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Rapid report

Epigenetic contribution to successful polyploidizations: variation in global cytosine methylation along an extensive ploidy series in *Dianthus broteri* (Caryophyllaceae)

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Summary

- Polyploidization is a significant evolutionary force in plants which involves major genomic and genetic changes, frequently regulated by epigenetic factors. We explored whether natural polyploidization in *Dianthus broteri* complex resulted in substantial changes in global DNA cytosine methylation associated to ploidy.
- Global cytosine methylation was estimated by high-performance liquid chromatography (HPLC) in 12 monocytotypic populations with different ploidies (2×, 4×, 6×, 12×) broadly distributed within *D. broteri* distribution range. The effects of ploidy level and local variation on methylation were assessed by generalized linear mixed models (GLMMs).
- *Dianthus broteri* exhibited a higher methylation percent (~33%) than expected by its monoploid genome size and a large variation among study populations (range: 29.3–35.3%). Global methylation tended to increase with ploidy but did not significantly differ across levels due to increased variation within the highest-order polyploidy categories. Methylation varied more among hexaploid and dodecaploid populations, despite such cytotypes showing more restricted geographic location and increased genetic relatedness than diploids and tetraploids.
- In this study, we demonstrate the usefulness of an HPLC method in providing precise and genome reference-free global measure of DNA cytosine methylation, suitable to advance current knowledge of the roles of this epigenetic mechanism in polyploidization processes.

Introduction

The potential contributions of epigenetics to plant adaptation and evolution have been thoroughly outlined, but their impact on natural populations remains understudied (e.g. Grant-Downton & Dickinson, 2006; Jablonka & Raz, 2009; Hirsch *et al.*, 2013; Kilvitis *et al.*, 2014). Polyploidization, that is, the possession of at least three complete sets of chromosomes, has long been recognized as a major evolutionary force in plants. Whole genome duplication events, frequently associated to hybridization, have occurred extensively in plants and have been related to phenotypic innovation and speciation (Madlung, 2013; Soltis *et al.*, 2014; Vanneste *et al.*, 2015). The proximate molecular mechanisms by which polyploidy becomes feasible are far from being completely understood (Soltis *et al.*, 2010). Nevertheless, successful polyploidization most likely involves both structural and functional alterations, which are intimately associated to epigenetic regulation of the activity of transposable elements (TEs) and silencing of redundant genes (Osborn *et al.*, 2003; Soltis *et al.*, 2010; Hegarty *et al.*, 2011; Madlung & Wendel, 2013; Tayalé & Parisod, 2013; Song & Chen, 2015).

At the molecular level, epigenetic processes comprise interdependent DNA and histone reversible covalent chemical modifications, which concurrently alter chromatin structure and may affect genome stability and individual phenotype without modifications of the nucleotide sequence (see e.g. Hirsch *et al.*, 2013, for a review). In plants, DNA cytosine methylation is an important epigenetic mechanism required for normal individual development, involved in responses to environmental factors, and with potential for transgeneration persistence (Finnegan, 2010; Hirsch

et al., 2013). Methylated cytosines can be found in different sequence contexts (CG, CHG, CHH; where H can be any nucleotide except G), each associated to different genomic positions (gene bodies, promoters, TEs, etc.), and involving the action of different demethylase and methyltransferase families and small non-coding RNAs (Finnegan, 2010; Hirsch *et al.*, 2013). Relative relevance of cytosine methylation at different nucleotide contexts varied across species, likely associated to important differences in genome features among them (Alonso *et al.*, 2016; Springer *et al.*, 2016).

