Intraspecific genome size variation in *Dactylis glomerata* and *Hordeum spontaneum*: ... (něco úderného) :-)

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Abstract

Background and aims. *Dactylis glomerata* and *Hordeum spontaneum* are two species with previously documented genome size variation. 1. In *Dactylis glomerata*, a frequently cited study of Reeves et al. (1998) reported a negative association of genome size with site altitude. Our aim was to reinvestigate this report using the same accessions, and explain the variation using climatic data or infraspecific taxonomic traits. 2. For in *Hordeum spontaneum*, environmentally induced proliferation of transposable elements was reported, but without an apparent impact on genome size estimations. Our aim was to study the variation in this species on a sharp climatic gradient and explain it by fitting appropriate climatic variables.
Methods. Relative nuclear DNA amount was estimated using FCM with DAPI staining. Climatic variables were extracted from the global WORLDCLIM model. In *D. glomerata*, maximum leaf width was recorded as a proxy of the phenotype traits.
Results. *D. glomerata* samples varied by 1.11 fold in genome size and the variation was proven by double-peaks. Genome size and leaf width showed a strong positive correlation together and both also with annual precipitation and the geographical distribution. Genome size in *H. spontaneum* varied 1.02 fold.
Conclusions. The previously estimated relationship of genome size and altitude in *Dactylis glomerata* was not confirmed, thus this hypothesis of the abovementioned study of Reeves et al. should be rejected.
Higher genome size was found in Spanish populations, where the distribution ranges of subsp. *glomerata* and subsp. *izcoi* overlap. In *Hordeum spontaneum*, a detailed assessment of its genome size variation is still beyond the actual limits of flow cytometric measurements.

Introduction

The period of doubts about the existence and extent of intraspecific genome size (GS onwards) variation, mostly caused by imprecise GS estimates (reviewed by Greilhuber 2005, Šmarda et Bureš 2010), is already over. Nowadays, evidence for taxa with actual variation accumulates again (e.g. Šmarda et Bureš 2006, Leong-Škorničková 2007, Achigan-Dako 2008, Balao 2009, Cires 2010, Benor et al. 2011). When proper measurement conditions are met (i. e. internal standardization, cytosolic compounds avoidance, more in the review by Loureiro et al. 2010), flow cytometry (FCM onwards) yields reliable data, in suitable amounts and timespans, hence allowing detailed population screening in a single species. Distinct individuals can be examined in a simultaneous measurement, where, even relatively small differences in GS can be proven, depending on tissue properties.

Proximate causes of GS variation have been largely uncovered (e.g. Bennetzen et al. 2005), as well for the increases and decreases of the GS. However, the ultimate causes of GS variation remain still unknown. That's why observation studies, bringing new hypotheses on the constraints and advantages of specific GSs are still needed. In such studies, environmental conditions are used to as first-choice explanatory variables. In the background, there are general theories as the nucleotypic theory (Bennett, 1971) or an analogy of the Large genome constraint hypothesis (Knight et al. 2005), but independent of phylogenetic relations when studied at intraspecific level.

Because the response of plants to abiotic factors is obviously species-dependent, the most suitable objects of such a study are individuals from a single species. Congeneric species usually differ in genome size as in ecological preferences, and several studies have already pointed to potential causal relations among them (see e.g. Bottini et al. 2000, Albach et Greilhuber 2004). Intraspecific GS variation is believed not to be a very common feature of plant taxa (see above), nevertheless only few studies so far have examined species in deep at the population level (because not species, but populations evolve), with respect to e. g. demographic processes, species history, or the effects of its distribution range (citace). The observed variation might result from gradualistic as well as punctuacionalistic processes: transposable element proliferation (e.g. Ambrozova 2011), single nucleotide deletions and insertions and recombinations (e.g. Kirik et al. 2000) etc.. Which extent of GS variation has still the form of a neutral, slightly deletrious or advantageous mutation and what extent poses real constraints on the plants' life, remains also unclear. An effect of stabilizing selection towards a species-specific optimum was documented in conditions of sharp intreaspecific competition in experimental conditions (Šmarda et al. 2010).

Dactylis glomerata

Orchard grass is a perennial herb, cultivated worldwide as forage crops. Its Eurasian wild relatives form a taxonomic complex of diploid, tetraploid and one hexaploid subspecies (reviewed in Jogan, 2002), although taxonomic ranks of some of them might differ according to different authorities. A frequent production of unreduced gametes (Lumaret et Barrientos, 1990) causes gene flow among sympatric subspecies and also the formation of autopolyploids. In small areas, autotetraploids grow in sympatry with their parental diploids and are not reproductively isolated from them (e.g. Bretagnolle and Thompson, 2001, Gauthier et al. 1997). Apart from these local diploid-tetraploid complexes, there are two widely distributed tetraploid subspecies in continental western Europe, subsp. *glomerata* and subsp. *hispanica*, very variable, and probably due to convergent evolution, they form rather a morphological continuum or cline along the climatic gradient from temperate to mediterranean (Borrill, 1961). These tetraploids are supposed to originate via hybridization of two diploids, but only one parental species is known in both events (Stebbins et Zohary 1959, Mizianty 1991). Other hypotheses discuss their autopolyploid origin are reviewed by Mizianty (1990). Interestingly, local adaptation in ecological preference, was recorded in transplanting experiments in *D. glomerata* (Gauthier, Lumaret et Bedecarratas 1996), even regardless the taxonomic rank (Joshi et al. 2001).

GS investigations in Dactylis glomerata

First single estimates of GS from the early era of GS investigations are reviewed by Vilhar et al. (2002). Population-based studies within one ploidy level have been carried out by Creber et al. (1994) and Reeves et al. (1998) using densitometry with Feulgen staining. The study assessed 19 accessions from three altitudinal gradients in SW Europe and the reported 1.33-fold intraspecific variation was one of the highest values published in that period. Moreover, a significant negative correlation of GS and altitude was found, hence, there emerged a question about the puissant ecological constraint, that shapes this relation. However, the cold hydrolysis protocol published in the study of Creber et al. (1994) and utilized as well in the latter (Reeves et al. 1998), was later criticized (Greilhuber et Baranyi, 1999). Nevertheless, Reeves's study has been cited as a proper example of GS variation until recently (e.g. Chen et al. 2010, Xie et al. 2010, Benor et al. 2011). A comparative experiment was done in Slovenia, on an altitudinal transect along the Krvavec mountain. Only a 1.021-fold variation was found using Feulgen-stained image densitometry and no relation of GS to altitude was observed. (Vilhar et al. 2001). Do these discrepancies result from

genetic (intrinsic) differencies in the populations studied and the variation in the Slovenian populations is not present despite a sharp ecological gradient? Or was the high variation observed by Reeves et al. (1998) an artifact of unnacurate methodology?

