

Environmental differences are correlated with the distribution pattern of cytotypes in *Veronica* subsection *Pentasepalae* at a broad scale

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Received: 30 April 2019 Returned for revision: 16 September 2019 Editorial decision: 26 October 2019 Accepted: 1 November 2019
Published electronically 2 November 2019

- **Background and Aims** The distribution of cytotypes and its potential correlation with environmental variables represent a cornerstone to understanding the origin and maintenance of polyploid lineages. Although many studies have addressed this question in single species at a regional scale, only a few have attempted to decipher this enigma in groups of closely related species at a broad intercontinental geographical scale. Here, we consider approx. 20 species of a diploid–polyploid complex (*Veronica* subsect. *Pentasepalae*) of recent and rapid diversification represented in Europe and North Africa to study the frequency and distribution of cytotypes and their relationship to environmental variables.
- **Methods** A total of 680 individuals (207 populations) were sampled. Ploidy levels were determined using flow cytometry. Ecological differentiation among cytotypes was tested using climatic and environmental variables related to temperature, precipitation, vegetation and biogeographical region, among others, and by performing univariate and multivariate (constrained principal coordinates analysis) analyses.
- **Key Results** Four ploidy levels (2x, 4x, 6x and 8x) were found and genome downsizing was observed to occur within the group. Plants of different ploidy level are ecologically differentiated, with hexaploids and octoploids occurring in wetter and colder habitats with a higher seasonality than diploids. A south to north distribution pattern was found, with diploids occupying southern refugial areas and octoploids being more frequent in northern regions of Europe above the permafrost boundary.
- **Conclusions** The distribution of cytotypes can be explained by ecological differentiation, the geographical position of refuge areas during the Quaternary climatic oscillations as well as by ice and permafrost retreat patterns. The Balkan Peninsula constitutes the most important contact zone between cytotypes. This work provides the first comprehensive ploidy screening within *V.* subsect. *Pentasepalae* at a broad scale and indicates that polyploidy and genome downsizing might have contributed to the colonization of new habitats in a recently diverged polyploid complex.

Key words: Cytotype distribution patterns, ecological differentiation, genome downsizing, historical biogeography, minority cytotype exclusion, ploidy level, polyploidy, Quaternary glaciations, *Veronica* subsection *Pentasepalae*.

INTRODUCTION

Polyploidy or whole-genome duplication (WGD) plays a major role in the evolution of angiosperms and other eukaryotes (Stebbins, 1950; Otto and Whitton, 2000; Wendel, 2000; Soltis *et al.*, 2015). Molecular studies have demonstrated that repeated rounds of polyploidization have modelled the evolutionary history of all flowering plants since their origin (Jiao *et al.*, 2011; Wendel, 2015). While some authors consider polyploids as ‘evolutionary dead-ends’ (Mayrose *et al.*, 2011; Arrigo and Barker, 2012), other studies associate polyploidy

with high diversification rates (Fawcett *et al.*, 2009; Soltis *et al.*, 2014; Meudt *et al.*, 2015; Kellogg, 2016; Levin and Soltis, 2018; Han *et al.*, 2019). Genome downsizing, i.e. the reduction of DNA per cell without numerical loss of chromosomes (Leitch and Bennet, 2004; Ramsey and Ramsey, 2014), is likewise ubiquitous in angiosperms and has also been correlated with an increased diversification rate in polyploid complexes (Kraaijeveld, 2010; Meudt *et al.*, 2015). Indeed, recent work has corroborated the contribution of genome downsizing to the origin of physiological novelties that lead

to the diversification of flowering plants and their expansion to new ecological habitats (Pandit *et al.*, 2014; Simonin and Roddy, 2018). Furthermore, changes in genome size may affect different aspects of polyploids regarding ecology, physiology, reproduction (e.g. meiosis) or genetic patterns (Bennett and Smith, 1972; Cavalier-Smith, 1978; Gregory, 2001; Leitch and Bennett, 2004; Comai, 2005; Pandit *et al.*, 2014; Simonin and Roddy, 2018).

The frequency and geographical distribution of cytotypes are important for exploring the origins of polyploids and their maintenance in nature (Duchoslav *et al.*, 2010). Differences in cytotype distribution patterns have been explained by different but often complementary processes. The adaptive evolutionary scenario assumes that the successful persistence of polyploids is determined by ecological factors (Rodríguez, 1996; Husband and Schemske, 1998; Ramsey, 2011; Fowler and Levin, 2016). Under this hypothesis, novel phenotypic, physiological and genetic combinations associated with polyploidy – mainly allopolyploidy – seem to be responsible for different ecological tolerances (Paun *et al.*, 2011; Ramsey, 2011) or broader geographic ranges of polyploids, as compared with diploid congeners (Hijmans *et al.*, 2007; Meimberg *et al.*, 2009). Thus, genome duplication may allow polyploids to expand to new habitats previously unavailable to their diploid counterparts (Stebbins, 1984, 1985; Thompson and Lumaret, 1992; Maceira *et al.*, 1993; te Beest *et al.*, 2012). Numerous studies that have recently assessed this hypothesis have found ecological differences among ploidy levels (see Ramsey and Ramsey, 2014; Mairal *et al.*, 2018). However, other studies failed to detect habitat differentiation among cytotypes, suggesting that niche differentiation patterns are not universal (Glennon *et al.*, 2014; Visser and Molofsky, 2015; Marchant *et al.*, 2016; Castro *et al.*, 2018, 2019). The lack of ecological niche differentiation among cytotypes might occur due to several reasons. First, ecological divergence may arise with time and, thus, the effects of WGD on ecological differentiation can be obscured when studies are focused on a single species, in which new cytotypes may have not had enough time to diverge (e.g. Schranz *et al.*, 2012; Godsoe *et al.*, 2013). Secondly, differences between cytotypes could occur at a microenvironmental scale, thus being difficult to detect by the large-scale climate data, particularly when the ranges of the different cytotypes overlap (Glennon *et al.*, 2014; Kirchheimer *et al.*, 2016). Thirdly, broader geographical scale studies have suggested that species' ranges are also influenced by their phylogenetic history (Levin, 2000; Losos, 2008; Burns and Strauss, 2011) due to the inheritance of ecological preferences (Martin and Husband, 2009). According to this idea, phylogenetically related taxa would be prone to occupy more similar environments due to the so-called phylogenetic niche conservatism (Harvey and Pagel, 1991; Lord *et al.*, 1995).

