Molecular Ecology (2009) **18**, 2659–2667 doi: 10.1111/j.1365-294X.2009.04216.x

Genetic differentiation and reproductive isolation of a naturally occurring floral homeotic mutant within a wild-type population of *Capsella bursa-pastoris* (Brassicaceae)

STEFFEN HAMEISTER, BARBARA NEUFFER and WALTER BLEEKER
Department of Botany, University of Osnabrueck, Barbarastraße11, D-49076 Osnabrueck, Germany

Abstract

Apart from the common floral architecture in Brassicaceae, variation in flower morphology occurs in several genera within the family and is considered to affect speciation processes. We analysed genetic differentiation and flowering time variation of two floral variants of *Capsella bursa-pastoris*, the *Spe* variant and the wild-type, which occur sympatrically in a vineyard in southwest Germany. The *Spe* variant is characterized by an additional whorl of stamens instead of petals and was formerly classified as an independent taxon '*Capsella apetala*' Opiz. Amplified fragment length polymorphism and allozyme analysis revealed a substantial genetic differentiation of the two floral variants and a higher genetic variation within the wild-type subpopulation compared with the *Spe* subpopulation. The low genetic variation in the mutant provided evidence of a recent local origin or recent introduction. Flowering time analysis indicated that, within the analysed population, the *Spe* variant flowers significantly later than the wild-type ($P < 0.001$). We conclude that the evolution and persistence of *Spe* within a wild-type population is facilitated by high selfing rates and been enhanced by a shift in flowering phenology. Hence, our data provide substantial evidence that the *Spe* phenotype has established itself as an isolated entity within a wild-type population and may thus serve as a model for the analysis of the evolutionary significance of homeotic mutants in wild populations.

Keywords: AFLP, Cruciferae, floral variants, flowering time, population structure, sympatric differentiation

Received 3 October 2008; revision received 23 March 2009; accepted 30 March 2009

Introduction

Within the mustard family (Brassicaceae), the genus *Capsella* comprises at least two diploid species [*C. rubella* Reut., *C. grandiflora* (Fauché & Chaub.) Boiss.] and *C. bursa-pastoris* (L.) Medik. which is tetraploid. A large amount of data for wild populations of *Capsella* species has been published (e.g. Baskin & Baskin 1989; Hurka & Neuffer 1991, 1997; Neuffer & Hurka 1999; Hawes *et al*. 2005). The fact that *Capsella* is one of the closest relatives of the molecular model plant *Arabidopsis thaliana* (L.) Heynh. (Al-Shehbaz *et al*. 2006) has recently made this genus a very attractive target for the study of evolutionary processes which occur in natural populations. In this context, the persistent occurrence of an 'apetalous' variant of *C. bursa-pastoris* in natural populations is of considerable interest as it might be another promising tool for evolutionary studies (Hintz *et al*. 2006; Nutt *et al*. 2006; Theißen 2006). This floral variant was first described about 200 years ago (Opiz 1821) and considered as an independent species named '*Capsella apetala*' Opiz. Flowers of the plants observed by Opiz (1821) were characterized by 10 instead of six stamens (decandric), indicating that petals are not fully lost but transformed into additional stamens (Fig. 1). Recently, Nutt *et al*. (2006) used the term '*Stamenoid petals*' (*Spe*) to describe the changed flower morphology. The variant is now interpreted as a floral homeotic mutant, which is possibly caused by co-dominant alleles of a single locus (Nutt *et al*. 2006). Applying the 'ABC model' proposed by Coen & Meyerowitz (1991) for floral

Correspondence: S. Hameister, Fax: +49-541-969-2845; E-mail: hameister@biologie.uni-osnabrueck.de

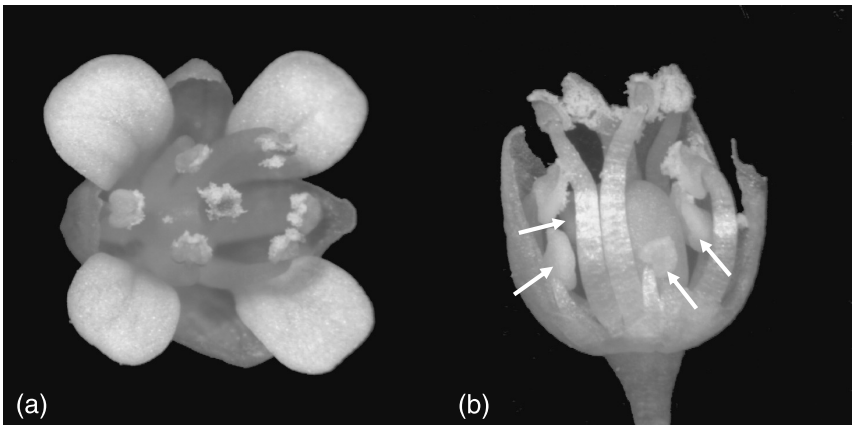

Fig. 1 Single *Capsella bursa-pastoris* flowers of: (a) wild-type with four showy petals and six stamens, and (b) the *Spe* variant with petals transformed into additional stamens (marked with arrows).

