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Radiative evolution of polyploid races of the Iberian carnation *Dianthus broteri* (Caryophyllaceae)

Francisco Balao[1]*, Luis M. Valente[2]*, Pablo Vargas[2], Javier Herrera[1] and Salvador Talavera[1]

[1]Departamento de Biología Vegetal y Ecología, Universidad de Sevilla, Apdo. 1095, E-41080 Sevilla, Spain; [2]Real Jardín Botánico de Madrid, CSIC, Plaza de Murillo 2, 28014 Madrid, Spain

Author for correspondence:
Francisco Balao
Tel: +34954559887
Email: fbalao@us.es

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Summary

• The micro-evolutionary mechanisms that drive large-scale radiations are not completely understood, partly because of a shortage of population-level studies aimed at identifying putative causes of rapid evolutionary change. The *Dianthus broteri* complex, representing the largest polyploid series known to date for any species in the genus (2×, 4×, 6× and 12× cytotypes), belongs to a lineage that was recently found to have diversified at unusually rapid rates.

• We used a combination of genome sequencing (internal transcribed spacer (ITS), plus chloroplast DNA (cpDNA) regions *trnH-psbA, psbA-trnK* and *trnK-matK*) and amplified fragment length polymorphism (AFLP) fingerprinting in 25 populations to infer the evolutionary history of extant polyploid races.

• The haplotype, ribotype and AFLP reconstructions showed a star-shaped arrangement suggesting a pattern of radiative evolution. The major, widespread haplotype occurred at all ploidy levels, whereas 20 minor haplotypes were restricted to single populations and cytotypes. In addition, AFLP analyses retrieved well-supported cytogeographic groups: six clades were clearly differentiated in terms of ploidy level and geography. Molecular data indicate that gene flow among different cytotypes is rare or nonexistent.

• Our study supports a scenario of rapid diversification in carnations in which autopolyploidy and allopolyploidy, in interaction with geography and/or isolation, have played prominent roles.

Introduction

During the past decade, several rapid radiations of plant lineages have been documented (Linder, 2008), revealing that the processes leading to extant plant diversity may be remarkably dynamic. Despite an abundance of studies, the causes of 'explosive' diversification still remain obscure (Coyne & Orr, 2004). In particular, the micro-evolutionary mechanisms that drive large-scale radiations are incompletely understood, partly because of a shortage of population-level studies with the explicit aim of identifying putative causes of rapid evolutionary change in species from young hyperdiverse lineages**.**

Polyploidy, a major evolutionary force in plants (Ramsey & Schemske, 1998; Otto, 2007; Leitch & Leitch, 2008; Paun *et al.*, 2009; Soltis & Soltis, 2009), is often invoked as a potential driver of angiosperm radiations. Genome duplication events are thought to have played an important role in generating plant diversity both directly – speciation events are often associated with polyploidization (Wood *et al.*, 2009) – and indirectly – polyploid lineages often display above-average rates of diversification (Soltis & Soltis, 2009; Soltis *et al.*, 2009). Indeed, the radiation of several species-rich lineages of plants has been partially attributed to polyploidization (e.g. Barrier *et al.*, 1999; Guo *et al.*, 2005; Jordon-Thaden & Koch, 2008; Blöch *et al.*, 2009). However, the role of polyploidy in shaping population structuring in such lineages has rarely been addressed.

Dianthus L. (Caryophyllaceae), a genus of > 300 species centred in the Mediterranean Basin, is a good system in which to study the role of polyploidy in evolutionary radiation. Polyploidization seems to occur readily in *Dianthus*, and speciation often takes place through hybridization and genome duplication (Carolin, 1957; Weiss *et al.*, 2002).

*These authors contributed equally.

A recent phylogenetic study of the genus revealed that rates of diversification in Mediterranean *Dianthus* have been exceptionally high (Valente *et al.*, 2010), and raised the question of whether a combination of polyploidy and geographical speciation may have driven cladogenesis in the group. In this context, a 'zoom in' approach, in which the genetics of a particular group of *Dianthus* is studied in detail, could provide valuable insights into the causes of large-scale radiations.

Here, we focus on *Dianthus broteri* s.l. as a model system in which to study the evolutionary radiation in polyploid angiosperms. *Dianthus broteri* is a well-defined Mediterranean polyploid group of perennial, xenogamous (but self-compatible) herbs pollinated by hawkmoths (F. Balao, unpublished data). This complex is endemic to the Iberian Peninsula, where it occurs mainly on calcareous soils but can also inhabit dolomitic and siliceous areas in the south and east of the Peninsula, from altitudes of 1800 m to coastal sand palaeodunes. According to a recent study of chromosome numbers and genome size (Balao *et al.*, 2009), *D. broteri* represents the most extensive polyploid series known to date for the genus. Diploids, triploids, tetraploids, hexaploids and dodecaploids occur, but cytotypes rarely if ever coexist in the same population, and have distinct geographical distributions: dodecaploids occur only in certain areas of the lower Guadalquivir River valley; hexaploids are restricted to arid localities in south-eastern Spain; tetraploids are distributed from Portugal to north-eastern Spain; diploids are scattered in two disjunct areas in mountain ranges of Portugal and Spain. An autopolyploid origin has been suggested for most of these cytological races (Balao *et al.*, 2009), but precise genetic data are required for a deeper understanding of their evolutionary history.

