Int. J. Plant Sci. 180(5):000–000. 2019.

1058-5893/2019/18005-00XX$15.00 DOI: 10.1086/703127

GENOME SIZE VARIATION IN A HYBRIDIZING DIPLOID SPECIES COMPLEX IN *ANACYCLUS* (ASTERACEAE: ANTHEMIDEAE)

Alicia B. Agudo,* Rubén Torices,†,‡ João Loureiro,‡ Sílvia Castro,‡,§ Mariana Castro,‡ and Inés Álvarez[1,*]

*Departamento de Biodiversidad y Conservación, Real Jardín Botánico, Consejo Superior de Investigaciones Científicas, Plaza de Murillo 2, Madrid 28014, Spain; †Area of Biodiversity and Conservation, Universidad Rey Juan Carlos, Tulipán s/n, Móstoles, Madrid E-28933, Spain; ‡Department of Life Sciences, Centre for Functional Ecology, University of Coimbra, Calçada Martim de Freitas, Coimbra 3000-456, Portugal; and §Botanic Garden of the University of Coimbra, Calçada Martim de Freitas, Coimbra 3000-456, Portugal

Editor: Oscar Rocha

Premise of research. Interspecific hybridization was hypothesized to explain the remarkable floral phenotype variation found in overlapping areas of distribution for several *Anacyclus* species. We aimed to investigate genome size in natural populations of three diploid *Anacyclus* species with special emphasis in their contact areas to explore patterns of variation as additional evidence supporting current hybridization in these areas.

Methodology. Flow cytometry was used to estimate the genome size of 564 individuals of the species complex of *A. clavatus*, *A. homogamos*, and *A. valentinus* from 30 sites. Additionally, genome size variation of 173 first-generation synthetic hybrids between these three species was also studied and compared with the estimates obtained in sympatric sites.

Pivotal results. Differences in genome size between *A. clavatus* and *A. valentinus* were significant in nonoverlapping areas of species distribution, whereas in overlapping areas, the variation increased, preventing a clear differentiation between species. In sympatric sites of *A. clavatus* and *A. valentinus*, individuals with intermediate genome sizes between them were also observed and were significantly similar to those obtained from the first-generation experimental hybrids between these species. Genome sizes of *A. clavatus* and *A. homogamos* did not differ enough to allow discrimination between these species.

Conclusions. The patterns of genome size variation observed in sympatric populations of *A. clavatus* and *A. valentinus* support the occurrence of current gene flow between these species and the existence of contact areas in overlapping distribution areas where phenotypic and genomic variation increases.

Keywords: Compositae, flow cytometry, genome evolution, homoploid hybridization.

Online enhancement: supplemental appendix.

Introduction

Reproductive isolation and ecological differentiation between hybrids and parental lines leading to homoploid hybrid speciation (i.e., speciation via hybridization without a change in chromosome number) has been considered rare in plant evolution (Rieseberg 1997; Abbott et al. 2013; Yakimowski and Rieseberg 2014). However, the existence of gene flow and homoploid hybrid swarms between closely related species seems to be relatively common (Nieto Feliner et al. 2017). In overlapping distribution areas between species that may hybridize, the existence of large phenotypic variation together with genetic diversification may precede adaptive radiation and speciation (Abbott et al. 2013; Seehausen 2013; Yakimowski and Rieseberg 2014; Abbott 2017).

Although homoploid hybrid speciation may be evidenced by genetic markers (Arnold et al. 1991; Rieseberg 1991; James and Abbott 2005; Pan et al. 2007; Sherman and Burke 2009; Brennan et al. 2012), studies of cytogenetics, reproductive biology, and ecology of the involved species are fundamental to understanding the mechanisms behind the speciation process. For instance, in *Helianthus* L., different chromosomal rearrangements are involved in the partial reproductive isolation of homoploid hybrids (Lai et al. 2005). Contrarily, in the homoploid hybrid *Iris nelsonii* Randolph, karyotype differences did not contribute substantially to the isolation between this species and its progenitors, and ecological barriers were suggested to be the main determinant for the absence of gene flow (Taylor et al. 2013).

As the amount of nuclear DNA is characteristic of a particular species, this character has been considered increasingly

[1] Author for correspondence; email: ines@rjb.csic.es.

Manuscript received September 2018; revised manuscript received February 2019; electronically published April 26, 2019.

useful in the fields of systematics, ecology, and plant evolution (García et al. 2004; Kron et al. 2007; Loureiro et al. 2010; Greilhuber and Leitch 2013; Suda et al. 2015; Vallejo-Marín and Hiscock 2016; Rey et al. 2017). The advent of more robust and high-throughput techniques such as flow cytometry (FCM) has allowed not only the study of genome size at the population level with the screening of a large number of individuals, but also a more accurate evaluation and interpretation of genome size differences (either absolute or relative) among the analyzed individuals (Vallejo-Marín et al. 2016; Chrtek et al. 2017; Castro et al. 2018; Oberprieler et al. 2018). Cases of homoploid hybridization may particularly benefit from a population-level survey of genome size when differences are sufficient to be detected by current methods (Loureiro et al. 2010).

The evolution of genome size is a highly dynamic process. The major mechanisms responsible for genome changes in homoploid hybrid plants include changes in chromosomal structure and changes in the number of copies of transposable elements (Bennetzen 2002; Leitch and Bennett 2004). The detection of homoploid hybrids using genome size is challenging and, technically, requires that the parental taxa differ sufficiently in genome size (by at least 7%; see Loureiro et al. 2010). Genome size studies focusing on homoploid hybrid speciation or on the contact zones of homoploid hybrids are still scarce. In plants, some of those studies report intermediate but nonoverlapping genome sizes between the hybridizing species (Jeschke et al. 2003; Trucco et al. 2005, 2006), whereas others show intermediate overlapping genome size values (Šiško et al. 2003; Mahelka et al. 2005; Bennert et al. 2011). Still, in other cases, the genome size of the hybrids is closer to that of the progenitor species with smaller genome (Bureš et al. 2004), whereas in others the established homoploid hybrid species present even more nuclear DNA content than the parents, possibly indicating a positive selection for this trait in the habitats where they occurred (Baack et al. 2005). Indeed, correlations between intraspecific genome size variation and type of habitat were already observed in both diploid (Kolář et al. 2009) and polyploid hybrids (McIntyre 2012), suggesting a potential adaptive role of genome size in these populations.

Anacyclus L., a genus belonging to the Anthemideae tribe of the Asteraceae, is composed of nine species of mostly annual herbs, predominantly distributed in anthropic western Mediterranean habitats (Humphries 1979; Vitales et al. 2018). The species *A. clavatus* (Desf.) Pers., *A. valentinus* L., and *A. homogamos* (Maire) Humphries present distinct floral phenotypes and adjacent distributions, where sympatric populations and individuals showing intermediate floral phenotypes can be observed (fig. 1). Hybridization between these three species was considered the cause of the existence of individuals with intermediate floral characters (mainly related to the length of ray florets; Humphries 1979, 1981). It was documented that the artificial crosses between the self-incompatible species *A. clavatus*, *A. homogamos*, and *A. valentinus* were highly successful and produced hybrids with intermediate morphologies as well (Humphries 1981). Several studies focused on the cytogenetics and reproductive biology of *Anacyclus* (Nagl and Ehrendorfer 1974; Schweizer and Ehrendorfer 1976; Humphries 1981) reported remarkable differences in nuclear DNA content among some species (from 9.58 pg in *A. homogamos* to 16.04 pg in *A. radiatus* Loisel.), whereas the somatic chromosome number ($2n = 2x = 18$) was invariant. Apart from this, little is known about the relationships among these species. The most complete phylogenetic analyses included *Anacyclus* in the tribe Anthemideae within a clade integrated by several genera of the subtribes Anthemidinae and Matricariinae (Oberprieler 2004; Oberprieler et al. 2007, 2009; Vitales et al. 2018). Although in these phylogenies there is no strong support for the relationships between *A. clavatus* and *A. valentinus*, they seem to be more closely related to each other than either of them is to *A. homogamos*. However, to document gene flow between species, a more exhaustive sampling and population genetic markers are needed. The use of microsatellite markers developed for *A. clavatus* (Agudo et al. 2013) allowed for the documentation of the occurrence of current hybridization between *A. clavatus* and *A. valentinus* in sympatric sites (Agudo 2017). Furthermore, other studies focused on the 45S rDNA site-number variation in these species also supported the existence of current hybridization in their overlapping areas of distribution (Rosato et al. 2017).