organ identity, the aberrant phenotype of *Spe* could be explained by ectopic expression of class C genes in the second floral whorl rather than class A genes (Hintz *et al*. 2006; Nutt *et al*. 2006). This assumption is supported, as *Spe*-like phenotypes are known in transgenic *A. thaliana* (Jack *et al*. 1997), in which the class C gene *AGAMOUS* (*AG*) is expressed in the second floral whorl under control of a class B gene *APETALA3* (*AP3*) promoter. Such altered expression patterns might be based on minor genetical changes in a single or just a few loci, thus the *Spe* variant might benefit the controversy concerning non-gradual evolution of phenotypic novelties. The impact of minor genetical modifications has already been shown, for example, the origin of maize (Doebley *et al*. 1995), the loss of ray floret in *Senecio* (Comes 1998) and flower colour variants in *Mimulus* (Bradshaw & Schemske 2003). Further studies in *Mimulus* propose that mutations with drastic effects might trigger reproductive isolation and facilitate rapid speciation (Bradshaw *et al*. 1995). However, empirical studies and information about naturally occurring (homeotic) mutants in stable populations in the wild are rare.

In this context, the re-discovery of the *Spe* variant in a vineyard in southwest Germany by Reichert (1998) might promote the ongoing debate. This flourishing population with tens of thousands of individuals is characterized by sympatric occurrence of the homeotic mutant with wild-type *C. bursa-pastoris*. A major question regarding the co-existence of *Spe* and wild-type individuals is how the floral variant could be maintained within a wild-type population. High rates of self-fertilization in *C. bursa-pastoris* undoubtedly facilitate prezygotic isolation. Outcrossing rates are low in *C. bursa-pastoris* and vary between 0–20% (Shull 1929; Hurka & Neuffer 1997). The altered flower morphology of the decandric variant might even strengthen self-fertilization in *Spe* as pollinator attractants (i.e. petals) are lost. Also, a shift in the pollinator assemblage might be another consequence. Due to the increased number of stamens, the *Spe* variant provides more pollen which might favour pollen-eating insects. Furthermore, variation in flowering time has led to ecotypic differentiation in *C. bursa-pastoris*, allowing for fine-scaled adaptation in various environments (Neuffer & Hurka 1986; Neuffer & Hurka 1999; Linde *et al*. 2001). A shift in flowering phenology has also been reported for artificial homeotic *Arabidopsis* mutants (Borner *et al*. 2000; Yu *et al*. 2002; Michaels *et al*. 2003). Hence, beside selfing, differences in flowering time might be an additional mechanism explaining sympatric occurrence of *Spe* and wild-type plants.

The natural occurrence of a floral homeotic mutant of *C. bursa-pastoris* within a wild-type population offers the unique opportunity to elucidate the significance of homeotic mutants with respect to population structure and ecological differentiation. Three major questions are addressed: (i) Is the morphological discrimination of *Spe* and wild-type reflected in a genetic differentiation? (ii) What is the extent of genetic variation within *Spe* and wild-type subpopulations? (iii) Are there differences in flowering phenology which may promote prezygotic reproductive isolation? Amplified fragment length polymorphisms (AFLP) and the allozyme aspartate aminotransferase (AAT) have been used as molecular markers. Differences in the onset of flowering between both variants have been analysed in a greenhouse experiment. The significance of the sympatric occurrence of *Spe* and wild-type is discussed in an evolutionary context.

Materials and methods

Plant material

The studied population is located in intensively cultivated vineyards close to Gau-Odernheim, about 25 km southwest of Mainz (Rhinehessen, Germany; Reichert 1998). The sampling site is characterized by a tremendous abundance of *Capsella bursa-pastoris* as it is the predominant species in between the rows of the vine plantations. Among tens of thousands individuals of *C. bursa-pastoris*, approximately 10% show the *Spe* phenotype. Seed material was collected in May 2005 at 15 sampling sites over a total area of 2.5 km². From each

site, mature silicles were harvested from 25 individuals. Sowing and cultivation were carried out from March to June 2007 in a greenhouse under controlled conditions (12 h illumination/day: min 14 °C–max 30 °C; night: min 10 °C). In total, 191 individuals (103 wild-type; 88 *Spe*) were available for analyses.

