom is 40 m a.s.l., the SFS inclines 20–40°, and the NFS inclines 20–30°. The 'African' slope, SFS, is covered by open park forest of evergreen *Ceratonia siliqua*-*Pistacia lentiscus*, with savanna-like grassland dominant. In contrast, the 'European' slope, NFS, is covered by a dense Mediterranean garrigue of evergreen *Quercus calliprinos* and deciduous *Pistacia palaestina* (Nevo et al., 1999). Geology (Upper Cenomanian Limestone; Karcz, 1959), regional Mediterranean climate (mean annual rainfall ~600 mm, potential evapotranspiration 1700 mm, mean August and January temperature 28°C and 13°C, respectively; Anonymous, 1970) and pedology (terra rossa: Nevo et al., 1998) are the same on both slopes. Due to differences in geographic orientation the SFS is significantly warmer, less humid and more insolated than the NFS, and its microclimates vary more (Pavlícek et al., 2003). The microclimatic differences are reflected in sharper differences in local biodiversity. The SFS is on average richer in 'terrestrial' species and displays higher genetic diversity than the NFS, which is richer in 'aquatic dependent' taxa (Nevo, 1995, 1997, 2001).

### MODEL SPECIES

The bird's-foot trefoil (*Lotus peregrinus* L.) belongs to the very large family Fabaceae (also known as family Leguminosae), subfamily Faboideae previously called Papilionoideae. This large, diverse (trees, shrubs, and herbs), and important (both ecologically and economically) family includes over 643 genera and 18,000 species and is of cosmopolitan distribution (Simpson, 2006). *L. peregrinus* L. is a self-compatible monoecious species regarded as a self-pollinator (Heyn, 1966; Heyn et al., 1995). However, several bees observed on flowers of *L. peregrinus* by Madmony (1991) indicate that out-crossing also might be present. As a matter of fact, *Lotus carmeli* described from Mt Carmel by Boissier (1872) is sometimes today mentioned as *L. peregrinus* var. *carmeli* (Boissier, 1872). Revision of the taxonomic status of this variety is needed, as suggested earlier (Heyn, 1966; Chrtková-Žertová, 1971). Heyn (1966) described tetraploidy in both *L. peregrinus* var. *carmeli* and *L. peregrinus* var. *peregrinus* from Israel, but our material is diploid (2n = 14). *Lotus peregrinus* is significantly more abundant on the SFS than on the NFS at EC I (Cohen et al., 2003).

### COLLECTION OF SAMPLES

Ten adult plants of *L. peregrinus* were collected shortly before flowering from each of six stations along a 300 m horizontal transect on the two opposite slopes of EC I. The stations are located 120 m (SFS1 and NFS7), 90 m (SFS2 and NFS6) and 60 m (SFS3 and NFS5) above sea level. The plants were transplanted to pots in the greenhouse in the Institute of Evolution, University of Haifa, and kept

TABLE 1. C-values in *L. peregrinus* at Evolution Canyon I, lower Nahal Oren, Israel

| Station | No. of genotypes | No. of samples* | DNA average | DNAmin | DNAmax |
|---|---|---|---|---|---|
| SFS1 | 10 | 84 | 2.553±0.0497 | 2.42 | 2.63 |
| SFS2 | 10 | 87 | 2.541±0.0552 | 2.39 | 2.64 |
| SFS3 | 10 | 81 | 2.552±0.0560 | 2.44 | 2.66 |
| NFS5 | 10 | 81 | 2.542±0.0503 | 2.46 | 2.67 |
| NFS6 | 10 | 78 | 2.536±0.0552 | 2.45 | 2.65 |
| NFS7 | 10 | 87 | 2.553±0.0648 | 2.46 | 2.71 |
| SFS | 30 | 252 | 2.549±0.0538 | 2.39 | 2.66 |
| NFS | 30 | 246 | 2.544±0.0575 | 2.45 | 2.71 |
| 'EC' | 60 | 498 | 2.546±0.0557 | 2.39 | 2.71 |

*No. of samples include repetitions of the same genotypes; ± SD.

under standard conditions to flower and produce seeds. The seeds were collected and used for subsequent experiments.

### PLANT MATERIAL AND CULTIVATION

Three seeds of each genotype of *L. peregrinus* sent from the Institute of Evolution, University of Haifa, were cultivated in a greenhouse under standard conditions at the Department of Botany, Faculty of Science, Palacký University, Olomouc-Holice, Czech Republic. Unfortunately, in 12 genotypes only 2 seeds germinated, and in one genotype only one seed germinated (Tab. 1). Young healthy intact leaves were sampled from each plant to estimate genome size when the seedlings were seven weeks old.