Hordeum spontaneum

Wild barley has been extensively studied in order to explore genetic variation of breeding resources of this closest wild relative of a major crops – Hordeum *vulgare*. *H. spontaneum* and H. *vulgare* are diploid (2n=2x=14). According to the narrowness of a taxonomic concept applied both are distinguished as subspecies of H. *vulgare* (subsp. *vulgare* and subsp. *spontaneum*) or species. Genetic variation with respect to environmental conditions in *H. spontaneum* has been assessed repeatedly and the results differ according to the markers used and the sampling scale. Forster (1997) found specific AFLP markers for salt tolerance and SSR markers for drought tolerance. Ivandic et al. (2002) found microsatellite markers with large-scale geographical interpretation, but no specific ecological relations. Huang (2002) conducted micro-scale screening of SSR on the area of one mountain (Mt. Tabor) and found a non-random distribution of genotypes reflecting heat and edaphic stress. More recent papers agree on markers for salt and drought tolerance, used for breeding purposes – RAPD, AFLP, SSR, rDNA, SNP and QTL loci (reviewed in Nevo et Chen, 2010). We can thus assume that ecological preferences of particular populations *H. spontaneum* are genetically fixed.

GS investigations in Hordeum spontaneum

One of the first studies of GS variation in populations of *H. spontaneum* was performed using FCM by Kankanpaa et al. (1996). Nine ecologically distinct populations, each represented by a single accession, showed a 1.13-fold variation but failed to reflect any significant environmental pattern. However, these GS estimates (i) lacked verifications using double peaks and (ii) lacked information about the quality of measurements, expressed by CVs. The sample peak, as it can be seen in the Figure 1 in the concerned paper, is clearly bimodal and asymmetric, which could indicate that conditions for proper measurements of small variation in GS were not met. Finally (iii) the possible variation inside the populations was not considered.
The data of Kankanpaa et al. (1996) was later compared to the results of quantification of transposable elements BARE-1, but did not show any significant relationship to the copy number. Even the relations of GS and the environment were denoted just as trends (Vicient et al. 1999). The connection of BARE1 copy number to the site conditions was later confirmed in a micro-scale experiment in the Evolution Canyon, nonetheless without a relation to the almost invariable GSs of the samples (Kalendar et al. 2000). Minor importance is assigned to little GS variation in the works of Eilam et al. (2007) and Jakob et al. (2004); both of these studies are based on FCM on a limited number of accessions (12 and 4, respectively). An other geographically mid-scale FCM study analyzed 97 accessions from 10 Israeli populations and found a 1,05-fold variation in GS. This variation was significantly associated to January temperature (Turpeinen, Kumala et Nevo, 1999). However, the observed variation was not proven by double-peaks.
The putative mechanism behind the shifts in GS in the genus *Hordeum* is BARE-1 proliferation (Vicient et al. 1999) and their subsequent environmentally induced removal by LTR-LTR recombination (Schulman et Kalendar, 2005), which manifests itself as an excess of solo-LTRs. BARE-1 removal and consequentially solo-LTR abundance is associated with micro-scale gradients of environmental conditions (Kalendar et al. 2000). It is therefore rather paradoxical that the effect of GS changes originating from an environmentally induced mechanism seems not to be observable on the whole-genome level using a standard methodology of GS estimation (Kalendar et al. 2000).

Aims of this study

Our aim was to study intraspecific GS variation using two models with well doccumented (see citations below) GS variation: orchard grass (*Dactylis glomerata*) and wild barley (*Hordeum spontaneum*) on a relative broad ecological scale in wild conditions without strong intraspecific competition.
We attempted to re-investigate the same accessions[1] of *Dactylis glomerata*, as had used Reeves et al. (1998) in order to (I) confirm or reject the GS variation in this species and its relation to altitude. Further, we (II) searched for more precise explanatory variables, since "altitude" is not only an ecological factor per se, but rather a complex of more, correlated and uncorrelated variables - temperature, moisture, exposition, UV irradiation etc. (Korner, 2007). A key question for *D. glomerata* was also, (III) whether the observed variation is not attributable to taxonomic heterogeneity. In *Hordeum spontaneum*, we aimed (IV) to complete the link between the known environmentally induced variation in transposable elements and GS estimates by the means of precise FCM measurements. Similarly to *D. glomerata*, (V) to propose more precise environmental explanatory variables to this variation at the widest altitudinal range limited only by ecological preferences of the studied species..

Material and methods

Sampling

Identical 19 accessions of *Dactylis glomerata* as in the experiment of Reeves et al. (1998) were used. Seeds were kindly provided by the Genetic Resources Unit of the Institute of Biological, Environmental and Rural Sciences, Aberystwyth, UK (IBERS onwards).
The seeds originated from three geographically distant mountain transects (French transect: Massif Central and Cottian Alps, Italian transect: Sicily, Spanish transect: Galicia), sample coordinates used in our analyses are based on those recorded by the original collectors. See Table 1 for details.
Seeds of *Hordeum spontaneum* were collected in the wild, at 7 sites along the altitudinal gradient of a steeply elevating mountain range in the Golan Heights, near to Mt. Hermon, Israel (from 80 to 1600 m a. s. l. on a ca. 10 km distance). Site coordinates were recorded in the field using a GPS instrument (Garmin). See Table 2 for details.

Cultivation

Seeds were sown into pots filled with common gardening soil, kept in a greenhouse and watered regularly. Populations were given random running numbers, individuals from the same population within a plot were distinguished by letters. Pots were placed into plots in a latin square arrangement in order to avoid potential influence of nonhomogenities in cultivation conditions. After two months, the pots were transferred from the greenhouse to open-air cultivation.
From each population, on average 9–10 individuals were obtained (see Tables 1 and 2).
While none of the plants flowered, voucher specimen were not taken. Approximate taxonomic identification of the samples was done using leaf lamina width measurements.

FCM

All the samples were analyzed using the DAPI fluorescent dye. Previous studies of *Festuca pallens* (e. g. Šmarda and Bureš, 2006) indicated high resolution of FCM measurements with AT- selective

[1] As they are conserved in the IBERS collection.