Molecular markers

AFLP analysis. Genomic DNA was isolated from fresh leaves (100 mg) using the Invisorb Spin Plant Kit (Invitek). DNA concentration was quantified and the quality assessed by gel electrophoresis (0.8% agarose). AFLP procedure (Vos *et al*. 1995) followed the AFLP Plant Mapping protocol by Applied Biosystems with minor modifications: restriction of DNA (0.5 μg) and ligation to double-stranded adaptors was performed in a single reaction (2 h at 37 °C). *Eco*RI and *Mse*I (5 U respective 1 U per reaction) were used to digest DNA. For ligation and amplification, kits available from ABI were used. For selective amplification, 5 μL of preselective amplification product, 0.05 μM *Eco*RI and 0.25 μM *Mse*I primer, 2 mM $MgCl_2$, 0.1 U Biotherm *Taq*-Polymerase (GeneCraft) were used. Cycle parameters were in accordance with the ABI guide. Based on a primer screening, the combinations *Eco*RI-ACA/*Mse*I-CAC, *Eco*RI-AAG/*Mse*I-CAC, *Eco*RI-ACC/*Mse*I-CTA were chosen for our study. Amplified products were separated on an ABI PRISM 377 sequencer (Applied Biosystems) with GeneScan-500 Rox as internal standard. After editing raw data in GeneScan 3.1 (Applied Biosystems), fragment sizes were estimated using GenoTyper 2.1 (Applied Biosystems). The evaluation for presence (1) or absence (0) of fragments was carried out manually. Scoring of presence/absence of bands was performed by two persons independently, and the inferred genetic distance matrices were tested for correlation applying a Mantel-test in GenAlEx 6.0 (Peakall & Smouse 2006).

Allozyme studies. The AAT (EC 2.6.1.1) was included, as it is known to be highly informative for population genetics in *C. bursa-pastoris* (Neuffer & Hurka 1999). Two additional enzyme systems (glutamate dehydrogenase; GDH; EC 1.4.1.4 and leucine aminopeptidase LAP; EC 3.4.11.1) provided no further information for this study. Fresh rosette leaves (0.7 g) were harvested from 10-week-old plants and stored at –80 °C until further analyses. Extracts were prepared on ice in 1.4 mL chilled extraction buffer (0.160 M Tris, 0.107 M glycin, pH 8.0; measured at 22 °C). For native electrophoresis, 50 μL samples were separated on 7.5% polyacrylamide gels (19:1 acrylamide : bisacrylamide). Overnight staining was carried out according to Wendel & Weeden (1989). Interpretation of allozyme variation followed Hurka *et al*. (1989). In tetraploid *C. bursa-pastoris*, three duplicated *Aat* loci can be distinguished: *Aat*-1A/B, *Aat*-2A/B (both extra plastidic), and *Aat*-3A/B (plastidic). Former AAT studies revealed that the inheritance of allozymes is disomic (Hurka *et al*. 1989). As a consequence, heterozygous individuals are barely distinguishable due to the overlapping intra- and interlocus bands of the duplicated loci, especially as no progeny approach was included in this study. Therefore, the various allozymes were coded as dominant characters (presence/absence) and the resulting multilocus phenotypes were used in subsequent analyses of population differentiation (see below).

Data analysis. For the AFLPs, Nei's (1973) gene diversity (*H*), the Shannon index (*I**; Lewontin 1972), and the percentage of polymorphic loci were calculated using PopGene 1.32 (Yeh *et al*. 1997) and AFLPsurv 1.0 (Vekemans 2002). Differences in molecular diversity between wild-type and *Spe* were tested with a Student's *t*-test based on mean values of *H* and *I** for each AFLP locus. For further studies of genetic differentiation of *Spe* and wild-type plants, a combined data matrix of AFLP and allozyme data has been used (Table S1, Supporting information). The allozyme data have thus been analysed as dominant markers, comparable to approaches for polyploid plant species (e.g. Bleeker & Hurka 2001). Calculation of genetic distance and principal coordinate analysis (PCO) was performed in MVSP 3.13 (Kovach Computing System). The nearest-neighbour clustering method was applied using the Nei and Li similarity coefficient (Nei & Li 1979) for binary data and the Euclidean distance for PCO. Genetic variation at three hierarchical levels (among floral variants, within floral variants among (15) sampling sites, within sampling sites) was estimated by analysis of molecular variance (AMOVA) as implemented in Arlequin 3.1 (Excoffier *et al*. 1992). The re-allocation procedure in AFLPOP 1.1 (Duchesne & Bernatchez 2002) was used to analyse the frequency of successful (re-) allocation to predefined source populations (*Spe*, wild type) based on the molecular data set. AFLPOP computes the likelihood at which each individual derives from each source population on the basis of band frequencies of dominant markers. The allocation and re-allocation procedures in AFLPOP may be applied to diploid as well as polyploid populations since they do not assume a specific mode of marker inheritance. Re-allocation of individuals to a source population was interpreted as successful when it was at least 100 times more likely to belong to that population than to the other (minimum log-likelihood difference, MLD = 2). A model-based clustering approach was performed using Structure 2.1 (Pritchard *et al*. 2000). For data entry, absent markers were considered to be homozygous (00), and present markers to be either hetero- (10) or homozygous (11). According to the Structure manual for input of dominant data, present markers (11/10) were coded as 1;-9 and absent markers (00) as 2;-9. Structure uses Markov chain Monte Carlo (MCMC) algorithms to assign individuals to predefined numbers of clusters *K*. Structure had

