The DNA methylation level against the background of the genome size and t-heterochromatin content in some species of the genus *Secale L*

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Methylation of cytosine in DNA is one of the most important epigenetic modifications in eukaryotes and plays a crucial role in the regulation of gene activity and the maintenance of genomic integrity. DNA methylation and other epigenetic mechanisms affect the development, differentiation or the response of plants to biotic and abiotic stress. This study compared the level of methylation of cytosines on a global (ELISA) and genomic scale (MSAP) between the species of the genus *Secale*. It was analyzed whether the interspecific variation of cytosine methylation was associated with the size of the genome (C-value) and the content of telomeric heterochromatin. MSAP analysis showed that *S. sylvestre* was the most distinct species among the studied rye taxa, however, the results clearly indicated that these differences were not statistically significant. The total methylation level of the studied loci was very similar in all taxa and ranged from 60% in *S. strictum* ssp*. africanum* to 66% in *S. cereale* ssp*. segetale*, which confirmed the lack of significant differences in the sequence methylation pattern between the pairs of rye taxa. The level of global cytosine methylation in the DNA was not significantly associated with the content of t-heterochromatin and did not overlap with the existing taxonomic rye relationships. The highest content of 5-methylcytosine was found in *S. cereale* ssp*. segetale* (83%), while very low in *S. strictum* ssp. *strictum* (53%), which was significantly different from the methylation state of all taxa, except for *S. sylvestre*. The other studied taxa of rye had a similar level of methylated cytosine ranging from 66.42% (*S. vavilovii*) to 74.41% in (*S. cereale* ssp. *afghanicum*). The results obtained in this study are evidence that the percentage of methylated cytosine cannot be inferred solely based on the genome size or t-heterochromatin. This is a significantly more complex issue.

1   **The DNA methylation level against the background of the genome size and t-**

2   **heterochromatin content in some species of the genus *Secale* L.**

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14   **Abstract**

15   Methylation of cytosine in DNA is one of the most important epigenetic modifications in

16   eukaryotes and plays a crucial role in the regulation of gene activity and the maintenance of

17   genomic integrity. DNA methylation and other epigenetic mechanisms affect the development,

18   differentiation or the response of plants to biotic and abiotic stress. This study compared the level

19   of methylation of cytosines on a global (ELISA) and genomic scale (MSAP) between the species

20   of the genus *Secale*. It was analyzed whether the interspecific variation of cytosine methylation

21   was associated with the size of the genome (C-value) and the content of telomeric

22   heterochromatin. MSAP analysis showed that *S. sylvestre* was the most distinct species among

23   the studied rye taxa, however, the results clearly indicated that these differences were not

24   statistically significant. The total methylation level of the studied loci was very similar in all taxa

25   and ranged from 60% in *S. strictum* ssp. *africanum* to 66% in *S. cereale* ssp. *segetale*, which

26   confirmed the lack of significant differences in the sequence methylation pattern between the

27   pairs of rye taxa. The level of global cytosine methylation in the DNA was not significantly

28   associated with the content of t-heterochromatin and did not overlap with the existing taxonomic

29   rye relationships. The highest content of 5-methylcytosine was found in *S. cereale* ssp. *segetale*

30   (83%), while very low in *S. strictum* ssp. *strictum* (53%), which was significantly different from

31   the methylation state of all taxa, except for *S. sylvestre*. The other studied taxa of rye had a

32   similar level of methylated cytosine ranging from 66.42% (*S. vavilovii*) to 74.41% in (*S. cereale*

33   ssp. *afghanicum*).

34   The results obtained in this study are evidence that the percentage of methylated cytosine cannot

35   be inferred solely based on the genome size or t-heterochromatin. This is a significantly more

36   complex issue.

37 INTRODUCTION

38   Among epigenetic mechanisms, DNA methylation, histone modifications, chromatin or

39 RNAi rearrangements can be distinguished, which are responsible for the regulation of gene

40 expression and the maintenance of genomic integrity. All these mechanisms can affect the

41 phenotype of the organism without altering the DNA sequence (Grant-Dowton & Dickinson,

42 2005; Jablonka & Raz, 2009; *Hirsch, Baumberger & Grossniklaus,* 2013; Alonso et al., 2015). In

43 plants, changes in the level of cytosine methylation may affect the viability, fertility, inbred status

44 or herbicide resistance (Santo et al., 1990; Johannes et al., 2009; Verhoeven et al., 2010; Herrera

45 & Bazaga, 2011; Vergeer, Wagemaker & Ouborg, 2012).

46   Epigenetic mechanisms, particularly through DNA methylation, may also affect

47 macroevolution by the potential impact on diversification rate and speciation. In addition,

48 methylated cytosines are more frequently mutated than the unmethylated ones (Nachman &

49 Crowell, 2000; Ossowski et al., 2010; Alonso et al., 2015). Therefore, methylation can influence

50 the frequency of point mutations and favor the occurrence of evolutionary changes (McClintock,

51 1984; Gorelick, 2003). The analysis of the level of global methylation of cytosines is the first step

52 in evaluating the role of this epigenetic mechanism in the evolution of non-model organisms

53 (Rozhon et al., 2008). Interspecific variation in the cytosine global methylation is not well

54 understood, although some studies showed significant differences between species (Finnegan et

55 al., 1998; Bender, 2004; Alonso et al., 2015). There is no information available whether

56 interspecies differences in the level of cytosine global methylation are associated with changes

57 resulting from their evolution, e.g., the size of the genome. The size of plant genome is a species-

58 specific trait, and its enlargement in the course of evolution resulted primarily from an increase in

59 the number of copies of repetitive sequences (Bennett & Leitch, 2012; Leitch & Leitch, 2013;

60 Alonso et al,. 2015) thus, theoretically, one could expect an increase in the level of methylation.

61 Repetitive sequences are mainly present in the heterochromatin regions. It is within

62   heterochromatin areas, such as telomeric, centromeric and pericentromeric regions (Li et al.,

63   2008; Zilberman et al., 2007) where high levels of DNA methylation are observed, while they are

64   lower in the euchromatin. Transposable elements (TEs), the amplification of which may lead to

65   an increase in the size of the genome are such repeated sequences. Usually they are highly

66   methylated, thus they can not be active, which protects genome integrity (Fujimoto et al., 2008;

67   Sasaki et al., 2011; Kawanabe et al., 2012). Under stress conditions, silencing of TEs can be

68   abrogated, they can transpose, which especially in the case of retroelements can cause a

69   significant increase in the number of copies in the genome. After stress cessation, they are

70   silenced again. If a TE is inserted in the coding region, it can inhibit the expression of a given

71   gene (Kakutani, 2002; Tsukahara, 2009) or neighboring genes (Fernandez et al., 2010; Fujimoto

72   et al., 2006; Hollister & Gaut, 2009; Lippman et al., 2004; Naito et al., 2009), because the

73   heterochromatin state can spread to adjacent regions. Through this mechanism, genome expands

74   and the level of global methylation changes (Bird, 1995; Federoff, 2000; Federoff, 2012; Grover

75   & Wendel, 2010). Since the distribution of transposable elements varies even among closely

76   related species, it can be assumed that TE polymorphisms affect the epigenetic variability (Saze

77   & Kakutani, 2007; Kawanabe et al., 2012).

78   There is an assumption that in rye evolution, from the time when *Secale* diverged from the

79   common ancestor with wheat, there was an increase in the DNA content, which was associated

80   with the addition of heterochromatin. It resulted in the formation of multiple copies of the same

81   DNA fragments (Bennett & Smith, 1976). Comparisons of repetitive DNA sequences suggested

82   that rye, barley, wheat and oat had a common ancestor, and therefore, at the beginning of the 70s

83   of the twentieth century scientists began to determine the phylogenetic relationship and

84   interspecies similarity in the genus *Secale* (Flavell, Rimpau & Smith,1977*)*. Despite years of

85   research on the genus *Secale*, they have not been fully determined. The reason for this may be

86   that it is believed that in rye many evolutionary pathways exist (Tarkowski, 1983). Hence, there

87   is a need to search for different methods to verify the proposed systems of classification and

88   phylogenetic relationships. It is now believed that studying the pattern of DNA methylation is

89   very important in explaining genome (Kalisz & Purugganan, 2004). As mentioned above, the

90   evolution of the genus *Secale* proceeded with increasing amounts of heterochromatin, mainly

91   telomeric heterochromatin. This is clearly correlated with the size of their genomes. *Secale*

92   *sylvestre* has the lowest amount of t-heterochromatin (*Sing* & Röbbelen, 1975), and has the

93   smallest genome (7.23 pg) (Bennett & Smith, 1976*)* among the species of rye. *Secale cereale* is

94   considered evolutionarily the youngest species, having the most t-heterochromatin (*Sing* &

95   Röbbelen, 1975) and the largest genome (8.28 pg) (Bennett & Smith, 1976*)* among *Secale*. It is

96   then interesting whether the results of DNA methylation will confirm this relationship. Or

97   perhaps they will demonstrate the complexity of the process of DNA methylation and indicate

98   that the evolution of rye is not solely based on heterochromatin formation, but is associated with

99   a different methylation, which results in significant phenotypic differences between species.

100   The MSAP (Methylation-Sensitive Amplification Polymorphism) method was used to

101   compare the methylation status of the DNA fragments on a genomic scale between rye taxa. This

102   technique is based on the use of methylation-sensitive enzymes and the selective amplification of

103   restriction fragments obtained. MSAP combines the advantages of AFLP, which allows to

104   visualize multiple markers per sample, and to analyze cytosine methylation at the restriction site

105   (Baurens et al., 2003). MSAP is one of the latest methods, which has already been used in studies

106   of genome differentiation in cotton, banana, barley, Arabidopsis, tobacco and wild emmer wheat

107   (Baurens et al., 2003; Li et al., 2009; Yang et al., 2011). ELISA was another method used to

108   determine the level of global methylation in rye.

109   We analyzed the data concerning global (ELISA) and genomic scale cytosine methylation

110   (MSAP) in eight taxa of the genus *Secale*, together with information on the size of their genome

111   and the amount of t-heterochromatin. The aim of the research was to answer the question whether

112 the cytosine methylation variation was consistent with phylogenetic relationships in the genus

113 *Secale* and whether it was evolutionarily related to the size of the genome and the amount of

114 telomeric heterochromatin.

115 MATERIALS AND METHODS

116 **Plant material. -** The following taxa of *Secale* were included in the study: *Secale cereale* L. cv.

117 Dankowskie Zielonkawe, *Secale cereale* ssp. *afghanicum*, *Secale cereale* ssp. *segetale*, *Secale*

118 *strictum*, *Secale strictum* ssp. *africanum*, *Secale strictum* ssp. *kuprijanovii*, *Secale sylvestre* and

119 *Secale vavilovii*.

120 Caryopses of all taxa mentioned above were obtained from Botanical Garden of Polish Academy

121 of Science (Warsaw, Poland).

122 **DNA isolation. -** Total genomic DNA was isolated from 7-day-old seedlings using the DNeasy

123 Plant Mini Kit (Qiagen, Valencia, USA) according to the manufacturer's instruction. Each DNA

124 extract was prepared from 30 randomly selected seedlings. The quality and quantity of the DNA

125 samples was assessed by 0.8 % agarose electrophoresis in 1×TBE buffer and measurements done

126 using Nanodrop 2000c spectrophotometer (Thermo Scientific, Vilnius, Lithuania).