Several methods have been developed to analyze global cytosine methylation level and compare cytosine methylation patterns across samples (see e.g. Laird, 2010; Kim *et al.*, 2014 for a review). The simultaneous quantification and location of cytosine methylation allows detailed interpretation of molecular processes related to gene expression and TE activity in genomes differing in complexity (Diez *et al.*, 2014; Springer *et al.*, 2016). However, this requires a high-quality annotated reference sequence for whole-genome scanning (BC-seq and WGSBS), which is not readily available for most nonmodel plants (Laird, 2010; Schrey *et al.*, 2013; Kim *et al.*, 2014). Thus, less thorough molecular analyses applied to a wider number of plant species have been instrumental to unraveling the magnitude of intraspecific variation in DNA methylation patterns of wild growing plants, its relationship with functional and fecundity-related plant phenotypic traits and its role in ecological adaptation to contrasting environmental conditions (e.g. reviewed in Schrey *et al.*, 2013; Medrano *et al.*, 2014; Verhoeven *et al.*, 2016). Methods based on high-performance liquid chromatography (HPLC) provide a global estimation of the percentage of total genomic cytosines that are methylated and do not require any previous knowledge of the species genomic features (Fraga & Esteller, 2002; Lisanti *et al.*, 2013; Alonso *et al.*, 2015, 2016). Such quantitative methods do not reveal patterns of methylation variation at specific sequence contexts or positions, do not differentiate between coding and noncoding sequences and do not detect subtle differences in the methylation status of individual genes. However, they are recommended for genome-wide quantification because of its global assessment, accuracy and reproducibility (Fraga & Esteller, 2002; Lisanti *et al.*, 2013) and have been successfully used to infer macroevolutionary patterns in vertebrates (Varriale, 2014) and plants (Alonso *et al.*, 2015). The quantitative global methylation value is, thus, a genomic feature such as genome size, which can be interpreted as an individual phenotypic trait whose variation within a species can be also significant (Alonso *et al.*, 2016). In this study we will apply this method to uncover whether, within a species, natural populations with different ploidy may quantitatively differ in genome-wide cytosine methylation.

As already stated, epigenetic regulation is instrumental to successful polyploidization events by silencing redundant genes and regulating the activity of TEs (Osborn *et al.*, 2003; Hegarty *et al.*, 2011; Madlung & Wendel, 2013; Tayalé & Parisod, 2013; Song & Chen, 2015). Complementary insights can be gained by analyzing epigenome and transcriptome features both in early generations of synthesized polyploids and also in naturally stabilized polyploids with more complex evolutionary histories (Paun *et al.*, 2007; Springer *et al.*, 2016). On the one hand, induced polyploids should contribute to the understanding of how different epigenetic mechanisms solve redundancy and genome regulation during the highly disturbing and stochastic nature of the early stages (Parisod *et al.*, 2010; Madlung & Wendel, 2013; Hollister, 2015; Springer *et al.*, 2016). Interpretation of these experiments should take into account that, in allopolyploids, hybridization rather than genome doubling *per se*, appears to be a major cause of epigenetic changes (Doyle *et al.*, 2008). On the other hand, the analysis of natural polyploids may contribute to reveal the genomic features of the stabilized polyploids, successfully established in the wild after the strong selective pressures affecting the initial stages (Parisod *et al.*, 2010; Madlung & Wendel, 2013, and references cited therein). In natural populations of polyploid species, variation in patterns of cytosine methylation contributes to explain functional plasticity and local adaptation (Paun *et al.*, 2010; Róis *et al.*, 2013; Schulz *et al.*, 2014) and has been proposed as a relevant factor for invasion success of polyploids (Ainouche *et al.*, 2009). However, the relationships between magnitude and patterns of cytosine DNA methylation and ploidy within a species remains largely unexplored. In *Actinidia chinensis*, a low genetic and epigenetic differentiation was observed between diploid, tetraploid and hexaploid individuals growing intermixed along an altitudinal gradient although epigenetic markers were more closely linked to the altitude where each sampled individual lived than to ploidy (Liu *et al.*, 2015). We are not aware of any other study analyzing intraspecific variation in global cytosine methylation across wild growing plants differing in ploidy.

The question addressed in this study is whether natural polyploidization events result in substantial genome-wide methylation changes. For this aim, we analyzed global cytosine methylation in *Dianthus broteri*, a perennial herb with an extensive polyploid series, to uncover whether natural populations with increased number of sets of chromosomes may quantitatively differ in global cytosine methylation. Our study species is particularly interesting because it encompasses diploid, triploid, tetraploid, hexaploid and dodecaploid individuals that do not usually coexist and are most likely the result of several autopolyploidization events (Balao *et al.*, 2009). Analysis of related organisms of several ploidies should be powerful to detect either a quantitative trend along the series or specific changes associated to any particular ploidy. However, absence of significant differences in global methylation should not be interpreted as evidence of similarity in methylation patterns because magnitude and patterns of cytosine methylation provide complementary but not identical information (Alonso *et al.*, 2016).