Table 1 Frequency (in percentage) of allozyme genotypes for aspartate aminotransferase (AAT) in wild-type (*Wt*) and mutant (*Spe*) phenotypes. The genotypes are displayed as detected alleles for each of the three duplicated loci (A/B). For each flower type, the predominant genotype is denoted in bold numbers and (.) indicates absent genotypes

Genotype	Locus 1 A/B	Locus 2 A/B	Locus 3 A/B	*Wt* ($n = 103$)	*Spe* ($n = 88$)
I	11 11	11 11	33 55	**55.3**	3.4
II	11 11	11 44	33 55	14.6	9.1
III	11 11	11 44	11 55	8.7	**84.1**
IV	11 33	11 11	33 55	7.8	1.1
V	11 11	11 11	11 55	1.9	1.1
VI	11 33	11 44	11 55	1.0	1.1
VII	11 33	11 11	11 55	5.8	.
VIII	11 11	11 11	22 33	1.9	.
IX	11 33	11 44	33 55	1.9	.
X	11 11	11 44	22 33	1.0	.

originally been developed for analysing diploid populations. Here it is applied to an allotetraploid species with a disomic mode of inheritance that behaves like a diploid during chromosomal pairing in meiosis. We hypothesized the existence of two separate populations ($K = 2$, no-admixture model), representing *Spe* and wild-type. Various test runs revealed that a burn-in period of 30 000 followed by 300 000 iterations is suitable for our data. We also tested whether $K = 2$ is the most likely number of K's by performing several independent runs for $K = 1$–16. We calculated the slope (m) between two successive likelihood values for K [$m = \ln \Pr(X \mid K_2) - \ln \Pr(X \mid K_1)/K_2 - K_1$], to detect the real number of K indicated by a decrease in slope. This is in accordance with the estimation of $L'(K)$ given in Evanno *et al*. (2005) which they expanded to the ad hoc statistic ΔK.

Flowering time

The onset of flowering was evaluated as a putative mechanism which promotes prezygotic isolation. Progenies of 16 wild types and 10 *Spe* individuals (families) were cultivated in a greenhouse under the same conditions as described above. Five individuals per family were analysed on average (122 individuals in total). The opening of the first flower bud was defined as the onset of flowering and recorded in days after sowing. Mean, standard deviation (SD), range (R) and coefficients of variation (cV) were calculated separately for wild type and *Spe* individuals. A student's t-test was used to assess whether the family means of the two groups differ significantly. All calculations were performed using SPSS 15.0.

Results

Molecular markers

The analysis of three duplicated *Aat* loci revealed obvious differences between the two floral variants in the quantity and frequency of observed genotypes. In total, 10 different multilocus genotypes were detected. While all 10 multilocus genotypes were recorded in the wild-type, only six of them were present in the *Spe* variant (Table 1). In the *Spe* variant, genotype III dominated with a frequency of 84.1%. Three of the remaining five genotypes were observed only once. A higher variation was detected in wild-type *Capsella bursa-pastoris*, the most common genotype I was identified in 55.3% of the samples. Another third were set up by three additional genotypes, genotype II with a frequency of 14.6%, the *Spe*-specific genotype III with less then 9% and genotype IV with 7.8% (Table 1).

In the AFLP analysis, three primer combinations yielded a total of 81 reliable bands (AFLP loci), 47 (58%) of them were polymorphic within the analysed population. A Mantel-test revealed high consistency between two genetic distance matrices, generated independently by one of the authors (S.H.) and a former colleague ($R^2 = 0.73$; $P < 0.001$, see Fig. S1, Supporting information). The extent of AFLP variation within the *Spe* and wild-type subsamples was in accordance with the allozyme data as the molecular diversity was higher in wild-types for all indices (Table 2). The percentage of polymorphic loci (PLP) varied from 83.0% in *Spe* to 93.6% in wild-types. Nei's gene diversity for *Spe* was $H = 0.229$ (± 0.129) and for wild-types $H = 0.330$ (± 0.137). The Shannon information index ranged from $I^* = 0.374$