In the present study, our goal was to investigate patterns of genome size variation in natural populations of *A. clavatus*, *A. homogamos*, and *A. valentinus* in congruence with the hypothesis of current hybridization between these species in their contact areas. To achieve this, genome size was estimated inside and outside overlapping areas, including sympatric sites, and compared with synthetic first-generation (F_1) hybrids.

Material and Methods

Study System

This study is focused on the annual species *Anacyclus clavatus*, *A. homogamos*, and *A. valentinus*, which differ mainly in the type of peripheral florets in the capitulum and their morphology (Humphries 1979; Bello et al. 2013; fig. 1). The species delimitation follows Humphries's taxonomic treatment with few modifications based on the revision of the genus in *Flora iberica* (Álvarez 2019). Following this criterion, *A. clavatus* presents rayed capitula formed by around eight to 15 peripheral female flowers that display white ligules of 0.5–1.5 cm in length (rays; fig. 1*A*). By contrast, in *A. valentinus*, the capitula are discoid (nonrayed) because the peripheral female flowers present very short white or yellow ligules that do not form rays (i.e., up to 0.3 cm) and are usually hidden behind the involucral bracts (fig. 1*B*). *Anacyclus homogamos* also shows discoid capitula, but in this case all flowers are tubular and bisexual (fig. 1*C*). The discoid appearance of the capitula in *A. valentinus* can lead to confusion with *A. homogamos*, unless detailed observations on peripheral florets are made (fig. 1*B*, 1*C*). These three species grow in similar anthropogenic disturbed habitats. Under this taxonomic criterion, and based on our own field observations of 290 populations plus 586 herbarium specimens, we determined the areas of distribution for these species in the western Mediterranean (fig. 2). *Anacyclus clavatus* occurs all over the Mediterranean Basin; *A. valentinus* is known from coastal areas of the Iberian Peninsula, southern France, northern Morocco, and Algeria; and *A. homogamos* is mainly restricted to the Middle Atlas region in northern Morocco. Based on five herbarium

Fig. 1 Capitula of *Anacyclus* studied species showing different floral phenotypes. *A*, Rayed capitulum in *A. clavatus* with showy white female flowers. *B*, Discoid capitulum in *A. valentinus* where female flowers are not visible. *C*, Discoid capitulum in *A. homogamos* where all flowers are bisexual. *D*, *Clavatus*-like intermediate phenotype with white female flowers shorter than in typical *A. clavatus*. *E*, *Valentinus*-like intermediate phenotype with white female flowers longer than in typical *A. valentinus*. *F*, A new phenotype described in Bello et al. (2013) with yellow tubular female flowers.

specimens (Humphries 1979; Álvarez 2019), *A. homogamos* was also observed in a few scattered sites along the Iberian Mediterranean coast. However, an exhaustive search for this species across all these sites was not successful, and we therefore could not confirm its current existence in the Iberian coast.

The areas of overlapping distribution between the species were confirmed by the presence of sympatric populations (i.e., populations in which at least two species coexist), where it was also common to find individuals with intermediate floral phenotypes between the coexisting species (fig. 1*D*, 1*E*). In addition, populations of different species (i.e., *A. clavatus* and *A. valentinus*) separated by few kilometers were frequently found in these areas, and on some occasions intermediate or new floral phenotypes were also observed (Bello et al. 2013; fig. 1*F*). Although the limits of the overlapping zones are not clearly defined, we arbitrarily considered to be outside an overlapping area when only individuals of a unique species (i.e., unique floral phenotype) were observed within a 30 km radius. Considering this, the whole area of distribution of *A. valentinus* is mostly overlapped with part of the distribution areas of *A. clavatus* and *A. homogamos* (fig. 2).

Plants

A total of 564 individuals from 30 sites were included to assess genome size variation (table B1; tables B1–B4 are available online). Generally, 10–19 individuals per site were analyzed, except for most of the sympatric sites and sites selected for experimental crosses in which a more extensive sampling was performed. *Anacyclus clavatus* was represented in a total of 12 sites (i.e., four sites outside overlapping areas and eight inside overlapping ones, of which four were sympatric sites with *A. valentinus*). In *A. homogamos*, seven out of eight sites were outside overlapping areas with the other two species, while *A. valentinus* was present in 10 sites plus the four in sympatry with *A. clavatus* (fig. 2). Along with our previous field survey, we identified the southern and eastern Iberian coast as hot spots for floral phenotype variation in *Anacyclus* (i.e.,