In the present study, we adopted a combined molecular-marker approach to investigate the origins of the *D. broteri* complex using data from the nuclear ribosomal internal transcribed spacer (ITS) region, chloroplast DNA (cpDNA) and amplified fragment length polymorphism (AFLP). Our specific goals were to gain insights into the micro-evolutionary processes that drive plant species radiations; to investigate the origin of the different cytotypes; and to identify the role of polyploidy in the evolution of this taxon.

Materials and Methods

Sampling

During the summers of 2006 and 2007 we sampled 245 individual plants from *Dianthus broteri* s.l. populations (including *Dianthus broteri* Boiss. & Reuter, *Dianthus inoxianus* Gallego and *Dianthus valentinus* Willk.) distributed across the whole range of this complex in Iberia (Fig. 1). A total of 25 populations, sampled in Portugal (populations 1–4), southern Spain (populations 5–16) and eastern Spain (populations 17–25), were studied. Details and ecological parameters of the sampling sites are provided in Supporting Information Table S1.

Fresh leaves were collected in the field and stored in silica gel until DNA extraction. Levels of ploidy were obtained from a previous study (Balao *et al.*, 2009). Voucher specimens for all populations were deposited in the Herbarium of the University of Seville (Seville, Spain).

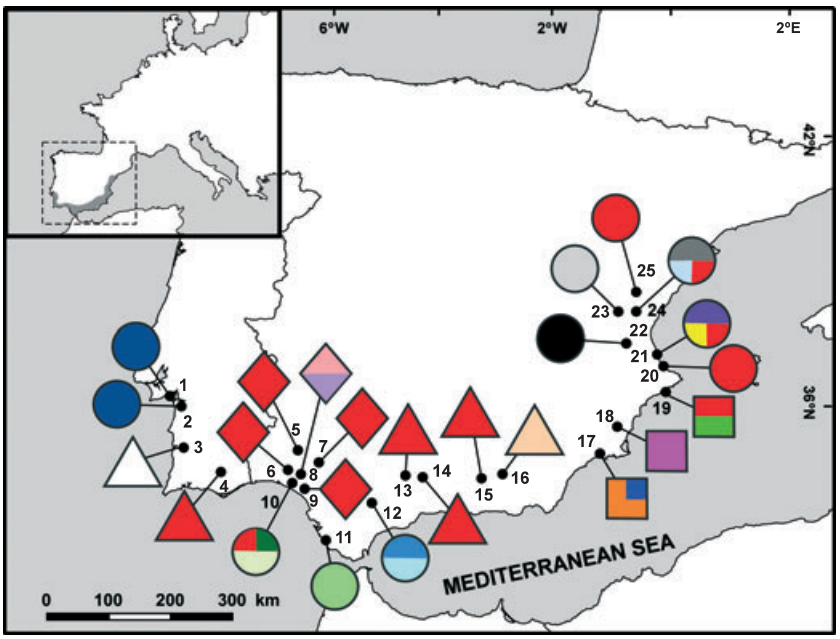

Fig. 1 Distribution of the 25 *Dianthus broteri* populations sampled. Ploidy levels are indicated by: triangles, diploids (2×); circles, tetraploids (4×); squares, hexaploids (6×); diamonds, dodecaploids (12×). Colours indicate chloroplast DNA (cpDNA) haplotype frequencies. The inset shows the distribution range of *D. broteri* in the Iberian Peninsula.

DNA extraction and sequencing

Total genomic DNA was extracted using the cetyl trimethyl ammonium bromide (CTAB) method (Doyle & Doyle, 1987; with modifications following Ortiz *et al.*, 2007) at facilities of the Biology Research Services (CITIUS) of the University of Seville. A pilot study was performed to find the most variable DNA sequences among 12 different molecular markers, and the nuclear ribosomal internal transcribed spacer (ITS1-5.8S-ITS2) and three chloroplast regions (*trn* (*tRNA*)*H-psbA*, *psb* (*photosystem II*)*A-trnK* and *trnK-matK* (*maturase*)) were identified as the most suitable markers. These regions were sequenced for a representative subset of 100 plants (four individuals per population). Primer sequences for polymerase chain reaction (PCR) amplification were obtained from White *et al.* (1990), Johnson & Soltis (1994), Demesure *et al.* (1995) and Liston *et al.* (1996). Sequencing was conducted on an Applied Biosystems Prism Model 3700 DNA analyser (Applied Biosystems, Foster City, CA, USA).

Sequences were proofread, assembled into contigs and trimmed of ambiguous ends in Geneious Pro 4.7.6 (Drummond *et al.*, 2009). Ambiguous nucleotides were represented by IUPAC symbols. Alignment was performed manually. The three chloroplast matrices were concatenated and a parsimony network was constructed using TCS version 1.21 (Clement *et al.*, 2000), with gaps treated as missing data and a 95% connection limit. A second parsimony network based on the ITS sequences was built with the same settings.