Table 2 Flowering time differentiation and AFLP diversity indices of wild-type (*Wt*) and decandric (*Spe*) individuals. A subsample of individuals for molecular analysis was also considered for the onset of flowering. Values given in parentheses are range (r) and standard deviation (SD). Asterisks indicate that differences between *Wt* and *Spe* are highly significant

	Onset of flowering				Molecular diversity (AFLPs)			
	n	Mean (SD)	cV	Min-max (r)	n	Nei's gene diversity H (SD)	Shannon index I^* (SD)	*PLP*
Wt	78	61.79 (± 9.43)	19.17	41–93 (52.0)	103	0.329 (± 0.136)	0.499 (± 0.165)	93.6
Spe	49	81.56 (± 10.61) ***	14.01	59–101 (42.0)	88	0.229 (± 0.129) ***	0.374 (± 0.171) ***	83.0

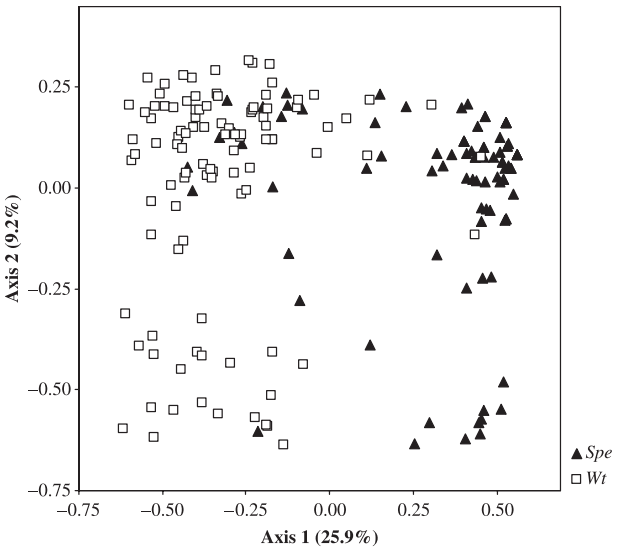

Fig. 2 Principal coordinates analysis (PCO) based on pairwise genetic distances inferred from a distance matrix using Nei and Li's coefficient. Analysis was performed for a combined data set including 47 AFLP markers and eight allozymes of two *Capsella bursa-pastoris* floral phenotypes which occur sympatrically. The first axis separates wild-types (*Wt*) and the floral homeotic mutant '*Stamenoid petals*' (*Spe*) into distinct clusters.

Table 3 Analysis of molecular variance (AMOVA) among and within two floral variants (*Spe*, wild-type) which occur sympatrically in the Gau-Odernheim population. Variance components are significant at $P < 0.001$ (3000 permutations)

	d.f.	Sum of squares	Variance components	Percentage of variation
Among floral variants	1	294.17	2.87	27.35
Among sampling sites	28	462.76	1.73	16.52
Within floral variants	161	947.24	5.88	56.13
Total	190	1704.17	10.48	

Table 4 Percentage of successful re-allocations of wild-type (*Wt*; $n = 103$) and '*Stamenoid petals*' (*Spe*; $n = 88$) individuals (AFLPOP) and results of individual-based assignment into $K = 2$ clusters using Structure (no-admixture model). Both analyses are highly consistent and only 4.2% in AFLPOP respective 2.1% in Structure were not allocated with a significant probability to a specific cluster

	AFLPOP		Structure	
	to *Wt*	to *Spe*	Cluster 1 (*Wt*)	Cluster 2 (*Spe*)
Wt	93.2	3.9	96.1	3.0
Spe	15.9	78.4	19.3	79.6

(± 0.171) in decandric individuals to 0.499 (± 0.165) in wild-type *C. bursa-pastoris* (Table 2). The differences in molecular diversity between wild-type and *Spe* were significant referring to a *t*-test (*H*: $P < 0.001$; *I**: $P < 0.001$) based on mean values of *H* and *I** for each AFLP locus.

Figure 2 shows the results of a principal coordinates analysis based on a combined data set comprising eight allozymes and 47 polymorphic AFLP loci. The first two axes accounted for 35.1% of the total variation. Axis 1 (25.9%) separated the *Spe* variant from the wild-type subsample. A few individuals were placed intermediate between these two groups. The second axis (9.2%) did not provide a further resolution regarding the separation of the floral variants (Fig. 2). The result of the AMOVA confirmed a considerable differentiation among the two subsamples within the Gau-Odernheim population: 27.4% of the total variation was expressed among the two floral variants, 56.1% of the variation was expressed within the subsamples (Table 3). Variation among sampling sites within floral types (16.5%) was lower than variation among floral types (27.4%).

