

Microgeographic Genome Size Differentiation of the Carob Tree, *Ceratonia siliqua*, at ‘Evolution Canyon’, Israel

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- **Background and Aims** We tested whether the local differences in genome size recorded earlier in the wild barley, *Hordeum spontaneum*, at ‘Evolution Canyon’, Mount Carmel, Israel, can also be found in other organisms. As a model species for our test we chose the evergreen carob tree, *Ceratonia siliqua*.
- **Methods** Genome size was measured by means of DAPI flow cytometry.
- **Key Results** In adults, significantly more DNA was recorded in trees growing on the more illuminated, warmer, drier, microclimatically more fluctuating ‘African’ south-facing slope than in trees on the opposite, less illuminated, cooler and more humid, ‘European’ north-facing slope in spite of an interslope distance of only 100 m at the canyon bottom and 400 m at the top. The amount of DNA was significantly negatively correlated with leaf length and tree circumference. In seedlings, interslope differences in the amount of genome DNA were not found. In addition, the first cases of triploidy and tetraploidy were found in *C. siliqua*.
- **Conclusions** The data on *C. siliqua* at ‘Evolution Canyon’ showed that local variability in the C-value exists in this species and that ecological stress might be a strong evolutionary driving force in shaping the amount of DNA.

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Key words: *Ceratonia siliqua*, carob, DNA content, flow cytometry, genome size, ‘Evolution Canyon’.

INTRODUCTION

In general, the C-value paradox (no observed relationship between genome size diversity and organism complexity) is a largely overlooked factor in evolution and a matter of controversy (e.g. Elder and Turner, 1995). Among the different theories proposed to explain this phenomenon, two basic groups can be distinguished (Gregory, 2001). First, there are neutral mutation pressure theories (e.g. Ohno, 1972; Doolittle and Sapienza, 1980; Orgel and Crick, 1980) which state that genome size differences are due to a balance between an upward mutation pressure acting to increase DNA content in contrast to the tolerance of the ‘host’ cell to the building-up of functionless ‘junk’ DNA (Gregory, 2001). Secondly, there are selective optimal DNA theories (e.g. Orgel *et al.*, 1980; Cavalier-Smith, 1982) in which DNA is considered to play both a qualitative and a quantitative role in the determination of organism fitness, with C-values reflecting the adaptive optimized outcome of selection (Gregory, 2001).

Ecogeographically correlated intraspecific variability in genome size is documented by many authors in several plant species, including *Poa annua* (Grime, 1983), *Milium effusum* (Bennett and Bennett, 1992), *Dactylis glomerata* (Reeves *et al.*, 1998) and *Sesleria albicans* (Lysák *et al.*, 2000). For critical reviews of intraspecific variability of genome size see Ohri (1998) or Greilhuber (1998), and for its ecogeographical implications see Grime and Mowforth (1982) and Bennett (1987).

The ‘Evolution Canyon’ (EC) project involves evaluating the relative importance of different evolutionary forces and outcomes on local biodiversity differentiation (see reviews in Nevo, 1995, 1997, 2001). In wild barley, *Hordeum spontaneum*, growing in Israel, it seems that larger genome size, due to retrotransposon activity, may enable these plants to cope more effectively with aridity stress locally at EC as well as regionally (Vicent *et al.*, 1999; Kalendar *et al.*, 2000).

To determine whether local interslope differences in genome size are more general, as our model species we chose the carob, *Ceratonia siliqua*, a perennial tree species characterized by great longevity, slow growth and cross-pollination, which contrasts to the annual, quick-growing and mostly self-pollinating *H. spontaneum*.