Materials and Methods

Study species and sites

Dianthus broteri Boiss. & Reuter (Caryophyllaceae) is a perennial herb endemic to the south and east of the Iberian Peninsula. The species occurs mainly on calcareous and dolomitic areas, from altitudes of 1700 m to coastal sand palaeodunes, where it represents a relatively rare component of xerophytic scrub formations. Along

its distribution range, *D. broteri* shows an extensive polyploid series including diploids, triploids, tetraploids, hexaploids and dodecaploids, which do not usually coexist within a site (Balao *et al.*, 2009). Tetraploidy is the most common and widespread cytotype, in contrast to the other cytotypes that exhibited more restricted geographic distributions (Supporting Information Fig. S1; Balao *et al.*, 2009). Tetraploids inhabit mainly on calcareous soils but also on dolomitic and siliceous substrates associated to either coastal or mountaineous areas in the south and east of the Iberian Peninsula (Fig. S1). By contrast, diploids appear in two disjunct areas associated to different mountain ranges (up to 1700 m above sea level (asl) of Portugal and Spain with characteristic cold and rainy winters; hexaploids are restricted to the semiarid lowlands in east of Spain (< 200 m asl); and dodecaploids (also identified as *D. inoxianus*) are endemic to a small littoral area (< 100 m asl) of the Doñana National Park in south Spain where they inhabit dry sandy soils (Fig. S1; Balao *et al.*, 2009). For the present purposes, 12 monocytotypic populations with different ploidies were selected (Table 1) and five individuals per population were analyzed. Most study populations belong to the same genetic group based on previous analyses of neutral molecular markers (amplified fragment length polymorphism, AFLP), except all dodecaploids and the tetraploid population in the eastern region that belonged to two separate genetic groups (Balao *et al.*, 2010). The plant material used here is a subset of the larger collection analyzed in Balao *et al.* (2009, 2010, 2011), where additional information of study populations can be found.

Field sampling

Populations were visited in summer when individuals were bearing both flowers and fruits that made them more conspicuous. During the daytime, leaves from each reproductive plant were collected separately in numbered individual containers, dried at ambient temperature in silica gel and preserved in this way by periodic replacement of the desiccant until processing. This fast drying method is the current standard for plant molecular DNA analyses of field-collected samples, providing good yield of high quality DNA in many species (Carrió & Roselló, 2014, for a review), including *D. broteri* (Balao *et al.*, 2010).

Laboratory methods

Dry leaves were subsequently homogenized to a fine powder using a Retsch MM 200 mill. Total genomic DNA was extracted using Qiagen DNeasy Plant Mini Kit, including RNAse treatment. A 100 ng aliquot of DNA extract was digested with 3 U of DNA Degradase PlusTM (Zymo 71 Research, Irvine, CA, USA), a nuclease mix that degrades DNA to its individual nucleoside components. Digestion was carried out in a 40 µl volume at 37°C for 3 h, and terminated by heat inactivation at 70°C for 20 min. Two independent replicates of digested DNA per sample were processed to estimate global cytosine methylation (Table S1 contains the full data). Selective derivatization of cytosine moieties with 2-bromoacetophenone under anhydrous conditions and subsequent reverse phase HPLC with spectrofluorimetric detection, were conducted. The percentage of total cytosine methylation on each replicated sample was estimated as $100 \times 5mdC/(5mdC + dC)$, where 5mdC and dC are the integrated areas under the peaks for 5-methyl-2′-deoxycytidine and 2′-deoxycytidine, respectively (see Lopez-Torres *et al.* [2011] and Alonso *et al.* [2016] for details).