In order to investigate whether there was significant genetic structuring within the Iberian Peninsula (more specifically, southern vs eastern populations), we estimated the nearest-neighbour statistic (S_{nn}; Hudson, 2000) which is appropriate for diverse haplotype data sets with small sample sizes. Permutation tests with 1000 replicates were performed in DnaSP v5 (Librado & Rozas, 2009). The distribution of among-haplotype pairwise differences (i.e. the 'mismatch distribution' of Rogers & Harpending, 1992) was examined for evidence of past spatial and demographic expansions. This was done by comparing the observed distribution of mismatches with distributions obtained using models of either sudden demographic expansion or sudden spatial expansion (Ray *et al.*, 2003; Excoffier, 2004).

AFLP fingerprinting

We analysed 245 individuals from 25 populations. The AFLP procedure followed the protocol established by Gaudeul *et al.* (2000), with modifications (Escudero *et al.*, 2008). An initial screening of selective primers was performed on six individuals from five populations (one individual was replicated), using 33 primer combinations with three selective nucleotides. In order to choose the most replicable combinations, a second screening was then run on eight individuals from four randomly chosen populations (one individual replicated per population) employing the 25 best primer combinations. The four primer combinations selected for the selective PCR were: *Eco*RI-ACC (FAM)/*Mse*I-CAA; *Eco*RI-AAC (VIC)/*Mse*I-CGA; *Eco*RI-ACT (FAM)/*Mse*I-CAT; and *Eco*RI-AAC (VIC)/*Mse*I-CCT.

For each individual, 0.5 μl of 6-FAM-labelled and 0.5 μl of VIC-labelled selective PCR products were combined with 0.5 μl of GeneScan 500 LIZ (Applied Biosystems) and 13.5 μl of formamide. The mix was run on a capillary sequencer (ABI 3730; Applied Biosystems), and the GeneMapper™ software application (version 3.4; Applied Biosystems) was used to score amplified fragments 100–500 bp in length. An automated allele binning protocol and posterior manual review were performed. To calculate the error rate of the method, replicates of the AFLP protocol were conducted on 13 individual plants (5.3% of the total).

AFLP scores were treated as a binary (presence/absence) variable, incorporated into a data matrix, and imported into r software (R Development Core Team, 2008). To assess the genetic diversity in each population, the total number of fragments scored ($Frag_{tot}$), the number of private fragments ($Frag_{priv}$) and the percentage of polymorphic fragments ($\%_{pol}$) were determined. Additionally, the Rarity 1 Index (equivalent to the frequency of down-weighted marker values; i.e. DW sensu Schönswetter & Tribsch, 2005) was calculated using AFLPdat (Ehrich, 2006). Nei's gene diversity (H_j) was measured in aflp-surv version 1.0 (Vekemans *et al.*, 2002), and genetic diversity within plant groups was estimated from band richness by the rarefaction method (Kalinowski, 2004) using HP-Rare version 1.0 (Kalinowski, 2005). Our estimates are based on two (randomly chosen) populations within each group, and eight individuals per population. Group-based DW estimates were obtained by nonparametric bootstrapping of plant individual values (not population averages) for each group. The average and confidence interval (bias-corrected and accelerated (BC_a); Efron & Tibshirani, 1986) were calculated from 1000 repetitions.

AFLP-based population and individual relatedness

Groups of genetically similar individuals were identified graphically with a principal coordinate analysis (PCoA) of their genetic distances (1 – Jaccard similarity). Subsequently, a second PCoA was performed using the chord distance matrix (single-locus chord distance; Cavalli-Sforza & Edwards, 1967) among populations based on allele frequency data (*adegenet* and *vegan* packages in r software version 2.8.0; Jombart, 2008; Oksanen *et al.*, 2010).

Among-population fixation indices (F_{ST}) were computed using aflp-surv version 1.0 (Vekemans *et al.*, 2002) and

10 000 matrices obtained by bootstrapping. These were then used to build a neighbour-joining tree (with PHYLIP software; Felsenstein, 2005). The hidden genetic structure of populations was studied by Bayesian analysis of plants clustering into genetically divergent groups using BAPS version 5.1 (Corander & Marttinen, 2006). The program was run with the maximal number of groups (K) set to 1–25 (i.e. the number of populations), and each run was replicated five times. The partition with the highest log-marginal likelihood was plotted onto the neighbour-joining tree.

AFLP molecular variance analysis

The partitioning of variance among PCoA plant groups was studied with molecular variance analysis (AMOVA) as implemented in ARLEQUIN 3.11 (Excoffier *et al.*, 2005). Variance partitioning was also investigated in the groups retrieved in the neighbour-joining analysis. To find the best-fit model, and to compare models with different numbers of parameters, we used the corrected Akaike information criterion (AICc) (Halverson *et al.*, 2008). Variance components were tested for significance using an exact non-parametric test with 10 000 permutations.