### ISOLATION AND STAINING OF NUCLEI

Since the standard genome size of *Lotus peregrinus* was previously reported, the appropriate internal standard was chosen from the list of flow cytometry reference standards recommended by Doležel et al. (1992). The most suitable candidate was tomato (*Lycopersicon esculentum* cv. Stupicke) with 2C-value 1.96 pg (PI), because the G1 peak of the second available standard, soybean (*Glycine max*), was close to the G1 peak of the target. Plant tissue (~20 mg) from the analyzed sample was chopped with a razor blade into small pieces together with the standard in a Petri dish containing 0.5 mL OTTO I buffer composed of 0.1 M citric acid and 0.5% Tween 20. The suspension of isolated nuclei was filtered through nylon (pore size 40 μM). Then 50 μM RNAse, and 50 μL PI (propidium iodide) stock solution was added and the suspension was incubated for 60 min at room temperature. Then 1 mL OTTO II composed of 0.4 M $Na_2HPO_4$ was added. The suspension was incubated for ~5 min at room temperature. After incubation, each sample was run on a flow cytometer.

### FLOW CYTOMETRY

The relative fluorescence of the nuclei was measured with a PAS flow cytometer (Partec GmbH, Münster, Germany) equipped with an argon ion laser. The instrument was calibrated before each set of measurements with calibration beads (Partec, Germany). The gain was adjusted so that the fluorescence peak of the reference standard was placed on channel 75 of the 512-channel scale. The flow rate did not exceed 50 fluorescent events per second. At least 5,000 nuclei were analyzed in each sample. Each plant was measured three times on three different days by the same operator, and a different leaf was used for each analysis.

### DATA ANALYSIS AND ESTIMATION OF 2C-VALUE

The 2C DNA nuclear content of the samples was calculated as follows:

$$\text{Sample 2C relative DNA content} = \frac{\text{Sample peak mean}}{\text{Standard peak mean}} \times \text{2C DNA content of the standard}$$

### STATISTICAL ANALYSIS

Data on nuclear DNA content were analyzed with NCSS 97 software (Statistical Solutions Ltd., Cork, Ireland) and Statistica for Windows ver. 5.0 (StatSoft. Inc.). One-way ANOVA and multiple range tests (Tukey's test) were employed to analyze the variation of mean 2C-values.

## RESULTS

### GENERAL INTERPRETATION OF DATA

Sixty genotypes (genotype defined as seeds collected from one plant) from EC I were studied. Ten genotypes were analyzed from each station (Tab. 1). Flow

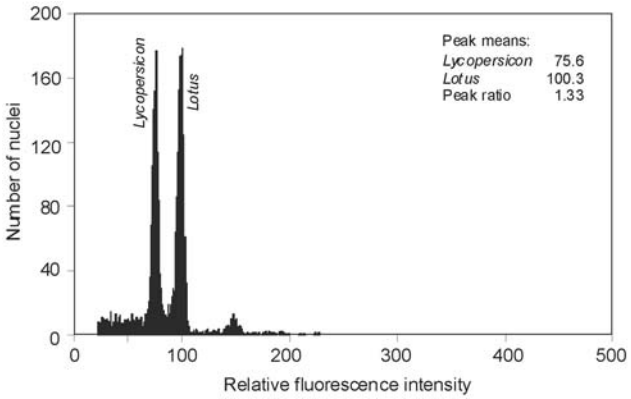


**Fig. 1.** Histogram of relative fluorescence intensity obtained by simultaneous flow cytometric analysis of PI-stained *Lotus peregrinus* nuclei and nuclei of the tomato. Absolute nuclear DNA content of *Lotus peregrinus* was equal to 1.96 × 1.33 = 2.6 pg.

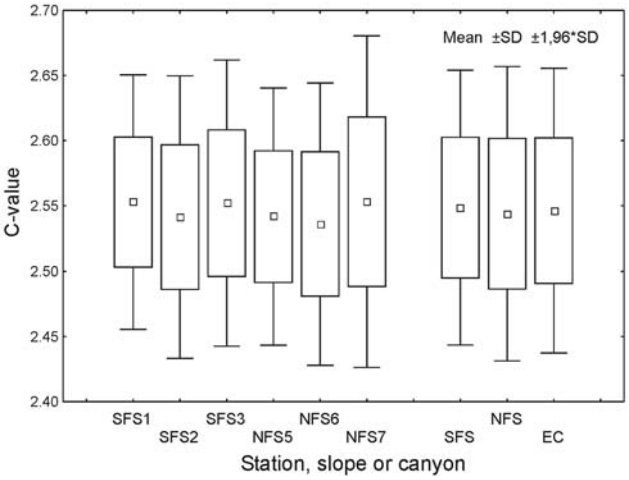


**Fig. 2.** Mean 2C-values obtained in *L. peregrinus* at Evolution Canyon I (EC I) per station and slope and for EC I. ±SD – standard deviation representing 68% of variability; ±1.96*SD – standard deviation representing 95% of variability.