Nonetheless, certain distribution patterns (e.g. higher number of polyploids inhabiting higher latitudes) can also be generated by other processes, such as biogeographic history (Avisé, 2000; Duchoslav *et al.*, 2010; Paule *et al.*, 2017). For example, the climatic oscillations that occurred during the Quaternary have induced range shifts of plant species that found refuge at lower latitudes or altitudes (Hewitt, 1999; Thompson, 2005). The existence of new and unexploited areas after ice retreat probably represented an opportunity for polyploids to expand

and establish outside the range of their diploid counterparts (Stebbins, 1950, 1984; Brochmann *et al.*, 2004; Marchant *et al.*, 2016). Thus, the distribution of cytotypes may also be explained by the geographical position of the refuge areas, as well as by ice and permafrost retreat patterns (van Dijk *et al.*, 1992; Mandáková and Münzbergová, 2006; Duchoslav *et al.*, 2010). Importantly for the analysis of distribution patterns in European plants, permafrost reached even the lowland regions at mid-latitudes within Europe during the last permafrost maximum (LPM; Fig. 1), and active mountain glaciers were present in the central and southern mountain ranges of Europe (Fig. 1). Apart from migration patterns out of refuge areas, Quaternary climatic oscillations might have triggered secondary contacts among otherwise geographically isolated taxa (Stebbins, 1984, 1985; Brochmann *et al.*, 2004; Kadereit, 2015). Accordingly, the biogeographic history of species may have been responsible for the origin of allopolyploid taxa and/or mixed ploidy populations in secondary contact areas.

The distribution of cytotypes can also be explained by other locally operating processes, such as the frequency-dependent mating success (Ramsey and Schemske, 1998). According to the minority cytotype exclusion (MCE) model, the reproductive disadvantage of the minority cytotype would lead to its extinction from the population (Levin, 1975). However, several mechanisms may counteract MCE, allowing neopolyploids to establish. On the one hand, neopolyploids may persist outside the range of the parental cytotype by eco-geographical differentiation, which will result in different cytologically uniform regions (Levin, 1975). On the other hand, mixed-ploidy populations have been reported for many plant species (e.g. Husband and Schemske, 2000; Duchoslav *et al.*, 2010; Bardy *et al.*, 2010, 2011; Krejčíková *et al.*, 2013; McAllister *et al.*, 2015; Etterson *et al.*, 2016; Čertner *et al.*, 2017), suggesting the existence of mechanisms that enable the persistence of a new cytotype within the population of origin. Genome duplication may promote the occurrence of reproductive barriers among cytotypes within the same populations (Ramsey, 2011), favouring assortative mating. These include changes in selfing (Fausto *et al.*, 2001; Comai, 2005), shifts in flowering time (Fowler and Levin, 1984), pollinator preferences (Thompson and Merg, 2008) and/or ecological differences at a microscale (Ramsey, 2011), which alone, or in concert, can contribute to the coexistence of cytotypes at the local scale. Cytotype coexistence can also be favoured by asexual reproduction (Paule *et al.*, 2011), clonal propagation (Baldwin and Husband, 2013), a better competitive ability of neopolyploids (Keeler and Davis, 1999) and/or a higher resistance to herbivory (Segraves and Anneberg, 2016). Finally, mixed-ploidy populations originated by autopolyploid events (in the so-called primary contact zones) can also be maintained by the recurrent production of unreduced gametes (Rodríguez, 1996), a process which is in turn influenced by environmental stress (McHale, 1983; Hao *et al.*, 2013).

In this study, we focus on the diploid–polyploid complex *Veronica* subsection *Pentasepalae*. This monophyletic group is composed of approx. 20 phylogenetically closely related species with ploidy levels ranging from $2x$ to $8x$, and more rarely $10x$ (Peev, 1972; Rojas-Andrés *et al.*, 2015). It is a recently diverged polyploid complex [mean crown age of 2.87

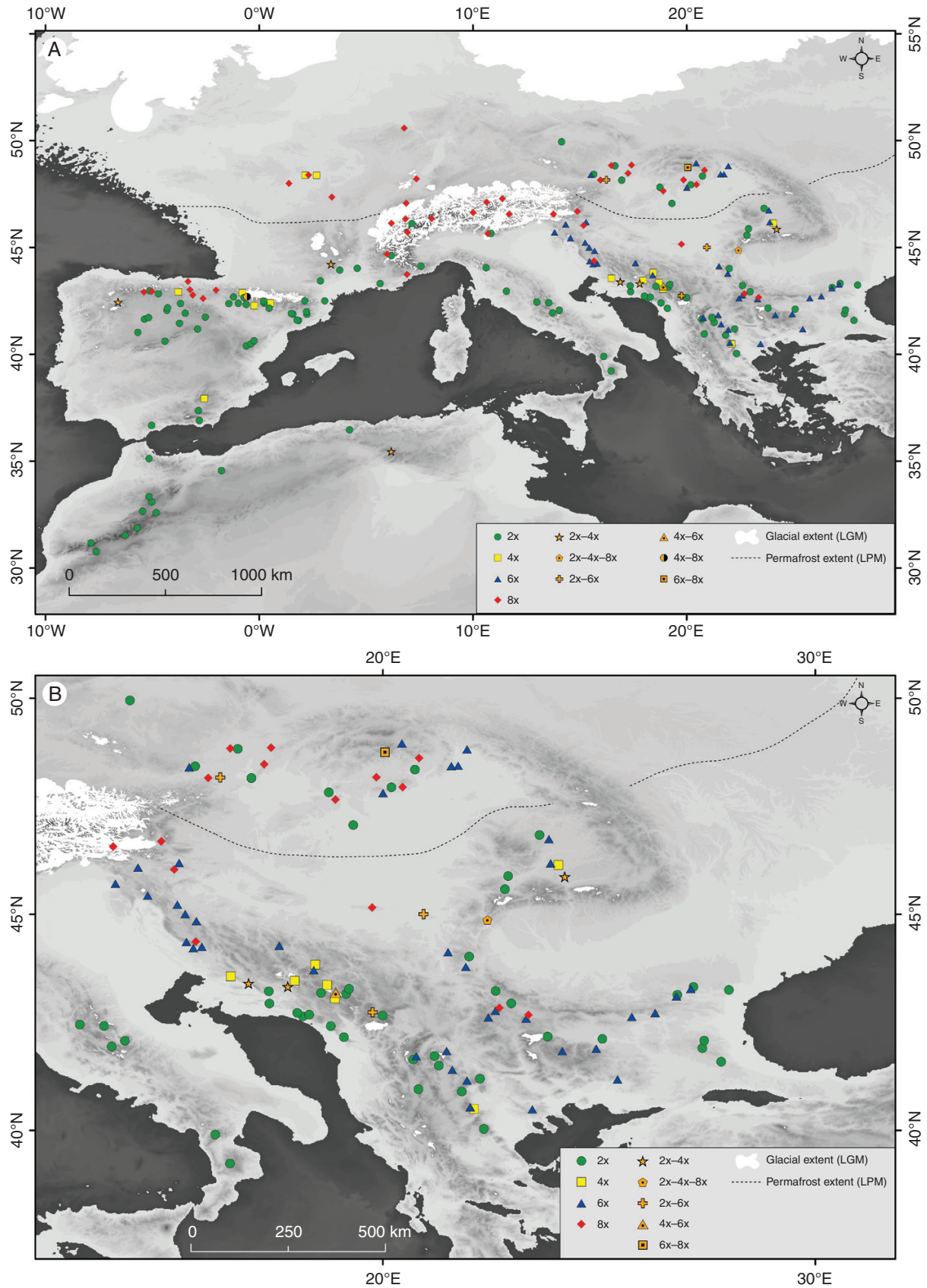


FIG. 1. Map showing the geographical distribution of cytotypes of *Veronica* subsect. *Pentasepalae* during the last glacial maximum (LGM). (A) General view of the study area. The line indicates the limit of the continuous permafrost during the last permafrost maximum (LPM) according to Vanderberghe *et al.* (2014). (B) Detail of the Balkan Peninsula.