As a cautionary remark, all our analyses based on allelic frequencies explicitly assume Hardy–Weinberg equilibrium and polysomic inheritance. Unfortunately, the effect of ploidy level on AFLP allelic frequencies is unknown, and a theoretical basis and practical methodology for dealing with this problem are not yet available (unlike, for instance, in microsatellite or isoenzyme studies; e.g. Bruvo *et al.*, 2004; De Silva *et al.*, 2005; Obbard *et al.*, 2006).

Results

cpDNA and ITS sequences

The combined cpDNA sequence alignment contained 2283 nucleotide base pairs, including 37 variable sites. The cpDNA haplotype network presented a star-like topology in the combined data set (Fig. 2a), with two unresolved loops caused by recurrent mutations at sites 1193 and 2224. *Dianthus broteri* showed a single major haplotype (45% of samples) which, as inferred from the networking analysis, was ancestral and occurred at all ploidy levels (2×, 4×, 6× and 12×). In addition, there were 20 minor haplotypes (one to seven mutation steps from the ancestral haplotype; see Table S2) that were mostly unique to a single population, except for haplotype B, which occurred in populations 1 and 2 (Fig. 1). In the majority of populations (72%), all four sampled individuals shared the same haplotype. The probability that all (21) haplotypes had been sampled was 80.5% (95% confidence interval: 21–22 haplotypes; Dixon, 2006).

The permutation tests in DNASP revealed a highly significant geographic signal (S_{nn} statistic = 0.791, $P < 0.001$), following division of the plastid data set into two partitions (southern vs eastern populations). The observed mismatch distribution (Supporting Information Fig. S1) was not consistent with a demographic expansion model (Harpending's raggedness index = 0.08, $P = 0.002$). Instead, it was compatible with a model of spatial expansion ($P = 0.65$), with the following parameter estimates (95% confidence intervals): $\tau = 10.80$ (6.43–15.88), $\theta = 0.51$ (0–1.65) and $M = 2.95$ (0.80–8.55).

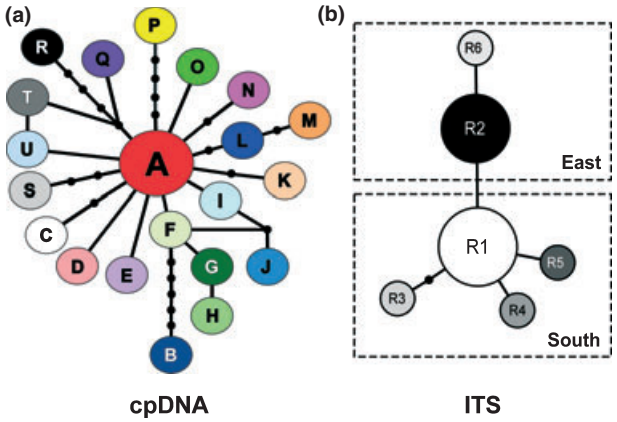

Fig. 2 Parsimony networks of (a) chloroplast DNA (cpDNA) haplotypes and (b) internal transcribed spacer (ITS) ribotypes of *Dianthus broteri* populations estimated by statistical parsimony. Colours indicate different haplotypes (coded as in Fig. 1), and circle sizes are proportional to frequencies.

The aligned ITS region was 629 bp long. Sequences were virtually constant and only six sites were polymorphic. As shown in Fig. 2(b), there were two major ribotypes (R1 and R2), and four minor ones. Based on a G → T substitution at site 473, we divided populations into two geographical groups, 'south' (populations 1–16) and 'east' (populations 17–25), with the exception of population 18, which had individuals with both ribotypes. For details on ribotype distributions within and among populations, see Tables S1 and S3.

AFLP variation

The AFLP analysis of 245 individual from 25 populations resulted in 1175 scored fragments. Most of these (99.8%) were polymorphic and only two were monomorphic. Random replicates had tolerable locus reproducibility (94.1 ± 0.03%; mean ± SE here and below). On average, diploid plants had 112.9 ± 3.5, tetraploids 107.1 ± 1.4, hexaploids 119.8 ± 3.6 and dodecaploids 140.8 ± 2.0 fragments. Fragment numbers increased with ploidy level (Spearman's rank correlation $r = 0.45$, $n = 245$, $P < 0.001$), but if dodecaploids were excluded from analysis the correlation was no longer significant ($r = 0.11$, $n = 196$, ns).