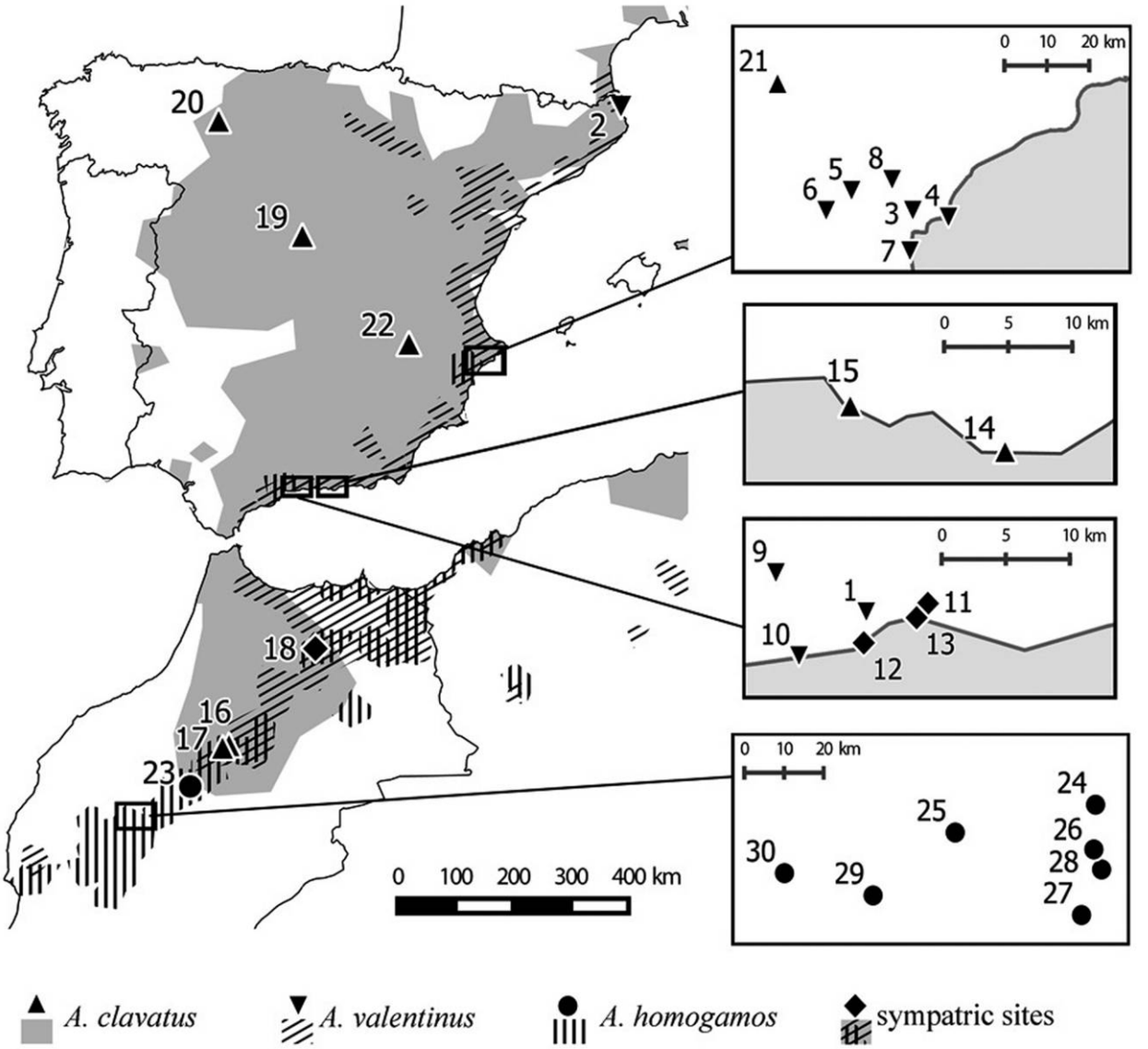

Fig. 2 Distribution of *Anacyclus clavatus*, *A. homogamos*, and *A. valentinus* in the western Mediterranean. Areas indicate potential distributions based on previous taxonomic revisions (Humphries 1979; Álvarez, forthcoming) and our own field observations. Each site included in the study is indicated by a number. Symbols represent the species present in each site. The map was generated using QGIS 2.8 (http://www.qgis.org) ©QGIS Development Team.

high variation in ligule length, number, and color, and presence of semitubular to tubular ligules), and therefore these areas were sampled in more detail (fig. 2).

Six individuals cultivated from seeds collected at sites 2, 14, and 29 were selected as mother plants for the experimental crosses. At flowering, each mother plant was hand pollinated to receive two treatments, one per capitulum: (1) self-incompatibility test (i.e., pollen from the same individual) and (2) interspecific pollination (i.e., pollen from one of the other species, one capitulum per species). All treated capitula were bagged before anthesis until seeds were collected. Viable seeds obtained from these treatments were germinated and cultivated in the greenhouse. Finally, a total of 173 individuals (i.e., 21–38 per treatment, except for the self-incompatibility test in which no viable seeds were obtained) were included for FCM estimation of genome size.

Fresh leaf tissue of all individuals was collected either directly in the field and maintained at 4°C until FCM analysis (usually within 2 or 3 d) or from greenhouse-cultivated plants obtained from seeds collected in natural populations or after the experimental crosses. Field sampling was carried out haphazardly with a minimum distance of 5 m between individuals. For plant cultivation, a minimum set of 30 seeds per population or type of cross were sown. The outermost winged achenes were preferentially used since they show higher and faster germination rates (Torices et al. 2013).

Genome Size Estimations Using Flow Cytometry

Genome size was estimated by FCM following the procedure for nuclear isolation in Galbraith et al. (1983). In brief, nuclei were released by chopping 0.5 cm^2 of fresh leaf material from each individual of *Anacyclus* together with 0.5 cm^2 of leaf tissue of the internal standard, *Vicia faba* L. "Inovec" (2C = 26.9 pg; Doležel et al. 1998), with a razor blade in a petri dish containing 1 mL of the woody plant buffer described in Loureiro et al. (2007; i.e., 0.2 M Tris.HCl, 4 mM $MgCl_2.6H_2O$, 1% Triton X-100, 2 mM EDTA $Na_2.2H_2O$, 86 mM NaCl, 10 mM metabisulfite, 1% PVP-10, pH adjusted to 7.5 and stored at 4°C). The nuclear suspension was filtered through a 30 μm nylon filter. Afterward, nuclei were stained with 50 $\mu g\ mL^{-1}$ of propidium iodide (Fluka, Buchs, Switzerland), and 50 $\mu g\ mL^{-1}$ of RNAse (Fluka) were added to avoid staining of double-stranded RNA. After an incubation period of 3–5 min, samples were analyzed in a Partec CyFlow Space flow cytometer (Partec GmbH, Görlitz, Germany) equipped with a 532-nm green, solid-state laser, operating at 30 mW. After the initial analyses, the amplifier system was set to a constant voltage and gain. Each session, prior to analysis, the instrument's stability and linearity were checked with fluorescent beads. Results were acquired using Partec FloMax 2.4d (Partec) in the form of four graphics: fluorescence pulse integral in linear scale (FL); forward light scatter (FS) versus side light scatter (SS), both in logarithmic (log) scale; FL versus time; and FL versus SS in log scale. To analyze only intact nuclei, the FL histogram was gated using a polygonal region defined in the FL versus SS cytogram. The stability of each sample was controlled by the analysis of the results of the FL versus time; also, the possible presence of secondary metabolites was evaluated in the FL versus SS cytogram. At least 1300 particles were analyzed per *Anacyclus*'s G_1 peak. As a quality criterion, a coefficient of variation (CV) of <5% was achieved in 85% of the samples. When G_1 peaks presented higher CV values, samples were discounted, and a new sample was prepared.

In all cases, two G_1 peaks were obtained, one from the *Anacyclus* sp. nuclei and the other from standard nuclei, with minor to negligible G_2 peaks, and the measurements obtained were reproducible. Two replicated independent runs were made per individual, ensuring that a variation lower than 2% was obtained for each individual. Additionally, in order to discard that genome size variation was due to differences in the amount of secondary compounds among species, populations, and individuals, several combined flow histograms were prepared.

The genome size in mass units (2C in pg; Greilhuber et al. 2005) was assessed using the following formula: sample 2C nuclear DNA content (pg) = (sample G_1 peak mean/*V. faba* G_1 peak mean) × genome size of *V. faba*.

Statistical Analyses

We explored our results following recommendations in Zuur et al. (2010) to ensure that our data met the assumptions of linear modeling. Differences among species, sites, phenotypes within sympatric sites, and experimental crosses were assessed using analysis of variance. Additionally, we evaluated whether the genome size varied in those sites that were located inside or outside overlapping areas. All models were fitted in R (R Core Team 2015). Genome size differences between different levels of each factor were tested using least-square means comparison tests from the lsmeans package, by applying the Tukey's honest significant difference adjustment (Lenth and Hervé 2015).

Results

Genome Size in Natural Sites

A whole analysis of genome size by species showed high variation and complex patterns. Excluding sympatric sites, the genome sizes observed in *Anacyclus valentinus* differed significantly from those of the two other studied species ($n = 392$, $F_{2, 22} = 33.95$, $P < 0.0001$; table 1), whereas differences between *A. clavatus* and *A. homogamos* were not statistically significant. Intraspecific variation was observed in all species (figs. 3, B1; figs. B1, B2 are available online). *Anacyclus clavatus* showed the highest CV, 6.31%, which is almost two- to threefold the variation observed within the other two species, ranging from 8.52 to 11.09 pg/2C. However, in this case, in sites outside overlapping areas (i.e., sites 19–22), the variation decreases dramatically to a CV of 1.71% (table 1). Moreover, the range of values differs significantly than those observed inside overlapping areas ($n = 140$, $F_{1, 69} = 75.62$, $P < 0.0001$; table 1). In *A. valentinus*, the intraspecific variation was also relatively high (CV = 3.58%), but no comparison of outside versus inside overlapping areas was possible since all sites sampled for this species were located inside overlapping areas with *A. clavatus*. Finally, although *A. homogamos* showed the lowest CV (2.16%) of the three species, the values obtained outside versus inside overlapping areas of distribution were still significantly different ($n = 92$, $F_{1, 69} = 16.09$, $P = 0.0001$).