Data analyses

The variation in global cytosine methylation was analyzed by using generalized linear mixed models (procedure MIXED; SAS Institute, 2008) including ploidy, and population nested within ploidy as fixed categorical effects. Individual was entered as a random effect to account for nonindependence of the replicated analyses. The intraclass correlation coefficient, which estimates consistency of the replicated analyses, was 0.8745. The interpretation of a significant nested effect was accomplished by using the slice option, which tests the equality of simple effects of one factor (i.e. population) for a given level of the other factor (i.e. ploidy). In addition, as ploidy can

Table 1 Location and features of the 12 populations of *Dianthus broteri* studied along the distribution range in the southern Iberian Peninsula, from the Atlantic Portuguese-Spanish coast (southwest [SW] region) to the easternmost Mediterranean coastal locations (east [E]), and including also three intermediate populations located in the Baetic ranges (south [S])

Locality	Code	Region	Latitude	Longitude	Altitude (m)	Ploidy	Methylation (%)
1. Troia	259/06	SW	38°25′N	8°49′W	15	4×	32.74 ± 1.09
2. São Brás de Alportel	213/06	SW	37°09′N	7°50′W	218	2×	31.55 ± 1.83
3. Valverde	239/07	SW	37°30′N	6°40′W	250	12×	35.25 ± 2.08
4. Moguer	328/06	SW	37°09′N	6°49′W	24	12×	33.53 ± 1.37
5. Doñana, Puntal	FA/05	SW	36°58′N	6°26′W	6	12×	29.33 ± 1.52
6. Doñana, Peladillo	333/06	SW	37°05′N	6°35′W	41	4×	32.93 ± 1.26
7. Ronda	827/05	S	36°45′N	5°10′W	672	4×	33.89 ± 2.68
8. Zafarraya1	335/06	S	36°59′N	4°11′W	1025	2×	32.53 ± 1.31
9. Orgiva	338/06	S	36°53′N	3°24′W	370	2×	33.93 ± 1.01
10. Cartagena	280/07	E	37°35′N	0°57′W	210	6×	34.47 ± 1.58
11. San Miguel de Salinas	276/07	E	37°58′N	0°45′W	54	6×	30.50 ± 4.00
12. Alcublas	250/07	E	39°50′N	0°41′W	950	4×	34.37 ± 1.03

Ploidy data as reported by Balao *et al.* (2009). Global cytosine methylation was analyzed in five individuals per locality, mean ± standard deviation (SD) is shown. For exact geographical location see Supporting Information Fig. S1.

be more precisely understood as a quantitative factor variable in which levels are ordered ($2 < 4 < 6 < 12$) and unequally spaced (i.e. difference between 12 and 6 levels was larger) orthogonal polynomial contrasts were used to analyze the effect of this factor. Accordingly, 2× level was contrasted against the other three levels in proportion to their difference in genome copies (i.e. using the contrast coefficients −1; 0.1818; 0.2727; 0.5455 for 2×, 4×, 6×, and 12×, respectively). The exclusion of the individual with the minimum percentage of methylation, which was outlier within the distribution of study samples (see Fig. 1), improved model adjustment but did not qualitatively alter the results (analyses not shown). Averages (± standard error [SE]) are hereafter shown.

Results

The percentage of cytosine methylation in *D. broteri* leaves averaged 32.92% (SE ± 0.22). Across the studied individuals DNA methylation ranged between 23.96% and 37.79%, the minimum value, obtained from the hexaploid population at San Miguel de Salinas, being an outlier within the study sample (Fig. 1).

Contrary to our expectations, cytosine methylation did not significantly differ between ploidies when considered either as unordered categorical classes ($F_{3,60} = 0.89$, $P = 0.45$) or when contrasted against the diploid level according to their proportional difference in genome copies ($F_{1,60} = 0.04$, $P = 0.84$). A significant population nested effect ($F_{8,60} = 5.28$, $P < 0.0001$) suggested that differences across populations of each ploidy were not constant (Fig. 2). The sliced analyses indicated that the three diploid populations studied did not differ in the percent of cytosine methylation (32.67 ± 0.43%; $F_{2,60} = 1.95$, $P = 0.15$). The four tetraploid populations neither differed among them in average methylation (33.48 ± 0.37%; $F_{3,60} = 0.82$, $P = 0.49$). By contrast, the two hexaploid populations recorded different methylation levels (32.49 ± 1.12%; $F_{1,60} = 10.70$, $P = 0.0018$) and the three dodecaploid populations were widely different in methylation percentage (Fig. 2; 32.71 ± 0.78%, $F_{2,60} = 12.59$, $P < 0.0001$), including the two extreme populations studied.