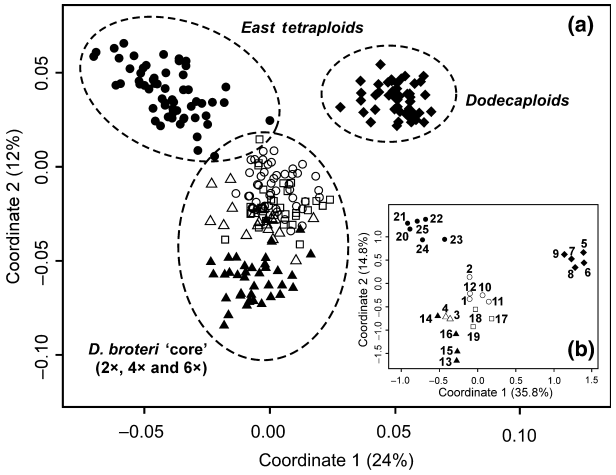

Fig. 3 Principal coordinate analysis (PCoA) of amplified fragment length polymorphism (AFLP) data. (a) An ordination based on the matrix of Jaccard similarities among 245 individuals of *Dianthus broteri*. (b) Among-population relationships based on the chord distance matrix. Individuals and populations are labelled according to geographical provenance and ploidy level: open triangles, southwestern (SW) diploids; black triangles, southern diploids; open circles, southern tetraploids; black circles, eastern tetraploids; squares, hexaploids; and black diamonds, dodecaploids. Numerals next to symbols refer to populations as illustrated in Fig. 1 and described in Supporting Information Table S1.

AFLP individual and population relatedness

Results of the principal coordinate analysis (PCoA) are shown graphically in Fig. 3. The first axis divided the data set into three distinct clusters: the largest one was heterogeneous (the *D. broteri* 'core') and comprised the southern 2× and 4× individuals, along with eastern 6× plants; the second cluster was very homogeneous and consisted exclusively of the dodecaploid plants; the third cluster was composed of eastern tetraploids only. Axes 2 and 3 of the PCoA involved only the *D. broteri* 'core' and separated, respectively, the southern diploids and the southern tetraploids. Even more clearly than the individual-based analysis, the population-based PCoA (Fig. 3b) revealed the same three groups.

The Bayesian analysis again underlined the three geographical/ploidy-based aggregates (Fig. 4a). Using this approach all individuals could unambiguously be assigned to one of the three groups, and only six plants had some (low) probability of being 'misplaced'. Additional analyses were performed only on the 'core' group (Fig. S2), and on the core group plus eastern tetraploids (Fig. S3). In these cases, PCoA showed a complex genetic pattern within the 'core' group, in which only the eastern tetraploids were clearly separated from the others. As for BAPS, again two distinct groups were revealed (Fig. S3b), but only if eastern tetraploids were included.

Fig. 4(b) depicts the neighbour-joining phylogram based on AFLP data. Internal relationships were generally poorly resolved (bootstrap value (BS) < 50), and the resulting phylogram was thus distinctively star-shaped with short branch lengths. However, populations were clearly structured into five distinct and highly supported (BS > 83) clades corresponding to geography/ploidy level. Dodecaploids were retrieved as a well-supported group, broom-like in outline as a result of its short secondary branches. The two southwest diploid populations did not form a strongly supported clade (BS < 50).

Genetic diversity

The analysis of molecular variance (Table 1) revealed that most (up to 78.5%) variation occurred within populations. Plant groups as defined by PCoA explained 9.6% of variation, whereas neighbour-joining clustering was slightly more efficient (11.3%) and groups also had a better fit (AIC_c = 1008).

Estimates of genetic diversity for the studied populations are presented in Table 2. For all measurements of diversity (H_j, DW and $Frag_{priv}$), the highest values were those of the

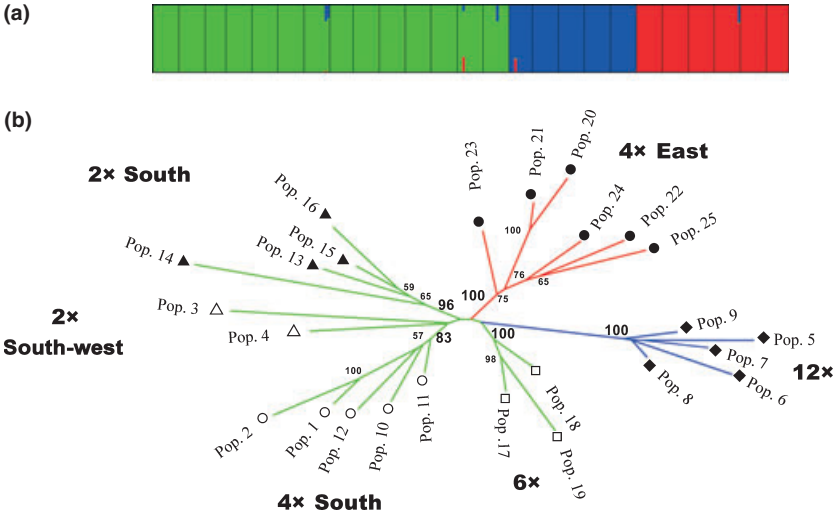

Fig. 4 (a) Bayesian analysis of population structure, with individual *Dianthus broteri* plants grouped according to highest log-marginal likelihood partition (three groups). (b) Neighbour-joining analysis of 25 populations of *D. broteri* based on pairwise among-population fixation index (F_{ST}) of amplified fragment length polymorphism (AFLP) data. Populations are labelled as in Fig. 1, and branches are colour-coded as in (a). Numerals by nodes are bootstrap values > 50.