cytometry was used to estimate absolute nuclear DNA content, with tomato (*Lycopersicon esculentum* cv. Stupicke) as internal reference standard. On average, 8.3 representative plant samples of each genotype were analyzed. The histograms of the relative fluorescence intensity obtained by the described method had a distinct major peak at channel 75, corresponding to the G1 nuclei (2C-value) of the standard, a minor peak at channel 150 representing nuclei of the analyzed standard in the G2 phase (4C-value), and another distinct major peak corresponding to the G1 nuclei (2C-value) of the target. The presence of dominant peaks representing G1 nuclei is typical for leaf tissues in the majority of higher plants. No nuclei with DNA content higher than 4C were observed (Fig. 1). The mean 2C-value of all samples was 2.546, and ranged from 2.39 to 2.71 (Tab. 1, Fig. 2). The mean 2C-value was slightly higher for plants from the SFS (2.549) than from the NFS (2.544) (Tab. 1, Fig. 2). The appropriateness of parametric statistics to analyze the obtained data is confirmed by the lack of significant deviations from homogeneity of variance (Levene test of homogeneity of variance: a) between the slopes, F = 0.236, p = 0.71; b) or between the six stations, F = 1.72, p = 0.15), and direct observation of the distribution graphs indicated no deviation from a normal distribution.

### INTERSLOPE DIFFERENCES IN 2C-VALUES OF *L. PEREGRINUS*

The test of means against the mean of analyzed genotypes (mean of analyzed genotypes = 2.5462, df = 59, t-value = 0.000098, p = 0.9999) at EC I did not reject the Ho hypothesis that all genotypes are equal to the sample mean. The t-test did not reject the Ho hypothesis that the slopes are equal as regards 2C-value (df = 58, t-value 0.729, p = 0.47). The same test did not reject the Ho hypothesis that the stations are equal (Tab. 2). Regression analysis of 2C-value did not show any significant interslope or interstation differences either [F (2.57) = 0.347, $p_{slopes}$ = 0.48, $p_{stations}$ = 0.68].

### DIPLOIDY VERSUS TETRAPLOIDY

We checked four plants used in the analysis for ploidy level. All of them (accessions 2–54–5, 2–50–2, 6–53–5, 6–24–2) were diploid (2n = 14). Aneuploidy was observed in some cells. All plants tested by flow cytometry were diploid (see Fig. 1). In addition, twenty plants grown in the greenhouse had from 1 to 3 flowers in the inflorescence and yellow-brown to dark brown seeds, indicating that they belong to *L. peregrinus peregrinus* (for details on morphological differences see, e.g.: Boissier, 1872; Heyn, 1966; Chrtková-Žertová, 1971).

## DISCUSSION

The adaptive significance of nuclear DNA variation in angiosperms, the causative factors influencing genome size, and the adaptive consequences for an organism in given environmental conditions have been widely discussed (Ohri, 2005). The main aim of this study was to estimate absolute nuclear DNA content in the species *Lotus peregrinus*, and to critically assess the presence and extent of local intraspecific 2C-value variation within this species. The measurements of genome size in *Lotus peregri-*

TABLE 2. Pairwise comparison of C-values in *L. peregrinus* between stations at Evolution Canyon by t-test

| Station combination | *p* | Station combination | *p* |
|---|---|---|---|
| SFS1 × SFS2 | 0.41 | SFS2 × NFS7 | 0.40 |
| SFS1 × SFS3 | 0.76 | SFS3 × NFS5 | 0.36 |
| SFS1 × NFS5 | 0.30 | SFS3 × NFS6 | 0.18 |
| SFS1 × NFS6 | 0.16 | SFS3 × NFS7 | 0.77 |
| SFS1 × NFS7 | 0.95 | NFS5 × NFS6 | 0.47 |
| SFS2 × SFS3 | 0.50 | NFS5 × NFS7 | 0.24 |
| SFS2 × NFS5 | 0.98 | NFS6 × NFS7 | 0.13 |
| SFS2 × NFS6 | 0.68 | | |