DAPI dye for determination of intraspecific GS variability. Measurements were carried out at the Institute of Botany and Zoology, Masaryk University, Brno. Sample suspensions were prepared according to the Otto procedure (Otto, 1990). Small amounts of the lamina of a just developed leaf of the standard and the sample plant were co-chopped in a Petri dish with 2 x 0,5 ml of cool Otto-I buffer. The suspension was filtered trough a CellTricks filter and Otto-II buffer with fluorescent stain was added. Stained sample suspensions were kept in the dark until their measurement.
All samples were measured in a random order within each measurement set. For *Hordeum spontaneum*, three repetitions of sample measurements were performed, each in one day, and their values averaged. All the measurements were performed on the same machine, PA-1 (Ploidy Analyser, Partec), 3 000 cells included in one run. Samples of *Dactylis glomerata* were measured on PA-1, with 5000 cells in one run.
The standard used for *Dactylis glomerata* on DAPI was one individual of *Pisum sativum* 'Ctirad' (9,09 pg, Doležel et al. 1998), in subsequent base composition verification and for *Hordeum spontaneum* it was a single tuft of *Festuca rupicola* (14,18 pg, Šmarda et al. 2007). Relative GS was calculated as the ratio of sample mean to standard mean. If possible the difference in GS within the populations was confirmed by coprocessing two distinct samples and by bimodal histograms produced in analyses.

Measurement accuracy and repeatibility were proven (i) by the correlation of measurement sets and (ii) by the correlations of differences among single measurements and the two peaks in analysis ofcoprocessed samples. To exclude potential seasonal variation in secondary compounds (sensu Walker et al. 2005) , a random chosen subset of 43 samples was measured three months later using the same protocol in the case of *Dactylis glomerata* . To exclude the putative intraspecific variation in base composition these additional analysis was done simultaneously with propidiumiodide and tested for correlation with DAPI. These simultaneous measurements were done on two machines Cy-Flow ML (Partec) equipped by HBO lamp or 100 mW green laser (Cobolt) respectivelly. A standard and sample suspension was prepared in a double volume (2x 0.5 ml), divided into two tubes after the filtration and the halves were stained with a DAPI or propidiumiodide Otto-II buffer, respectively.

Environmental data

Climatic data were downloaded from the global climatic model WORLDCLIM (Hijmans et al. 2005) and processed in GRASS GIS (GRASS Development Team, 1999- 2007)[2] . A list of WORLDCLIM variables and their values is included in Appendix 1.
Site altitudes, slope steepness and aspect were recorded from the ASTER high-resolution digital elevation model (NASA & METI, 2010). These three components plus site latitude were used to estimate the potential annual direct incident radiation and heat load of the site (McCune et Keon, 2002) in a calculation spreadsheet by M. Chytrý (www....). These calculations were done only for localities of *Hordeum spontaneum*, having high-resolution coordinates available.

Leaf lamina width measurements

Fresh middle leaves, directly taken from the experimental garden, were scanned on an office image scanner. High-resolution images (xxx dpi) were obtained and measured using a tool in the GIMP

[2] WORLDCLIM is based on records of temperature and precipitation from meteorological surveys covering the whole Earth. Data are extrapolated to a high spatial resolution of a square kilometer and in the case of Europe, which is relatively densely covered by climatic stations, the model provides a very good approximation of the climatic conditions. Average, maximum, and minimum temperature and precipitation sums for every month are supplemented with 19 derived “bioclimatic” variables. These describe the yearly minima, maxima and variation of temperature and precipitation, thus providing additional biologically meaningful explanatory variables.

image manipulation program (www...). Leaves were chosen at random, one per individual. The width was measured at the widest part of the lamina.

Statistical treatment

All statistical analyses were performed using the R environment (R Development Core Team, 2010).
The normality of distribution was tested with Shapiro-Wilk test. The homogeneity of variances in populations was tested with Fligner-Kileen test. Differences between populations were tested with a one-way ANOVA, differing populations were identified with the Tukey HSD post-hoc test. The influence of environmental variables was tested with linear regression. Due to small numbers of populations in most cases, our datasets did not met the statistical assumptions of quadratic or multiple regression models.
Data sets with non-normal distributions were analyzed analogically with Kruskal-Wallis test, Wilcoxon rank sum test and Spearman correlation. When using multiple pairwise comparisons, Holm correction was applied.
Sets of environmental variables from the WORLDCLIM model were simplified using Principal component analysis (PCA, rda() function in vegan package (citace)), representative axes were selected using the broken stick model (PCAsignificance() function in BiodiversityR package (citace)). Selected axes were interpreted according to the correlated environmental variables and site scores on these axes were taken as environmental predictors.

Spatial autocorrelation of GS among populations was tested using Mantel test. Geographic distances were estimated as simple Euclidean distances of the two localities compared.
The level of statistical significance was set at alpha = 0.05.

Datasets analyzed
According to the normality of distribution, the GS of every population was characterized by four mostly independent variables: (1) its mean *(H. spontaneum)* or median *(D. glomerata).* To test large genome constrains hypothesis, we included (2) the mean of three highest values , (3) mean of three lowest values and (4) the "median position" (calculated as MP = (median – minimum) / (maximum – median), where a value >1 points to a more negatively skewed distribution, values < 1 point to a more positively skewed distribution).
In order to test the relation of GS to detailed environmental characteristics of the *Hordeum spontaneum*, we divided the dataset (100 individuals) into subpopulations according to proximity and slope orientation. Subpopulations including two or less samples were omitted from the analysis. We obtained 13 subopoulations (67 individuals), for which mean genome size was used . For detailed information see Appendix 2.

Results

Flow cytometry

The mean CV of GS measurements for *Hordeum spontaneum* samples and standards with DAPI was 1.35. For *Dactylis glomerata* with DAPI the mean CV was 1.75 and 3.45 with PI. Low CV values and peak symmetry indicate the absence of a harmful influence of cytosolic compounds. Relative GS values in arbitrary units are listed in Tables 3 and 4.

Dactylis glomerata

We found a 1.118-fold variation within tetraploid samples of *Dactylis glomerata* and a maximum 1.084-fold variation inside a population. One population from Spain (Ribadavia, 400 m a. s. l.) exhibited a diploid GS compared to the rest of the samples. One population from France (Chanac, 450 m a. s. l.) suffered from very low seed germination capacity and yielded only three seedlings, one probably diploid and two tetraploid. These two populations were excluded from further analyses. Two populations, that were originally labelled as *D.glomerata* subsp. *hispanica* (see Table 1), did not differ in GS.