127 **MSAP. -** 500 ng of genomic DNA was digested with 1 µl of FastDigest® *Eco*RI (Fermentas,

128 Vilnius, Lithuania) in a total volume of 50 µl for 20 min at 37 °C. After *Eco*RI inactivation for 5

129 min at 80 °C each sample was divided into two separate series: one for *Hpa*II, one for. The DNA

130 samples were digested with 1 µl of FastDigest® *Hpa*II (Fermentas) and 1 µl of FastDigest®

131 *Msp*I (Fermentas) in a total volume of 50 µl for 30 min at 37 °C. Enzymes were inactivated by

132 incubation for 15 min at 65 °C. *Eco*RI and *Hpa*II/*Msp*I adapters (Table 1) were prepared by

133 mixing 50 µM oligonucleotides in equal volumes and incubation at the following profile: 10 min

134 at 65 °C, 10 min at 37 °C, 10 min at 25 °C, 10 min at 4 °C. The restricted DNA was added the

135 ligation mixture [2 µM Eco and Hpa/Msp adapter, 1 × ligase buffer, 0.2 mM dATP, 5U T$_4$ DNA

136 ligase (Fermentas)] and incubated for 3 h at 37 °C. The samples were diluted ten times, and 5 µl

137   of the diluted DNA was added to the preamplification mixture. The total volume of 50 µl of

138   preamplification mixture also contained: 10 µM Hpa+A/C primer (Table 1), 10 µM Eco+A

139   primer (Table 1), 1×polymerase buffer, 0.2 mM dNTP, 2.5 mM MgCl$_2$, 2 U DNA Polymerase

140   (DyNAzyme^{TM}, Finnzymes, Espoo, Finland). The PCR conditions were as follows: initial

141   denaturation of 1 min at 94 °C; 25 cycles – 30 s at 94 °C, 60 s at 60 °C, 60 s at 72 °C; final

142   elongation of 5 min at 72 °C. The PCR products were diluted 20 times and 5 µl of the diluted

143   sample was added to 15 µl of selective amplification mixture. The final concentrations of the

144   components in the mixture were: 4 µM Hpa selective primer (Table 1), 4 µM Eco selective

145   primer (Table 1), 1 x polymerase buffer, 0.2 mM dNTP, 2.5 mM MgCl$_2$, 0.5 U DNA Polymerase

146   (DyNAzyme^{TM}, Finnzymes). The PCR conditions were as follows: initial denaturation of 1 min at

147   94 °C; 12 cycles – 30 s at 94 °C, 30 s at 60 °C, 60 s at 72 °C; 22 cycles – 30 s at 94 °C, 30 s at 56

148   °C, 60 s at 72 °C final elongation of 30 min at 72 °C. In total 35 different combinations of

149   selective primers were tested, out of which 9 combinations were chosen for the final analysis

150   (Table 1).

151   PCR products were separated on a 6.5 % denaturing polyacrylamide gel. 100 ml of the gel was

152   prepared from RapidGel^{TM}-XL-6 % Liquid Acrylamide (Affymetrix, Cleveland, USA) containing

153   6 % modified acrylamides, 7 M Urea, 89 mM Tris, 89 mM boric acid and 2 mM EDTA, and to

154   this solution 1000 µl of 10 % APS and 100 µl of TEMED was added. Electrophoresis in 1×TBE

155   buffer was carried out on the Sequi-Gen GT Sequencing Cell (Bio-Rad, Hercules, USA) with the

156   Power Pac^{TM} (Bio-Rad) as a power supply unit. The gel was pre-electrophoresed for 1 h (100 W,

157   2000 V, 50 mA). Loading buffer (98 % formamide, 20 mM EDTA, 0,01 % bromophenol blue,

158   0,01 % xylene cyanol) was added to the samples (12 µl per 20 µl), the mixture was denatured at

159   95 °C for 5 min, placed in ice for 10 min. 7 µl of the mixture from each samples were loaded in

160   the wells. Simultaneously with the samples the pBR322 DNA/*Bsu*RI (*Hae*III) marker

161   (Fermentas) was prepared and loaded onto gel. Electrophoresis was carried out for 2.5 h (100 W,

162   2000 V, 50 mA). After electrophoresis bands were detected by silver staining technique using

163   Silver Sequence™ DNA Sequencing System (Promega, Madison, USA), according to

164   manufacturer's protocol. The documentation and analysis of the gels were done by means of GS-

165   800 Calibrated Densitometer (Bio-Rad) and Quantity One® ver. 4.6.9 (Bio-Rad) software.

166   MSAP procedure was repeated - three biological replicates were performed. Reproducible,

167   clearly distinguishable fragments were scored in a form of binary matrix as either present (1) or

168   absent (0) for the samples. MSAP markers were classified as different types: 11, 01, 10, or 00

169   accordingly to the presence (1) or absence (0) of the corresponding amplified fragment in the

170   digestion reactions with *Hpa*II and *Msp*I, respectively.

171   Assessment of methylation level (%) of Methylation-Susceptible Loci (MSL), Principal

172   Coordinate Analyses (PCoA) and Neighbor-Joining tree were done by means of R program (R

173   version 3.2.2) with msap package.

174   UPGMA (unweighted pair group method using arithmetic averages algorithm was used for

175   cluster analysis in order to show the relationships between taxa. Dice Index was computed in

176   FreeTree (Pavlicek, Hrdá & Flegr, 1999) program and the dendrogram was visualized in

177   TreeView (Page, 1996) program. Bootstrapping with 1000 repetition was done in Free Tree

178   program. The data from double matrix including: 11, 01, 10 and 00 types were transformed into

179   single 1/0 matrix, in which 1 represented any type of methylated fragment (types: 01, 10 or 00)

180   and 0 represented unmethylated fragment (type 11).

181   The analysis of differences between the taxa (*U*-values) was based on the suggestion of Wang et

182   al. (2014):

183     $p = \dfrac{y1 + y2}{n1 + n2}$;

184   q = 1-p;

185 $\quad \delta_{p1\text{-}p2} = \quad \sqrt{(pq(1/n1)+1/n2)}$;

186 $\quad \mathrm{U} = \dfrac{p1 - p2}{\delta_{p1-p2}}$

187 where n1 represents the total number of fragments of a given sample, n2 the total fragments of

188 another sample, y1 the total number of methylated fragments of a given sample values, y2 the

189 total number of methylated fragments in another sample, p1 the proportion (%) of methylated

190 fragments of a given sample values and p2 the proportion (%) of methylated fragments in another

191 sample.

192 **Giemsa staining. -** Roots from 2-day-old seedlings were treated with 0.05% colchicine for 3 h at

193 room temperature in the dark prior to fixation in ethanol-glacial acetic acid (3 : 1). Roots were

194 macerated in a mixture of 4 % (w/v) pectinase (Fluka), 6 % (w/v) hemicellulase (Sigma-Aldrich,

195 St. Louis, USA) and 4 % (w/v) cellulase (Sigma-Aldrich) in 0.01 M citric acid − sodium citrate

196 buffer (pH 4.8), for 3 h at 37 °C . Roots were washed in distilled water. Each root tip were

197 dissected on a single microscope slide, a drop of 45 % acetic acid was added and the root tip was

198 squashed under a cover glass. Preparations were heated for 20 min at 47 °C. The cover slip was

199 removed after freezing over dry ice, and the slides were air-dried overnight. Chromosomes were

200 stained with Giemsa reagent according to Darvey and Gustafson (1975) and Merker

201 (1976). Preparations were analyzed under Eclipse E600 microscope (Nikon, Tokyo, *Japan*). NIS

202 Elements ver. 3.00 SP7 (Nikon) software was used to measure: total chromosomes lengths, arms

203 lengths and telomeric C-bands lengths. Based on the measurements the percentage of total

204 telomeric heterochromatin content in the genome of each *Secale* taxon was computed. The

205 Kruskal-Wallis test was performed in Statistica software ver. 12.

206 **ELISA. -** The 5-mc DNA ELISA Kit (Zymo Research, Irvine, USA) was used to detect global 5-

207 methylocytosine in the DNA. 100 ng of each sample DNA was used in the assay. Four replicates

208   of each sample were prepared in a single experiment, and the experiment was repeated three

209   times. The analysis was performed according to the manufacturer's instruction. Adequate controls

210   included in the were used in the experiments. Plates were read in the Infinite® M200 Pro

211   microplate reader (Tecan, Crailsheim, Germany). Magellan™ ver. 7.0 (Tecan) software was used

212   to generate the standard curve and to quantify the percentage of 5-mC in DNA samples. The

213   Tuckey test was performed in Statistica software ver. 12.

214   Flow cytometry. - 20 mg of coleoptiles from 7-day-old seedlings were chopped in 1 ml of ice-

215   cold nuclei isolation buffer (45 mM $MgCl_2 \cdot 6H_2O$, 20 mM MOPS, 0,1 % (v/v) Triton X-100, 1 %

216   PVP; pH 7.0) supplemented with propidium iodide (Sigma-Aldrich; 50 µg/mL) and ribonuclease

217   A (Sigma-Aldrich; 50 µg/mL). The homogenate was filtered through a 42-mm nylon mesh into a

218   1.5 ml tube and incubated for 10 min at ice. *Secale cereale* was used as an internal standard. The

219   measurements were done at the rate 20 – 50 nuclei. For each sample, fluorescence in at least 7000

220   was measured using BD FACSCalibur™ flow cytometer (Becton Dickinson, San Jose, USA). The

221   mass of the genome was calculated from the formula below:

$$Sample\,2\,C\,value\,(pg) = Reference\,2\,C\,value \times \frac{Sample\,2\,C\,mean\,peak\,position}{Reference\,2\,C\,mean\,peak\,position}$$

222   Reference 2C value for *Secale cereale* = 16.55 pg (Bennett & Smith, 1976).

223   Histograms were analyzed using the CellQuest™ Pro (Becton Dickinson) software. The results

224   were estimated using Tuckey test (Statistica software ver. 12).

225   RESULTS

226   **MSAP. -** Methylation profiling by MSAP relies on the use of methylation-sensitive enzymes. In

227   this work we used a pair of enzymes, *Msp*I and *Hpa*II, which recognize the 5'-CCGG-3'

228   sequence, but they cut the DNA depending on the methylation state of that sequence: *Msp*I will

229   not cut the DNA if the external cytosine is fully methylated or hemimethylated. *Hpa*II will not cut

230 the DNA when the cytosine is fully methylated, and it cuts the DNA when the external cytosine is

231 hemimethylated (Table 2). Therefore, the application of this pair of enzymes in MSAP allows to

232 specify 4 different methylation states of the 5'-CCGG-3' sequence: type 11 – no methylation, type

233 01 – methylated CpG sequence, type 10 – hemimethylated sequence or methylated CpCpG

234 sequence, type 00 – hypermethylation (Table 2). In type 4, it is also possible that no band in a

235 given taxon does not result from changes in the methylation state, the reason may be the lack of

236 this sequence. Hence, the MSAP analysis may introduce some error. However, based on the

237 conducted experiments, including less clear bands omitted in this analysis, it could be said that

238 band patterns of the examined rye taxa were very similar to each other, and even if the analysis

239 was burden with the above-described error, it was small. Before we performed the MSAP

240 analysis, some preliminary tests with the classical AFLP technique had been done (data not

241 published). We calculated the Dice coefficient for each pair of species/subspecies. The values

242 were high (0.89 – 0.95), thus we decided that MSAP analysis would be reliable. The values of the

243 coefficient were not surprising as from ISSR and IRAP analyses, which allow to study the

244 polymorphism of highly variable inter-microsatellite and inter-retrotransposon sequences, the

245 values were not low, 0.48 – 0.85 (IRAP) and 0.39 – 0.82 (ISSR) (Achrem et al., 2014). We were

246 also aware that MSAP can analyze a small quantity of methylated cytosines in the genome that is

247 why we also complemented with global methylation data, which shed more light on the variation

248 of methylation in *Secale*.

249 To ensure the robustness of the results, the analysis included only the most evident and repetitive

250 fragments in the size range 50–500 nt. In total, 149 loci were selected of all the reactions

251 performed with 9 primer combinations. Among these loci, 98 methylation-susceptible loci (MSL)

252 and 51 non-methylated loci (NML) were distinguished. The number of polymorphic MSL was 14

253 (14% of total MSL) and the NML was 7 (14% of total NML). The level of methylation of MSL

254 loci is shown in Fig. 1, which demonstrates that type 01 (internal methylated cytosine) and type

255   00 (hypermethylation) fragments are most frequent among rye taxa, reaching approximately 43%

256   and 38%, respectively. Type 11 fragments were definitely the least frequent (approx. 7%), and

257   were followed by slightly more frequent type 10 – (approx. 12%).

258   Both the MSL analysis alone (Fig. 2) and the combined analysis of MSL and NML loci (Fig. 3)

259   showed that *S. sylvestre* was the most distinct from other species. *Secale vavilovii* also showed

260   greater epigenetic variation in relation to the taxa belonging to the species *S. cereale* and *S.*

261   *strictum*. The total methylation level of the studied loci was very similar in all taxa (Fig. 4). The

262   *U* values were calculated between all taxa to verify whether the differences in the level of

263   methylation are statistically significant. Higher U values indicated greater differences between

264   taxa, but only U values > 1.96 [$U_{(0.05)}$ = 1.96] would mean statistically significant differences. The

265   U values in the studied rye taxa ranged between 0 for *S. sylvestre and S. vavilovii, S. sylvestre* and

266   *S. cereale, S. cereale* and *S. vavilovii* and 1.25 for *Secale s.* ssp. *africanum* and *S. c.* ssp. *segetale*,

267   indicating that the methylation pattern did not differ significantly between any pair of taxa (Table

268   3). Dice coefficients confirmed this high similarity (Table 3), as it ranged between pairs of

269   individual taxa from 87% (*S. sylvestre* and *S. s.* ssp. *kuprijanovii*) to 96% (*S. cereale* and *S. s.* ssp.