As expected, and resembling their morphological diversity in sympatric sites as a whole (i.e., sites where *A. clavatus* and *A. valentinus*, and/or individuals showing their intermediate phenotypes coexist), genome size variation, measured as CV, was significantly higher than that in other sites within overlapping areas for any of the three species ($n = 28$, $F_{1, 26} = 8.59$, $P = 0.007$; table 1). However, this variation was not evenly distributed across sites (figs. 3, 4). Whereas site 12 achieved the highest overall CV (7.83%) showing the most divergent values, the remaining sympatric sites (i.e., 11, 13, and 18) presented lower variation and values similar to those of the sites in overlapping areas (fig. 4; table B2). Moreover, genome size within site 12 revealed significant differences between species and phenotypes (i.e., similar values were obtained for *A. valentinus* and individuals with intermediate phenotypes vs. those for *A. clavatus*), showing a bimodal distribution. In contrast, in the remaining sympatric sites, genome size values and variation did not differ significantly across species and phenotypes, and their distributions were unimodal (table 1; fig. 4).

Genome Size Variation in Intra- and Interspecific F_1 Crosses

All F_1 synthetic hybrids between *A. clavatus*, *A. valentinus*, and *A. homogamos* showed intermediate genome sizes compared

Table 1

Genome Size (2C Value) Variation Observed In the Species and Areas Studied

Species/phenotypes	Mean ± SD (pg)	Min (pg)	Max (pg)	CV (%)	*n*	Sites
Anacyclus clavatus	9.97 ± .63 A[1]	8.52	11.09	6.31	140	8
	10.53 ± .18 a[2]	10.16	11.09	1.71	61	4
	9.40 ± .23 b[3]	8.52	10.18	3.34	79	4
	9.46 ± .31[4]	8.36	10.72	4.50	54	4
A. homogamos	10.10 ± .22 A[1]	9.57	10.74	2.16	92	8
	10.22 ± .15 a[2]	9.86	10.74	1.47	82	7
	9.93 ± .20 b[3]	9.57	10.21	2.01	10	1
A. valentinus	8.36 ± .30 B[1]	7.70	9.26	3.58	160	10
	9.00 ± .35[4]	8.14	9.90	6.38	46	3
Intermediate	9.03 ± .36[4]	8.03	10.20	6.87	72	3

Note. Different letters after the mean values indicate significant differences ($P < 0.05$) according to the least-squares means comparison test. Lowercase letters are used to show within-species differences between outside and inside overlapping areas, whereas uppercase letters show statistically different mean 2C values between the whole distributions of the three species. Superscript numbers indicate the following: 1 = all sites, excluding the sympatric ones; 2 = sites outside overlapping areas; 3 = sites inside overlapping areas, excluding the sympatric ones; 4 = sympatric sites. Note that only intermediate phenotypes between *Anacyclus clavatus* and *Anacyclus valentinus* were analyzed. Min = minimum value; max = maximum value; *n* = no. individuals analyzed; sites = no. sampled sites.

with their respective parental intraspecific crosses (fig. B2) and were significantly different in each case (i.e., *A. valentinus* × *A. clavatus*, $n = 132$, $F_{3,128} = 168$, $P < 0.0001$; *A. valentinus* × *A. homogamos*, $n = 132$, $F_{3,128} = 64.4$, $P < 0.0001$; *A. clavatus* × *A. homogamos*, $n = 109$, $F_{3,105} = 359.7$, $P < 0.0001$). Mean 2C values of synthetic hybrids were not significantly affected by the direction of the crosses, although some differences in minimum and maximum values and in the ranges of variation were found (table B3; fig. B2). The genome size values of the synthetic hybrids between *A. clavatus* and *A. valentinus* were similar to those of their corresponding natural sympatric sites, values that are intermediate respecting the nonsympatric sites of *A. valentinus* and *A. clavatus* outside overlapping areas (fig. 3).

Discussion

The genome size values estimated were within the range of variation found in other Asteraceae and in other diploid members of the tribe Anthemideae using similar techniques (Garnatje et al. 2001; Bennett and Leitch 2012). Other genome size estimates for *Anacyclus clavatus*, *A. homogamos*, and *A. valentinus* found in the literature fall mostly within the range of our values (i.e., 10.48 pg/2C for *A. clavatus* in Nagl and Ehrendorfer 1974; 9.58 pg/2C for *A. homogamos* in Humphries 1981) or are very similar (i.e., 11.55 pg/2C for *A. clavatus* in Humphries 1981; 7.41 pg/2C for *A. valentinus* in García et al. 2013). The exceptions found were the reported values for one individual of *A. valentinus* from Morocco (10.54 pg/2C) and one cultivated individual of *A. clavatus* (12.71 pg/2C in Humphries 1981) and for one individual of *A. valentinus* from Liège, Belgium (11.40 pg/2C in Nagl and Ehrendorfer 1974). The differences in the methods and sampling used in these previous works, including the methodology and standards used (i.e., Feulgen photometry with *Allium cepa* L. as reference standard) and the samples origin (i.e., cultivated or escaped from cultivation), prevent a valid comparison with our data. In all these cases, both authors reported a chromosome number of 2n = 18 for all samples, and therefore, at least in these cases, trisomy or presence of B chromosomes may be discounted as being responsible for the differences observed. In the case of the *A. valentinus* sample reported by Humphries (1981), its geographic origin within an overlapping area of distribution with *A. homogamos* in Morocco does not allow us to discount a possible hybrid origin of the sample or even a misidentification.

Our results revealed a geographic pattern of variation in genome size across sites and species (fig. 3) that cannot be explained by differences in the number of chromosomes. Although here we did not report chromosome numbers, in a recent work on 45S rDNA site-number variation in *Anacyclus* using a similar sampling (Rosato et al. 2017), only two deviant individuals of *A. valentinus* (2n = 19) from one population were recorded out of 196 individuals from 47 populations in all *Anacyclus* species. In line with this, invariant chromosome number (2n = 18) without exception was observed in a survey of 33 published works (Rice et al. 2015) that reported chromosome counts for 113 individuals of several *Anacyclus* species, comprising 16 individuals of *A. clavatus*, six of *A. homogamos*, and 14 of *A. valentinus*, representing their whole areas of distribution. Other factors, such as the presence of varying amounts of secondary metabolites in each sample that might affect the fluorescence signal, were also discounted as the cause of genome size variation based on the results of combined FCM histograms (fig. 5). As expected, multiple peaks were observed in the combined histograms prepared with individuals of *A. valentinus* and *A. clavatus* (fig. 5*A*), with individuals of *A. clavatus* from sites inside versus outside overlapping areas of distribution (fig. 5*B*) and with progenitors and F_1 synthetic hybrids (fig. 5*C*). Therefore, other causes, such as the number of copies of transposable elements (Bennetzen 2002; Leitch and Bennett 2004), rather than chromosome number