Table 1 Analysis of molecular variance (AMOVA) of amplified fragment length polymorphism (AFLP) data of *Dianthus broteri* under different grouping criteria

Criterion (*K*); AIC_c	Source of variation	df	Variation (%)[1]
No grouping (*K* = 1); 1033.5	Among populations	24	21.47
PCoA groups (*K* = 3); 1017.5	Among groups	2	9.62
	Among populations	22	14.77
	Within populations	220	75.61
Neighbour joining (*K* = 6); 1008.4	Among groups	5	11.27
	Among populations	19	11.52
	Within populations	220	77.21

K, number of groups; AICc, corrected Akaike's value.
[1]$P < 0.001$ in all cases.

two disjunct diploid areas. The lowest values corresponded to eastern tetraploids.

Table 3 presents estimates of genetic diversity in the cytogeographic groups of the neighbour-joining phylogram (Fig. 4b). In terms of fragment number, band richness, or DW value, the least diverse group was again that of eastern tetraploids. Maximum band richness occurred in southern diploids, which also had the highest DW value.

Discussion

Incipient radiation in *Dianthus broteri*

The genus-wide phylogenetic analysis by Valente *et al.* (2010) proposed a recent origin for *D. broteri*, and placed the species in a remarkably young (0.9–2.1 million yr old) Eurasian lineage that has diversified at unusually rapid rates. The low variation of ITS sequences detected in the present study supports the hypothesis of a recent origin for this species. In 100 individuals sampled, we found very few variable sites, particularly when compared with other intraspecific studies on Mediterranean angiosperms (e.g. Gaudeul, 2006; Koch *et al.*, 2006). Nevertheless, despite being a young species, *D. broteri* has produced at least 21 plastid haplotypes, a minimum of four cytotypes (Balao *et al.*, 2009), and has colonized a wide variety of habitats and soils in the Iberian Peninsula. Our results therefore provide the first molecular evidence for rapid, radiative divergence at the intraspecific level in carnations.

The haplotype, ribotype and AFLP reconstructions showed a star-shaped arrangement with unusually short internal branches (which would translate into a multichotomy in a phylogenetic analysis), suggestive of radiative evolution (Figs 2, 4). Such a network configuration is similar to that found in ecological races of North American *Achillea*

Table 2 Genetic diversity indices in *Dianthus broteri* populations from amplified fragment length polymorphism (AFLP) analysis

Population	*n*	$Frag_{tot}$	$\%Frag_{pol}$	$Frag_{priv}$	H_j	DW	Ploidy	AFLP group
Pop. 1; Troia	10	373	93.83	5	0.089 ± 0.005	4.8	4	4×, south
Pop. 2; Comporta	8	269	87.73	3	0.071 ± 0.004	3.4	4	4×, south
Pop. 3; Monte Clérigo	10	407	92.87	10	0.104 ± 0.005	7.4	2	2×, south-west
Pop. 4; São Brás de Alportel	10	450	98.89	21	0.115 ± 0.005	8.4	2	2×, south-west
Pop. 5; Valverde	10	400	90.75	4	0.105 ± 0.005	5.8	12	12×
Pop. 6; Moguer	10	398	88.19	1	0.099 ± 0.005	4.9	12	12×
Pop. 7; Hinojos	10	373	91.96	4	0.093 ± 0.005	4.3	12	12×
Pop. 8; Doñana, Acebrón	9	459	95.21	4	0.113 ± 0.005	4.6	12	12×
Pop. 9; Doñana, Puntal	10	392	90.05	2	0.096 ± 0.005	4.1	12	12×
Pop. 10; Doñana, Peladillo	10	361	91.69	7	0.085 ± 0.004	4.6	4	4×, south
Pop. 11; Chiclana	10	404	94.06	5	0.097 ± 0.005	5.0	4	4×, south
Pop. 12; Ronda	10	315	88.57	2	0.072 ± 0.004	3.7	4	4×, south
Pop. 13; Zafarraya 1	9	394	94.67	5	0.105 ± 0.005	6.4	2	2×, south
Pop. 14; Zafarraya 2	10	266	87.97	6	0.060 ± 0.004	3.3	2	2×, south
Pop. 15; Orgiva	10	421	95.01	16	0.103 ± 0.005	7.4	2	2×, south
Pop. 16; Laroles	10	309	90.29	3	0.073 ± 0.004	3.8	2	2×, south
Pop. 17; Cartagena	10	359	93.31	4	0.087 ± 0.005	4.2	6	6×
Pop. 18; San Miguel de Salinas	10	388	97.68	7	0.098 ± 0.005	5.1	6	6×
Pop. 19; Peñón de Ifach	10	356	89.04	3	0.087 ± 0.005	4.9	6	6×
Pop. 20; Albufera de Valencia 1	10	245	90.61	1	0.059 ± 0.004	2.5	4	4×, east
Pop. 21; Albufera de Valencia 2	9	258	91.47	7	0.064 ± 0.004	3.4	4	4×, east
Pop. 22; Lliria	10	313	93.29	7	0.076 ± 0.004	4.0	4	4×, east
Pop. 23; Sierra de Espadán	10	358	91.34	9	0.090 ± 0.005	4.8	4	4×, east
Pop. 24; Alcublas	10	352	92.61	5	0.085 ± 0.004	3.6	4	4×, east
Pop. 25; Azuebar	10	274	91.61	9	0.072 ± 0.004	5.2	4	4×, east

n, number of plants analysed; $Frag_{tot}$, total number of fragments, $\%Frag_{pol}$, percentage of polymorphic fragments; $Frag_{priv}$, number of private fragments. H_j, Nei's genetic diversity; DW, rarity index. DNA ploidy levels were estimated by flow cytometry in Balao *et al.* (2009). AFLP groups are those of Fig. 4(b).