The observed differences in GS were verified in simultaneous measurements of two samples, which resulted in bimodal histogram, if the difference in GS exceeded 3,5 %. (see Figure 1). This verification was done for 9 pairs of samples, each pair from the same population. Differences in GS found in individual measurements were correlated to those got in simultaneous measurements (Pearson correlation coefficient $r = 0.861$, $p = 0.0003$ (Figure 2). GS values observed with DAPI staining were highly correlated with estimates done with PI staining ($r = 0.925$, $df = 36$, $p < 0.0001$) and with DAPI estimates done in a different season ($r = 0.916$, $df = 36$, $p < 0.0001$).

The data did not have a normal distribution even after a transformation (Shapiro-Wilk test), but the variance in populations washomogeneous (Fligner-Killeen test). Population medians significantly differred (Kruskal-Wallis test, $\chi^2_{16}=10.578$, $p < 0.0001$), however only one population from Italy was clearly separated from the nearly continuous variation of the rest (pairwise Wilcoxon test). .

The median of the Spanish transect populations was significantly different from the Italian and French transect populations (K-W test: $\chi^2_2= 53.151$, $p < 0.0001$) (see Figure 3). On a regional scale, significant differences have been found also within the populations in France (K-W test: $\chi^2_4= 32.221$, $p < 0.0001$), where the outlying population wass the one from the Alps (Orcières, 2 500 m a. s. l.) (Figure 5). Spatial autocorrelation touched the limit of significance (Mantel test with Spearman ρ, $p=0.049$).

Hordeum spontaneum

In this species a 1.022-fold variation was found. This difference was too small to be testable by the means of double peaks, the simultaneous measurements resulted in a just slightly broader single peak. Neither the verification with PI staining was attempted, because the observed variation is beneath the technical limitation of this method.
The data had a normal distribution, the variance inside the populations was homogeneous (same statistics as above). When the 100 samples were grouped according to the site altitude, 11 population means significantly differ (ANCOVA, $F10 = 3.936$, $p < 0.001$), however, none of them is really distinct. When 67 samples were grouped according to tslope exposition, 13 subpopulation

means did not differ significantly. No spatial autocorrelation among the populations was present (p=0.26).

Relation to environment

Dactylis glomerata

When all *Dactylis glomerata* transects were put together, PCA of environmental variables revealed that the first component is correlated with mean August temperature, the second with annual precipitation and the third with mean temperature of the wettest quarter. Median GS of the populations was strongly correlated with annual precipitation (r=0.499, p=0.0022) and similarly with the related variables on the second axis. A similar result was obtained for the means of three largest and three smallest values, respectively (Figure 4).
Because the analysis of variance indicated an overall difference in genome size among the three transects, that could be attributed also to large-scale spatial differentiation, we performed PCA and correlation analyses for any transect separately, despite the low number of populations. On the French transect, median GS was correlated with temperature variation ($r = 0.917$, $df = 3$, $p = 0.029$). On the Spanish transect, mean GS wasnegatively correlated with latitude (Spearman $\rho = -0.91$, $p = 0.005$) and the median positionwas correlated with precipitation in the driest quarter (Spearman $\rho = 0{,}93$, $p = 0{,}003$).
Analyses that would reflect slope and exposition of the localities were not performed because of the absence of appropriate data (low-resolution coordinates only).

Hordeum spontaneum

First three PCA axes for the localities of *H. spontaneum* were correlated with mean annual temperature, temperature variance and September precipitation respectively. Neither of these environmental parameters was correlated with GS and related measures of population GS heterogeneity. For subpopulations according to slope exposition, the most important environmental variables were mean annual temperature, temperature variance and incident radiation. The median position was negatively correlated with incident radiation (r=-0.741, df=11, p= 0.0038) and further variables. The mean of three largest genomes was correlated positively with mean annual temperature, however, the significance level was surpassed by the correction for multiple correlations.

Dactylis glomerata leaf width

Leaf width ranged from 1.4 mm to 8.5 mm. Mean width (Table 4) significantly differed among the transects (Kruskal-Wallis test, p=0.005), with Spanish populations having the broadest leaves (Figure 4). Mean leaf width of the populations was significantly correlated with median GS (r=0.865, p<0.0001). The correlation was also present in the Spanish transect itself. In the French and Italian populations together the relation was driven by the population with smallest GS (Montevago, 80 m a. s. l.), which has also the narrowest leaves. Similarly as for GS, leaf width was correlated with annual precipitation (r=0.786, p<0.001). (see Figure 3,4).

Discussion

GS variation in Dactylis glomerata

Compared to the study of Slovenian populations (Vilhar et al. 2002), we found higher GS variation in the present study.
In comparison with the study of Reeves et al. (1998), our variation is lower, although the same accessions were used. Both series of GS estimations are correlated ($r=0.471$, $p=0.047$), despite the data in the previous study are seeming partly underestimated (Figure 5). A significant relation of GS to altitude indicated by Reeves et al. (1998) could not be confirmed either. In adition, the significance of this relation was overestimated by Reeves et al. (l. c.) due to an incorrect use of Pearson correlation (the independent variable does not have a normal distribution); a more correct Spearman ρ (or a transformation of the independent variable) would result in significance approximately $p=0.03$.

The discrepancies in estimations might result partly from (i) methodological inaccuracies of previous estimations, (ii) sample identification mismatch and possibly also from (iii) seed multiplication procedures.
(i) Methodological inaccuracies resulted from probably incorrectly estimated cold hydrolysis curves and imprecise temperature control, as Greilhuber and Baranyi (1999) have pointed out. The misuse of Feulgen densitometry lead already to numerous erroneous GS estimates in the past (e.g. Greilhuber 1998, 2005). FCM, used in our study, is a robust technique yielding more reliable results (Greilhuber 2008), when some “quality standards” are met (Doležel et al. 2007, Loureiro et al. 2010). High correlations of our measurement repetitions prove it (see Results).