Mya (1.37–4.53 Mya); Meudt *et al.*, 2015] mainly distributed over Europe and North Africa, which allows a study at a large geographical scale, enabling us to have the appropriate spatial and environmental data resolution for the model system. All these attributes make *V. subsect. Pentasepalae* an ideal system to evaluate whether independent polyploidization events within a single monophyletic lineage have led to ecological differentiation. For this, an extensive screening on ploidy levels by flow cytometry was performed for the whole subsection without considering taxonomy (i.e. irrespective of the assignment of individuals to particular taxa under this rank) and statistical analyses were used to address the following questions. (1) What are the frequencies and geographic distribution of cytotypes within *V. subsect. Pentasepalae*? (2) Do the polyploids show genome downsizing? (3) Are there environmental variables associated with the occurrence of different ploidy levels? (4) Which processes can explain the currently observed cytotype distribution patterns?

MATERIALS AND METHODS

Study group and sampling strategy

Veronica subsection *Pentasepalae* is a group of perennial herbs with a very branched rhizome indicating that, besides sexual reproduction, asexual vegetative propagation is common in the group. Little is known about the mating system of these plants, whose fruits and seeds lack dispersal organs or evident adaptations to anemochory or zoochory. Both xenogamy and self-pollination have been demonstrated to occur within the group (Scheerer, 1937; Scalone *et al.*, 2013, 2014). Regarding its evolutionary history, much progress has been made in the last years, yet there are still many open questions. Although some species of *V. subsect. Pentasepalae* have had time to diverge as independent lineages, reproductive isolation among others seems to be weak, as evidenced by hybridization and/or introgression patterns shown by some morphologically intermediate populations of uncertain taxonomic identity (Rojas-Andrés *et al.*, 2015; Rojas-Andrés and Martínez-Ortega, 2016; Padilla-García *et al.*, 2018). While it has been possible to identify the auto- or allopolyploid genesis of some populations, the origin of many polyploids is still not well understood or needs further confirmation (Padilla-García *et al.*, 2018). Genome designation of some polyploids would require extensive genome sequencing to find nucleotide polymorphisms that correlate with ancestral genomes and/or the use of advanced molecular cytogenetic methods such as genomic or fluorescent *in situ* hybridization. Thus, based on the rapid evolution under comparable environmental and historical conditions, we considered these taxa to be closely related, yet independent and equivalent enough for the purpose of our study. In addition, the existence of some narrow endemic species, for which a very low number of populations is known, hampers a robust statistical analysis at the species level. Given these limitations, our study is designed as an initial ploidy screening to explore cytotype distribution patterns within the subsection at an inter-continental geographical scale and their relationship with environmental conditions.

With this aim, a total of 680 individuals from 207 populations (Supplementary Data Table S1) of *V. subsect. Pentasepalae* were collected throughout Europe and North Africa (Fig. 1). Given that the area covered by our study is >2 million km², we tried to find a trade-off between the number of populations analysed and individuals per population. Therefore, we sampled >200 populations and, in general, three individuals per population, which provides an exceptional overview, usually unavailable for most polyploid complexes. Leaf material was collected in the field and immediately stored in silica gel for flow cytometric analyses. Geographical co-ordinates for each sampling location were registered using GPS. Vouchers were deposited at the herbaria MGC, SALA and MA (acronyms following Thiers, 2017).

Genome size and ploidy level estimations

Ploidy levels were estimated by flow cytometry and related to the chromosome counts available from previous studies (Martínez-Ortega *et al.*, 2004; Albach *et al.*, 2008; Delgado *et al.*, 2018). Direct chromosome counts were previously available for 29 sampled populations, so the corresponding data were incorporated in this study. For 18 of these 29 populations, individuals from the same origin were included in our flow cytometric measurements to ensure correspondence between chromosome number and genome size estimations. Genome size was estimated using silica gel-dried leaves for a total of 668 individuals from 196 populations (Supplementary Data Table S1). In total, 207 populations were included in the data analyses (196 plus 11 with chromosome counts only).

Leaf tissue from each individual was chopped together with fresh leaf material from an internal standard using a sharp razor blade in a Petri dish containing 1.1 mL of Woody Plant Buffer (WPB; Loureiro *et al.*, 2007). Depending on the sample C-value and standard availability, *Solanum pseudocapsicum* (2C = 2.589 pg; Tensch *et al.*, 2010), *Zea mays* L. ‘CE-777’ (2C = 5.43 pg; Lysak and Dolezel, 1998), *Pisum sativum* ‘Ctirad’ (2C = 9.09 pg; Dolezel *et al.*, 1998) and *Pisum sativum* ‘Kleine Rheinländerin’ (2C = 8.84 pg; Greilhuber and Ebert, 1994) were used as internal standards. A 1 mL aliquot of the nuclear suspension was filtered through a 48 µm nylon gauze, mixed with RNase (Sigma) to a final concentration of 0.15 mg mL⁻¹ and digested at 37 °C for 30 min. A total of 450 µL of the nuclear suspension was then mixed with 2 mL of the propidium iodide (PI) staining solution (60 µg mL⁻¹ PI in doubled-distilled water), incubated for at least 10 min and analysed. For each individual, one run of 5000 counts was made on a CyFlow SL (Partec GmbH, Münster, Germany) equipped with a solid-state laser featuring blue excitation at 488 nm.

For each sample, an estimate of genome size was obtained using the following formula: Genome size estimate (pg) = (*Veronica* G1 peak mean/Reference standard G1 peak mean) × Reference standard genome size. Samples were classified as diploid, tetraploid, hexaploid or octoploid according to the estimates of genome size and previous chromosome counts (Martínez-Ortega *et al.*, 2004; Albach *et al.*, 2008; Delgado *et al.*, 2018). Sixty-two per cent of the measurements presented a sample coefficient of variation (CV) of G1 peaks below 5 %.

For the remaining samples, CV values were between 5 and 9 %, even after sample repetition, most probably because the material was stored in silica gel for several months or years before being analysed and/or because of the presence of cytosolic compounds. As it was possible to assign ploidy levels unambiguously for all samples, these values were accepted for the estimation of ploidy levels. However, only measurements with CVs <5 % were considered to evaluate genome size values among different ploidy levels (Supplementary Data Table S2).

Environmental variables

Environmental parameters for 207 populations were extracted using ArcGIS 10.1 (ESRI, Redlands, CA, USA) from different sources (Supplementary Data Table S3). Precipitation and temperature were obtained from WorldClim (<http://www.worldclim.org/current>) (Hijmans *et al.*, 2005) and additional environmental parameters related to vegetation, slope, solar radiation, human footprint, soil type, land cover and biogeographical region were also included in the analyses. Spatial resolution and sources for all environmental parameters are given in Supplementary Data Table S3. Geographical data were directly obtained from GPS. We could not add any information regarding potential vegetation for the populations of North Africa given that no map of potential vegetation was found for this area. In total, 33 layers were considered: 19 related to precipitation and temperature, five to vegetation index, one to tree cover, one to slope, two to solar radiation, one to human footprint, one to soil type, one to potential natural vegetation, one to land type and one to biogeographical region (Supplementary Data Table S3).