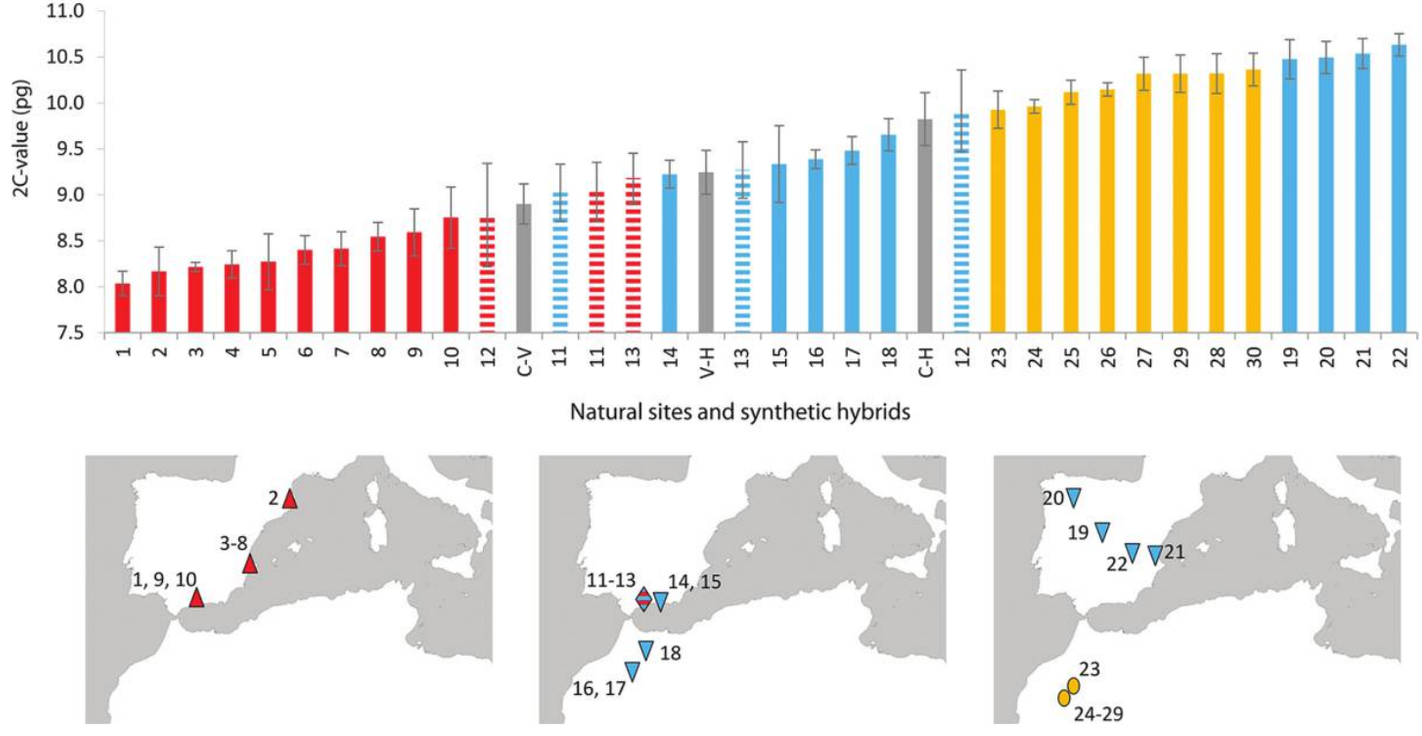

Fig. 3 Mean 2C values from all sites and from F_1 intraspecific experimental crosses. Solid bars represent sites of *Anacyclus clavatus* (blue), *A. homogamos* (yellow), *A. valentinus* (red), and the F_1 experimental crosses (gray) between *A. clavatus* × *A. valentinus* (C-V), *A. valentinus* × *A. homogamos* (V-H), and *A. clavatus* × *A. homogamos* (C-H). Striped bars represent *A. clavatus* (blue) and *A. valentinus* (red) in sympatric sites. Maps represent the location of the populations sampled. The maps were generated with QGIS 2.8 (http://www.qgis.org) ©QGIS Development Team.

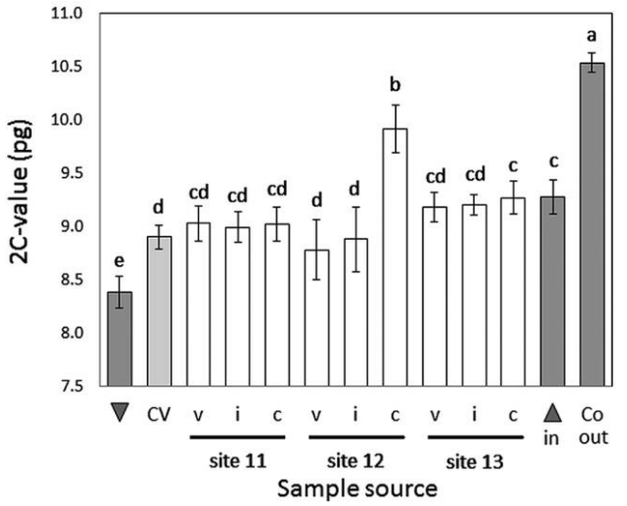

Fig. 4 Mean 2C values from *Anacyclus clavatus* and *A. valentinus* sites and from their F_1 interspecific experimental crosses. White bars represent sympatric sites, dark gray bars are nonsympatric sites, and the light gray bar (CV) includes the F_1 experimental hybrids. Symbols indicate species/phenotypes as follows: *A. clavatus* (c, Co, and upward-pointing arrowhead), *A. valentinus* (v and downward-pointing arrowhead), and intermediate phenotypes between *A. clavatus* and *A. valentinus* (i). Sites inside (in) and outside (out) overlapping areas are independently represented.

and technical issues, should be the responsible for the variation observed.

The geographic pattern of the genome size variation observed might be explained by the purifying and/or positive natural selection of a particular genome size on specific environment. In sympatric sites, current hybridization between these self-incompatible and interfertile species (Humphries 1981) must be the main process involved in the generation of such high variability. Over time, both the phenotype and genome size that provide the best fitness for a particular environment, if any, will be selected. Therefore, in current contact zones a high variation in both phenotype and genome size is expected to be observed, whereas in sites where gene flow between species occurred in the recent past (i.e., potentially all overlapping areas of distribution) such variation may be present, although to a lesser extent. Within the large range of variation in these areas, intermediate values for *A. clavatus* and *A. valentinus* were also found, which were remarkable in sympatric sites (fig. 3). Intermediate genome size values were also observed in sympatric sites of other hybridizing species (Trucco et al. 2005; Bennert et al. 2011). Additionally, the values recorded in sympatric sites of *A. clavatus* and *A. valentinus* were similar to those obtained in the F_1 interspecific synthetic crosses between these two species, which is similar to what has been observed as well in other plant genera such as *Amaranthus* L. (Jeschke et al. 2003; Trucco et al. 2006), *Cucurbita* L. (Šiško et al. 2003), and *Elytrigia* Desv. (Mahelka et al. 2005).

The characterization of contact zones is complex and requires multiple sources of evidence and a deep knowledge of the study system (Abbott 2017). Based on genome size and focusing on sympatric sites between *A. clavatus* and *A. valentinus*, different models of contact zones might be fitted. For example, for sites 11 and 13 (fig. 4), similar genome sizes were found independently of the phenotype (i.e., *clavatus*-like, *valentinus*-like, or intermediate between these two) according to the bounded hybrid superiority model (Moore 1977), whereas for site 12 a significant higher genome size of the *clavatus*-like phenotype might be explained under a tension hybrid zone model (Barton and Hewitt 1985)—although as a whole, a mosaic hybrid zones model (Harrison and Rand 1989) cannot be discounted. Although additional and fine analyses are required to test these models, it is clear that in overlapping areas of species distribution where hybridization might have occurred both phenotypic and genome size variation are found. A similar geographic pattern of variation for the 45S rDNA site-number in *A. clavatus* and *A. valentinus* illustrated current hybridization as the main process involved (Rosato et al. 2017). The contrasting values for *A. clavatus* inside versus outside overlapping areas might be due to a floral phenotype selection in contact zones or to a more successful type of backcrosses to *A. clavatus*, whereas a selection for a genome size is not clear. This might explain the significant lower genome sizes of individuals assigned to *A. clavatus*, which actually may correspond to hybrid lines (i.e., backcrosses to *A. clavatus*) between *A. clavatus* and *A. valentinus* in overlapping areas or, alternatively, to a positive selection of lower genome sizes and *clavatus*-like phenotype in these areas.