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(ii) Partly, the incongruence results from lapses in sample identification: both in accession labelling (it is very improbable, that ploidy level heterogeneity would be overlooked) and in correct determination of the subspecies (which is not always a trivial task). Besides, taxonomy itself may provide a hypothesis explaining the variation, since multiple subspecies of *D. glomerata* can be expected on the transects studied (see Jogan 2002 for a distribution review) in a pattern which concurs with genome size distribution. In France the transect crosses the ranges of the nominal subsp. *glomerata*, subsp. *hispanica* Roth, subsp. *slovenica* (Domin) Domin (all tetraploid) and subsp. reichenbachii (Hausm.) Stebbins and Zohary in the east, in which also tetraploids develop: albeit these occupy different micro-habitats, gene flow to subsp. *glomerata* was confirmed (Gauthier et al. 1998, Gauthier et al. 1999). In Spain, populations of subsp. *glomerata* and tetraploids of the Galician subsp. *izcoi* S.Ortiz & Rodr. Oubiña are present and gene flow among them was also suggested (Lindner et Garcia, 1997). In southern Italy, the nominal subspecies occurs only as introduced, subsp. *hispanica* or *D. marina* Borrill are native. Furthermore, all populations of *D. glomerata* from all three regions might be related to cultivated lineages and not necessarily to the local races, although they have been collected on semi-natural sites. Any investigations on GS differentiation among infraspecific taxa of *D. glomerata* have not been carried out yet. Our results do not suggest any among the accessions previously labelled as subsp. *hispanica* and the rest, however, the sample identity might be discussed, since the habitus of the plants does not match the subtle nature of typical subsp. *hispanica*. On the contrary, the lower GS value of the Italian population of narrow-leaved plants, without subspecific determination, points to evident taxonomic heterogeneity. We also hypothesize, that the overall higher GS of the Spanish populations has a taxonomic significance, but this question remains open to further study. This topic will be discussed more together with leaf width measurement results. Unfortunately, a clear determination of our samples' taxonomic status could not be performed, because the majority of the plants did not flower in cultivation.

(iii) The differences in GS estimations could be also seen a result of processes happening during the sample multiplications of the collections. As *D. glomerata* is predominantly alogamous (Lundquist, 1969), the populations in the collection might change slightly their genetic structure (Sackville

Hamilton, 1997). Anyway, simple accession contamination is highly improbable (I. D. Thomas, pers. comm.).

Dactylis glomerata, environmental correlations and leaf width

Leaf width was studied as a proxy of morphological differentiation among our populations, as it was obviously very variable. Quantitative determination characteristics of the subspecies are very variable and overlap, however, there is a trend to decrease in size: from the most robust subsp. *glomerata* to subsp. *slovenica,* subsp. *izcoi*, subsp. *reichenbachiana* till the smallest subsp. *hispanica* (Stebbins et Zohary 1959, Ortiz et Rodriguez-Oubiña 1993, Mizianty 1997). The order in plant height is similar to that for leaf width, only subsp. *izcoi* should have wider leaves than subsp. *glomerata*. As we found plants with the broadest leaves among the Spanish populations, we can corroborate the hypothesis of gene flow from the Galician race present in our samples, and thus possible GS differentiation of this subspecies.
However, the relation of GS and leaf width is present even in the Spanish transect itself, thus it is worth more than an "only taxonomic" explanation. A finding, that a quantitative morphological trait correlates with GS is rather unusual. A similar phenomenon has been observed in *Silene latifolia*, where flower size correlated with GS (Meagher et Costich, 2005). The authors suggest that epigenetic modifications, linked to genome reorganization after GS changement, are the indirect cause of this observation. Chung et al. (1998) published a correlation among *Glycine max* GS and leaf size, however, researchers doubted later this relation by stating soybean lineages as invariable in GS (Greiluber et Obermayer 1997, Greilhuber 1998, 2005).
Indeed, the explanation is linked here to the correlation with precipitation in both characters. Leaf width differentiation among dominant grassland species along the precipitation cline was recently documented e. g. by Oyarzabal et al. (2008), after all the occurrence of plants with narrower leaves in drier habitats is somehow expectable. Mizianty (1986), based on earlier studies, discusses a longitudinal gradient in *Dactylis* plants size and some qualitative morphological characters (e.g. lemma shape), which is equally a proof of the genetic nature of this phenomenon, and not only a result of "ecotypic" differentiation and site conditions influence. Certainly, there remains open the question which of the two characters studied (GS or leaf width) is subject to selection and which is influenced by the other one. GS influences organ size by via the cell size (firstly Bennett 1971, Jovtchev 2006), but the strength of the relationship decreases at higher levels of the phenotype (Knight et Beaulieu, 2008). We attempted to explain the leaf width variation by measuring cell size variation, but our results of guard cell length did not show any trend neither with GS, nor with leaf width (data not shown). Guard cell length is usually used because of being independent of water saturation of the tissue, however, in this case, cell width or a direct estimation of cell volume could be more convenient. Moreover, the 11 % increase in the three-dimensional cell volume would cause only a minor change in the one-dimensional cell length, and may thus interfere with the measurement error.
On the other hand, leaf width of the diploid population does not differ from the tetraploid ones, which suggest a different nature of the response to the environment among diploids and tetraploids.

GS variation in Hordeum spontaneum

GS in populations of *H. spontaneum* varied by 1.02-fold, which is largely in agreement with the previous estimations, if the extent of the studied area is considered: Turpeinen et al. (1999) recorded more variation on a larger area, oppositely Kalendar et al. (2000) found lower variation on a micro-scale.
The correlation of the mean position with incident radiation met the significance limit for multiple comparisons, thus a hypothesis can be drawn, that individuals with larger GSs prefer sites with less irradiation. Irradiation is closely linked to UV-exposition, which is able to induce TE activity

(Ramallo et al. 2009), in this case, the induction contributes probably to higher DNA removal via recombination of TE. Equally, higher irradiation might be stressful in itself and therefore inconvenient for larger genomes.

However, our study did not confirm the findings of Kalendar et al. (2000) in terms of GS, even if it encompassed a broader scale of environmental stress. We might still be facing the limitation of GS estimation accuracy, which does not discriminate enough precisely among such small quantities as single TE copies.

Environmental correlations

In this study, environmental correlations represent just a supplementary information to the GS estimates. If we wanted to study the influence of climatic variables on GS itself, we should indeed opt for a balanced experimental design with respect to environmental variables. The design of the study of Reeves et al. reflects mostly the altitude, thus the other variables do not cover the whole species niche. However, achieving a balanced design in such a study is not a trivial task. The influence of climate on plant GS evolution will be undoubtedly a matter of further studies.