Statistical analyses

All statistical analyses were performed using R 3.2.2 (R Development Core Team, 2015). Ecological differentiation among ploidy levels was first tested using univariate analyses. All variables were analysed to test their significance. Contingency tables were used for qualitative variables, whereas for quantitative variables analysis of variance (ANOVA) and the Kruskal–Wallis test were used (for parametric and non-parametric data, respectively). Significant variables in the overall test were subsequently analysed using Dunn’s test to check for significant differences among ploidy levels for each environmental parameter. The Bonferroni correction of $\alpha = 0.0003$ for multiple tests was applied to avoid type I errors (Shaffer, 1995). Although it is a very conservative method, it limits the number of significant models to more robust associations (Narum, 2006).

Environmental variables were subsequently subjected to multivariate analysis. Given that some variables might be highly correlated, analysis of correlation and reduction of collinearity are recommended to avoid type II errors and to find which variables are driving the system (Zuur *et al.*, 2010). Therefore, first, a Pearson product correlation was used to identify quantitative variables correlated at a level of $|r| \geq 0.8$. From each pair of correlated variables, one was excluded from subsequent analyses. However, looking at correlations only among pairs

of predictors is limiting because a linear dependence among three or more variables may exist. Thus, the variance inflation factor (VIF) on the environmental variables was applied, using 6.0 as a threshold value for acceptable collinearity (Chatterjee and Hadi, 2006). The VIF analyses were done with the R package ‘HH’ v. 1.4 (Heiberger, 2015), which was iteratively applied. Regarding association among qualitative variables, a Cramer’s V test was performed with the R package ‘DescTools’ v. 0.99.22 (Signorell *et al.*, 2017). None of the variables considered showed a high level of dependence (≥ 0.7).

After correlation analyses, the 33 variables initially considered were reduced to 17 variables representing climatic, vegetation, topographic, solar irradiance, anthropic, edaphic and biogeographical variability (Supplementary Data Table S3). Due to different types of descriptors (qualitative and quantitative), the Gower general coefficient of dissimilarity (Gower, 1985) was used to obtain a distance matrix with the R package ‘FD’ v. 1.0-12 (Laliberté *et al.*, 2014). The distance matrix was subjected to principal coordinates analysis (PCoA). The results obtained were subsequently subjected to constrained PCoA [distance-based redundancy analysis (db-RDA); Legendre and Anderson, 1999], where the PCoA scores of the localities containing the environmental information were linked to the ploidy levels (constraints). Calculations were performed with the R package ‘vegan’ v. 2.2-1 (Oksanen *et al.*, 2013). Given that the overall test was significant, pairwise tests between ploidy levels with reduced matrices containing only populations of the ploidy levels being tested were conducted to clarify which of the cytotypes are different from each other regarding environmental conditions. The significance level of the db-RDA was assessed by an ANOVA-like permutation test by which the original environmental matrix was randomly permuted 999 times and the results were compared with the real model (original environmental matrix). The Bonferroni correction was applied for multiple tests ($\alpha = \text{approx. } 0.001$). To identify the environmental variables that differ most between pairs of cytotypes from those significant models, we correlated each environmental variable value with the values of population scores in the first axis of the models: each one containing a pair of cytotypes. Pearson correlations were used for this purpose, and good correlation was considered when $|r| > 0.5$.

RESULTS

Genome size and cytotype diversity in *Veronica subsect. Pentasepalae*

Ploidy level estimates were obtained for 668 individuals from 207 localities. Genome size values obtained for individuals of *V. subsect. Pentasepalae* were arranged in four groups, corresponding to four different ploidy levels: 2x, 4x, 6x and 8x (Supplementary Data Fig. S1). Nuclear DNA contents (1C-values) ranged between 0.70 and 1.03 pg for diploids, 1.13 and 1.58 pg for tetraploids (with approx. 1.72 pg for a tetraploid population from north Spain), 1.71 and 2.06 pg for hexaploids, and 2.24 and 2.88 pg for octoploids (considering only measurements with CVs <5 %). The genome size of tetraploids was not double that of diploids, nor were hexaploids or octoploids three or four times, respectively, the

size of diploids (Supplementary Data Table S1; Fig. S1). The monoploid genome size (1Cx-value; Greilhuber et al., 2005) of tetraploids was 17.86 % lower than that of diploids, and 25.00 and 26.20 % lower in the case of hexaploids and octoploids, respectively (Supplementary Data Table S2). Most of the populations included a single cytotype (194 populations; 93.72 %), while there were differences in ploidy level among individuals collected at 13 (6.28 %) populations (Table 1). The latter are hereafter referred as mixed-ploidy populations. Among single ploidy populations (93.72 % of the total), diploid was the most frequent cytotype (49.76 %), followed by octoploids (18.84 %) and hexaploids (18.36 %), and finally by tetraploids (6.76%).

Distribution of cytotypes

Populations of *V. subsect. Pentasepalae* showed a clear geographic structuring of cytotypes (Fig. 1). Diploids were preferentially found at low latitudes, except for a few populations extending to central Europe. Tetraploids represented the rarest cytotype and were found in specific locations in the Balkan Peninsula, Spain, France and Algeria. Hexaploids were distributed towards the east and south-east of Europe, and octoploids were mainly found at higher latitudes in central Europe, with only some populations in the north of Spain and the Balkan Peninsula. The Balkan Peninsula exhibited the highest diversity in ploidy levels, and it is the only area where all cytotypes were found.

Despite low intra-population sampling, some mixed-ploidy populations were found (Table 1; Fig. 1). Seven of them were found in the Balkans and were composed of $2x + 4x$ (three), $2x + 6x$ (two), $4x + 6x$ (one) and $2x + 4x + 8x$ (one) cytotypes. Furthermore, six mixed-ploidy populations were found scattered in the remaining area, in north-eastern Algeria ($2x + 4x$), north-western Spain ($2x + 4x$), Austria ($2x + 6x$), Slovakia ($6x + 8x$), southern France ($2x + 4x$) and northern Spain ($4x + 8x$). No uneven ploidy levels were detected.

TABLE 1. Cytotype diversity found within sampled populations of *Veronica subsect. Pentasepalae*

Number of ploidy levels per population	Number of populations/frequency
1	194/93.72 %
2	12/5.79 %
3	1/0.48 %
Total	207
Populations containing one ploidy level	
$2x$	103/49.76 %
$4x$	14/6.76 %
$6x$	38/18.36 %
$8x$	39/18.84 %
Populations containing different ploidy levels	
$2x + 4x$	6/2.90 %
$2x + 6x$	3/1.45 %
$4x + 6x$	1/0.48 %
$4x + 8x$	1/0.48 %
$6x + 8x$	1/0.48 %
$2x + 4x + 8x$	1/0.48 %

Percentages are always calculated relative to the total number of sampled populations.