MATERIAL AND METHODS

Microsite

The microsite, named ‘Evolution Canyon’ I (EC I), located at Lower Nahal Oren, Mount Carmel, Israel (32°43’N, 34°58’E), is a Plio-Pleistocene canyon (Nevo, 1995). The opposite slopes, which run in an east–west direction, are separated by 100 m at the bottom and 400 m at the top. The ‘African’ south-facing slope (SFS) is covered by an open park forest of evergreen *Ceratonia siliqua* and *Pistacia lentiscus* with dominant savanna-like grassland. In contrast, the ‘European’ north-facing slope (NFS) is covered by a dense Mediterranean maquis of evergreen *Quercus calliprinos* and deciduous *Pistacia palaestina* (Nevo *et al.*,

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1999). Geology (Upper Cenomanian limestone; Karcz, 1959), regional Mediterranean climate (mean annual rainfall, approx. 600 mm; potential evapotranspiration, 1700 mm; and mean August and January temperatures 28 °C and 13 °C, respectively; Atlas of Israel, 1970), and pedology (terra rossa; Nevo *et al.*, 1998) are the same on both slopes. Due to differences in geographic orientation in the northern hemisphere, SFSs receive, on average, more radiation per year than NFSs of the same inclination (Ayyad, 1971). Indeed, the SFS of EC I is significantly more illuminated, warmer and drier, and microclimatically and spatiotemporally more fluctuating than the opposite NFS (Pavlíček *et al.*, 2003). These microclimate differences are reflected in strong local biodiversity differences. 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

Ceratonia siliqua (the carob or locust tree) is native to the eastern Mediterranean basin (Batlle and Tous, 1997). It is a large, evergreen, sclerophyllous, leguminous tree of slow growth, great longevity and an extended flowering period (Diamantoglou and Mitrakos, 1981). It is also resistant to drought and salinity (Lo Gullo and Rosso, 1986). The carob has been growing in the eastern Mediterranean since long before the start of agriculture was documented. It is known to have been growing in what is now Israel as early as 43 000 BC (Weinstein, 1982). At EC I, the carob has been present since around 6000–4000 BC (Noy, 1977). Some authors believe that the carob originated from southern Arabia and not from the eastern Mediterranean (Schweinfurth, 1894; Zohary, 1973). This is supported by the fact that *C. oreothauma*, the only known carob-related species, is thought to have one of two origins, either in Oman in south-east Arabia or around the Horn of Africa in Somalia (Hillcoat *et al.*, 1980). Currently, the carob is cultivated widely in other regions of the world with a Mediterranean climate (Vardar *et al.*, 1980). Relative to other taxa of the subfamily Caesalpinioideae, carob flowers are anomalous in being initially bisexual with one sex usually suppressed during late development (Tucker, 1992), and by the lack of petals. The trees are usually dioecious but can be monoecious, and occasionally hermaphroditic (Linskens and Scholten, 1980; Batlle and Tous, 1988). Male and female flowers both contain nectar, although not in equal amounts, and insects play an important role in pollination (Retana *et al.*, 1990; Ortiz *et al.*, 1996). The seeds' hard testa is an effective protective structure allowing them to survive fire, and being passed through the guts of large animals which aid dispersal (Ortiz *et al.*, 1995). It has also resulted in a persistent seed-bank being built up in the soil. To date, the reported number of chromosomes in the carob has been $2n = 24$ (de Almeida, 1948; Frahm-Leliveld, 1957; Berger *et al.*, 1958; Goldblatt, 1981; Yeh *et al.*, 1986; Arista and Talavera, 1990). However, in our study we found one case each of triploidy and of tetraploidy (see Results). No intersexual differences should exist in

genome size since both sexes have the same number of identical chromosomes and the sex chromosome(s) is not present.

Sample collection

The 44 seedlings analysed for genome size were collected on 15 April 2003 at EC I. They were collected directly from under the mother trees, transferred together with soil to polyethylene bags, sealed, kept at a low temperature, and delivered to the Department of Botany, Masaryk University, Brno. Measurements took place on 22 April 2003. The 81 leaf samples from the mother trees were collected on 30 and 31 May 2003, following the same procedure as for the seedlings. Measurements took place on 9 and 10 June 2003. At the same time as collecting leaves, trunk circumferences were measured. If a tree had more than one trunk, the circumference of the thicker trunk was measured. The two pods subsequently used to measure intra-tree variability were collected on 18 September 2003. Pod 1 was sampled from a main trunk with a circumference of 230 cm, and pod 2 from a young trunk with a circumference of 10 cm from the same tree. The seeds from the pods were later extracted and germinated in a greenhouse at the Department of Botany, Masaryk University, Brno. The DNA genome size was measured in the seedlings.