Conclusions

Our study submits observations of two species with previously reported GS. For both of them, the variation was confirmed. In *Dactylis glomerata*, we did not confirm the previously described relation of GS to altitude. Nevertheless, we propose hypotheses, that the variation is partially linked to taxonomic differentiation of the populations on the transects studied. As a whole, the species' GS reflects the annual precipitation, which is corroborated by the same pattern in leaf maximum width. GS investigations in the *Dactylis glomerata* subspecies might thus be a matter of further study, notably the galician subsp. *izcoi*, where a trend to overall higher GS, compared to other possible taxa, is expected.
In *Hordeum spontaneum*, we confirmed only little GS variation, which did not reflect the climatic pattern as it could be expected, based on previous results of environmentally induced TE proliferation and removal. We argue, that this is due to methodological limitations in measurement accuracy.
Both species exhibited different responses to the ecological factors studied. In general, the correlations indicate avoidance of stressful conditions by plants with larger genomes. For *D. glomerata*, stress is linked with drought, for *H. spontaneum*, with irradiation. But only results of transplanting experiments would confirm or reject these hypotheses.

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Figures and Tables:

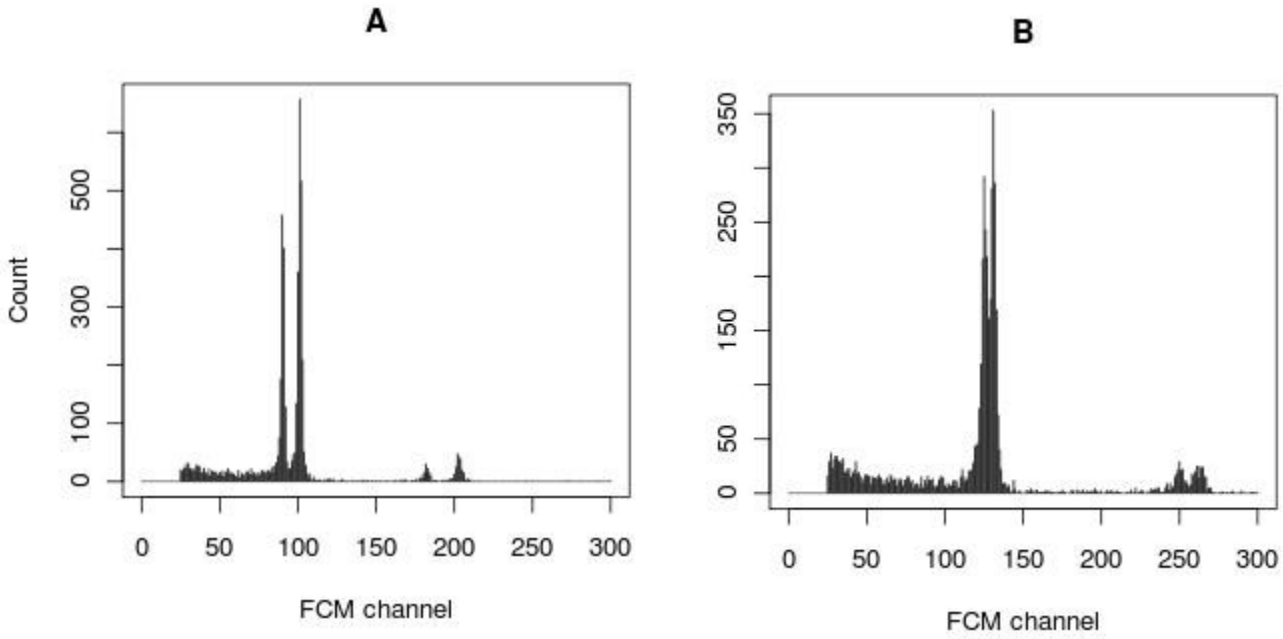

Figure 1: FCM peaks A - maximum variation among the populations, 11 %, B - maximum variation inside a population - 8 %.

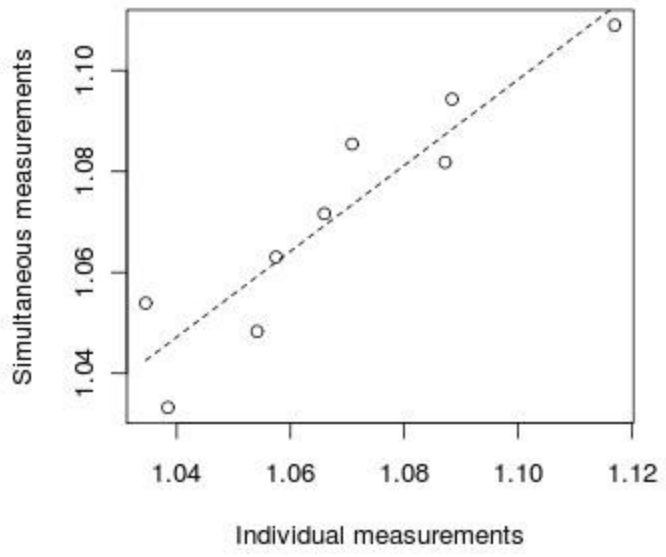

Figure 2: Relative genome size values obtained in individual measurements are highly correlated with those obtained in simultaneous measurements of two samples.