Relationships between environmental variables and ploidy level distribution

Univariate analysis From the 33 environmental variables analysed, 14 variables related to temperature, precipitation, vegetation index, potential natural vegetation and biogeographical region were found to be significantly associated with the distribution of ploidy levels (Supplementary Data Table S4; Figs S2, S3 and S4). Diploids and tetraploids occur in areas characterized by higher values of isothermality (Bio 3) in comparison with hexaploids (Supplementary Data Fig. S2A), whereas hexaploids occur in areas characterized by higher temperature seasonality (Bio 4) compared with the areas where diploids, tetraploids and octoploids are present (Supplementary Data Fig. S2B). Diploids occur in areas where the maximum temperature of the warmest month (Bio 5) is higher than in those places occupied by octoploids (Supplementary Data Fig. S2C), and where the minimum temperature of the coldest month (Bio 6), the mean temperature of the driest quarter (Bio 9) and the mean temperature of the coldest quarter (Bio 11) are higher than in those locations where hexa- and octoploids are found (Supplementary Data Fig. S2D–F). Locations occupied by tetra-, hexa- and octoploids are characterized by higher values of precipitation of the driest month (Bio 14) in comparison with those where diploids are present (Supplementary Data Fig. S2G). Hexa- and octoploids are found in locations where the precipitation of the warmest quarter (Bio 18), as well as the average and range values of NDVI (normalized difference vegetation index) are higher than in those areas occupied by diploids (Supplementary Data Fig. S2H, I, L). Likewise, values of the standard deviation of NDVI are higher in areas occupied by hexa- and octoploids than in those occupied by di- and tetraploids (Supplementary Data Fig. S2J). Regarding NDVI annual maximum, significant differences were found between areas occupied by diploids and hexaploids (Supplementary Data Fig. S2K).

Finally, the distribution of cytotypes was found to be significantly affected by the potential vegetation type and biogeographic region (Supplementary Data Fig. S3). Diploids occur in areas with different potential types of vegetation, but they tend to be more frequent in thermophilous mixed deciduous broadleaved forests, with 35 % of populations occurring in this potential vegetation type, followed by potential Mediterranean sclerophyllous forests and scrub (17 %) and potential mesophytic broadleaved deciduous and mixed broadleaved/conifer forests (17 %). Tetraploids are preferentially found in areas with potential thermophilous mixed deciduous broadleaved forests (43 %) and potential mesophytic broadleaved deciduous and mixed broadleaved/conifer forests (35 %). Hexaploids and octoploids are mainly found in potential mesophytic broadleaved deciduous and mixed broadleaved/conifer forests (65 and 50 %, respectively) and to a lesser extent in potential thermophilous mixed deciduous broadleaved forests (28 and 26 %, respectively). Regarding biogeographical regions, diploids occur preferentially in the Mediterranean region, whereas octoploids are mainly found in alpine regions. Hexaploids are preferentially distributed in the continental region and tetraploids are evenly distributed in the Mediterranean and alpine regions.

Multivariate analysis The overall and paired tests showed that plants of different ploidy levels are ecologically differentiated,

with the largest niche differences between di- and hexaploids (Tables 2 and 3; Fig. 2). Significant differences were also found between di- and octoploids, and between tetra- and hexaploids. In general, diploids occur predominantly in areas characterized by higher isothermality (Bio 3), and higher values of mean temperature of the driest and coldest quarter (Bio 9 and Bio 11, respectively), as compared with those areas where hexaploids and octoploids grow. Tetraploids tend to occupy locations with similar climatic characteristics to that of diploids [i.e. high isothermality (Bio 3) and high values of mean temperature during the driest quarter (Bio 9)]. No significant differences were found between the areas occupied by hexaploids and octoploids, defined by high levels of precipitation both during the warmest quarter (Bio 18) and during the driest month (Bio 14), as well as vegetation index (NDVI) values. Areas occupied by hexaploids were also characterized by higher temperature seasonality (Bio 4) and higher tree coverage (Tree Cover) than those of diploids and tetraploids.

DISCUSSION

Ploidy levels and estimates of genome size in V. subsection Pentasepalae

This study represents the first comprehensive ploidy screening for *V. subsect. Pentasepalae* covering its whole geographic distribution in Europe and North Africa. The results indicate that diploids are the most common cytotype in the group, whereas the tetraploid cytotype is the least frequent. Although previously reported by chromosome counts (Peev, 1972; Strid, 1986), the decaploid level was not detected, which indicates that this cytotype is not frequent in nature.

Genome size values range between 0.70 and 2.88 pg (1C-value), fitting in the middle range of genome sizes known for *Veronica* (Meudt et al., 2015). As found for other groups of the genus, comparison of monoploid genome size values (1Cx-values) obtained for *V. subsect. Pentasepalae* show that the polyploids have experienced genome downsizing with respect to the diploids (Supplementary Data Fig. S1). It is also worth noting that hexaploids and octoploids show stronger genome downsizing and are more widespread than tetraploids, which are the least frequent cytotype within the group. Thus,

TABLE 2. Summary of the multivariate analyses using constrained PCoA (db-RDA) applied to environmental variables recorded in populations of *Veronica* subsect. *Pentasepalae*

Model	Trace (first first axis)	F	P
Overall model	1.212	5.93	<0.001
2x vs. 4x	0.161	1.819	0.047
2x vs. 6x	0.969	11.638	<0.001
2x vs. 8x	0.759	9.441	<0.001
4x vs. 6x	0.384	4.596	<0.001
4x vs. 8x	0.195	2.346	0.016
6x vs. 8x	0.148	1.851	0.046

Significance level was assessed by an ANOVA-like permutation test by which the environmental matrix was randomly permuted 999 times.

P-values in bold are significant after Bonferroni correction ($P < 0.001$).

polyploidy and genome downsizing might be associated with the successful colonization of new habitats in *V. subsect. Pentasepalae*. Although Meudt et al. (2015) found no significant genome downsizing within *V. subsect. Pentasepalae*, these results are not in contradiction to those obtained here. These authors considered DNA loss for a sub-set of our sampling across the whole genus and with respect to the ancestral genome size of the genus *Veronica*, while here genome downsizing is detected for the polyploid taxa within the subsection (Supplementary Data Fig. S1).

The genome size of one population from north-western Spain (Supplementary Data Table S1; population 129) was slightly higher than most of the genome size values of the tetraploid group (Supplementary Data Table S1; Fig. S1). We are, nevertheless, confident that this population is tetraploid. Previous analyses showed an autopolyploid origin of these tetraploids (Martínez-Ortega et al., 2004) from diploids that show a relatively higher genome size (0.96 pg) compared with other diploids from the subsection (0.85 pg). Thus, higher values for those tetraploids are expected. The tetraploid level assigned to these individuals is also based on chromosome counts for other tetraploid individuals from the same area (Martínez-Ortega et al., 2004).