270   *kuprijanovii*, *S. cereale* and *S. c.* ssp. *segetale*, *S. c.* ssp. *segetale* and *S. s.* ssp. *kuprijanovii*).

271   Since the rye taxa investigated in this study belonged to 4 species, it was very interesting how

272   epigenetically similar to each other were subspecies within the species. Such analysis was

273   conducted and the graphical representation is shown in Fig. 2. Fig. 2a shows the variation

274   between the taxa without grouping them in species, while Fig. 2b shows the variation between

275   species. Dendrogram (Fig. 2c) shows the relationship between 8 rye taxa grouped into 4 species.

276   The results clearly indicated that epigenetic similarity between species was sufficiently

277   significant so that the subspecies of *S. cereale* and *S. strictum* did not group together. The

278   bootstrapping analysis confirmed this result (Fig. 3). Low values obtained indicated that the

279 presented clustering of taxa was unreliable, and only *S. sylvestre* was definitely different from

280 other taxa, but these differences were not statistically significant.

281 **Giemsa staining. -** Thirty preparations of root tips of each rye taxa were prepared in order to

282 determine the total percentage of telomeric heterochromatin. Five preparations were analyzed in

283 detail, in which 9 best metaphase plates were selected and the following measurements were

284 performed in them: the overall length of each chromosome, short and long arm length, the length

285 t-heterochromatin bands in individual chromosomes. On this basis, the total percentage of

286 telomeric heterochromatin was calculated in the whole genome of each taxon. The results showed

287 that the content of t-heterochromatin was varied (Fig. 4): the lowest amount of t-heterochromatin

288 was found in *S. sylvestre* (6.18%) (Fig. 6B), *S. s.* ssp. *africanum* (6.25%), *S. strictum* (7.18%) and

289 *S. s.* ssp. *kuprijanovii* (7.72%). No significant differences in the content of t-heterochromatin

290 were found between these taxa (Table 4). More t-heterochromatin was present in the genomes of

291 *S. c.* ssp. *segetale* (10.94%), *S. c.* ssp. *afghanicum* (11.90%), *S. cereale* (12.29%) and *S. vavilovii*

292 (13.39%) (Fig. 6A). In this case, also no significant differences were recorded between these

293 taxa. Therefore, these *Secale* taxa could be divided into two groups, with a relatively lower and

294 higher content of t-heterochromatin. In contrast to the MSAP method, the results of which

295 showed that there are no significant differences between all taxa, and the taxa of the species *S.*

296 *cereale* and *S. strictum* did not cluster together, the analysis of the content of t-heterochromatin

297 confirmed the currently accepted taxonomic relationships. Admittedly, species that were the most

298 different from others in the MSAP analysis, here also had the lowest (*S. sylvestre*; 6.18%) and the

299 highest (*S. vavilovii*; 13.39%) t-heterochromatin contents. While the remaining taxa in the group

300 with smaller amounts of heterochromatin-t belonged to the species *S. strictum*, and in the group

301 with a higher t-heterochromatin content they belonged to the species *S. cereale*. Nevertheless,

302 there were no statistically significant differences found in the t-heterochromatin content between

303 *S. s.* ssp. *kuprijanovii* and *S. c.* ssp. *segetale* and *S. c.* ssp. *afghanicum*.

304   **ELISA. -** In total, 12 measurements of the global cytosine content were performed in the genome

305   of each of the rye taxa. Five taxa had a similar level of methylated cytosine (Fig. 4) and no

306   significant differences were observed (Table 4) between *S. c.* ssp. *afghanicum* (74.41%), *S. s.* ssp.

307   *africanum* (69.52%), *S. cereale* (69.80%), *S. s.* ssp. *kuprijanovii* (67.05%) and *S. vavilovii*

308   (66.42%). Slightly lower level of methylation was detected in *S. sylvestre* (61.09%) and it only

309   differed from the methylation state of *S. c.* ssp. *afghanicum* of the above-listed taxa. Very high

310   content of 5-methylcytosine (82.86%) was found in *S. c.* ssp. *segetale*. In this case, it has a

311   significantly higher level of methylation compared to the genomes of all the other taxa, with the

312   exception of *S. c.* ssp. *afghanicum*. On the other hand, extremely low levels of DNA methylation

313   was present in *S. strictum* (52.56%) and differed significantly from the methylation status of all

314   taxa besides *S. sylvestre*.

315   The level of global cytosine methylation in DNA, as opposed to the total t-heterochromatin in the

316   genome, did not overlap with the current taxonomic relationships. Within three taxa belonging to

317   the species *S. cereale*: *S. cereale*, *S. c.* ssp. *afghanicum* and *S. cereale* differed significantly from

318   *S. c.* ssp. *segetale*, while in the taxa belongong to *S. strictum*: subspecies *S. strictum* subsp.

319   *strictum* were different from the other two subspecies *S. s.* ssp. *africanum* and *S. s.* ssp.

320   *kuprijanovii*.

321   It is not surprising that the results of methylation analysis of the CCGG sequence by MSAP did

322   not overlap with global analysis of cytosine methylation by ELISA, as in plant genomes, in

323   addition to the methylation of CG sequences, CNG and CNN sequence methylation also occurs

324   (Johnson et al., 2007; Meyer, 2011; González et al., 2013). Thus, it was possible that the CpG

325   methylation was similar in the genomes of all *Secale* taxa tested, while such high differences in

326   the level of 5-methylcytosine were due to differences in the CNG and CNN methylation. In

327   addition, the MSAP method allowed to examine only selected loci in a limited number, which

328   might not give a complete picture of epigenetic variation.

329   **Flow cytometry. -** The DNA content in the nucleus of the studied *Secale* taxa was the lowest in

330   *S. s.* ssp. *africanum* (14.76 pg) and *S. s.* ssp. *kuprijanovii* (15.23 pg), in which it did not exceed

331   16 pg. All other taxa had the 2C value above 16 pg, with the largest amount of DNA measured in

332   *S. vavilovii* (Fig. 5). It was this rye species that differed significantly from *S. s.* ssp. *africanum* (P

333   < 0.001) and *S. s.* ssp. *kuprijanovii* (P = 0.007). In other cases, there were no significant

334   differences in the DNA content in the nucleus (P > 0.05).

335   DISCUSSION

336   DNA methylation is involved in many important processes in plants, including the

337   regulation of gene expression, gene imprinting or growth and differentiation of plants (Finnegan

338   et al., 1998). This is a dynamic process, differences in which depend on the degree of

339   development or tissue type. The MSAP technique was used in the study to determine the

340   polymorphism methylation level between species of the genus *Secale*. Methylation of the CCGG

341   sequence was evaluated by using the combination of *Msp*I and *Hpa*II restriction enzymes. The

342   level of methylation in the test species was high and ranged from 60 to 66%. Very similar

343   methylation level was determined by MSAP in *Triticum turgidum* ssp. *dicoccoides* (Venetsky et

344   al., 2015). However, using the same method, Shaked et al. (2001) found that the level of cytosine

345   methylation in *T. turgidum* ssp. *durum* reached only 35%. The results showed a higher degree of

346   DNA methylation in *Secale* taxa compared with the species of the genus *Cycas* (Sea-Eung, 2011)

347   or species such as *Brassica oleracea* (Salmon et al., 2008), *Elaeis guineensis* (Jaligot et al., 2004)

348   or *Arabidopsis thaliana* (Cervera, Ruiz-García & Martínez-Zapater, 2002), which may be caused

349   by a higher number of repeated sequences (up to 92%) in the genomes of species of the genus

350   *Secale* (Flavell, Rimpau & Smith,1977).

351   Both the analysis of MSL alone and combined analysis of MSL and NML loci showed

352   that *S. sylvestre* was the most epigenetically distinct species compared to other species studied,

353 which was consistent with previous studies on the phylogeny of rye (Reddy, 1990; Del Pozo,

354 1995; De Bustos & Jouve, 2002; Chikmawati, Skovmand & Gustafson, 2005; Shang, 2006;

355 Achrem, Kalinka & Rogalska, 2014). The dissimilarity of this species in relation to other *Secale*

356 taxa was confirmed by molecular analysis of plastid DNA (Murai, Naiyu & Tsunewaki, 1989;

357 Petersen & Doebly, 1993), repetitive sequences (Cuadrado & Jouve, 2002; Shang et al., 2006;

358 Zhou et al., 2010; Achrem, Kalinka & Rogalska, 2014), 18S − 5.8S − 26S rDNA (De Bustos &

359 Jouve, 2002) or AFLP (Chikmawati, Skovmand & Gustafson,2005). The cited studies confirm the

360 hypothesis of Singh and Röbbelen (1975) and Bennett, Gustafson & Smith (1977), that *S.*

361 *sylvestre* is most likely phylogenetically the oldest among *Secale* species. Cuadrado and Jouve

362 (2002), when examining the distribution of SSR sequences in the chromosomes of different taxa

363 of rye, found that *S. sylvestre* separated earlier from the other species of the genus *Secale*. The

364 lack of 480 bp family sequences in this species, indicated that this sequence could be amplified in

365 the evolution of the genus *Secale* after the divergence of this taxon from a common ancestor. It

366 can be concluded that the amplifications and deletions of repetitive sequences in the genomes of

367 rye formed a basis of the evolutionary pathway of this genus (Cuadrado & Jouve, 2002), which

368 also affected the level of DNA methylation. However, the differences between *S. sylvestre* and

369 other rye taxa, demonstrated by the MSAP technique, were not statistically significant. It seems

370 that genus *Secale* is not an exception. Epigenetic distance calculated on the basis of MSAP

371 among three diploid *Limonium* species (*L. nydeggeri, L. ovalifolium, L. lanceolatum)* was very

372 low. It ranged from 0.002 to 0.029 between pairs of individual species *(Róis et al., 2013).*

373 The higher epigenetic diversity of *S. vavilovii* compared to other rye taxa was surprising,

374 however, as in the case of *S. sylvestre*, it was not statistically significant. The results confirmed

375 that this species was very similar to *S. cereale* (Table 3), and confirmed the assumption that they

376 might share a common ancestor. This thesis was also consistent with the study of Ren et al.

377 (2011) and Bobolik-Brągoszewska et al. (2015), where this species was even classified as a

378 subspecies of *S. cereale*. Osabe et al. (2014) observed different methylation levels at a low

379 genetic variation in the genotypes of cotton, which were grown under the same environmental

380 conditions over many generations. Similar results in other crops suggest the involvement of

381 methylation changes, compensating for the absence of genetic variation (Fang et al., 2008; Fang

382 et al., 2010).

383 The use of the MSAP method alone does not provide a complete methylation picture. This

384 technique is limited by recognizing methylation only at the CCGG sites and not entirely reliable

385 detection of hypermetylated regions. In plants, DNA methylation is not restricted to CpG islands

386 only, as it occurs in three sequence contexts – CG, CHG and CHH (H = A, C or T) (Bender,

387 2004; Salmon et al., 2008; Kim et al., 2015), which are characterized by a specific pattern of

388 inheritance and have their own regulatory pathways. However, numerous literature data suggest

389 that the highest level of methylation is usually obtained in the CG context, intermediate in the

390 CHG context, and the lowest level of methylation is observed in CHH (Feng et al., 2010; Eichten

391 et al., 2016; Osabe et al. 2014; Salmon et al., 2008). On this basis, MSAP results can be

392 considered to be meaningful when comparing epigenomes.

393 Another issue that should be raised here is that MSAP patterns might be in certain circumstances

394 ambiguous (Fulneček & Kovařík, 2014). According to Fulneček et al. (2002) or Cokus et al.

395 (2008) the most frequent status of CCGG methylation is C^{m}CGG, less frequent ^{m}C^{m}CGG while

396 ^{m}CCGG is the rarest. It is consistent with our results. However, as a consequence, in the regions

397 where CCGG motifs are closely located, in of some *Hpa*II-*Eco*RI fragments internal C^{m}CGG

398 might be located. The digestion of *Hpa*II-*Eco*RI by *Msp*I would cause to the misinterpretation of

399 the MSAP pattern (Fulneček & Kovařík, 2014). Thus, as the interpretation of type 11 and type 01

400 patterns is not complicated, the patterns of 10 fragments may be more ambiguous. Considering

401 this, we might expect, that the calculated methylation level based on MSAP, may not perfectly

402 correspond to the actual methylation level of analyzed fragments. On the other hand, it did not

403   affected the assessment of the *Secale* taxa similarity because the MSAP patterns were strikingly

404   similar.