Genome size measurement

A ploidy analyser (PA-1, Partec GmbH, Münster, Germany) equipped with a HBO-100 mercury arc lamp was used to measure genome size. Sample preparations were carried out in a two-step procedure (Otto, 1990; Doležel and Göhde, 1995) in the Laboratory of Flow Cytometry, Department of Botany, Masaryk University, Brno. One specimen of *Luzula luzuloides* was used as a standard to measure the relative amount of DNA in leaves of adult trees (Barlow and Nevin (1976) reported a haploid DNA content of $C = 1.15$ pg for *L. luzuloides*) and one specimen of *Medicago sativa* was used to measure the relative amount of DNA in seedlings (Bennett (1972) reported a haploid DNA content of $C = 1.75$ pg for *M. sativa*). Samples from a measured individual (0.5 cm² of leaf blade from seedlings or 0.5 cm of leaf petiole from adult trees) and from a reference standard (0.5 cm² of leaf blade from *M. sativa* or 1.0 cm² of leaf blade from *L. luzuloides*) 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 % Tween20 adjusted to 200 mL and filtered through a 0.22-µm filter). Another 0.5 mL Otto I buffer was then added before the solution was filtered through nylon cloth (50 µm mesh size). For DNA staining, 2 mL of Otto II buffer (0.4 M disodium hydrogenphosphate dodecahydrate) including DAPI (4',6-diamidino-2-phenylindole; 4 µg mL⁻¹ final concentration) was used. DAPI is known to form fluorescent complexes with natural double-stranded DNA, showing fluorescence specificity preferentially for AT-bases, and is more useable for the estimation of small infraspecific differences of DNA content than, for example, ethidium bromide. For leaves

TABLE 1. Mean relative genome size of adult *Ceratonia siliqua* trees according to slopes and stations at 'Evolution Canyon' I, Mount Carmel, Israel

Station/slope	Sample size (<i>n</i>)	Mean relative genome size (± s.d.)	Mean tree circumference (cm)	Mean leaf length (cm)*
SFS1	13	0.572 (0.003)	49.5	36.4
SFS2	15	0.576 (0.003)	33.8	34.6
SFS3	14	0.572 (0.004)	51.8	37.1
NFS5	11	0.565 (0.005)	99.1	65.3
NFS6	13	0.567 (0.003)	41.1	42.0
NFS7	13	0.570 (0.005)	44.0	43.7
Mean				
SFS 'African' slope	42	0.573 (0.004)	44.6	36.0
NFS 'European' slope	37	0.568 (0.005)	59.4	50.3

* Data from Nevo *et al.*, 2000.

from adult trees, which contain a higher amount of secondary substances, we used only leaf petioles, not leaf blades as in seedlings. Therefore, samples from adults were incubated for 1 h (instead of 2–5 min for seedlings), and centrifuged for 15 min at 1100 g. Each sample was measured three times and the calibrated average value was included in the statistical analysis. Calibrated average relative DNA content R_{sample} was calculated using the following formula:

$$R_{\text{sample}} = \frac{\sum_{i=1..3} (1/\text{CV}_{\text{sample}i} \times \text{F_DAPI}_{\text{sample}i} / \text{F_DAPI}_{\text{reference}i})}{\sum_{i=1..3} (1/\text{CV}_{\text{reference}i} \times \text{F_DAPI}_{\text{reference}i})}$$

where CV is variance coefficient and F_DAPI is relative fluorescence channel (mean of peak) for both sample and reference in the first, second and third measurements ($i = 1..3$), i.e. each measurement of relative DNA content $R_{\text{sample}i} = \text{F_DAPI}_{\text{sample}i} / \text{F_DAPI}_{\text{reference}i}$ is included in the calculation of the average with weight equal to $1/\text{CV}_{\text{sample}i} \times 1/\text{CV}_{\text{reference}i}$. All measurements for both seedlings and adult trees were carried out in 2 days. Samples from the SFS and the NFS of EC I were measured alternatively. For the repeat control measurements of triploid and tetraploid samples, diploid plants of *C. siliqua* were used as the reference standard. For the adult plants we used a two-step procedure with one-step buffers, which are usually successful for plants with secondary compounds, i.e. tris-MgCl₂ buffer (Pfosser *et al.*, 1995) and lysis buffer LB01 (Doležal *et al.*, 1989), in combination with the following conditions: centrifuge time 5 or 15 min, $g = 290$ or 1100, addition of polyvinylpyrrolidone (PVP 40) in concentrations of 10, 20 or 30 g L⁻¹ to buffer. All these methods yielded poorer results than the two-step procedure in combination with centrifuging.