Two different approaches were employed in order to further analyse genetic differentiation of the floral variants. AFLPOP was used to test for the frequency of successful re-allocation of all individuals to their flower type-specific source population (*Spe* or wild-type). In total, 93% of the wild-type individuals and 78% of the *Spe* individuals were successfully re-allocated with log-likelihood differences > 2 (Table 4). Only 4% of the wild-type individuals and 16% of the *Spe* individuals were allocated to the incorrect source population. Eight individuals (4.2%) were not allocated based on the applied criterion (MLD = 2). Additionally, individual-based assignment to a given number of clusters *K* (without prior population information) was performed using Structure. The most likely number of clusters *K* of individuals has been estimated based on 10 independent runs for $K = 1$–16 using the procedure described in Evanno *et al*. (2005). Adopting the transformation of calculated $\ln \Pr(X \mid K)$ into ΔK, the most probable number of populations was detected for $K = 2$. By inferring the slope for all estimates of *K*'s, the maximum estimate was detected again for two clusters, supporting our assumption of two populations being the most likely characterization (Fig. S2, Supporting information). Under settings of two populations ($K = 2$) and a minimum assignment probability of 0.95, one cluster includes 96.1% of the wild-type individuals and the second cluster includes about 80% of the *Spe* individuals, respectively (Table 4). Only 3% of wild-types and 19% of *Spe* individuals respectively, were assigned to the contrary cluster.

Flowering time

The onset of flowering has been analysed in a *t*-test based on mean scores of 16 wild-type ($n = 73$) and 10 decandric ($n = 49$) families (122 individuals in total). Under controlled greenhouse conditions, a shift in flowering phenology was

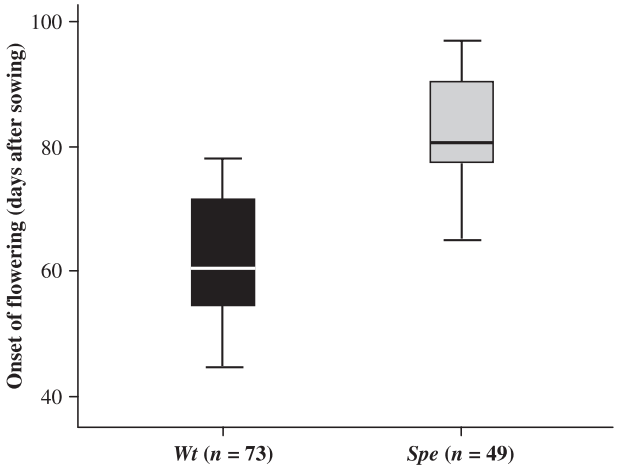

Fig. 3 Boxplot of the number of days until flowering in two *Capsella bursa-pastoris* floral phenotypes which occur sympatrically. Family mean scores of wild-type (*Wt*) and '*Stamenoid petals*' (*Spe*) are separated in their onset of flowering significantly ($P < 0.001$).

detected between both variants, as the onset of flowering was significantly later in *Spe* compared with wild-types ($P < 0.001$). First wild types of *C. bursa-pastoris* started to bloom 41 days after sowing. Considering a range of 52 days, the latest onset of flowering was detected after 93 days. In contrast, first *Spe* individuals started flowering 59 days after sowing. With a range of 42 days, the latest onset of flowering was documented after 101 days. The mean number of days until flowering was 62.8 days in the wild-type and 81.6 days in *Spe*, revealing a temporal difference of 19 days (Fig. 3).

Discussion

AFLP and allozyme data provided evidence for a genetic differentiation within the Gau-Odernheim population, which coincides with the phenotypic discrimination of *Capsella bursa-pastoris* wild-type and the homeotic mutant. These sympatric morphotypes are further differentiated in their flowering time, facilitating prezygotic isolation of the floral variants. Hence, our findings shed light on the evolutionary significance of the homeotic mutant occurring in the wild.

Genetic differentiation of Spe *and wild-type*

Genetic differentiation within the large *C. bursa-pastoris* population in Gau-Odernheim reflects variation in flower morphology (*Spe vs.* wild-type) rather than a spatial discrimination of different sampling sites (Fig. 2, Table 3). The genetic diversity within the wild-type subsample was higher compared with that of *Spe* which may be explained by the regional colonization of *C. bursa-pastoris*. We hypothesize that multiple introductions and subsequent hybridization among the different source populations may have resulted in high genetic diversity, assuming the local occurrence of *C. bursa-pastoris* probably 12 centuries ago, when agricultural land use like wine-growing was initiated in the area. Additionally, the anthropogenic disturbance by ploughing the soil will maintain the genetic diversity by resurrecting seeds from soil bank (Mahy *et al*. 1999; Morris *et al*. 2002). In contrast to the variation observed in the wild-type, genetic variability within the *Spe* subsample was lowered by one third. This reduced heterogeneity in *Spe* may be explained either by a rather young origin of *Spe* within the population, or by a recent introduction of a single or a few *Spe* individuals. The establishment and persistence of such an initial genotype may be facilitated by high rates of selfing. However, selection pressure is reduced in disturbed habitats (Bosbach & Hurka 1981) and mechanical processing in vineyards may have led to further seed dispersal within the vineyards.