405   The ELISA technique was applied to complement MSAP results and elucidate global methylation

406   in the species of the genus *Secale*. Determination of global methylation level of all cytosines in

407   the DNA has its own drawbacks, as it does not provide information about the genomic position of

408   methylated cytosines. However, knowledge of the global methylation may reflect functional

409   changes in the genome, such as mutations, genomic stability, gene expression changes or

410   chromosomal rearrangements (McClintock, 1984; Steward et al., 2002; Feschotte & Pritam,

411   2007; Rozhon et al., 2008; Bonchev & Parisod, 2013; Alonso et al., 2015). The level of global

412   methylation turned out to be very high and ranged from 53% in *S. strictum* to 83% in *S. c.* ssp.

413   *segetale*. In most species, as expected, it was higher than that obtained using MSAP, with the

414   exception of species, such as *S. strictum* and *S. sylvestre.* It is possible that the CpG methylation

415   was similar in the genomes of all *Secale* taxa tested, while such high differences in the level of 5-

416   methylcytosine were due to differences in the CNG and CNN methylation. In addition, the MSAP

417   method allows to examine only selected loci in a limited number, which may not give a complete

418   picture of epigenetic variation. The obtained variability in DNA methylation patterns among the

419   tested taxa of rye was at a rather low level. However, it showed that in the process of evolution,

420   each taxon accumulated epialleles and faithfully passed them on from generation to generation,

421   which resulted in differences in the obtained percentages and changes in DNA methylation

422   patterns between the taxa.

423   DNA methylation is an epigenetic mechanism that is directly related to the

424   heterochromatin fraction in the genome. Thus the evolutionary increase of genome sizes by

425   heterochromatin addition affects the complexity of the genome and must be associated with

426   increasing levels of global cytosine methylation in DNA (Fedoroff, 2012). This was confirmed by

427   the study of Alonso et al. (2015) concerning the correlation between global methylation and the

428 size of the genomes of 54 angiosperm species. The evolution of the species *Secale* proceeded

429 with the addition of heterochromatin, especially telomeric heterochromatin, and consequently an

430 increase in genome size. Therefore, in this study, the results of DNA methylation were

431 complemented with the data on genome size and the amount of t-heterochromatin of individual

432 taxa. This hypotheses were confirmed by the results of MSAP, and ELISA analyses, obtained in

433 the taxon *S. c.* ssp. *segetale*. It was characterized by the highest methylation percentage, which

434 positively correlated with a high DNA content – 16.07 pg (2C), and large blocks of telomeric

435 heterochromatin, which constituted as much as 11%. This relationship was similar in *S. c.* ssp.

436 *afghanicum* and *S. cereale*. Hence it would seem that with increasing genome size, the level of

437 DNA methylation will also increase. However, the results obtained in this study are evidence that

438 the percentage of methylated cytosine can not be inferred solely based on the genome size or t-

439 heterochromatin. This is a significantly more complex issue. Alonso et al. (2015) demonstrated

440 that methylation level increased slower than genome size. These authors concluded that during

441 evolution when an increase in DNA content occurred, the content of methylated cytosine steadily

442 decreased (Alonso et al., 2015). This may be due to the reduced percentage of repetitive

443 sequences in the genome as compared with its size or due to methylation density of repetitive

444 sequences. This study demonstrated that *S. s.* ssp. *africanum* had the smallest genome (2C –

445 14.76 pg), and it also had the lowest percentage of telomeric heterochromatin (6%). However, in

446 contrast to the expected value, the global methylation was high and reached 70%. This can be

447 explained by the fact that it is an endemic species, which occurs only in South Africa and its

448 evolution could be different compared to other rye taxa or subspecies *S. strictum* (Hammer,

449 1990). However, this example confirms that the increasing genome size in *Secale* does not

450 necessarily means higher level of DNA methylation. Lower than the expected value of the global

451 methylation was detected in *S. vavilovii* (66%) at high DNA (2C – 16.66 pg) and t-

452 heterochromatin contents (13%). This variation in the level of DNA methylation may be due to

453   changes resulting from the adaptive process of plants, wherein the methylation is an important

454   epigenetic mechanism (Sea-Eung, 2011. The process of DNA methylation/demethylation may

455   affect the inhibition or activation of a gene or the entire range of different genes in different

456   environment in order to adapt the species to changing biotic and abiotic conditions. Many

457   literature data showed that changes in the level of genome methylation occurred even after short-

458   term exposure of plants to pathogens, low temperature or drought (Steward et al., 2002; Lukens

459   & Zhan, 2007; Peng & Zhang, 2009; Verhoeven et al., 2010; Grativol*, Hemerly & Ferreira,* 2012;

460   Alonso et al., 2014). Therefore, differences in DNA methylation may be caused by the adaptation

461   of organisms to a particular environment (Finnegan, 2001). This was the conclusion drawn from

462   MSAP and TMD (transposon methylation display) analyses of natural population of emmer

463   wheat (*T. turgidum* ssp. *dicoccoides*), in which the level of DNA methylation was modified by the

464   influence of the environmental factors (Venetsky et al., 2015). This also may be the reason of

465   such a low level of global DNA methylation in *S. strictum* (53%), as compared to other taxa, and

466   even its subspecies, despite the high DNA content (2C - 16.22 pg). The lack of relationships

467   between the t-heterochromatin content and the level of global cytosine methylation in *Secale* may

468   be also explained by the fact, that not only DNA methylation but also other epigenetic

469   mechanisms are involved in maintaining the constitutive heterochromatin state (Soppe et al.,

470   2002). The evidence comes from the studies on barley, in which differences in chromosomal

471   distribution of constitutive heterochromatin and 5-mC pattern were found (Castiglione et al.

472   2008).

473   Additionally, genome size enlargement could occur in various species through the

474   accumulation of other types of sequences, which would be associated with their different

475   methylation levels. A large part of the genome size variation is caused by the differences in the

476   presence and amplification of transposable elements, especially retrotransposons (Bennetzen et

477   al., 1998; Pearce et al., 1996; Vincient et al., 2001; Schulman, Flavell & Ellis, 2004; Grover &

478   Wendel, 2010). Mobile genetic elements are often the main target of DNA methylation in plants

479   (Hirochika, Okamoto & Kakutani, 2000; Kato et al., 2003; Salmon et al., 2008, because they

480   should be silenced to ensure the stability and integrity of the genome. Thus changes in DNA

481   methylation can be related to the presence or absence of transposable elements in the genome. It

482   is associated with a different number of transposable elements, as well as the specificity of certain

483   transposable elements that could be subject to amplification in the genome of a particular species

484   after the evolutionary separation. It should be noted that epigenomic changes may occur under

485   stress, particularly changes in the level of DNA methylation and activation of transposable

486   elements. Especially in the case of retrotransposons it can be connected with a significant

487   increase in genome size (Kim & Zilberman, 2014; Mirouze & Vitte, 2014; Hollister et al., 2011;

488   Eichten et al., 2012; Eichten et al., 2013; Eichten et al., 2016). Although many examples show

489   variation resulting from the absence or presence of TEs, leading to changes in DNA methylation

490   (Gaut & Hollister, 2009; Hollister et al., 2011; Eichten et al., 2012; Eichten et al., 2013; Eichten

491   et al., 2016; Ahmed et al., 2011), there are studies showing a minimal relationship between the

492   variation of transposable elements and methylation (Li et al., 2015). They may result for example

493   from the fact that insertions and deletions of TEs concern heterochromatin regions, leading to

494   minimal changes in overall DNA methylation patterns (Eichten et al., 2016).

495   It is believed that since the genetic activity of TEs in plant is strictly dependent on their

496   methylation, reducing their methylation with expanding genome size may increase the chances of

497   genetic, phenotypic or evolutionary effects of TE action in plants with larger genomes (Banks &

498   Federoff, 1989; Bonchev & Parisod, 2013; Alonso et al., 2015).

499   CONCLUSIONS

500   The genome enlargment and heterochromatinization can influence the global methylation level. However,

501   such dependency did not apply to each studied *Secale* taxon. Gathering of various types of sequences may

502 result in the increase of the DNA quantity in the genome, but as not all of them are highly methylated, the

503 differences in the DNA methylation level are not surprising. Additionally, various events during the

504 evolution of *Secale* taxa could affect their epigenomes. The results obtained in this study are evidence

505 that the percentage of methylated cytosine cannot be inferred solely based on the genome size or

506 t-heterochromatin. This is a significantly more complex issue.

507 ABBREVIATIONS

508 MSAP, Methylation-Sensitive Amplification Polymorphism; ELISA, enzyme-linked

509 immunosorbent assay; MSL, methylation-susceptible loci; NML, non-methylated loci; PCoA,

510 Principal Coordinate Analyses; IRAP, inter-retrotransposon amplified polymorphism; TE,

511 transposable elements; MOPS, 3-(*N*-morpholino)propanesulfonic acid, TBE, Tris-borate-EDTA;

512 APS, ammonium persulfate; TEMED, 1,2-Bis(dimethylamino)ethane.

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517 REFERENCES

518 1. Achrem M, Kalinka A, Rogalska S. 2014. Assessment of genetic relationships among Secale

519 taxa by using ISSR and IRAP markers and the chromosomal distribution of the AAC

520 microsatellite sequence. Turkish Journal of Botany 38:213-225. DOI:10.3906/bot-1207-26.

521   2.   Ahmed I, Sarazin A, Bowler C, Colot V, Quesneville H. 2011. Genome-wide evidence for

522   local DNA methylation spreading from small RNA-targeted sequences in Arabidopsis. Nucleic

523   Acids Research 39:6919–6931. DOI: 10.1093/nar/gkr324

524   3.   Alonso C, Pérez R, Bazaga P, Herrera CM. 2015. Global DNA cytosine methylation as an

525   evolving trait: phylogenetic signal and correlated evolution with genome size in angiosperms.

526   Frontiers in Genetics 6, 4. DOI: 10.3389/fgene.2015.00004.

527   4.   Alonso C, Pérez R, Bazaga P, Medrano M, Herrera CM. 2014. Individual variation in size and

528   fecundity is correlated with differences in global DNA cytosine methylation in the perennial

529   herb Helleborus foetidus (Ranunculaceae). American Journal Botanical 101:1309-1313. DOI:

530   10.3732/ajb.1400126.

531   5.   Banks J, Fedoroff N. 1989. Patterns of developmental and heritable change in methylation of

532   the   Suppressor-mutator   transposable   element.   Developmental Genetics   10:425-437.

533   DOI: 10.1002/dvg.1020100604.

534   6.   Baurens P, Bonnot F, Bienvenu D, Causse S, Legavre T. 2003. Using SD-AFLP and MSAP to

535   assess CCGG methylation in the banana genome. Plant Molecular Biology 21:339-348. DOI:

536   10.1007/BF02772583.

537   7.   Bender J. 2004. DNA methylation and epigenetics. Annual Review of Plant Biology. 55:41-

538   68. DOI: 10.1146/annurev.arplant.55.031903.141641.

539   8.   Bennett M, Leitch I. 2012. Angiosperm DNA C-values database. (release 8.0, Dec. 2012), in:

540   http://www.kew.org/cvalues/.

541   9.   Bennett MD, Smith JB. 1976. Nuclear DNA amounts in angiosperms. Philosophical

542   transactions of the Royal Society B 274:227–274. DOI: 10.1098/rstb.1976.0044.

543   10. Bennett MD, Gustafson JP, Smith JB. 1977. Variation in nuclear DNA in the genus Secale.

544   Chromosoma 61:149–176. DOI:10.1007/BF00327398

545   11. Bennetzen J, Sanmiguel P, Chen M, Tikhonov A, Francki M, Avramova Z. 1998. Grass

546   genomes. Proceedings of the National Academy of Sciences of the United States of America

547   95:1975-1978.

548   12. Bird A. 1995. Gene number, noise reduction and biological complexity. Trends in Genetic

549   11:94-100. DOI: 10.1016/S0168-9525(00)89009-5.

550   13. Bird A, Wolffe AP. 1999. Methylation-induced repression-belts, braces, and chromatin. Cell

551   99:451–454.

552   14. Bolibok-Brągoszewska H, Targońska M, Bolibok L, Kilian A, Rakoczy-Trojanowska M.

553   2015. Genome-wide characterization of genetic diversity and population structure in Secale.

554   BMC Plant Biology 14:184. DOI: 10.1186/1471-2229-14-184.

555   15. Bonchev G, Parisod C. 2013. Transposable elements and microevolutionary changes in

556   natural   populations.   Molecular   Ecology Resources 13:765-775.   DOI: 10.1111/1755-

557   0998.12133.