Although the absence of odd ploidies and aneuploidy in *V. subsect. Pentasepalae* could be due to the low intra-population sampling, our results are consistent with the lack of aneuploids or intermediate ploidies in >200 chromosome counts made to date for *V. subsect. Pentasepalae* (Krpáč, 2000; Martínez-Ortega et al., 2004; Albach et al., 2008; chromosome numbers from the literature are available at: https://www.researchgate.net/publication/258769259_cariologia2013; Delgado et al., 2018). Experimental crosses carried out by Scheerer (1949) demonstrated that normal seeds (in number and morphology) were only produced from crosses between plants of the same ploidy level. Thus, as previously suggested for other plant species (Duchoslav et al., 2010), it is possible that intermediate ploidies or aneuploids have never been detected within *V. subsect. Pentasepalae* due to their non-viability. For example, gametic barriers are usually strong in inter-cytotype crosses and may include pollen–pistil interactions (e.g. Husband et al., 2002) and/or or genomic imprinting (e.g. unbalanced ratio of maternal to paternal genomes in endosperm tissue; Grossniklaus et al., 2001). The absence of triploids and other odd ploidies should be further confirmed by analysing a higher number of individuals per population and the reproductive interactions between ploidy levels to evaluate whether strong reproductive (pre- or post-zygotic incompatibilities) or ecological barriers (caused by habitat differentiation) exist among cytotypes as is the case for other species (Lumaret et al., 1987; Lumaret and Barrientos, 1990; Bretagnolle and Thompson, 1996; Thompson, 2005; Kolář et al., 2009; Husband et al., 2016).

Patterns of cytotype ecogeographic distribution

Cytotypes of *V. subsect. Pentasepalae* show a general pattern of geographic distribution at a broad scale, with diploids preferentially distributed in southern Europe and North Africa, and octoploids in central and northern areas of Europe (Fig. 1A). Additionally, hexaploids are geographically restricted

TABLE 3. Survey of environmental variables which are best correlated ($|r| > 0.5$) with occurrence of ploidy levels in constrained PCoA (db-RDA) applied to environmental variables recorded in populations of *Veronica subsect. Pentasepalae*

2x (-) vs. 6x (+)			2x (-) vs. 8x (+)			4x (-) vs. 6x (+)		
Code	Variable	<i>r</i>	Code	Variable	<i>r</i>	Code	Variable	<i>r</i>
Bio 18	Precipitation of warmest quarter	0.78	Bio 18	Precipitation of warmest quarter	0.86	Bio 4	Temperature seasonality	0.67
NDVI ran	Vegetation index (range)	0.77	NDVI ran	Vegetation index (range)	0.75	NDVI ran	Vegetation index (range)	0.67
NDVI av	Vegetation index (average)	0.63	NDVI av	Vegetation index (average)	0.67	NDVI av	Vegetation index (average)	0.57
Bio 4	Temperature seasonality	0.58	Bio 14	Precipitation of driest month	0.61	Tree cover	Tree cover	0.55
Tree cover	Tree cover	0.55	Bio 3	Isothermality	-0.61	Bio 9	Mean temperature of driest quarter	-0.57
Bio 14	Precipitation of driest month	0.55	Bio 11	Mean temperature of coldest quarter	-0.67	Bio 3	Isothermality	-0.60
Bio 9	Mean temperature of driest quarter	-0.64	Bio 9	Mean temperature of driest quarter	-0.71			
Bio 11	Mean temperature of coldest quarter	-0.65						
Bio 3	Isothermality	-0.70						

Within each analysis, variables show the positive or negative correlation with the first axis. The sign of correlation coefficients corresponds to the position of the respective ploidy level along the first axis within each analysis.

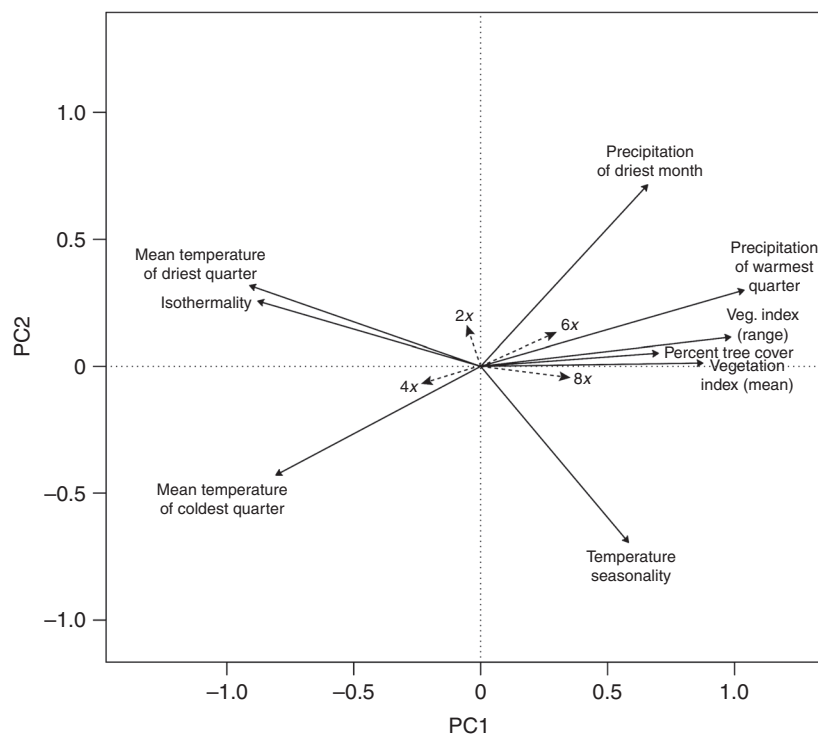


FIG. 2. Constrained PCoA (db-RDA) showing the first and the second axes. Vectors of the environmental variables that were found to be correlated with the distribution of ploidy levels at $|r| > 0.5$ were used as supplementary data to help in the interpretation of the ordination.

to eastern and south-eastern Europe, while tetraploids are restricted to specific areas without clear biogeographic distribution patterns. The complex geographic distribution of cytotypes within *V. subsect. Pentasepalae* can be explained by different mechanisms: ecological differentiation of plants with different ploidy level, biogeographic history associated with the

Quaternary glaciations and other local-level processes. These processes, that are not mutually exclusive and might have acted in combination, are discussed in detail below.

Ecological differentiation among ploidy levels Univariate and multivariate analyses demonstrate a significant association

between the distribution of cytotypes and climatic variables related to temperature and precipitation, as well as vegetation variables (Fig. 2; Tables 2 and 3; Supplementary Data Table S4, Figs S2, S3A and S4A). Consistently, the values of these variables are congruent with the association of ploidy levels with specific biogeographical regions (Supplementary Data Figs S3B and S4B).