As a consequence of current hybridization between *A. clavatus* and *A. valentinus*, different floral phenotypes are produced that might be adaptive and thus influence plant fitness. Detecting hybrid variants involved in ecological adaptation has been considered a challenging task (Yakimowski and Rieseberg 2014). In the specific case of *A. clavatus* and *A. valentinus* crosses, floral traits showed large variation and have been commonly used for species delimitation (Humphries 1981; Bello et al. 2013; Álvarez 2019). In a similar system, two *CYC*-like genes involved in the development of ray flowers in the sunflower family were expressed in the nonrayed species *Senecio vulgaris* L. via natural introgression from the rayed *Senecio squalidus* L. (Kim et al. 2008). The resulting new hybrid variant was adaptive because the rayed *S. vulgaris* became more attractive to pollinators and thus presented a higher outcrossing rate (Abbott and Irwin 1988). In *Anacyclus*, the rayed phenotype is controlled by a similar set of genes (Bello et al. 2017), but unlike *S. vulgaris*, both *A. valentinus* and *A. clavatus* are self-incompatible species. Thus, the study of the adaptive significance of ray expression in the contact zones between these two species provides an opportunity to study the effect of self-incompatibility in the evolution of floral traits in homoploid hybrids.

Gene flow and hybridization might be occurring across overlapping areas of other *Anacyclus* species in Morocco. Although no sympatric sites between *A. homogamos* and *A. clavatus* or between *A. homogamos* and *A. valentinus* were found during our fieldwork, significant differences in genome size values across *A. homogamos* sites together with similarities observed between some natural populations and the F_1 offspring from crosses between *A. clavatus* and *A. homogamos* suggest that hybridization between these species might take place. However, in this case, identification of contact areas based on genome size

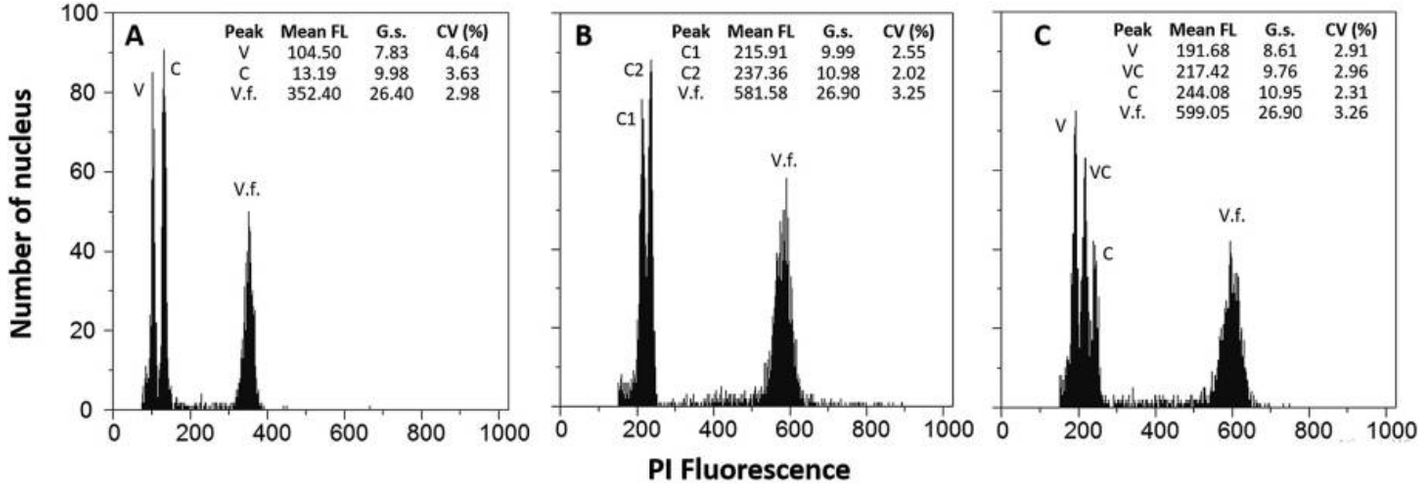

Fig. 5 Combined flow cytometry (FCM) histograms from different *Anacyclus* species and sites. *A*, Combined FCM histogram of one individual of *A. valentinus* (V) from site 3 and one individual of *A. clavatus* (C) from site 20. *B*, Combined FCM histogram of two individuals of *A. clavatus*, one from site 14 (C1) and the other from site 22 (C2). *C*, Combined FCM histogram of one synthetic interspecific hybrid between *A. valentinus* and *A. clavatus* (VC) and its progenitors *A. valentinus* (V) and *A. clavatus* (C). In all FCM histograms, the standard *Vicia faba* "Inovec" (V.f.) was included as reference.

differences is not possible, since genome sizes of the putative parents are quite similar (fig. 3).

The characterization of genome size of *A. clavatus* and *A. valentinus* confirms that the variability observed in floral phenotypes in overlapping areas of distribution is also reflected in terms of genome size and that the pattern of variation is congruent with the existence of current hybridization in contact zones. Although *Anacyclus* species are all diploid with identical chromosome numbers and their hybrids are thus homoploid, the significant differences in genome size between *A. clavatus* and *A. valentinus* allowed for the documentation of the wide range of genome size variation generated by hybridization in sympatric sites. The geographic pattern of distribution for the two divergent genome sizes present in *A. clavatus* suggests that environment might have a role in selection for this feature, although further studies on population genetics and ecological modeling are needed to confirm this hypothesis. Although no direct relationship between genome size, phenotype, and/or hybrid origin may be established individually, our investigations also confirmed the potential usefulness of FCM for a rapid assessment of genome size at the population level in homoploid hybrid systems in which genome sizes of the two progenitors differ significantly.

Acknowledgments

We thank Ana Afonso, Alberto Herrero, Daniel Montesinos, and Lucía de Soto for field and technical assistance and Gonzalo Nieto Feliner for the helpful discussion and suggestions. This work was cofunded by the Spanish Ministry of Science and Innovation (CGL2010-18039) and the European Regional Development Fund, as well as by grants of the Spanish Ministry of Economy and Competitiveness (BES-2011-048197; BVA 2010-0375) to A. B. Agudo and R. Torices, respectively. The Foundation for Science and Technology of Portugal financed the work of S. Castro (IF/01267/2013) and M. Castro (SFRH/BD/89617/2012).