*nus* showed distinct variability within the group of 60 genotypes collected from opposite slopes at EC I. The mean 2C-value of all samples was 2.546, with the maximum difference between individuals at 13.39% (0.32 pg). The observed 2C-value variation was not statistically significant, and had no correlation with altitude or with the position on the SFS or the NFS. One possible interpretation is that the populations on both slopes have stable genome size, and that this is not influenced by the ecogeographic gradient at EC I. On the other hand, increasing the sample size could yield statistically significant results. Although there are distinct interslope differences in microclimate at EC I (Pavlíček et al., 2003) they might be buffered by the plasticity of *L. peregrinus*. In such a closed region (a valley with two slopes), the expected high dispersal ability of *L. peregrinus* (Latin *peregrinus* = traveller) together with possible pollination could produce gene flow, preventing differentiation caused by differential natural selection. This explanation corresponds to the results obtained in adult carob trees *C. siliqua* (Bureš et al., 2004). The perennial carob tree has been selected by local ecogeographic conditions for a much longer time than the annual *L. peregrinus*. Low dispersal ability could influence genome size to enlarge as an adaptation to the local environment. Unfortunately, the dispersal ability of *L. peregrinus* was not studied at EC I, and there is no known vector transporting seeds between the slopes. From the data it can be inferred that *L. peregrinus* is not a strict self-pollinator in nature, and this warrants further study. It should be noted that related species from the same family, such as pea and soybean, are known for their genome size constancy (e.g., Greilhuber and Ebert, 1994; Baranyi and Greilhuber, 1995, 1996).

In earlier work, significantly more BARE–1 retrotransposon copies were found in *H. spontaneum* plants from the SFS than from the NFS, and the positive significant correlation between the number of copies and altitude (Kalendar et al., 2000) led us to suppose that genome size should also be higher in plants on the SFS than on the NFS. However, the interslope differences in genome sizes measured so far were not significant, though mean genome size was higher in the plants from the SFS than from the NFS (Kalendar et al., 2000). Bureš et al. (2004) found significantly higher genome size in adult carob trees growing in drier conditions on the 'African' SFS than on the more humid 'European' NFS, and concluded that the observed pattern of interslope differences in genome size might parallel the higher biodiversity present on the SFS than on the NFS as summarized by Nevo (1995, 1997, 2001). The common denominator of all interslope genetic differences at EC I, including genome size, was suggested to be environmental stress, which is regarded as the most probable driving force generating global-scale adaptive genome and phenome strategies, both microscale and macroscale (Nevo, 1995, 1997, 2001). Against this hypothesis, however, Price et al. (1981a,b, 1986) showed larger genome sizes in plants of the genus *Microseris* growing in wetter habitats than in those growing in drier conditions.

In light of the most recent studies, it should be stressed that almost all previous work on intraspecific C-value variation should be interpreted with caution. In most cases, repeated measurements, even with the same samples or genotypes, showed no variability exceeding measurement error. Greilhuber (2005) checked estimations of intraspecific C-value variability in *Dasypyrum villosum* by means of flow cytometry and refuted the suggestion of the 'plastic genome' phenomenon associated with fruit color previously reported (Cremonini et al., 1994; Frediani et al., 1994; Caceres et al., 1998). In the same way, Temsch and Greilhuber (2000, 2001) checked published results on interspecific variability of genome size in *Arachis hypogea* and *A. duranensis* by Singh et al. (1996). Importantly, rechecking of earlier work estimating intraspecific genome size variation in *Glycine max* (Graham et al., 1994; Rayburn et al., 1997) and in *Pisum sativum* (Guerra, 1983; Arumuganathan and Earle, 1991; Cavallini et al., 1993) confirmed the stability of genome size in these species, reflected in the absence of intraspecific genome size variation (Greilhuber and Ebert, 1994; Baranyi and

Greilhuber, 1995, 1996). In fact, genome stability was the reason why Doležel et al. (1992, 1998) proposed these two species as reference standards for flow cytometry. Presently, methodological specialists are trying to improve the flow cytometry procedure to minimize measurement error (e.g., Doležel and Bartoš, 2005). Undoubtedly there is intraspecific C-value variation in certain species; it might be associated with taxonomic heterogeneity caused by progressive speciation or else resulting from previously inaccurate taxonomic characterization (Murray, 2005).

Further work on EC I should focus on different plant genera that could react to the different microclimate conditions of the NSF and SFS, and also on plant genera that have the same characteristics as *L. peregrinus* (dispersal ability, annual). If the results are consistently repeated, there should be few concerns about methodology because any fault in methods would cause variations rather than constancy of measurement results (Greilhuber, 2005).

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