Diploids are associated with higher values of isothermality and temperature, whereas hexa- and octoploids are positively correlated with higher values of precipitation and vegetation indexes (Table 3). Niche divergence in hexaploids is also determined by tree coverage and high temperature seasonality (Fig. 2; Table 3). Accordingly, diploids are mainly found in the Mediterranean region and hexaploids in the continental region of southern and south-eastern Europe (Supplementary Data Fig. S3B). Octoploids occur mainly in the alpine region, in areas characterized by mild and humid summers with high vegetation coverage (Fig. 2; Supplementary Data Fig. S3B; Table 3). The occurrence of polyploids in colder and wetter environments than those of their diploid progenitors has been demonstrated in other plant groups, such as *Primula* sect. *Aleuritia* or *Tolmiea*, among others (Martin and Husband, 2009; Theodoridis et al., 2013; Visger et al., 2016; Muñoz-Pajares et al., 2018). These results contrast with the idea that polyploids are more tolerant to water stress (Levin, 2002; Li et al., 2009; Manzaneda et al., 2012; Paule et al., 2017) and show higher fitness than diploids in drought conditions (Ramsey, 2011). Due to larger cell sizes, polyploids usually have larger but fewer stomata, which reduce transpiration rates and water loss, enabling polyploids to inhabit dry environments (Li et al., 2009; Liu et al., 2011). However, this is not always the case, as found in *Tolmiea*, where stomata size and number did not differ between cytotypes (Visger et al., 2016). Within *V.* subsect. *Pentasepalae*, previous studies have shown that stomata were larger in tetraploid than in diploid plants (Brandt, 1961), but this study was restricted to a couple of taxa, and more exhaustive analyses should be performed including all the species from the subsection. Wider xylem diameters have also been found to improve water use efficiency in some polyploids (Pockman and Sperry, 1997; Maherali et al., 2009), but no information about this character is available in *Veronica* yet. Physiological and anatomical features related to drought stress need to be investigated in *V.* subsect. *Pentasepalae*, preferably coupled with reciprocal transplant studies to demonstrate adaptation of diploids to drier regions.

With respect to tetraploids, our analyses demonstrated that they are ecologically differentiated only with respect to hexaploids. Tetraploids occur mainly in two biogeographical regions: the Mediterranean (e.g. western coast of the Balkan Peninsula) and the alpine region (e.g. Pyrenees and Dinaric Alps) (Supplementary Data Figs S3B and S4B), which are the regions where diploids and octoploids, respectively, are mainly found. This overlap in the biogeographical region might explain the lack of differences in the ecological preferences of diploids vs. tetraploids, and tetraploids vs. octoploids. Despite the common assumption that WGD confers competitive advantages to polyploids (Maceira et al., 1993; te Beest et al., 2012; Thompson et al., 2014), this is not always the case. For instance, tetraploids of *Ranunculus kuepferi* Greuter & Burdet are outcompeted by diploids in warmer conditions (Kirchheimer

et al., 2016). Within *V.* subsect. *Pentasepalae*, the ecological requirements of high polyploids are different when compared with low ploidy cytotypes in the subsection. Moreover, tetraploids are found in a much smaller number of potential vegetation types than diploids and octoploids. It might be possible that tetraploids exhibit small advantages over diploids, and they are outcompeted by hexa- or octoploids. Further experiments comparing fitness rates of the different ploidy levels will be necessary to fully demonstrate whether the correlation found among environmental parameters and the distribution patterns of cytotypes observed is due to adaptive advantages of polyploids.

Our results demonstrate that polyploidy has been able to counteract phylogenetic niche conservatism in a recently diverged diploid–polyploid complex composed of numerous closely related species. Similar observations have been found in other polyploid species groups within particular genera of angiosperms (Brochmann and Elven, 1992; Theodoridis et al., 2013; Paule et al., 2017). Interestingly, subgenus *Pentasepalae* has supposedly evolved in xeric habitats from the Middle East, thus suggesting that the ancestors of *V.* subsect. *Pentasepalae* were adapted to xeric conditions (Albach et al., 2004). Polyploidization, alone or together with hybridization, could have enhanced environmental adaptations to cold and moist environments, which suggests that the ecological differentiation of cytotypes observed in the group might be explained by the adaptive evolutionary scenario.

Biogeographical history Species divergence at the diploid level within *V.* subsect. *Pentasepalae* has been estimated to have started by the end of the Pliocene–early Pleistocene (Meudt et al., 2015), with polyploids arising later, although no exact date for the origin of the polyploids is available yet. Thus, Quaternary glaciations have probably had an impact on species diversification as well as on their current distribution. In fact, the observed distribution of cytotypes in *V.* subsect. *Pentasepalae* partially conforms to patterns of glacial recolonization, with diploids or lower ploidy levels mainly occupying southern refugial areas and octoploids being more frequent in northern areas (Fig. 1). This south to north distribution pattern has been frequently shown in other plant polyploid complexes in Europe (e.g. van Dijk and Bakx-Schotman, 1997; Luttikhuisen et al., 2007; Balao et al., 2009; Bardy et al., 2010; Theodoridis et al., 2013; Kolář et al., 2016), while the opposite pattern seems to be quite uncommon (but see Durand, 1963). It has been recently proposed that at the time of the LGM, the southern extent of continuous permafrost (i.e. occurring everywhere in a given region) reached approximately the latitudes 44–47°N in Europe (Vandenbergh et al., 2014). Within *V.* subsect. *Pentasepalae*, octoploids are the main cytotype that is, nowadays, distributed in areas predicted to have been under permafrost during the LPM towards the end of the last ice age (Fig. 1). Thus, the permafrost extent, which is obviously related to Quaternary climatic oscillations, is probably also crucial to understand the geographical patterns observed nowadays for groups of plants distributed in Europe. The results obtained here suggest that octoploids of *V.* subsect. *Pentasepalae* might have been successful at establishing new populations in lowland regions that are assumed to have experienced continuous permafrost conditions (i.e. a mean annual air temperature of -8°C

or lower) during the LPM, and in mountain areas that became available after glacial retreat (Fig. 1). The fact that octoploids of *V. subsect. Pentasepalae* seem to be better colonizers in these environments could be related to the effects of WGD (i.e. genetic and physiological changes). This pattern has been found in other plant species such as *Achillea borealis* Bong. (Ramsey, 2011), among others (Brochmann et al., 2004; Wefferling et al., 2017). While many studies have demonstrated an influence of the ice retreat on the distribution patterns of polyploids (see references in Ramsey and Ramsey, 2014; Rice et al., 2019), more emphasis should be put on permafrost coverage as a driver that shaped the biogeographic patterns of polyploid groups in temperate regions of Europe.

Additionally, Quaternary climatic oscillations may have promoted recurrent events of polyploidization or repeated secondary contacts among otherwise geographically isolated taxa of *V. subsect. Pentasepalae* giving rise to homoploid and/or allopolyploid hybrids. The recurrent formation of polyploids has been demonstrated in multiple plant groups (Soltis and Soltis, 1999; McAllister and Miller, 2016), including species of the genus *Veronica* (Albach, 2007; Bardy et al., 2010). Within *V. subsect. Pentasepalae*, recent studies have indicated that octoploid plants from western Europe have probably resulted from multiple polyploidization events involving a tetraploid from western Europe (Padilla-García et al., 2018). A higher genetic variability derived from gene flow among new octoploid populations might have contributed to the successful establishment of these octoploid entities in new environments.