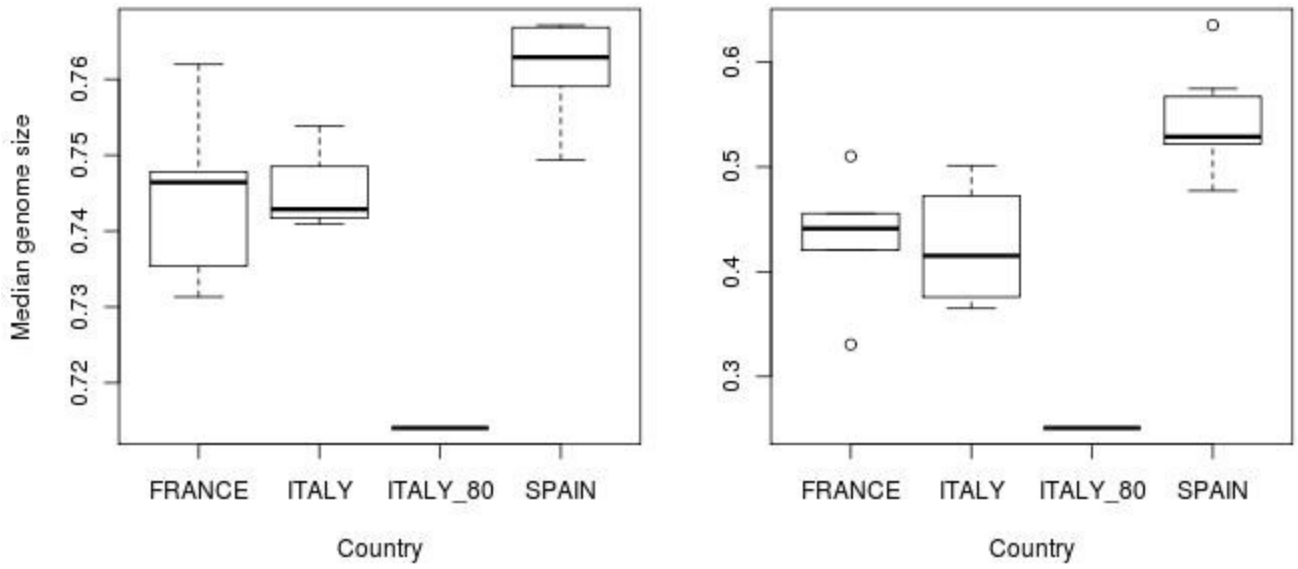

Figure 3: Genome size and leaf width variation in populations of *Dactylis glomerata*, grouped according to the country of origin. On the Italian transect, the population from 80 m a.s.l. with extremely narrow leaves and extremely small genome size plotted separately.

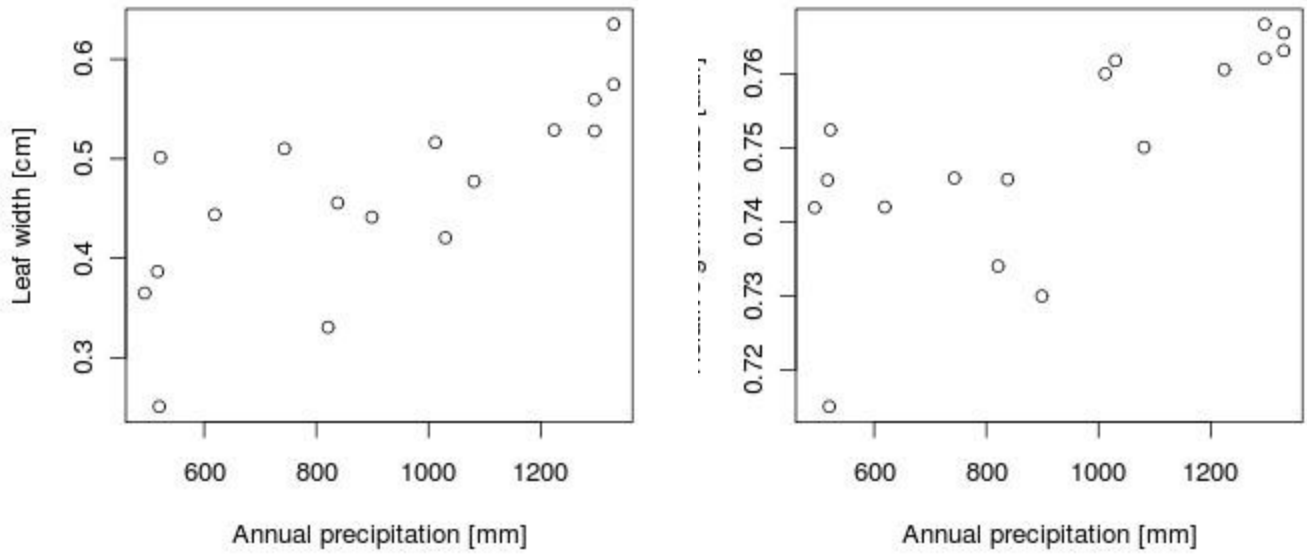

Figure 4: Correlation of annual precipitation with median genome size and mean leaf width in *D. glomerata*.

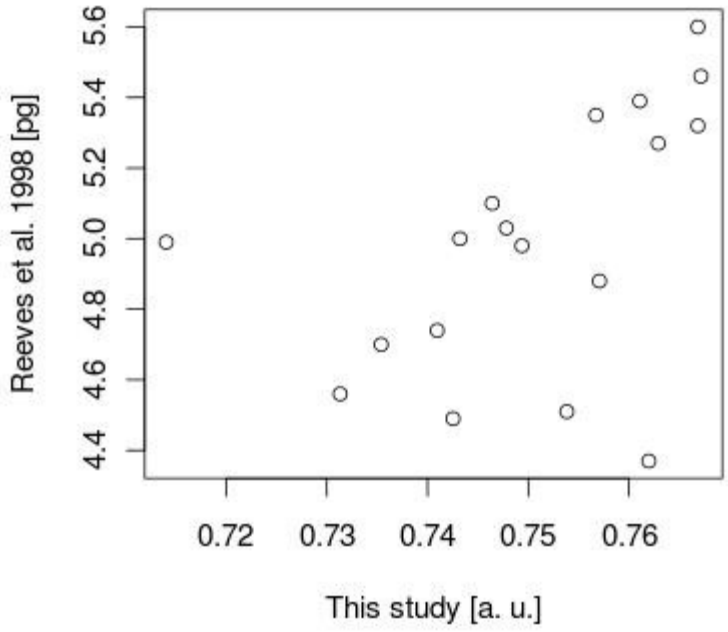

Figure 5: Comparision of both studies of *D. glomerata* accessions.

Table 1: Data from the IBERS collection for *Dactylis glomerata* accessions. *Country: F - France, I - Italy, S - Spain. ** Habitat status: SN - "semi-natural" habitat, W - "wild" habitat.