558   16. Castiglione MR, Venora G, Ravalli C, Stoilov L, Gecheff K, Cremonini R. 2008. DNA

559   methylation and chromosomal rearrangements in reconstructed karyotypes of Hordeum

560   vulgare L. Protoplasma 232: 215. DOI:10.1007/s00709-007-0275-6.

561   17. Cervera MT, Ruiz-García L, Martínez-Zapater JM. 2002. Analysis of DNA methylation in

562   Arabidopsis   thaliana   based   on   methylation-sensitive   AFLP   markers.   Molecular

563   Genetics and Genomics 268: 543–52. DOI: 10.1007/s00438-002-0772-4.

564   18. Chikmawati T, Skovmand B, Gust5afson JP. 2005. Phylogenetic relationships among Secale

565   species revealed by amplified fragment length polymorphisms. Genome 48:792-801. DOI:

566   10.1139/g05-043.

567 19. Cokus SJ, Feng S, Zhang X, Chen Z, Merriman B, Haudenschild CD, Pradhan S, Nelson SF,

568   Pellegrini M, Jacobsen SE. 2008. Shotgun bisulphite sequencing of the Arabidopsis genome

569   reveals DNA methylation patterning. Nature 452(7184): 215–219.

570 20. Cuadrado A, Jouve N. 2002. Evolutionary trends of different repetitive DNA sequences

571   during speciation in the genus Secale. The Journal of Heredity 93(5):339–345.

572   DOI: 10.1093/jhered/93.5.339.

573 21. Darvey NL, Gustafson JP. 1975. Identification of rye chromosomes in wheta-rye addition

574   lines and triticale by heterochromatin bands. Crop Science 15:239-243. DOI:

575   10.2135/cropsci1975.0011183X001500020029x.

576 22. De Bustos A, Jouve N. 2002. Phylogenetic relationships of the genus Secale based on the

577   characterisation of rDNA ITS sequences. Plant Systematics and Evolution 235(1):147-154.

578   DOI: 10.1007/s00606-002-0215-z.

579 23. Del Pozo JC, Figueiras AM, Benito C, De La Pelqa A. 1995. PCR derived molecular markers

580   and phylogenetic relationships in the Secale genus. Biologia Plantarum 37(4):481-489.

581 24. Eichten SR, Ellis NA, Makarevitch I, Yeh CT, Gent JI, Guo L, McGinnis KM, Zhang X,

582   Schnable PS, Vaughn MW, Dawe RK, Springer NM. 2012. Spreading of heterochromatin is

583   limited to specific families of maize retrotransposons. PLoS Genetics 8: e1003127. DOI:

584   10.1371/journal.pgen.1003127

585 25. Eichten SR, Briskine R, Song J, Li Q, Swanson-Wagner R, Hermanson PJ, Waters AJ, Starr

586   E, West PT, Tiffin P, Myers CL, Vaughn MW, Springer NM. 2013. Epigenetic and genetic

587   influences on DNA methylation variation in maize populations. Plant Cell 25:2783-2797.

588   DOI: 10.1105/tpc.113.114793.

589 26. Eichten SR , Stuart T , Srivastava A , Lister R , Borevitz JO. 2016. DNA Methylation profiles

590   of diverse Brachypodium distachyon aligns with underlying genetic diversity. bioRxiv. 2016.

591   DOI: 10.1101/gr.205468.116 biorxiv

592   27. Fang J, Song C, Zheng Y, Qiao Y, Zhang Z, Dong Q, Chao CT. 2008. Variation in cytosine
593   methylation in Clementine mandarin cultivars. The Journal of Horticultural Science and
594   Biotechnology 83:833–839. DOI: 10.1080/14620316.2008.11512469.

595   28. Fang JG, Song CN, Qian JL, Zhang XY, Shangguan LF, Yu HP, Wang XC. 2010. Variation of
596   cytosine methylation in 57 sweet orange cultivars. Acta Physiologiae Plantarum 32:1023–
597   1030. DOI: 10.1007/s11738-010-0491-0.

598   29. Fedoroff N. 2012. Transposable elements, epigenetics, and genome evolution. Science
599   338:758-767. DOI: 10.1126/science.338.6108.758.

600   30. Federoff N. 2000. Transposons and genome evolution in plants. Proceedings of the National
601   Academy of Sciences of the United States of America 97:7002–7007. DOI:
602   10.1073/pnas.97.13.7002

603   31. Feng S, Cokus SJ, Zhang X, Chen PY, Bostick M, Goll MG, Hetzel J, Jain J, Strauss
604   SH, Halpern ME, Ukomadu C, Sadler KC, Pradhan S, Pellegrini M, Jacobsen SE. 2010.
605   Conservation and divergence of methylation patterning in plants and animals. Proceedings of
606   the National Academy of Sciences of the United States of America 107:8689–8694. DOI:
607   10.1073/pnas.1002720107.

608   32. Fernandez L, Torregrosa L, Segura V, Bouquet A, Martinez-Zapater JM. 2010. Transposon-
609   induced gene activation as a mechanism generating cluster shape somatic variation in
610   grapevine. Plant Journal 61:545–557. DOI: 10.1111/j.1365-313X.2009.04090.x.

611   33. Feschotte C, Pritham E. 2007. DNA transposons and the evolution of eukaryotic genomes.
612   The Annual Review of Genetics 41:331-368. DOI: 10.1146/annurev.genet.40.110405.090448.

613   34. Finnegan EJ, Kovac KA. 2000. Plant DNA methyltransferases. Plant Molecular Biology
614   43:189–210. DOI:10.1023/A:1006427226972

615   35. Finnegan EJ, Genger RK, Peacock WJ, Dennis ES. 1998. DNA methylation in plants. Annual

616   Review   of Plant   Physiology and Plant   Molecular   Biology   49:223–247.   DOI:

617   10.1146/annurev.arplant.49.1.223

618   36. Flavell RB, Rimpau J, Smith DB. 1977. Repeated sequences DNA relationships in four cereal

619   genomes. Chromosoma 63:205-222. DOI:10.1007/BF00327450.

620   37. Fujimoto R, Kinoshita Y, Kawabe A, Kinoshita T, Takashima K, Nordborg M, Nasrallah ME,

621   Shmizu KK, Kudoh H, Kakutani T. 2008. Evolution and Control of Imprinted FWA Genes in

622   the Genus Arabidopsis. PLoS Genetetic 4(4):e1000048. DOI: 10.1371/journal.pgen.1000048.

623   38. Fujimoto R, Sugimura T, Fukai E, Nishio T. 2006. Suppression of gene expression of a

624   recessive SP11/SCR allele by an untranscribed SP11/SCR allele in Brassica self-

625   incompatibility. Plant Molecular Biology 61:577-587. DOI: 10.1007/s11103-006-0032-9.

626   39. Fulneček J, Matyasek R, Kovařík A. 2002. Distribution of 5-methylcytosine residues in 5S

627   rRNA genes in Arabidopsis thaliana and Secale cereale. Mol Gen Genomics 268(4):510–517.

628   40. Fulneček J, Kovařík A. 2014. How to interpret methylation sensitive amplified polymorphism

629   (MSAP) profiles? BMC Genetics 15:2.

630   41. González RM, Ricardi MM, Iusem ND. 2013. Epigenetic marks in an adaptive water stress-

631   responsive gene in tomato roots under normal and drought conditions. Epigenetics 8(8): 864–

632   872. DOI:10.4161/epi.25524.

633   42. Gorelick R. 2003. Evolution of dioecy and sex chromosomes via methylation driving Muller's

634   ratchet. The Biological Journal of the Linnean Society 80: 353-368. DOI: 10.1046/j.1095-

635   8312.2003.00244.x.

636   43. Grant-Downton RT, Dickinson HG. 2005. Epigenetics and its implications for plant biology.

637   1. The epigenetic network in plants. Annual Botany 96:1143–1164. DOI: 10.1093/aob/mci273.

638   44. Grativol C, Hemerly AS, Gomes Ferreira PC. 2012. Genetic and epigenetic regulation of

639   stress responses in natural plant populations. Biochimica et Biophysica Acta 1819:176–185.

640   DOI: 10.1016/j.bbagrm.2011.08.010.

641   45. Grover C, Wendel J. 2010. Recent insights into mechanisms of genome size change in plants.

642   Journal of Botany 382732. DOI: 10.1155/2010/382732.

643   46. Hammer K. 1990. Breeding system and phylogenetic relationships in Secale. Biologisches

644   Zentralblatt 109(1):45-50.

645   47. Herrera CM, Bazaga P. 2011. Untangling individual variation in natural populations:

646   ecological, genetic and epigenetic correlates of long-term inequality in herbivory. Molecular

647   Ecology 20:1675–1688. DOI: 10.1111/j.1365-294X.2011.05026.x.

648   48. Hirochika H, Okamoto H, Kakutani T. 2000. Silencing of retrotransposons in Arabidopsis and

649   reactivation by the ddm1 mutation. Plant Cell 12:357–369. DOI: 10.1105/tpc.12.3.357.

650   49. Hirsch S, Baumberger R, Grossniklaus U. 2013. Epigenetic variation, inheritance, and

651   selection in plant populations. Cold Spring Harbor Symposia on Quantitative Biology 77:97–

652   104. DOI: 10.1101/sqb.2013.77.014605.

653   50. Hollister JD, Gaut BS. 2009. Epigenetic silencing of transposable elements: a trade-off

654   between reduced transposition and deleterious effects on neighboring gene expression.

655   Genome Research 19:1419-1428. DOI: 10.1101/gr.091678.109.

656   51. Hollister JD, Smith LM, Guo Y-L, Ott F, Weigel D, Gaut BS. 2011. Transposable elements

657   and small RNAs contribute to gene expression divergence between Arabidopsis thaliana and

658   Arabidopsis lyrata. Proceedings of the National Academy of Sciences of the United States of

659   America 108:2322–2327. DOI: 10.1073/pnas.1018222108.

660   52. Jablonka E, Raz G. 2009. Transgenerational epigenetic inheritance: prevalence, mechanisms,

661   and implications for the study of heredity and evolution. The Quarterly Review of Biology

662   84:131–176. DOI: 10.1086/598822.

663   53. Jaligot E, Beule T, Baurens FC, Billotte N, Rival A. 2004. Search for methylation-sensitive

664   amplification polymorphisms associated with the "mantled" variant phenotype in oil palm

665   (Elaeis guineensis Jacq.). Genome 47:224-228. DOI: 10.1139/g03-085.

666   54. Johannes F, Porcher E, Teixeira FK, Saliba-Colombani V, Simon M, Agier N, Bulski A,

667   Albuisson J, Heredia F, Audigier P, Bouchez D, Dillmann C, Guerche P, Hospital F, Colot V.

668   2009. Assessing the impact of transgenerational epigenetic variation on complex traits. PLoS

669   Genetics 5:e1000530. DOI: 10.1371/journal.pgen.1000530.

670   55. Johnson LM, Bostick M, Zhang X, Kraft E, Henderson I, Callis J, Jacobsen SE. 2007. The

671   SRA methyl-cytosine-binding domain links DNA and histone methylation. Curr Biol. 17(4):

672   379–384. DOI:10.1016/j.cub.2007.01.009.

673   56. Kakutani T. 2002. Epi-alleles in plants: inheritance of epigenetic information over

674   generations. Plant Cell Physiology 43:1106–1111. DOI: 10.1093/pcp/pcf131.

675   57. Kalisz S, Purugganan MD. 2004. Epialleles via DNA methylation: Consequences for plant

676   evolution. Trends in Ecology and Evolution 9(6):309-314. DOI: 10.1016/j.tree.2004.03.

677   034.

678   58. Kankel MW, Ramsey DE, Stokes TL, Flowers SK, Haag JR, Jeddeloh JA, Riddle NC,

679   Verbsky ML, Richards EJ. 2003. Arabidopsis MET1 cytosine methyltransferase mutants.

680   Genetics 163:1109–1122.

681   59. Kato M, Miura A, Bender J, Jacobsen SE, Kakutani T. 2003. Role of CG and non-CG

682   methylation in immobilization of transposons in Arabidopsis. Current Biology 13, 421–426.

683   DOI: 10.1016/S0960-9822(03)00106-4.

684   60. Kawanabe T, Fujimoto R, Sasaki T, Taylor J, Dennis ES. 2012. A comparison of

685   transcriptome and epigenetic status between closely related species in the genus Arabidopsis.

686   Gene 506(2):301-309. DOI: 10.1016/j.gene.2012.07.003.

687   61. Kim MY, Zilberman D. 2014. DNA methylation as a system of plant genomic immunity.