Statistical analysis

The statistical program 'Statistica for Windows' version 5.0 (StatSoft Inc.) was used for most of the parametric and non-parametric tests. In order to test the effect of random events, the permutation test used was based on 500 000 random shufflings.

RESULTS

Interslope differences in genome size of adult trees

Two samples from the original data set were omitted because one was from a triploid plant and the other from a tetraploid (see below). All other sampled plants were diploid. The range of genome size variation detected among the diploid adult trees was 4.04 % relative to the smallest measured DNA content.

The mean relative genome size was greater on the SFS than on the NFS (Table 1). No deviation of the data from normality on the SFS or the NFS was detected either by visual examination of histograms or by Shapiro–Wilk's *W*-test (NFS: $W = 0.9845$, $P < 0.915$; SFS: $W = 0.9893$, $P < 0.978$). Mean variance was slightly higher on the NFS than on the SFS (see s.d. in Table 1), but not significantly different (Levene's test, $F = 1.8$, $P = 0.18$). Therefore, the results of both the normality and the variance tests allowed us to use parametric statistics. The *t*-test for independent samples showed significant interslope differences (t -value = 6.0457, d.f. = 77, $P < 0.001$). The significant results of the interslope differences were confirmed by permutation tests: the H_0 hypothesis of equal genome size between the slopes was rejected at $P = 2.10^{-6}$.

Additional evidence of interslope differences in genome size is given by comparison of SFS with NFS stations (Table 1). The mean relative genome size was significantly higher at the three SFS stations than at the three NFS stations (Mann–Whitney *U*-test, $P = 0.05$).

Interstation differences in adult trees

The pairwise comparisons were significant between SFS2 and NFS6 (t -value = 7.526, d.f. = 26, $P < 0.001$) and between SFS3 and NFS5 (t -value = 3.663, d.f. = 23, $P = 0.001$), but not between SFS1 and NFS7 (t -value = 1.290, d.f. = 24, $P = 0.209$). There seems to be an upslope trend of increasing genome size from the bottom to the top of the NFS (Table 1), but not on the SFS.

Multiple regression analysis

Multiple regression analysis indicates that the most important factor is slope ($\beta = 0.734$, $P = 0.0007$) and the

TABLE 2. Interslope differences in genome size of adult *Ceratonia siliqua* trees according to sex at 'Evolution Canyon' I, Mount Carmel, Israel

Slope	Sex	<i>n</i>	Mean (\pm s.d.)
SFS 'African'	Males	28	0.573 (0.001)
SFS 'African'	Females	14	0.574 (0.028)
NFS 'European'	Males	18	0.568 (0.001)
NFS 'European'	Females	19	0.567 (0.039)

second most important is tree circumference ($\beta = -0.186$, $P = 0.06$), the thicker-trunked trees having significantly smaller genomes. The stations appear not to play an important role ($\beta = 0.251$, $P = 0.25$). In this analysis we were not able to include leaf size since the data are only means per station.

Influence of sex

Sex does not seem to have influenced the interslope results of genome size. The relative genome size values of both sexes on both slopes were similar and both males and females on the SFS had larger genomes than those on the NFS (Table 2).

Correlation between genome size, tree circumference and leaf size

The relative genome sizes of adult trees were correlated with tree circumference. The negative correlation in all sampled trees at EC I was significant (Spearman rank order correlations, $n = 79$, $R_S = -0.2748$, $P = 0.0142$). A significant negative correlation was also found on the SFS ($n = 42$, $R_S = -0.2267$, $P = 0.0289$) but not on the NFS ($n = 37$, $R_S = -0.1402$, $P = 0.4079$). Circumferences of tree trunks on the SFS (mean circumference, 44.6 cm) were significantly smaller than those on the NFS (mean circumference, 59.4 cm) (one way ANOVA, $F = 4.01$, $P = 0.048$). There was a significant ($R_S = -0.9276$, $P < 0.05$) negative correlation between the mean relative genome contents of adult trees per station and mean leaf length per station (Table 1).