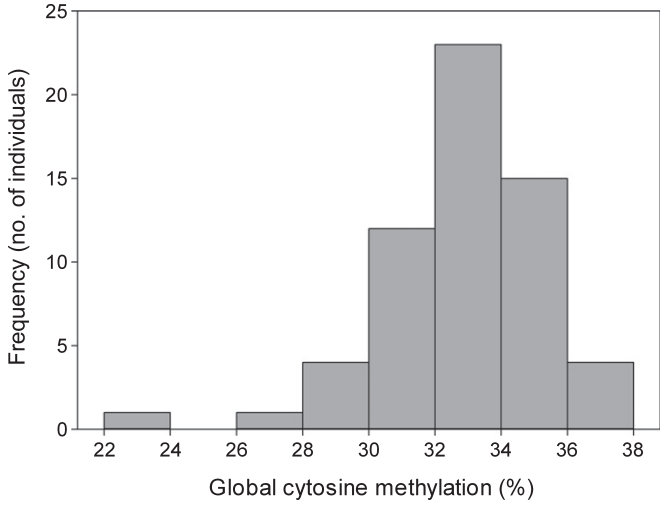

Fig. 1 Histogram of the natural variation in percent of global cytosine methylated in *Dianthus broteri* leaves ($n = 60$ individuals).

In cytotypes where differences between populations were statistically significant (hexaploid and docecaploid populations), we looked for a relationship between the average methylation percentage and site altitude, and found that it was positive and significant ($r_s = 0.90$, $P = 0.037$, $n = 5$).

Discussion

Cytosine methylation is the best understood mechanism for epigenetic regulation in higher plants with potential for transgeneration inheritance (Finnegan, 2010; Hirsch *et al.*, 2013). Global cytosine methylation has been recently revealed as a genomic feature, highly variable across plant species, with a strong phylogenetic signal, and evolutionary related to the monoploid genome size in Angiosperms (Alonso *et al.*, 2015). According to the data presented here, the global cytosine methylation in the leaves of *D. broteri* (*c.* 33%) is relatively high compared to the rest of Angiosperms, which coarsely range between 4% and 40% (Alonso *et al.*, 2015). Further, *D. broteri* leaves double the expected methylation percentage according to its monoploid genome size, which ranged between 0.78 pg and 1.00 pg (Balao *et al.*, 2009; Alonso *et al.*, 2015). Further analyses in other *Dianthus* species should help to better calibrate whether these outstanding results are specific to *D. broteri* or are characteristic of the entire *Dianthus* genus, a young and large genus, with exceptional endemic species richness associated to a fast and recent diversification process in the Mediterranean basin (Valente *et al.*, 2010).

With regard to the intraspecific variation in global cytosine methylation in *D. broteri*, the study populations ranged between 29.3% and 35.3% methylated cytosines. Such a 6% interval must be biologically relevant because it is in the range of differences obtained between contrasting ecotypes and growing conditions in other plant species (e.g. Cai & Chinnappa, 1999) and also equals in

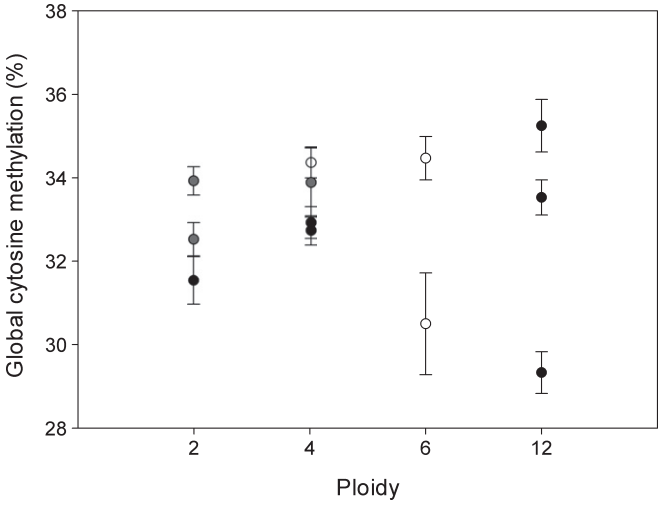

Fig. 2 Global cytosine methylation in *Dianthus broteri* leaves from different populations and ploidies. Each dot denotes the population mean based in values of five individuals, bars indicate ± standard error (SE) , and different colors refer to the geographic region (southwest [SW], black; south [S], grey; east [E], white). Only hexaploid and dodecaploid populations differed, respectively, in their average global cytosine methylation percentage (see text).