688   Trends Plant Science 19(5):320–326. DOI: 10.1016/j.tplants.2014.01.014.

689   62. Kim DY, Han YJ, Kim S, Song JT, Seo HS. 2015. Arabidopsis CMT3 activity is positively

690   regulated   by   AtSIZ1-mediatedsumoylation. Plant   Science 239:209–215.   DOI:

691   10.1016/j.plantsci.2015.08.003.

692   63. Leitch I, Leitch A. 2013. Genome size diversity and evolution in land plants. In: Greilhuber

693   J, Dolezel J, Wendel JF, ed. Plant genome diversity. Volume 2. Physical structure, behaviour

694   and evolution of plant genomes. New York: Springer, 307-322.

695   64. Li X, Wang X, He K, Ma Y, Su N, He H, Stolc V, Tongprasit W, Jin W, Jiang J, Terzaghi

696   W, Li S, Deng XW. 2008. High-resolution mapping of epigenetic modifications of the rice

697   genome uncovers interplay between DNA methylation, histone methylation, and gene

698   expression. Plant Cell 20(2):259-276. DOI:10.1105/tpc.107.056879.

699   65. Li XL, Lin ZX, Nie YC, Guo XP, Zhang XL. 2009. Methylation-sensitive amplification

700   polymorphism of epigenetic changes in cotton under salt stress. Acta Agronomica Sinica

701   35:588–596. DOI: 10.1007/s13258-015-0301-6.

702   66. Li Q, Gent JI, Zynda G, Song J, Makarevitch I, Hirsch CD, Hirsch CN, Dawe RK, Madzima

703   TF, McGinnis KM, Lisch D, Schmitz RJ, Vaughn MW, Springer NM. 2015. RNA-directed

704   DNA methylation enforces boundaries between heterochromatin and euchromatin in the maize

705   genome. Proceedings of the National Academy of Sciences of the United States of America

706   112:14728–14733. DOI: 10.1073/pnas.1514680112.

707   67. Lippman Z, Gendrel AV, Black M, Vaughn MW, Dedhia N, McCombie WR, Lavine K, Mittal

708   V, May B, Kasschau KD, Carrington JC, Doerge RW, Colot V, Martienssen R. 2004. Role of

709   transposable elements in heterochromatin and epigenetic control. Nature 430:471–476. DOI:

710   10.1038/nature02651.

711 68. Mcclintock B. 1984. The significance of responses of the genome to challenge. Science

712   226:792-801. DOI: 10.1126/science 15739260.

713 69. Merker A. 1976.The cytogenetic effect of heterochromatin in hexaploid triticale. Hereditas

714   83:215 – 222. DOI: 10.1111/j.1601-5223.1976.tb01586.x.

715 70. Meyer T. 2010. DNA methylation systems and targets in plants. FEBS Letters 13:2008–2015.

716   DOI: 10.1016/j.febslet.2010.08.017.

717 71. Meyer P. 2011. DNA methylation systems and targets in plants. FEBS Letters 58(13): 2008-

718   2015.

719 72. Mirouze M, Vitte C. 2014. Transposable elements, a treasure trove to decipher epigenetic

720   variation: insights from Arabidopsis and crop epigenomes. Journal of Experimental Botany

721   65:2801–12. DOI: 10.1093/jxb/eru120.

722 73. Murai K, Naiyu X, Tsunewaki K. 1989. Studies on the origin of crop species by restriction

723   endonuclease analysis of organellar DNA. III. Chloroplast DNA variation and interspecific

724   relationships   in   the   genus   Secale.   Japanese Journal   of Genetics 4:35-47.   DOI:

725   10.1266/jjg.64.35.

726 74. Nachman M, Crowell S. 2000. Estimate of the mutation rate per nucleotide in humans.

727   Genetics 156:297-304.

728 75. Naito K, Zhang F, Tsukiyama T, Saito H, Hancock CN, Richardson AO, Okumoto Y, Tanisaka

729   T, Wessler SR. 2009. Unexpected consequences of a sudden and massive transposon

730   amplification on rice gene expression. Nature 461:1130-34. DOI: 10.1038/nature08479.

731 76. Nei M, Li WH. 1979. Mathematical model for studying genetic variation in terms of

732   restriction endonucleases. Proceedings of the National Academy of Sciences of the United

733   States of America 76(10):5269-5273.

734   77. Osabe K, Clement JD, Bedon F, Pettolino FA, Zielkowski L, Llewellyn DJ, Finnegan EJ,

735   Wilson IW. 2014. Genetic and DNA methylation changes in cotton (Gossypium) genotypes

736   and tissues. PLoS One 9,e86049. DOI: 10.1371/journal.pone.0086049.

737   78. Ossowski S, Schneeberger K, Lucas-Lledo J, Warthmann N, Clark R, Shaw R, Weigel D,

738   Lynch M. 2010. The rate and molecular spectrum of spontaneous mutations in Arabidopsis

739   thaliana. Science 327:92-94. DOI: 10.1126/science.1180677.

740   79. Page RDM. 1996. TreeView: An application to display phylogenetic trees on personal

741   computers. Computer Applications in the Biosciences 12(4):357-358.

742   80. Pavlícek A, Hrdá Š, Flegr J. 1999. FreeTree – freeware program for construction of

743   phylogenetic trees on the basis of distance data and for bootstrap/jackknife analysis of the

744   trees robustness. Application in the RAPD analysis of genus Frenkelia. Folia Biologica (Praha)

745   45:97-99.

746   81. Pearce SR, Pich U, Harrison G, Flavell AJ, Heslop-Harrison JS, Schubert I, Kumar A. 1996.

747   The Ty1-copia group retrotransposons of Allium cepa are distributed throughout the

748   chromosomes but are enriched in the terminal heterochromatin. Chromosome Research 4:357-

749   364. DOI: 10.1007/BF02257271.

750   82. Petersen G, Doebley JF. 1993. Chloroplast DNA variation in the genus Secale (Poaceae).

751   Plant Systematics and Evolution 187(1-4):115-125. DOI: 10.1007/BF00994094.

752   83. Ren TH, Chen F, Zou YT, Jia YH, Zhang HQ, Yan BJ, Ren ZL. 2011. Evolutionary trends of

753   microsatellites during the speciation process and phylogenetic relationships within the genus

754   Secale. Genome 54:316-326. DOI: 10.1139/g10-121.

755   84. Rozhon W, Baubec T, Mayerhofer J, Scheid O, Jonak C. 2008. Rapid quantification of global

756   DNA methylation by isocratic cation exchange high performance liquid chromatography.

757   Analytical Biochemistry 375:354-360. DOI: 10.1016/j.ab.2008.01.001.

758   85. Róis AS, López CMR, Cortinhas A, Erben M, Espírito-Santo D, Wilkinson MJ, Caperta AD.

759   2013. Epigenetic rather than genetic factors may explain phenotypic divergence between

760   coastal populations of diploid and tetraploid Limonium spp. (Plumbaginaceae) in Portugal.

761   BMC Plant Biology. 13:205. DOI:10.1186/1471-2229-13-205.

762   86. Salmon A, Clotault J, Jenczewski E, Chable V, Manzanares-Dauleux MJ. 2008. Brassica

763   oleracea displays a high level of DNA methylation polymorphism. Plant Science 174: 61–70.

764   DOI: 10.1016/j.plantsci.2007.09.012.

765   87. Sano H, Kamada I, Youssefian S, Katsumi M, Wabiko H. 1990. A single treatment of rice

766   seedlings with 5-azacytidine induces heritable dwarfism and undermethylation of genomic

767   DNA. Molecular Genetics and Genomics 220:441-447. DOI: 10.1007/BF00391751.

768   88. Sasaki T, Fujimoto R, Kishitani S, Nishio T. 2011. Analysis of target sequences of DDM1s

769   in Brassica rapa by MSAP. Plant Cell Reports 30:81–88. DOI: 10.1007/s00299-010-0946-1.

770   89. Saze H, Kakutani T. 2007. Heritable epigenetic mutation of a transposon-flanked Arabidopsis

771   gene due to lack of the chromatin-remodeling factor DDM1. The EMBO Journal 26:3641-

772   3652. DOI: 10.1038/sj.emboj.7601788.

773   90. Schulman AH, Flavell AJ, Ellis TH. 2004. The application of LTR retrotransposons as

774   molecular markers in plants. Methods in Molecular Biology 260:145-173. DOI: 10.1385/1-

775   59259-755-6:145.

776   91. Sae-Eung C, Kanchanaketu T, Sangduen N, Hongtrakul V. 2012. DNA methylation and

777   genetic diversity analysis of genus Cycas in Thailand. African Journal of Biotechnology 11(4):

778   743-751. DOI: 10.5897/AJB11.2835.

779   92. Shaked H, Kashkush K, Ozkan H, Feldman M, Levy AA. 2001. Sequence elimination and

780   cytosine methylation are rapid and reproducible responses of the genome to wide

781   hybridization and allopolyploidy in wheat. Plant Cell. 13(8):1749–59.

782   93. Shang HY, Wei YM, Wang XR, Zheng YL. 2006. Genetic diversity and phylogenetic

783   relationships in the rye genus Secale L. (rye) based on Secale cereale microsatellite markers.

784   Genetics and Molecular Biology 29(4): 685-691. DOI: 10.1590/S1415-47572006000400018.

785   94. Singh RJ, Röbbelen G. 1977. Identification by Giemsa technique of the translocations

786   separating cultivated rye from three wild species of Secale. Chromosoma 59:217-229. DOI:

787   10.1007/BF00292779.

788   95. Soppe, WJJ, Jasencakova, Z, Houben, A, Kakutani, T, Meister, A, Huang, MS, Jacobsen, SE,

789   Schubert, I, Fransz, PF. 2002. DNA methylation controls histone H3 lysine 9 methylation and

790   heterochromatin assembly in Arabidopsis. EMBOJ 21:6549-6559.

791   96. Steward N, Ito M, Yamaguchi Y, Koizumi N, Sano H. 2002. Periodic DNA methylation in

792   maize nucleosomes and demethylation by environmental stress. Journal of Biological

793   Chemistry 277:37741–37746. DOI: 10.1074/jbc.M204050200.

794   97. Tarkowski C. 1983. Biology of rye. PWN Warsaw [in polish].

795   98. Tsukahara S, Kobayashi A, Kawabe A, Mathieu O, Miura A, Kakutani T. 2009. Bursts of

796   retrotransposition   reproduced   in Arabidopsis.   Nature   461:423–426.   DOI:

797   10.1038/nature08351.

798   99. Venetsky A, Levy-Zamir A, Khasdan V, Domb K, Kashkush K. 2015. Structure and extent of

799   DNA methylation-based epigenetic variation in wild emmer wheat (T. turgidum ssp.

800   dicoccoides) populations. BMC Plant Biology 15:200. DOI: 10.1186/s12870-015-0544-z

801   100.   Vergeer P, Wagemaker N, Ouborg N. 2012. Evidence for an epigenetic role in inbreeding

802   depression. Biology Letters 8:798-801. DOI: 10.1098/rsbl.2012.0494.

803   101.   Verhoeven KJF, Jansen JJ, van Dijk PJ, Biere A. 2010. Stress-induced DNA methylation

804   changes and their heritability in asexual dandelions. New Phytologist 185:1108-1118. DOI:

805   10.1111/j.1469-8137.2009.03121.x.

806 102. Vicient CM, Jaaskelainen MJ, Kalendar R, Schulman AH. 2001. Active retrotransposons

807   are a common feature of grass genomes. Plant Physiology 125:1283-1292. DOI: 10.1104/pp.

808   125.3.1283.

809 103. Wang J, Chen S, Qi X, Fang W, Guan Z, Teng N, Liao Y, Chen F. 2014. Rapid genetic and

810   epigenetic alterations under intergeneric genomic shock in newly synthesized Chrysanthemum

811   morifolium x Leucanthemum paludosum hybrids (Asteraceae). Genome Biology and

812   Evolution 6:247–259. DOI: 10.1093/gbe/evu008.

813 104. Yang L, Gaut BS. 2011. Factors that contribute to variation in evolutionary rate among

814   Arabidopsis genes. Molecular Biology and Evolution 28:2359-2369. DOI:

815   10.1093/molbev/msr058.

816 105. Zhou JP, Yang ZJ, Li GR, Liu C, Tang ZX, Zhang Y, Ren ZL. 2010. Diversified

817   chromosomal distribution of tandemly repeated sequences revealed evolutionary trends in

818   Secale (Poaceae). Plant Systematics and Evolution 287(1-2):49-56. DOI: 10.1007/s00606-

819   010-0288-z.

820 106. Zilberman D, Gehring M, Tran RK, Ballinger T, Henikoff S. 2007. Genome-wide analysis of

821   Arabidopsis thaliana DNA methylation uncovers an interdependence between methylation and

822   transcription. Nature Genetics 39:61-69. DOI: 10.1038/ng1929.