Beside differences in colonization history between both floral variants, reduced gene flow may further enhance the detected flower type-specific population structure. Although the split into two groups is evident, a few intermediates indicate occasional crossing events among these subpopulations, apparent as both clusters are not entirely separated in the PCO (Fig. 2). In line with this, a Bayesian clustering approach assuming admixture of two (sub-)populations (data not shown) identified only nine individuals (4.7%) that exposed almost equal posterior probabilities for either wild-type or *Spe* cluster. A field experiment may be useful to estimate relative outcrossing rates within wild-type and *Spe* respectively, *vs.* rates of crossings among the floral variants. For each *C. bursa-pastoris* phenotype, two inbred lines with known AAT genotypes will be surveyed during the vegetation period for flower visitations and subsequently, detection of heterozygotes in the progeny (AAT genotyping) may unravel putative differences in gene flow within and among the floral variants. Under the local conditions in the Rhinehessen wine-growing region, outcrossing rates in wild-type *C. bursa-pastoris* may be expected to increase to about 20%, as a dry and sunny climate is known to favour cross-fertilization (Hurka & Neuffer 1997).

Variation in flowering time promotes prezygotic isolation

Flowering time differences are an additional factor strengthening prezygotic isolation among *Spe* and wild-type and may explain their co-occurrence at the same location. Numerous studies indicate the importance of flowering time differences as a prezygotic isolation barrier (Stam 1983; Husband & Schemske 2000; Martin & Willis 2007). Such seasonal differences in flowering time may lead to occasional isolation, as reported in Wendt *et al*. (2002) for three sympatric species of *Pitcairnia* (Bromeliaceae). Additionally, variation in flowering time is often correlated with local adaptation (Stinchcombe *et al*. 2004; Hall & Willis 2006; Sandring *et al*.

2007) and even gives support for sympatric speciation in palms (Savolainen *et al*. 2006). While many studies revealed a decrease in gene flow among populations as a result of flowering time variation, our results indicate that this is valid for Gau-Odernheim on an intrapopulation level. However, although the mean number of days until the onset of flowering differed significantly, there was still an overlap in flowering period. Due to variation within the wild-type subpopulation, a few wild-types were late flowering like *Spe*. This may lead to occasional admixture between the two variants.

We conclude, that the flower type-specific population structure revealed by using molecular markers is maintained in complementary mechanisms: a differentiation in flowering time among the two variants and self-fertilization in general. With regard to the modified floral morphology in the *Spe* mutant, we argue, that outcrossing rates in the variant are strikingly lowered. The attraction of flower visitors is influenced by various factors, among them visual as well as olfactory cues (van Doorn 1997 and literature cited therein; Bradshaw *et al*. 1998). In *Spe*, both attractants are missing: petals are transformed into stamens and floral scents, which are often produced by petal cells (Pichersky & Gershenzon 2002), have not been detected in *Spe* plants but in the wild-type (J. Ziermann, M. Ritz, S. Hameister, C. Abel, M.H. Hoffmann, B. Neuffer & G. Theißen, unpublished). The latter study also revealed that the loss of corolla function in the *Spe* mutant is indeed followed by a reduced number of flower visitations, whereas the species assemblage was apparently not affected compared with the wild-type. Among the determined species, wild-bees and hoverflies are the most abundant species which visit flowers of *C. bursa-pastoris*. This is in line with former reports (Reichert 1998) and emphasizes the potential impact on outcrossing patterns, as both species groups are efficient pollinators.