Appendix A

Species, Localities, and Numbers for Voucher Specimens Used in the Present Study and Deposited in MA Herbarium

Anacyclus clavatus (Desf.) Pers. MOROCCO: Khenifra, Tighassaline (917192), Ouaoumana (917193); Taza (917220). SPAIN: Albacete, Chinchilla (917197); Alicante, Villena (917196); León (917195); Madrid, Soto del Real (917194); Granada, Carchuna (917190), Salobreña (917191); Málaga, Algarrobo (917215), Algarrobo Costa (917219), Vélez-Málaga (917217). *Anacyclus homogamos* (Maire) Humphries. MOROCCO: Azilal, Bin El Ouidane (917198); Marrakech, Ait Ben Ammar (917201), Asni (917204), Douar Ouriki (917200), Toufliht (917199), Wawizelt (917205); Ouarzazate, Col du Tichka (917203), Ighrem N'ougdal (917202). *Anacyclus valentinus* L. MOROCCO: Taza (917221). SPAIN: Alicante, Agost (917209), Alicante (792727, 917207), Cabo de Huertas (917208), Novelda (917210), San Vicent de Raspeig (917211); Girona, Castelló d'Empuries (792856); Málaga, Algarrobo (917214), Algarrobo Costa (917218), Benajarafe (917213), Iznate (917212), Torre del Mar (917206), Vélez-Málaga (917216).

Literature Cited

Abbott RJ 2017 Plant speciation across environmental gradients and the occurrence and nature of hybrid zones. J Syst Evol 55:238–258.

Abbott R, D Albach, S Ansell, JW Arntzen, SJE Baird, N Bierne, J Boughman, et al 2013 Hybridization and speciation. J Evol Biol 26:229–246.

Abbott RJ, JA Irwin 1988 Pollinator movements and the polymorphism for outcrossing rate at the ray floret locus in Groundsel, *Senecio vulgaris* L. Heredity 60:295–298.

Agudo A 2017 Evolution in *Anacyclus* L. (Anthemideae, Asteraceae). Analysis of the contact zone between *A. clavatus* (Desf.) Pers. and *A. valentinus* L. PhD diss. Universidad Autónoma de Madrid, Madrid.

Agudo A, FX Picó, I Álvarez 2013 Microsatellite primers in the weedy annual herb *Anacyclus clavatus* (Asteraceae) and four closely related species. Appl Plant Sci 1:1300043.

Álvarez I 2019 *Anacyclus* L. Pages 1777–1788 *in* C Benedí, A Buira, MB Crespo, A Quintanar, E Rico, C Aedo, eds. Flora Iberica XVI (III). Real Jardín Botánico CSIC, Madrid.

Arnold ML, CM Buckner, JJ Robinson 1991 Pollen-mediated introgression and hybrid speciation in Louisiana irises. Proc Natl Acad Sci USA 88:1398–1402.

Baack EJ, KD Whitney, LH Rieseberg 2005 Hybridization and genome size evolution: timing and magnitude of nuclear DNA content increases in *Helianthus* homoploid hybrid species. New Phytol 167:623–630.

Barton NH, GM Hewitt 1985 Analysis of hybrid zones. Annu Rev Ecol Syst 16:113–148.

Bello MA, I Álvarez, R Torices, J Fuertes-Aguilar 2013 Floral development and evolution of capitulum structure in *Anacyclus* (Anthemideae, Asteraceae). Ann Bot 112:1597–1612.

Bello MA, P Cubas, I Álvarez, G Sanjuanbenito, J Fuertes-Aguilar 2017 Evolution and expression patterns of *CYC/TB1* genes in *Anacyclus*: phylogenetic insights for floral symmetry genes in Asteraceae. Front Plant Sci 8:589.

Bennert HW, K Horn, M Kauth, J Fuchs, IS Bisgaard Jakobsen, B Øllgaard, M Schnittler, M Steinberg, R Viane 2011 Flow cytometry confirms reticulate evolution and reveals triploidy in central European *Diphasiastrum* taxa (Lycopodiaceae, Lycophyta). Ann Bot 108:867–876.

Bennett MD, IJ Leitch 2012 Plant DNA C-values database. http://data.kew.org/cvalues/.

Bennetzen JL 2002 Mechanisms and rates of genome expansion and contraction in flowering plants. Genetica 115:29–36.

Brennan AC, D Barker, SJ Hiscock, RJ Abbott 2012 Molecular genetic and quantitative trait divergence associated with recent homoploid hybrid speciation: a study of *Senecio squalidus* (Asteraceae). Heredity 108:87–95.

Bureš P, YF Wang, L Horova, J Suda 2004 Genome size variation in central European species of *Cirsium* (Compositae) and their natural hybrids. Ann Bot 94:353–363.

Castro M, S Castro, A Figueiredo, B Husband, J Loureiro 2018 Complex cytogeographical patterns reveal a dynamic tetraploid-octoploid contact zone. AoB Plants 10:ply012. https://doi.org/10.1093/aobpla/ply012.

Chrtek J, T Herben, R Rosenbaumová, Z Münzbergová, Z Dočkalová, J Zahradníček, J Krejčíková, P Trávníček 2017 Cytotype coexistence in the field cannot be explained by inter-cytotype hybridization alone: linking experiments and computer simulations in the sexual species *Pilosella echioides* (Asteraceae). BMC Evol Biol 17:87.

Doležel J, J Greilhuber, S Lucretti, A Meister, MA Lysák, L Nardi, R Obermayer 1998 Plant genome size estimation by flow cytometry: inter-laboratory comparison. Ann Bot 82(suppl):S17–S26.

Galbraith DW, KR Harkins, JM Maddox, NM Ayres, DP Sharma, E Firoozabady 1983 Rapid flow cytometric analysis of the cell cycle in intact plant tissues. Science 220:1049–1051.

García S, O Hidalgo, I Jakovljević, S Siljak-Yakovlev, J Vigo, T Garnatje, J Vallès 2013 New data on genome size in 128 Asteraceae species and subspecies, with first assessments for 40 genera, three tribes and two subfamilies. Plant Biosyst 147:1219–1227.

García S, M Sanz, T Garnatje, A Kreitschitz, ED McArthur, J Vallès 2004 Variation of DNA amount in 47 populations of the subtribe Artemisiinae and related taxa (Asteraceae, Anthemideae): karyological, ecological, and systematic implications. Genome 47:1004–1014.

Garnatje T, MA Canela, S García, O Hidalgo, J Pellicer, I Sánchez-Jiménez, S Siljak-Yakovlev, D Vitales, J Vallès 2001 GSAD: a genome size in the Asteraceae database. Cytom Part A 79:401–404.

Greilhuber J, J Doležel, MA Lysak, MD Bennett 2005 The origin, evolution and proposed stabilization of the terms "genome size" and "C-value" to describe nuclear DNA contents. Ann Bot 95:255–260.

Greilhuber J, IJ Leitch 2013 Genome size and the phenotype. Pages 323–344 *in* IJ Leitch, J Greilhuber, J Doležel, JF Wendel, eds. Plant genome diversity. Physical structure, behaviour and evolution of plant genomes. Springer, Vienna.

Harrison RG, DM Rand 1989 Mosaic hybrid zones and the nature of species boundaries. Pages 111–133 *in* D Otte, J Endler, eds. Speciation and its consequences. Sinauer, Sunderland, MA.

Humphries CJ 1979 A revision of the genus *Anacyclus* L. (Compositae: Anthemideae). Bull Br Mus Bot 7:83–142.

——— 1981 Cytogenetic and cladistic studies in *Anacyclus* (Compositae: Anthemidae). Nord J Bot 1:83–96.

James JK, RJ Abbott 2005 Recent, allopatric, homoploid hybrid speciation: the origin of *Senecio squalidus* (Asteraceae) in the British Isles from a hybrid zone on Mount Etna, Sicily. Evolution 59:2533–2547.

Jeschke MR, PJ Tranel, AL Rayburn 2003 DNA content analysis of smooth pigweed (*Amaranthus hybridus*) and tall waterhemp (*A. tuberculatus*): implications for hybrid detection. Weed Sci 51: 1–3.