Differential rate of cytotype formation Other processes affecting the mode and frequency of polyploid formation certainly influenced the cytotype distribution patterns of *V. subsect. Pentasepalae*. Thus, a crucial factor in the emergence of new cytotypes of *V. subsect. Pentasepalae* is the production rate of unreduced gametes. Previous studies support the link between environmental stress (i.e. extreme temperature, water stress, nutritional stress or herbivory) and the production of unreduced gametes (Brochmann and Elven, 1992; Ramsey and Schemske, 1998; Ramsey, 2007, Parisod et al., 2010). The presence of octoploids mainly in areas covered by permafrost during the LGM may be linked to increased rates of production of unreduced gametes under climatic fluctuation scenarios (Ramsey and Schemske, 1998; McAllister et al., 2015) which, in turn, would have favoured the recurrent formation of octoploids in the subsection (Padilla-García et al., 2018). It should be pointed out that a higher production of unreduced gametes under environmental stress might be common in all cytotypes. Consequently, it is worth noting, once again, that octoploids might have some ecological advantage over other cytotypes that allowed them to colonize central and northern Europe.

Mixed-ploidy populations

Despite the small intra-population sample size, we still observed cytotype variation in 6.27 % of populations (Table 1). The possibility that our results have underestimated the rate of cytotype admixture cannot be neglected since only three individuals per population were sampled for most of the

populations studied. However, subsequent analyses carried out in the western Balkans using an average of approx. 20 individuals per population found similar within-population homogeneous ploidy levels (N. López-González et al., University of Salamanca, Spain, unpubl. res.). According to the MCE model, the establishment of a new cytotype within a population is frequency dependent (Levin, 1975). Thus, the coexistence of different cytotypes within a population depends on, among other factors, the development of reproductive barriers, different microhabitat requirements, recurrent polyploidization events (through unreduced gamete production) or differential competitiveness among cytotypes counteracting the MCE (Levin, 1975; Husband and Sabara, 2004; Thompson, 2005; Collins et al., 2011; Husband et al., 2016; Čertner et al., 2017). Vegetative propagation is an important mode of expansion for plants of *V. subsect. Pentasepalae*. All species have a very branched rhizome that produces numerous adventitious roots and new vegetative shoots (Watzl, 1910). In addition, the production of unreduced gametes in sub-optimal ecological conditions might be an additional factor favouring the local coexistence of ploidy levels. It would be very interesting to explore to what extent and under which circumstances these factors are able to counteract MCE in *V. subsect. Pentasepalae*.

Five of the 13 mixed-ploidy populations found in our study are located in the western Balkans. The Balkan Peninsula, at the crossroads of several major floras, represents one of the most important hotspots of biodiversity in Europe (Griffiths et al., 2004). According to morphology, two of these mixed-ploidy populations (Supplementary Data Table S1; populations 5 and 114) consist of putative hybrids or taxonomically unresolved entities that may have resulted from secondary contacts among diverging lineages. They are found in the southern Dinaric Alps, an area of special topographic complexity that acted as a Quaternary microrefugium for plant species (e.g. Surina et al., 2011; Kutnjak et al., 2014). Thus, these mixed-ploidy populations are assumed to be the result of repeated admixture of lineages during the Quaternary climatic oscillations up until today, as seen for other morphologically intermediate populations of *V. subsect. Pentasepalae* from the western Balkans (Padilla-García et al., 2018). In contrast, spontaneous polyploidization (i.e. primary contact) might be responsible for the other two mixed-ploidy populations (Supplementary Data Table S1; populations 22 and 24), which have been demonstrated to have autopolyploid origins (Padilla-García et al., 2018). Altogether, these findings point to the existence of primary and secondary contact zones mainly in the Balkan Peninsula for *V. subsect. Pentasepalae*.

CONCLUSIONS

This work provides the first comprehensive ploidy screening within *V. subsect. Pentasepalae* at a broad scale and represents a solid baseline for future studies on polyploid evolution in this subsection. Four ploidy levels (2x, 4x, 6x and 8x) at different frequencies have been found in the group. Polyploids exhibit genome downsizing, suggesting that this process might also play a role in the evolution of the group and its expansion to new or alternative ecological niches. The geographic distribution of cytotypes can be explained by ecological differentiation

mainly between diploids and octoploids, and diploids and hexaploids, suggesting that high polyploids might cope better in colder and wetter regions. Our findings also indicate that phylogenetic niche conservatism has been overcome in a diploid–polyploid complex composed of closely related species. The extent of permafrost during the Quaternary glaciations, as well as a high production rate of unreduced gametes associated with present or historical stressful climatic conditions might also have influenced the frequency and distribution patterns of cytotypes in *V.* subsect. *Pentasepalae*. In particular, the distribution of octoploids could be related to the LPM extension. Cytotype coexistence is mainly detected in the western Balkans, indicating that it is an important contact zone among cytotypes. Future work should focus on these contact areas at a finer scale including higher intra-population sampling, reciprocal transplants, common garden experiments and synthetic polyploids, together with genomic analyses, to confirm if ecological differentiation of cytotypes is effectively caused by polyploidy.

SUPPLEMENTARY DATA

Supplementary data are available online at <https://academic.oup.com/aob> and consist of the following. **Figure S1**: observed and expected genome size values in *Veronica* subsect. *Pentasepalae*. **Figure S2**: box plots of the 12 quantitative environmental variables that are significantly associated with the distribution of cytotypes. **Figure S3**: relative frequencies of cytotypes in relation to the three most common vegetation types and the three most common biogeographical regions. **Figure S4**: distribution of cytotypes in relation to vegetation type, and biogeographical regions. **Table S1**: details of individuals of *Veronica* subsect. *Pentasepalae* included in this study. **Table S2**: mean and standard deviation of genome sizes for each ploidy. **Table S3**: environmental variables used in univariate and multivariate analyses. **Table S4**: associations between ploidy levels and environmental variables in populations of *Veronica* subsect. *Pentasepalae* found by the univariate analyses.

FUNDING

This work was supported by the Spanish Ministerio de Economía y Competitividad [projects CGL2009-07555 and CGL2012-32574]; the Spanish Ministerio de Ciencia e Innovación [PhD grants AP2008-03434 to B.R.A. and AP2010-2968 to N.L.G.]; and the University of Salamanca [PhD grant to N.P.G. co-financed by Banco Santander].

ACKNOWLEDGEMENTS

We thank A. Abad de Blas, S. Andrés, S. Barrios, A. Juan, V. Lucía, J. Peñas, D. Pinto, E. Rico, T. Romero, M. Santos and all other colleagues who helped to collect material for this study. We are particularly grateful to X. Giráldez who joined us during every field campaign. We thank S. Kempen and D. Pinto for help with flow cytometry. We thank Martin Lysak, Trude Schwarzacher and three anonymous reviewers for their constructive comments and suggestions, which greatly improved this work. M.M.O. and B.R.A. designed the research. M.M.O.,

B.R.A., N.L.G., N.P.G. and L.D. collected samples. B.R.A. and N.L.G. performed ploidy level estimations. M.P. performed statistical analyses. M.P., B.R.A., N.P.G., N.L.G. and L.D. prepared tables and figures. M.M.O. co-ordinated and supervised all these tasks, except for ploidy level estimations that were supervised by S.C., J.L. and D.C.A. N.P.G., B.R.A., M.P. and M.M.O. wrote the manuscript and all authors revised and actively improved the paper.

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