Table 1. The sequences of the oligonucleotides used in this study and the combinations of selective primers.

Type	Name/combination	Sequences
Adapters	Eco adapter	5'- CTCGTAGACTGCGTACC and 5'- AATTGGTACGCAGTCTAC
	Hpa/Msp adapter	5'- GATCATGAGTCCTGCT and 5'- CGAGCAGGACTCATGA
Preselective primers	Eco+A	5'- GACTGCGTACCAATTCA
	Hpa+A	5'- ATCATGAGTCCTGCTCGGA
	Hpa+C	5'- ATCATGAGTCCTGCTCGGC
Selective primers	Eco+AC	5'- GACTGCGTACCAATTCAC
	Eco+AG	5'- GACTGCGTACCAATTCAG
	Hpa+ATG	5'- ATCATGAGTCCTGCTCGGATG
	Hpa+ATT	5'- ATCATGAGTCCTGCTCGGATT
	Hpa+ACC	5'- ATCATGAGTCCTGCTCGGACC
	Hpa+ACG	5'- ATCATGAGTCCTGCTCGGACG
	Hpa+AAG	5'- ATCATGAGTCCTGCTCGGAAG
	Hpa+CTT	5'- ATCATGAGTCCTGCTCGGCTT
	Hpa+CGC	5'- ATCATGAGTCCTGCTCGGCGC
Selective primers pairs	Eco+AC and Hpa+ATG; Eco+AG and Hpa+ATG; Eco+AC and Hpa+ATT; Eco+AC and Hpa+ACC; Eco+AC and Hpa+ACG; Eco+AC and Hpa+AAG; Eco+AG and Hpa+CTT; Eco+AC and Hpa+CGC; Eco+AC and Hpa+CTT	

Table 2. The methylation status of the 5′-CCGG-3′ sequence base on the presence (1) or absense (0) of DNA restriction fragments (*Hpa*II and *Msp*I). C – unmethylated cytosine, ^{m}C - methylated cytosine.

							*Hpa*II	*Msp*I
C	C	G	G		type 11	unmethylated	1	1
G	G	C	C					
C	ᵐC	G	G		type 01	internal cytosine methylation	0	1
G	G	ᵐC	C					
ᵐC	ᵐC	G	G		type 10	hemimethylated	1	0
G	G	C	C					
or								
ᵐC	C	G	G					
G	G	C	C					
ᵐC	ᵐC	G	G		type 00	full methylation	0	0
G	G	ᵐC	ᵐC					

Table 3. The upper part of the table (grey cells) contains Dice coefficients (reflecting the epigenetic similarity among the rye taxons); the lower part (blue cells) contains the U - values (showing the differences between the rye taxons); both sets of d

	S. c. ssp. *afganicum*	*S. s.* ssp. *africanum*	*S. cereale*	*S. s.* ssp. *kuprijanovii*	*S. c.* ssp. *segetale*	*S. strictum*	*S. sylvestre*	*S. vavilovii*
S. c. ssp. *afganicum*		0,95	0,95	0,96	0,94	0,95	0,90	0,93
S. s. ssp. *africanum*	0.35		0,92	0,92	0,90	0,93	0,88	0,93
S. cereale	0.54	0.89		0,96	0,96	0,94	0,91	0,95
S. s. ssp. *kuprijanovii*	0.36	0.71	0.18		0,96	0,95	0,87	0,92
S. c. ssp. *segetale*	0.90	1.25	0.36	0.54		0,95	0,88	0,91
S. strictum	0.18	0.53	0.36	0.18	0.72		0,88	0,89
S. sylvestre	0.54	0.89	0.00	0.18	0.36	0.36		0,92
S. vavilovii	0.54	0.89	0.00	0.18	0.36	0.36	0.00	

Table 4. The upper part of the table (grey cells) contains P values from Kruskal-Wallis test for the differences in total t-heterochromatin content among the genomes of rye taxons; the lower part (blue cells) contains P values from Tukey test for the dif

	S. c. ssp. afganicum	S. s. ssp. africanum	S. cereale	S. s. ssp. kuprijanovii	S. c. ssp. segetale	S. strictum	S. sylvestre	S. vavilovii
S. c. ssp. afganicum		0,000032*	1,000000	0,331233	1,000000	0,007999*	0,000004*	0,132453
S. s. ssp. africanum	0,934268		0,000000*	0,526546	0,000016*	1,000000	1,000000	0,000000*
S. cereale	0,951740	1,000000		0,002697*	1,000000	0,000015*	0,000000*	1,000000
S. s. ssp. kuprijanovii	0,633481	0,998885	0,997694		0,226022	1,000000	0,175761	0,000003*
S. c. ssp. segetale	0,456476	0,035269*	0,042688*	0,005712*		0,004762*	0,000002*	0,198283
S. strictum	0,000137*	0,002251*	0,001789*	0,015542*	0,000118*		1,000000	0,000000*
S. sylvestre	0,035658*	0,459018	0,414978	0,832923	0,000138*	0,444195		0,000000*
S. vavilovii	0,530631	0,995110	0,991593	1,000000	0,003448*	0,024517*	0,899162	

Figure 1

Methylation level (%) of Methylation-Susceptible Loci (MSL) in *Secale* taxons.

Type 1 – unmethylated, type 2 – internal cytosine methylation, type 3 – hemimethylated, type 4 – full methylation.

Note: Auto Gamma Correction was used for the image. This only affects the reviewing manuscript. See original source image if needed for review.
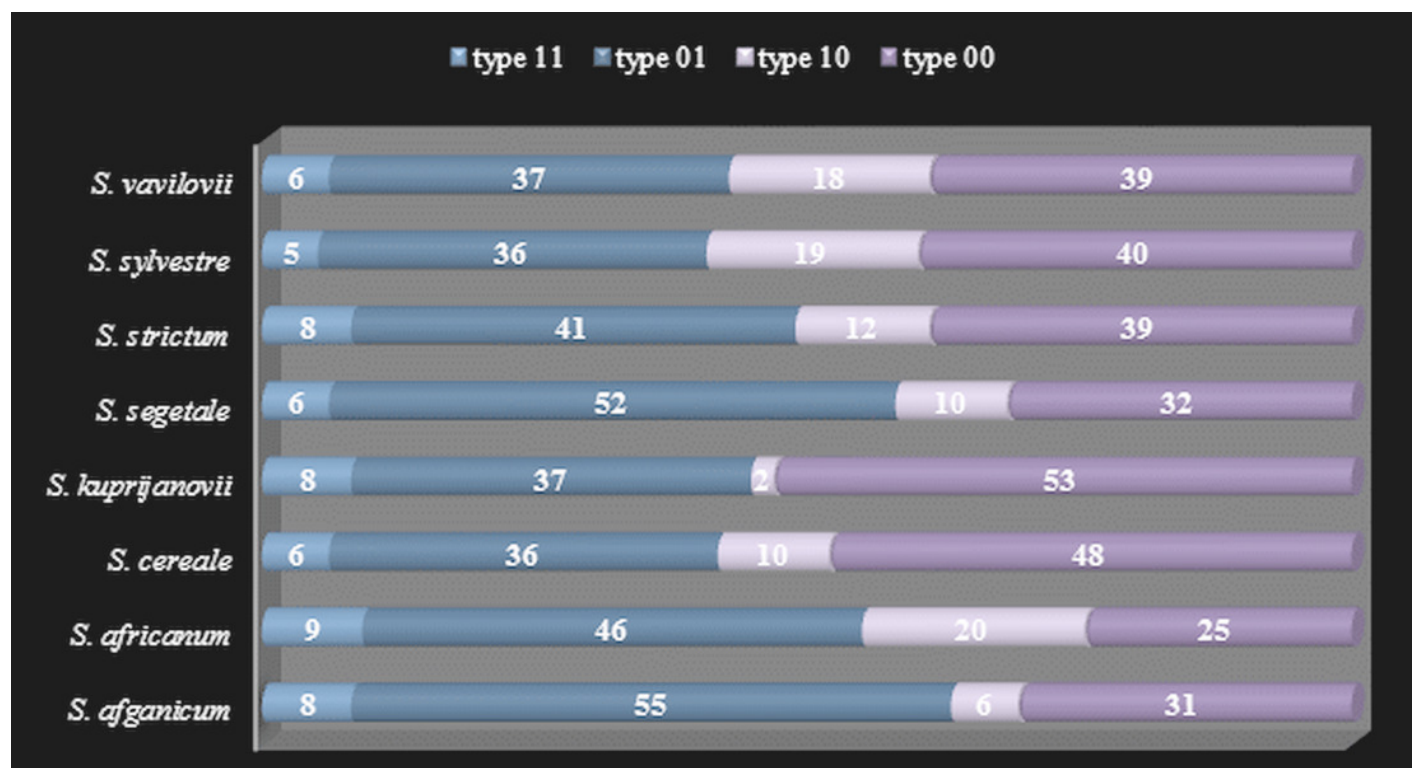
Figure 2

Principal Coordinate Analyses for epigenetic differentiation between the groups and Neighbor-Joining tree for epigenetic distances.

Principal Coordinate Analyses (PCoA) for epigenetic (MSL) differentiation between *Secale* taxons (A) and *Secale* species (B); (C): Neighbor-Joining tree of *Secale* species for epigenetic (MSL) distances; colors represent different taxons/species; (A), (B): C1 and C2 coordinates are shown with the percentage of variance explained by them, in (B) different point types represent subspecies from different species, species labels show the centroid for the points cloud in each species, ellipses represent the average dispersion of those points around their center, the long axis of the ellipse shows the direction of maximum dispersion and the short axis, the direction of minimum dispersion; cereale: *S. cereale*, *S. c.* ssp. *afghanicum, S. c.* ssp. *segetale;* strictum: *S. strictum, S. s.* ssp. *africanum, S. s.* ssp. *kuprijanovii*; sylvestre: *S. sylvestre*; vavilovii: *S. vavilovii*
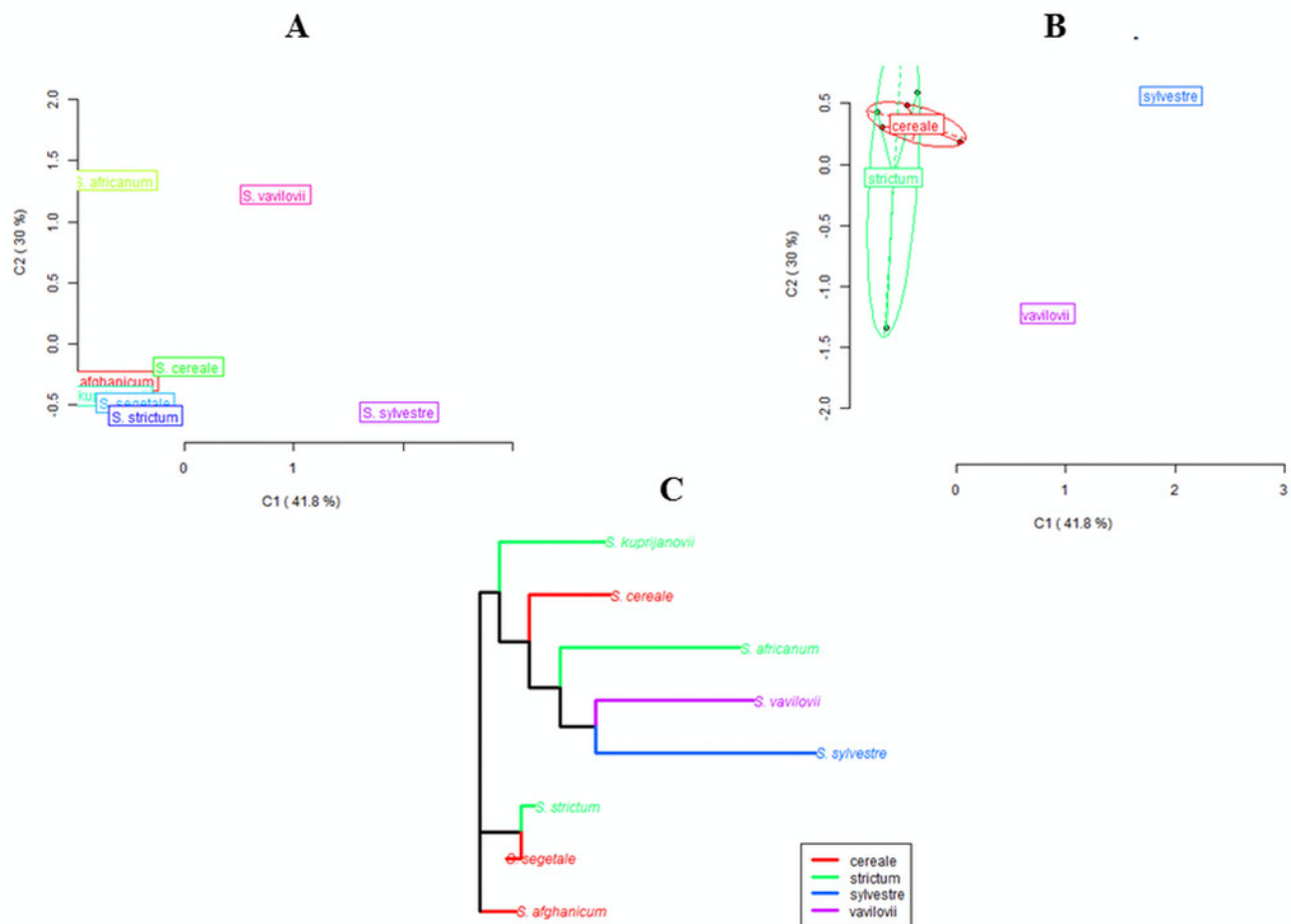
Figure 3

UPGMA dendrogram representing epigenetic relationships among rye taxons based on the data from MSAP analysis.