Table 3 Genetic characterization of *Dianthus broteri* cytogeographic groups

Cytogeographic groups	*N* (*n*)[1]	Fragments	Band richness[2]	DW[3]
2×, south-west	20 (2)	120 ± 6	1.37 ± 0.01[a]	7.9 (6.0–9.9)
2×, south	39 (4)	109 ± 4	1.29 ± 0.01[b]	5.2 (4.1–7.6)
4×, south	48 (5)	113 ± 2	1.27 ± 0.01[c]	4.3 (3.8–5.0)
4×, east	59 (6)	102 ± 4	1.24 ± 0.01[d]	3.9 (3.6–4.4)
6×, east	30 (3)	120 ± 4	1.29 ± 0.01[b]	4.7 (3.8–5.7)
12×, south	49 (5)	141 ± 2	1.30 ± 0.01[b]	4.7 (4.4–5.1)

Estimates of genetic diversity are reported as mean ± SE. Lowercase letters in a column indicate means not significantly different at $P < 0.05$.
[1]Number of plants (number of populations).
[2]Wilcoxon signed-rank test (Bonferroni-corrected).
[3]Average value (bias-corrected and accelerated bootstrap 95% confidence interval).
DW, rarity index.

millefolium (Ramsey *et al.*, 2008). Whereas in *A. millefolium* this was interpreted as a sign of rapid demographic expansion, in *D. broteri* the observed mismatch distribution suggests an expansion in range associated with historical/geographical factors (Excoffier, 2004). The fact that the ancestral cpDNA haplotype occurred throughout most of the distribution of this polyploid complex (Fig. 1) suggests that the range increase probably occurred early in the evolution of *D. broteri*. Following this initial stage of expansion, derived haplotypes would have evolved from the major haplotype in independent, but frequent, events. Importantly, most of the minor haplotypes were observed in all individuals in a population, indicating that such haplotypes can spread locally and are not solely the result of transient mutations that are soon eliminated.

Populations with derived haplotypes can be viewed as potential 'infant' lineages in the incipient radiation of *D. broteri*, analogous to young species in large-scale, genus-level radiations (Verheyen *et al.*, 2003; Coyne & Orr, 2004). Sharing of minor haplotypes between populations was very rare in *D. broteri*, an indication of the limited gene flow between recently colonized areas that constitutes an important prerequisite for speciation. Furthermore, the AFLP neighbour-joining phylogram retrieved six clades (Fig. 4) that were clearly differentiated in terms of ploidy levels and geography (Table 1). This again indicates that genotypes display a nonnegligible level of isolation.

Evolution of polyploids

Polyploid complexes are often the result of recurrent, independent genome duplication events that frequently lead to spatial coexistence of parental lineages with derived polyploids (Segraves *et al.*, 1999; Soltis & Soltis, 1999, 2000). Weiss *et al.* (2002) found that mixed populations are common in species of *Dianthus* section *Plumaria*, to which *D. broteri* belongs. However, in *D. broteri*, coexistence of different cytotypes within a population is very rare, which suggests that cytotypes evolved in single events (Balao *et al.*, 2009). Our molecular data support this view, at least for hexaploids and dodecaploids, which exhibit notable genetic relatedness within cytotypes (Fig. 4b) and are geographically restricted. Nevertheless, both plastid and nuclear markers indicate that the tetraploid cytotype is likely to have emerged more than once (see later in this section).

Although infrequent, polyploidization events in *D. broteri* seem to have had a variety of causes. We hypothesize that this extensive polyploid series has resulted from both autopolyploidy *and* allopolyploidy (or introgressive hybridization), a possibility that was proposed for *Dianthus* section *Plumaria* as a whole by Weiss *et al.* (2002). In our case, AFLP and ITS data (as well as morphological similarity) suggest that the tetraploids from the south originated by autopolyploidy from diploid plants (with which they share the monoploid DNA complement; Balao *et al.*, 2009). As for eastern tetraploids, they form a distinct AFLP cluster (Fig. 4a,b), differ from their southern counterparts in ITS and cpDNA sequences, and have a relatively small monoploid genome size (Table S1) suggestive of downsizing ('diploidization'; Parisod *et al.*, 2010). Overall, such evidence indicates that the eastern tetraploids are largely unrelated to southern tetraploids and are probably older, and that an allopolyploid origin cannot be ruled out.