Many other significant correlations between the relative mean genome size per station and environmental factors have been reported. Examples include significantly positive correlations ($R_S = 0.89$, $P < 0.02$) with mean temperature and illumination (both of which are significantly higher on the SFS: Pavlíček *et al.*, 2003) and significant negative correlation ($R_S = -0.89$, $P < 0.02$) with humidity (which is significantly higher on the NFS: Pavlíček *et al.*, 2003).

Seedlings

The range of DNA content variation among the seedlings was 3.93 %. The mean value was higher on the NFS than on the SFS (Table 3), but differences were not significant. Mean variance was slightly higher on the NFS than on the

TABLE 3. Mean relative genome size of *Ceratonia siliqua* seedlings according to slopes and stations at 'Evolution Canyon' I, Mount Carmel, Israel

Station/slope	<i>n</i>	Mean (\pm s.d.)
SFS1	9	32.782 (0.261)
SFS2	3	33.132 (0.161)
SFS3	14	33.070 (0.215)
NFS5	13	33.037 (0.261)
NFS6	3	33.382 (0.306)
NFS7	2	33.332 (0.019)
SFS 'African'	26	32.977 (0.263)
NFS 'European'	18	33.127 (0.286)

SFS (see s.d. in Table 3), but again differences were not significant (Levene's test, $F = 0.06$, $P = 0.81$). The permutation test did not reject the null hypothesis, as $P = 0.17$.

Intra-tree variability

Genome sizes in seven and eight seedlings that grew from the seeds of pod 1 (sampled from a main trunk with a circumference of 230 cm) and pod 2 (sampled from a young trunk with a circumference of 10 cm), respectively, were measured. The arithmetic mean, maximum and minimum for pod 1 seedlings were 1.703 ± 0.014 , 1.72, 1.68, and for pod 2 seedlings 1.702 ± 0.017 , 1.72, 1.68. As the values for seedlings from both pods were almost identical, the arithmetic means were not significantly different (t -value = 0.19, $P = 0.85$). No significant correlations were found either between seed weights and their DNA amounts (Spearman $R = 0.014$, $P = 0.96$) or between DNA amounts and seed positions in each pod ($R = 0.36$, $P = 0.43$ and $R = 0.31$, $P = 0.46$).

Chromosome numbers

During our investigation we discovered one triploid sample (Fig. 1) and one tetraploid sample (not shown) representing trees from SFS3 and NFS7, respectively. These are the first records of triploidy and tetraploidy in *C. siliqua*. While the genome size among diploids varies 1.04-fold, the amount of DNA in the triploid and tetraploid was, respectively, 1.545-fold and 2.054-fold more than in diploid plants with the smallest DNA content.

DISCUSSION

Interslope divergence in genome size

Our results on adult trees corroborate the results obtained on *Hordeum spontaneum* (Kalendar *et al.*, 2000), and show that differences in genome size at EC I are distributed non-randomly, the larger mean genome size being characteristic of the 'African' SFS and the smaller mean genome size of the 'European' NFS. Therefore, our results do not support the neutral mutation pressure theory(ies), including the

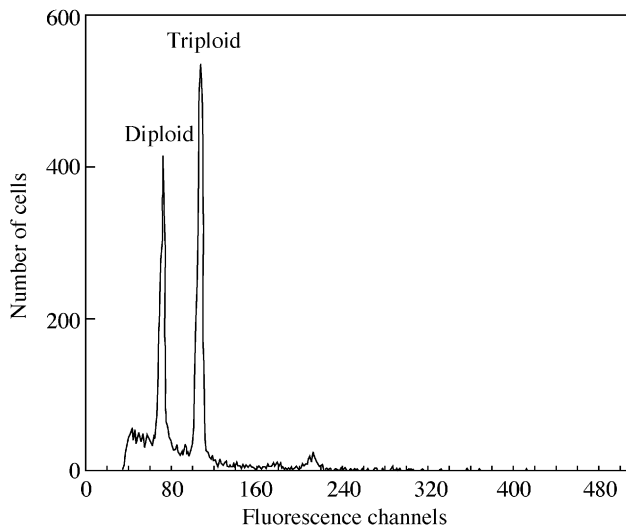


FIG. 1. Comparison of absorptions of diploid and triploid *Ceratonia siliqua* trees.