Country *	Altitude [m a.s.l.]	ID IBERS	Taxon	Habitat status **	Locality	Date of collection	Latitude	Longitude
F	450	BC 5734	*D. glomerata* L.	W	Chanac	unknown	44.467	3.3500
F	800	BC 5442	*D. glomerata* L.	W	Entrayques	30.7.1962	44.5667	2.9833
F	975	BC 5449	*D. glomerata* L.	W	Viarouges	31.7.1962	44.2500	2.8333
F	1040	BC 5545	*D. glomerata* L.	W	Laguole	30.7.1962	44.6833	2.8333
F	1320	BC 5446	*D. glomerata* L.	W	Aubrac	30.7.1962	44.6000	2.9833
F	2500	BC 5471	*D. glomerata* L.	W	Orcières, end of the road	4.8.1962	44.68333	6.3333
I	80	BC 7216	*D. glomerata* L.	SN	Montevago	16.6.1986	37.7167	12.9333
I	520	BC 7226	*D. glomerata* L.	W	Monterosso	20.2.1986	37.100	14.7667
I	700	BC 7227	*D. glomerata* L.	W	Ferla	21.2.1986	37.1333	14.9167
I	1050	BC 7245	*D. glomerata* subsp. *hispanica* Roth (Nyman)	SN	Mistretta	17.7.1986	37.8667	14.3833
I	1430	BC 7246	*D. glomerata* subsp. *hispanica* Roth (Nyman)	SN	Polizzi Generosa	18.7.1986	37.8667	14.0167
S	350	BC 6930	*D. glomerata* L.	SN	Santiso, reservoir	29.7.1977	42.8667	-8.05
S	400	BC 7041	*D. glomerata* f. galicia	SN	Ribadavia	26.7.1977	42.2500	-8.2167
S	550	BC 6924	*D. glomerata* L.	SN	Herrida	28.7.1977	42.5333	-8.1
S	650	BC 7003	*D. glomerata* L.	W	Linares	26.7.1977	42.4667	-8.2667
S	740	BC 6922	*D. glomerata L.*	SN	Herrida	28.7.1977	42.5333	-8.1
S	920	BC 6925	*D. glomerata* L.	SN	Requeiro	28.7.1977	42.4667	-8.2667
S	1000	BC 6949	*D. glomerata* L.	W	Cela, Reserva Nacional de Ancares	3.8.1977	42.7333	-7,0167
S	1120	BC 6974	*D. glomerata* L.	W	Pto. De Outeiro	6.8.1977	42.0167	-7,9833

Table 2: Locality details for *Hordeum spontaneum* collection.

Altitude (ASTER GDEM) [m a.s.l.]	Locality	Latitude	Longitude
87	Emek ha Chula	35.640222	33.216139
295	Banias	35.675222	33.235333
602	Qala'at Namrud	35.718139	33.245472
803	Qala'at Namrud 2	35.725167	33.252500
970	Majd al Shams	35.751889	33.257472
1236	Majd al Shams 2	35.754833	33.268000
1670	Har Chermon	35.769382	33.307541
267	Har Tavor - bottom	35.380028	32.692111
521	Har Tavor - top	35.389778	32.685194
677	Har Meron - bottom	35.436556	32.999361
1117	Har Meron - top	35.414220	32.991972

Table 3: Results summary for Dactyls *glomerata*. * LARGE - mean of three largesr values. ** SMALL - mean of three smallest vaules.

Country	Altitude [m a.s.l.]	No. of individuals	Median relative genome size [a.u.]	Previous estimations of absolute genome size [pg]	LARGE [a.u.] *	SMALL [a.u.] **	Mean leaf width [mm]
F	450	2 1	0.7567 ± 0.005 0.3924	5.35 ± 0.1			
F	800	10	0.7478 ± 0.012	5.03 ± 0.08	0.7589	0.7360	51.01 ± 14.23
F	975	9	0.7464 ± 0.009	5.10 ± 0.10	0.7570	0.7309	45.56 ± 10.57
F	1040	8	0.7343 ± 0.007	4.70 ± 0.01	0.7402	0.7270	33.05 ± 9.310
F	1320	10	0.7313 ± 0.007	4.56 ± 0.20	0.7356	0.7228	44.13 ± 6.770
F	2500	9	0.7620 ± 0.007	4.37 ± 0.07	0.7705	0.7514	42.05 ± 8.740
I	80	10	0.7141 ± 0.003	4.99 ± 0.07	0.7191	0.7151	25.09 ± 7.800
I	520	9	0.7410 ± 0.011	4.74 ± 0.07	0.7505	0.7320	36.50 ± 10.97
I	700	10	0.7432 ± 0.008	5.00 ± 0.07	0.7545	0.7337	38.67 ± 8.270
I	1050	9	0.7538 ± 0.009	4.51 ± 0.06	0.7602	0.7444	50.13 ± 18.76
I	1430	9	0.7425 ± 0.015	4.49 ± 0.05	0.7493	0.7352	44.38 ± 6.510
S	350	11	0.7611 ± 0.002	5.39 ± 0.04	0.7761	0.7463	52.88 ± 10.67
S	400	11	0.3917 ± 0.009	5.46 ± 0.04			50.51 ± 6.790
S	550	10	0.7668 ± 0.008	5.60 ± 0.04	0.7727	0.7567	63.52 ± 13.42
S	650	9	0.7629 ± 0.009	5.27 ± 0.03	0.7743	0.7454	52.78 ± 15.75
S	740	10	0.7668 ± 0.014	5.32 ± 0.02	0.7703	0.7572	57.47 ± 13.65
S	920	9	0.7671 ± 0.004	5.46 ± 0.03	0.7713	0.7621	55.95 ± 10.59

S	1000	9	0.7494 ± 0.010	4.98 ± 0.03	0.7611	0.7411	47.72 ± 7.450
S	1120	9	0.7571 ± 0.015	4.88 ± 0.02	0.7751	0.7425	51.63 ± 6.210

Table 4: Results summary for *Hordeum spontaneum*. * LARGE - mean of three largesr values. ** SMALL - mean of three smallest vaules.

Altitude [m a.s.l.]	No. of individuals	Mean relative genome size [a.u.]	LARGE * [a.u.]	SMALL ** [a.u.]
87	8	0.7436 ± 0.0056	0.749356	0.738502
295	9	0.7484 ± 0.0015	0.750154	0.746848
602	9	0.7476 ± 0.0031	0.750881	0.744243
803	9	0.7439 ± 0.0026	0.746527	0.741246
970	5	0.7455 ± 0.0020	0.746636	0.744370
1236	10	0.7470 ± 0.0023	0.749863	0.744407
1670	10	0.7435 ± 0.0026	0.746452	0.740276
267	9	0.7474 ± 0.0034	0.751020	0.743194
521	12	0.7494 ± 0.0026	0.752126	0.747070
677	9	0.7480 ± 0.0038	0.753108	0.743681
1117	11	0.7464 ± 0.0018	0.748584	0.744857