Kim M, ML Cui, P Cubas, A Gillies, K Lee, MA Chapman, RJ Abbott, E Coen 2008 Regulatory genes control a key morphological and ecological trait transferred between species. Science 322:1116–1119.

Kolář F, M Štech, P Trávníček, J Rauchová, T Urfus, P Vít, M Kubeová, J Suda 2009 Towards resolving the *Knautia arvensis* agg. (Dipsacaceae) puzzle: primary and secondary contact zones and ploidy segregation at landscape and microgeographic scales. Ann Bot 103:963–974.

Kron P, J Suda, BC Husband 2007 Applications of flow cytometry to evolutionary and population biology. Annu Rev Ecol Syst 38:847–876.

Lai Z, T Nakazato, M Salmaso, JM Burke, S Tang, SJ Knapp, LH Rieseberg 2005 Extensive chromosomal repatterning and the evolution of sterility barriers in hybrid sunflower species. Genetics 171:291–303.

Leitch IJ, MD Bennett 2004 Genome downsizing in polyploid plants. Biol J Linn Soc 82:651–663.

Lenth RV, M Hervé 2015 lsmeans: least-squares means. R package version 2.15. http://CRAN.R-project.org/package=lsmeans.

Loureiro J, E Rodriguez, J Doležel, C Santos 2007 Two new nuclear isolation buffers for plant DNA flow cytometry: a test with 37 species. Ann Bot 100:875–888.

Loureiro J, P Trávníček, J Rauchová, T Urfus, P Vít, M Štech, S Castro, J Suda 2010 The use of flow cytometry in the biosystematics, ecology and population biology of homoploid plants. Preslia 82:3–21.

Mahelka V, J Suda, V Jarolímová, P Trávníček, F Krahulec 2005 Genome size discriminates between closely related taxa *Elytrigia repens* and *E. intermedia* (Poaceae: Triticeae) and their hybrid. Folia Geobot 40:367–384.

McIntyre PJ 2012 Polyploidy associated with altered and broader ecological niches in the *Claytonia perfoliata* (Portulacaceae) species complex. Am J Bot 99:655–662.

Moore WS 1977 An evaluation of narrow hybrid zones in vertebrates. Q Rev Biol 52:263–278.

Nagl W, F Ehrendorfer 1974 DNA content, heterochromatin, mitotic index, and growth in perennial and annual Anthemideae (Asteraceae). Plant Syst Evol 123:35–54.

Nieto Feliner G, I Álvarez, J Fuertes-Aguilar, M Heuertz, I Marques, F Moharrek, R Piñeiro, et al 2017 Is homoploid hybrid speciation that rare? an empiricist's view. Heredity 118:513–516.

Oberprieler C 2004 On the taxonomic status and the phylogenetic relationships of some unispecific Mediterranean genera of Compositae-Anthemideae. I. *Brocchia*, *Endopappus* and *Heliocauta*. Willdenowia 34:39–57.

Oberprieler C, S Himmelreich, M Källersjö, J Vallès, LE Watson, R Vogt 2009 Tribe Anthemideae Cass. Pages 631–666 *in* VA Funk, ed. Systematics, evolution, and biogeography of Compositae. International Association for Plant Taxonomy, Vienna.

Oberprieler C, S Himmelreich, R Vogt 2007 A new subtribal classification of the tribe Anthemideae (Compositae). Willdenowia 37:89–114.

Oberprieler C, K Konowalik, A Fackelmann, R Vogt 2018 Polyploid speciation across a suture zone: phylogeography and species delimitation in S French *Leucanthemum* Mill. representatives (Compositae–Anthemideae). Plant Syst Evol 304:1141–155.

Pan J, D Zhang, T Sang 2007 Molecular phylogenetic evidence for the origin of a diploid hybrid of *Paeonia* (Paeoniaceae). Am J Bot 94:400–408.

R Core Team 2015 R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna.

Rey PJ, AJ Manzaneda, JM Alcántara 2017 The interplay between aridity and competition determines colonization ability, exclusion and ecological segregation in the heteroploid *Brachypodium distachyon* species complex. New Phytol 215:85–96.

Rice A, L Glick, S Abadi, M Einhorn, NM Kopelman, A Salman-Minkov, J Mayzel, O Chay, I Mayrose 2015 The chromosome counts database (CCDB): a community resource of plant chromosome numbers. New Phytol 206:19–26.

Rieseberg LH 1991 Homoploid reticulate evolution in *Helianthus* (Asteraceae): evidence from ribosomal genes. Am J Bot 78:1218–1237.

——— 1997 Hybrid origins of plant species. Annu Rev Ecol Syst 28:359–389.

Rosato M, I Álvarez, G Nieto Feliner, JA Rosselló 2017 High and uneven levels of 45S rDNA site-number variation across wild populations of a diploid plant genus (*Anacyclus*, Asteraceae). PLoS ONE 12:e0187131.

Schweizer D, F Ehrendorfer 1976 Giemsa banded karyotypes, systematics, and evolution in *Anacyclus* (Asteraceae-Anthemideae). Plant Syst Evol 126:107–148.

Seehausen O 2013 Conditions when hybridization might predispose populations for adaptive radiation. J Evol Biol 26:279–281.

Sherman NA, JM Burke 2009 Population genetic analysis reveals a homoploid hybrid origin of *Stephanomeria diegensis* (Asteraceae). Mol Ecol 18:4049–4060.

Šiško M, A Ivančič, B Bohanec 2003 Genome size analysis in the genus *Cucurbita* and its use for determination of interspecific hybrids obtained using the embryo-rescue technique. Plant Sci 165:663–669.

Suda J, LA Meyerson, IJ Leitch, P Pyšek 2015 The hidden side of plant invasions: the role of genome size. New Phytol 205:994–1007.

Taylor SJ, LD Rojas, SW Ho, NH Martin 2013 Genomic collinearity and the genetic architecture of floral differences between the homoploid hybrid species *Iris nelsonii* and one of its progenitors, *Iris hexagona*. Heredity 110:63–70.

Torices R, A Agudo, I Álvarez 2013 Not only size matters: achene morphology affects time of seedling emergence in three heterocarpic species of *Anacyclus* (Anthemideae, Asteraceae). An Jard Bot Madr 70:48–55.

Trucco F, MR Jeschke, AL Rayburn, PJ Tranel 2005 *Amaranthus hybridus* can be pollinated frequently by *A. tuberculatus* under field conditions. Heredity 94:64–70.

Trucco F, T Tatum, KR Robertson, AL Rayburn, PJ Tranel 2006 Characterization of waterhemp (*Amaranthus tuberculatus*) × smooth pigweed (*A. hybridus*) F_1 hybrids 1. Weed Technol 20:14–22.

Vallejo-Marín M, AM Cooley, MYQ Lee, MR Folmer, MR McKain, JR Puzey 2016 Strongly asymmetric hybridization barriers shape the origin of a new polyploid species and its hybrid ancestor. Am J Bot 103:1–17.

Vallejo-Marín M, SJ Hiscock 2016 Hybridization and hybrid speciation under global change. New Phytol 211:1170–1187.

Vitales D, G Nieto Feliner, J Vallès, T Garnatje, M Firat, I Álvarez 2018 A new circumscription of the Mediterranean *Anacyclus* (Anthemideae, Asteraceae) based on chloroplast and nuclear DNA markers. Phytotaxa 349:1–17.

Yakimowski SB, LH Rieseberg 2014 The role of homoploid hybridization in evolution: a century of studies synthesizing genetics and ecology. Am J Bot 101:1247–1258.

Zuur AF, EN Ieno, CS Elphick 2010 A protocol for data exploration to avoid common statistical problems. Methods Ecol Evol 1:3–14.