neutral theory of molecular evolution (Kimura, 1983). By contrast, they do indicate that some environmental factors might play an important role in generating and, perhaps, maintaining interslope divergence of genome size at EC I. It might also be possible to reject the nucleoskeletal theory, which suggests that nuclear size is selected to meet the needs of the cell for balanced growth (e.g. Cavalier-Smith, 1985, 1991), since leaf cells on SFS trees contain more DNA but are probably smaller than those on NFS trees.

Selection for higher genome size in carob?

Unfortunately, we do not know whether similarities between *H. spontaneum* and *C. siliqua* go beyond the similar interslope patterns in genome size at EC I. The most intriguing problem is that we did not find support for the interslope differences in seedling genome size. Conversely, our results might point to selection of seedling genome size in *C. siliqua* as suggested (but not proved) by the significant negative correlations between genome size and both tree circumference and leaf length. However, we cannot exclude the possibility that interslope differences may be caused by ontogenetic changes (e.g. by a differential transposition of somatically active transposable elements, which is known to occur in invertebrates and vertebrates: Emmons and Yesner, 1984; Woodruff *et al.*, 1999) or by associations of genome size changes with other ontogenetic processes. Also, it should be remembered that processes not sufficiently understood have led to environmentally induced changes of genome size during cell culturing (e.g. Blundy *et al.*, 1987; Quemada *et al.*, 1987; Zheng *et al.*, 1987) and that heritable changes within a single generation, involving changes both at the phenotypic level and in DNA, have been observed in annual flax (Cullis, 1990). However, there is no indication that parallel processes should necessarily occur in perennial trees.

Correlates of genome size

Many authors have studied, mainly at the cellular and physiological levels, the effect of DNA quantity on morphological features. Similar results to our findings of significant negative correlations between genome size and leaf size or tree circumference in *C. siliqua* have been found, for example, in leaf length of the grass *Dasypyrum villosum* (Caceres *et al.*, 1998). However, among legumes, Chung *et al.* (1998) detected a positive correlation between leaf size and nuclear DNA content in soybeans while Minelli *et al.* (1996) found a negative correlation between height of the main stem and nuclear DNA content in *Vicia faba*, a similar pattern to that detected between leaf size and tree circumference in *C. siliqua*. We can probably exclude from consideration the intra-plant differences in DNA contents, as described between seeds of *Helianthus annuus* (Cavallini *et al.*, 1989), as we did not find any significant intra-tree correlations between DNA content and the age of trunks, seed weight or seed position in the pod.

C. siliqua as a model to understand interslope biodiversity differences at EC I

The observed pattern of interslope differences in genome size probably fits the more general pattern of higher biodiversity on the 'African' SFS than on the 'European' NFS (Nevo, 1995, 1997, 2001). At the drier, more climatically stressful SFS we documented higher genetic variability (summarized in Nevo, 2001), a three-fold higher mutation rate in the fungus *Sordaria fimicola* (Saleem *et al.*, 2001), and a four-fold higher male recombination rate in *Drosophila melanogaster* (Derzhavets *et al.*, 1997). The common denominator of all genetic interslope differences, including genome size, could be ecological stress which, according to Nevo (2001), is the most probable driving force generating global-scale adaptive genome and phenome strategies at both microscales and macroscales, reinforcing homeostasis and fitness and suggesting continuity between microevolution and macroevolution.

Polyloidization in *C. siliqua*

Randomly naturally occurring triploids and tetraploids have been detected among Leguminosae trees in the genus *Acacia* (Blakesley *et al.*, 2002). With the carob tree, it may be interesting to investigate large-scale ploidy levels and possible ecological correlates.

Conclusions

Our data from *C. siliqua* at EC I, together with circumstantial evidence, indicate that ecology might be a strong evolutionary driving force in shaping quantities of DNA. In addition, we discovered that *C. siliqua* is not only diploid, but can also be triploid and tetraploid.

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