The numbers represent bootstrapping values.
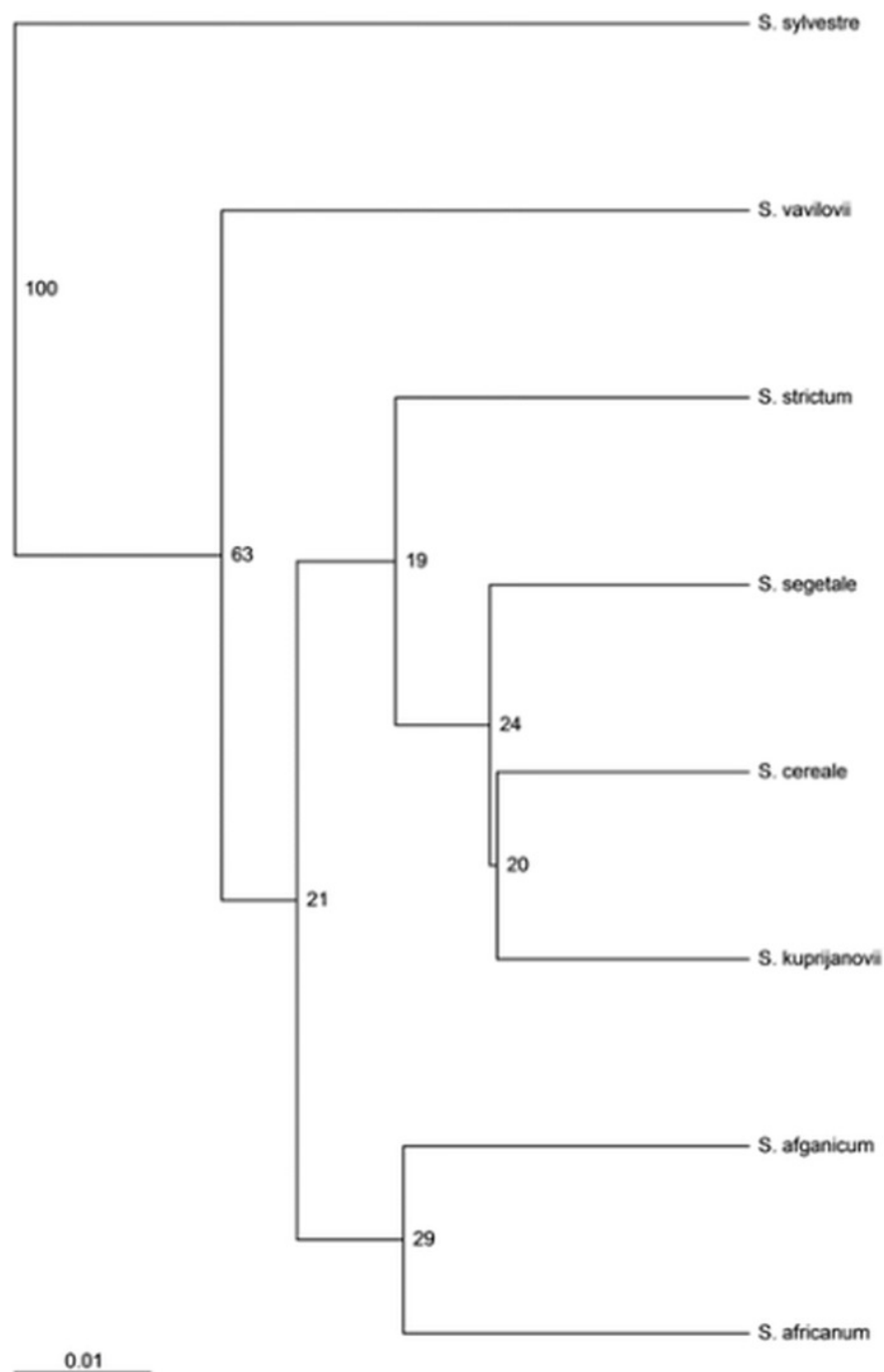
Figure 4

The comparison of t-heterochromatin content and cytosine methylation.

Total telomeric heterochromatin content (%) in rye genomes, level (%) of cytosine methylation at CCGG sites from MSAP analysis, and global cytosine methylation (%) from ELISA analysis.
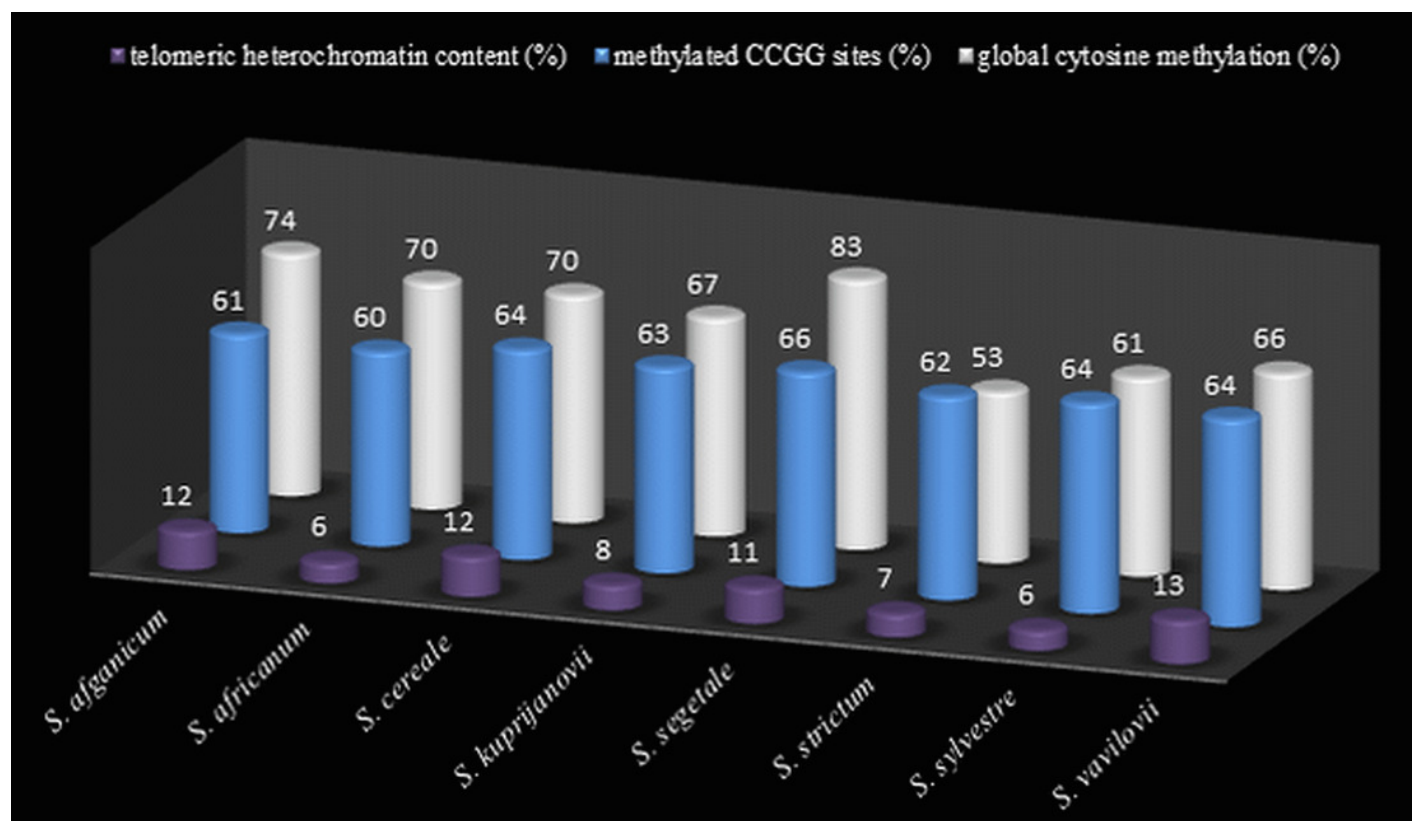
Figure 5

2C DNA nuclear content (pg).

* The 2C value for *Secale cereale* was used as reference value.

Note: Auto Gamma Correction was used for the image. This only affects the reviewing manuscript. See original source image if needed for review.
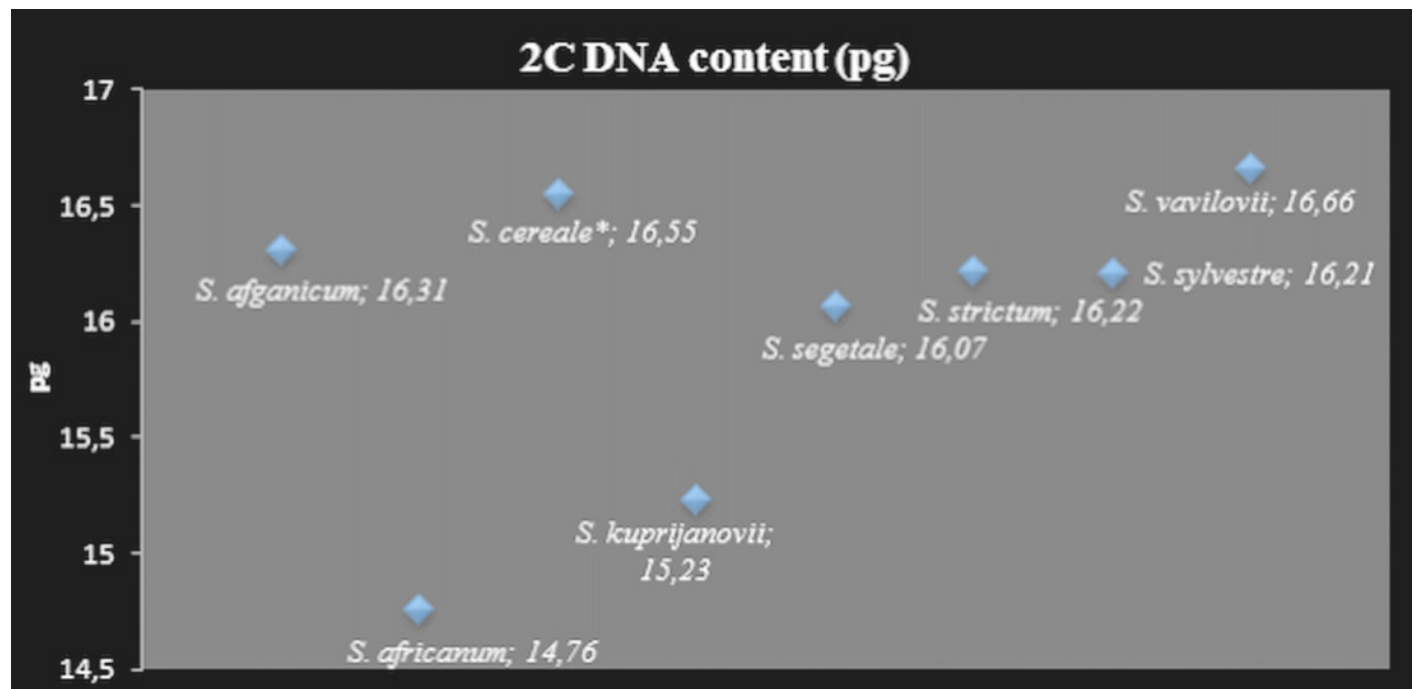
Figure 6

C-banded methaphase chromosomes of Secale, A: Secale vavilovii, B: Secale sylvestre.