The origin of the hexaploid cytotype is not clear-cut, as molecular markers yielded conflicting results. According to AFLP data, hexaploids are related to diploids and southern tetraploids (Fig. 3), whereas DNA sequences suggested a closer relationship with eastern tetraploids (Table S1). This apparent contradiction would be resolved if hexaploids had evolved by allopolyploidy, through hybridization of southern diploid and eastern tetraploids. This hypothesis receives additional support from the fact that both 'south' and 'east' ribotypes coexist in at least one hexaploid population (population 18). To gain insights into the process of hexaploid formation, we traced 6× AFLP fragments from putative diploid and tetraploid parents, following the method of Paun *et al.* (2006). We found that southern diploids contributed 21% of the fragments vs 19% for eastern tetraploids, whereas southern tetraploids and southwestern diploids contributed 5% and 3%, respectively. These proportions is more compatible with a complex origin (perhaps involving hybridization and back-crossing with diploids) than with strict allopolyploidy, which would have yielded a 1 : 2 proportion. A similar, multi-step process has been put forward to explain the origin of *Nicotina rustica* × *paniculata* allohexaploids (Lammerts, 1931) and *Senecio hoggariensis* (Kadereit *et al.*, 2006).

In *D. broteri*, the origin of the dodecaploid cytotype (which was considered an independent species, *D. inoxianus*, by some authors; e.g. Gallego, 1986) cannot be

established with certainty. Their distinctive clustering in PCoA and Bayesian analyses, allied with the fact that they present the smallest monoploid genome size (Table S1), suggests that the dodecaploids are largely unrelated to the remaining cytotypes. The remarkably low fixation index (data not shown) could be a sign of high gene flow and/or panmixis within this group, but additional molecular data (e.g. on microsatellites and single-copy genes) are needed before a hypothesis on its origin can be put forward.

Polyploidy and radiation

Polyploidy can contribute to rapid genetic divergence within a species if there is a considerable reduction of gene flow between neopolyploids and their parent cytotype(s) (Ramsey & Schemske, 2002). This is likely to have been the case in *D. broteri*. Indeed, the discrete clustering of independently evolved cytotypes (Fig. 4) suggests a scenario of limited gene exchange among populations with dissimilar chromosome numbers. This hypothesis is also supported by the fact that, following hand pollinations, interploidal crosses invariably fail (F. Balao, unpublished data).

Divergence among populations seems to have also been driven by an interaction between polyploidy and historical-geographical events. For example, the south–east genetic structuring revealed by the S_{nn} statistic (despite the lack of notable geographical barriers in the present day) could be a consequence of past climatic oscillations. Thus, diploid races with genetic diversity and DW typical of ancient populations are restricted to areas that have been identified as glacial refugia (Gómez & Lunt, 2006; Médail & Diadema, 2009). Eastern tetraploids also occur in glacial refugia (Salvador *et al.*, 2000; Petit *et al.*, 2002) and have very low DW (a characteristic of bottlenecked populations; Marhold & Lihová, 2006; Paun *et al.*, 2008). Occasional contact between diploids and tetraploids as a result of climatic oscillations cannot be ruled out, and could potentially have produced new 'hybrid' cytotypes, such as hexaploids.

A final means through which polyploidy could have contributed to rapid divergence is by providing increased genomic flexibility: both autopolyploids and allopolyploids are known to show greater genetic redundancy in comparison with diploids, which may represent an ecological advantage in rapidly changing environments such as the Mediterranean Basin (e.g. via greater colonizing ability; Parisod *et al.*, 2010). Furthermore, the putative advantages of polyploids over diploids could include lower rates of population extinction and increased diversification rates in the long term (Soltis *et al.*, 2009).

Concluding remarks

The bottom-up approach we have used sheds new light on the micro-evolutionary drivers of rapid diversification in plants. Provided that the results in *D. broteri* can be extrapolated to the remaining species in the Eurasian lineage of *Dianthus*, our study provides strong new evidence for a scenario of rapid diversification in carnations. Polyploidy and geography/history have played an active role in this case, but the question remains as to why carnations are so prone to genome-shaping events. Further studies could aim to address this question by comparing the mechanisms of polyploidization in *Dianthus* with those in other lineages.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Observed and expected mismatch distributions of *Dianthus broteri* haplotypes under (a) demographic expansion and (b) spatial expansion models.

Fig. S2 Principal coordinate analysis (PCoA) (a) and Bayesian analysis (b) of *Dianthus broteri* amplified fragment length polymorphism (AFLP) data in the 'core' group.

Fig. S3 Principal coordinate analysis (PCoA) (a) and Bayesian analysis (b) of *Dianthus broteri* amplified fragment length polymorphism (AFLP) data in the 'core' group plus eastern tetraploids.

Table S1 Details of the *Dianthus broteri* populations studied.

Table S2 Polymorphic sites for chloroplast DNA (cpDNA) regions (*trnK-matK*, *trnH-psbA* and *psbA-trnK*) of *Dianthus broteri* haplotypes, and GenBank accession numbers for each haplotype sequence.

Table S3 Polymorphic sites for the internal transcribed spacer (ITS) region in *Dianthus broteri* ribotypes, and GenBank accession numbers for each ribotype sequence.

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