magnitude the minimum global methylation so far reported in Angiosperms (Alonso *et al.*, 2015). Such differences among populations may result from either ploidy or local environmental conditions. Principally, we note that hexaploid and particularly dodecaploid populations were more variable in the methylation levels recorded than diploid and tetraploid populations. However, this is striking because both hexaploids and dodecaploids have significantly restricted geographic distributions and show notable genetic relatedness within cytotype (Balao *et al.*, 2010). By contrast, the geographically distant tetraploid populations analyzed did not differ in methylation level despite encompassing samples from two distinct genetic groups (Balao *et al.*, 2010). Such findings suggest that the local ecological factors more than genetic and geographic differentiation could underlie the observed epigenetic variability across populations within the same cytotype in *D. broteri*, as it has been found in other plant species (e.g. Lira-Medeiros *et al.*, 2010; Schulz *et al.*, 2014). The positive association between the average methylation and elevation in hexaploid and dodecaploid populations supports this view.

Epigenetic regulation plays critical roles during polyploidization as a new balance has to be created between the duplicated chromosomes. Differences in genomic features such as abundance of TE, monoploid genome size, and GC content may determine species-specific responses after polyploidization (Hegarty *et al.*, 2011; Springer *et al.*, 2016). Cytosine methylation must be essential along the initial genome perturbation and stabilization processes since either hyper- or hypo-methylation at specific loci could be vital for correct gene activity, and regulation of the activity of transposons (Osborn *et al.*, 2003; Parisod *et al.*, 2010; Tayalé & Parisod, 2013; Soltis *et al.*, 2014; Song & Chen, 2015). Thus, a quantitative assessment of genome-wide cytosine methylation can be useful to start a systematic analysis of changes associated to either experimentally synthesized polyploids or naturally stabilized polyploids. For instance, Lavania *et al.* (2012) reported an overall increase in global cytosine methylation with ploidy recorded by immunodetection after induced autopolyploidy in six species of aromatic grasses. Consistently, we report here a moderate steady increase in global cytosine methylation upon the natural polyploidization in *D. broteri*, particularly if the two most discordant populations at the highest-order polyploidy categories (Doñana Puntal and San Miguel de Salinas) were excluded (results not shown). Provided that cytosine methylation is also affected by environmental stress (reviewed in Chinnusamy & Zhu, 2009; Alonso *et al.*, 2015), our study cannot set apart how much the outstanding populations could be responding already to current local ecological factors, such recurrent and intensive herbivory (Herrera & Balao, 2015), more than to the long-term polyploidization process. Thus, in this particular species, differences among populations in global methylation may result from either ploidy (genomic) or environmental (ecological) variation, or a combination of both effects that should be tested experimentally because cytotypes do not naturally coexist within populations (Balao *et al.*, 2009).

Uncovering the distinctive roles that epigenetic regulation play at different phases of polyploid formation, stabilization and successful short- and long-term selection is a current challenge for plant evolutionary ecologists (Wendel, 2015). At interspecific level, the strong phylogenetic association between global cytosine methylation and monoploid genome size (Alonso *et al.*, 2015) supports the notion that cytosine methylation is an adaptive feature allowing the evolutionary increase in size and complexity of plant genomes, largely attributed to TEs and ancient whole-genome duplications (Fedoroff, 2012; Wendel, 2015; Springer *et al.*, 2016). Similarly, cytosine methylation should play a role in success of more recent genome duplication events but lack of data regarding variation in cytosine methylation in plant species with several ploidies precludes such analysis. The quantitative assessment of global methylation with methods that do not require a full genome sequence could contribute to fill this gap. In this study, we demonstrate the usefulness of an HPLC method as a quick global measure of DNA methylation analogous to flow cytometry for quickly measuring DNA content and ploidy level (Dolezel & Bartos, 2005).

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Author contributions

C.A. and F.B. designed the research and contributed to data analysis; F.B. conducted fieldwork sampling; P.B. and R.P. prepared, developed and process all lab analyses, and contributed to their interpretation; C.A. led the writing; all authors contributed to refining the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Map showing distribution ranges of different ploidy levels in *Dianthus broteri* complex and geographic location of the 12 populations studied.

Table S1 Global methylation data for all analyzed samples

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