Evolutionary significance of the Spe *variant of* C. bursa-pastoris

In an evolutionary context, the question arises whether the variation in flowering time is linked to the homeotic mutation explaining the formation of an initial *Spe* individual and its prezygotic isolation in a single step. Indeed, a shift in flowering time has been reported for artificial homeotic *Arabidopsis* mutants (Borner *et al*. 2000; Yu *et al*. 2002; Michaels *et al*. 2003). However, these mutations caused a shift to early flowering rather than to late flowering as in the *C. bursa-pastoris* variant. *Arabidopsis* mutants which show ectopic expression of *AGAMOUS*, the most probable candidate gene for the *Spe* phenotype, flower early (Mizukami & Ma 1997; Koornneef *et al*. 1998; Simpson & Dean 2002). Regarding the known differentiation of flowering ecotypes in *C. bursa-pastoris* (Neuffer & Hurka 1999; Linde *et al*. 2001), it is more likely that the *Spe* variant originated from a late flowering wild-type, either within the Gau-Odernheim population or elsewhere. Floral phenotypes show an overlapping range in the onset of flowering (Table 2), which may be another hint that the late flowering in *Spe* is not linked to the homeotic mutation. In line with this, preliminary findings of a quantitative trait loci analysis do not provide evidence for a linkage of a single *Spe* locus and a QTL for flowering time (Hameister *et al*. 2009, unpublished data). Isolation of candidate genes and analyses of expression patterns (in-situ hybridization) are underway (G. Theißen, personal communication) in order to further reveal the genetic basis of the *Spe* phenotype. Successful transformation as required for heterologous expression experiments in *C. bursa-pastoris* has been shown by Bartholmes *et al*. (2007).

Assuming a local origin, flowering time variation would represent a key factor for disruptive evolution in the Gau-Odernheim population. An initial selfing *Spe* individual could produce tens of thousands of seeds (Hurka & Neuffer 1991) which are easily spread in vineyards by intensive farming processes. As an alternative explanation, the *Spe* variant could have been introduced from elsewhere, leading to secondary contact and occasional hybridization. The decandric *C. bursa-pastoris* is currently known from Warburg (Germany), the surroundings of Vienna (Austria) and Russia, but only from a small number of individuals. In the proximity of the Gau-Odernheim vineyards, no further population has so far been discovered. However, a floristic survey from the early 19th century is of considerable interest (Becker 1828). At that time, a frequent occurrence of the decandric variant was reported in agricultural lands close to Frankfurt/Main, which is approximately 50 km away from Gau-Odernheim. Indeed, it is likely that the *Spe* variant was more common in the beginning of the 19th century (Opiz 1821; Trattinnick 1821; Becker 1828). Today, German floras do not distinguish this variant as an independent taxon and consequently it will not be recorded in floristic surveys. Thus, the geographical distance between the currently known locations of the decandric *C. bursa-pastoris* may be reduced by the existence of additional populations. In fact, based on molecular analysis for extant populations (S. Hameister, unpublished), we argue that multiple independent origins of the *Spe* variant are the most likely explanation for its disjunct distribution pattern.

In conclusion, the data presented provide substantial evidence that the *Spe* phenotype, formerly known as *Capsella apetala*, has established itself as an isolated entity within a wild-type population. '*Capsella apetala*' may indeed have the potential to represent an evolutionary novelty as proposed by Theißen (2000). The co-existence of *Spe* and wild-types for almost 20 years led Theißen (2006) to suggest *Spe* as a feasible example for non-gradualistic evolution, adopting the concept of 'hopeful monsters' founded by Richard

Goldschmidt (1940). With regard to the Gau-Odernheim population, one key question will be whether the differentiation is caused by the homeotic mutation, or if variation in flowering time is the driving force. Due to the coincidence of phenotypic and genetic differentiation, the Gau-Odernheim population represents a highly interesting model for studying evolutionary processes in sympatric plant populations.

Acknowledgements

The authors wish to thank C. Niepötter for independent AFLP scoring and U. Coja and R. Grupe for technical assistance. We are grateful to H. Hurka for valuable comments on the manuscript and to Lucille Schmieding for proofreading. Many thanks also to Günter Theißen for discussion in our cooperation project. We also thank Dr Christian Lexer and two anonymous reviewers for helpful comments on an earlier version of the manuscript. Financial support by the Deutsche Forschungsgemeinschaft DFG (Ne 314/7-1 and 2) is greatly acknowledged.

References

Al-Shehbaz IA, Beilstein MA, Kellogg EA (2006) Systematics and phylogeny of the Brassicaceae (Cruciferae): an overview. *Plant Systematics and Evolution*, **259**, 89–120.

Bartholmes C, Nutt P, Theißen G (2007) Germline transformation of Shepherd's purse (*Capsella bursa-pastoris*) by the 'floral dip' method as a tool for evolutionary and developmental biology. *Gene*, **409**, 11–19.

Baskin JM, Baskin CC (1989) Germination responses of buried seeds of *Capsella bursa-pastoris* exposed to seasonal temperature changes. *Weed Research*, **29**, 205–212.

Becker J (1828) *Flora der Gegend um Frankfurt am Main*. Brede, Offenbach, Germany.

Bleeker W, Hurka H (2001) Introgressive hybridization in *Rorippa* (Brassicaceae): gene flow and its consequences in natural and anthropogenic habitats. *Molecular Ecology*, **10**, 2013–2022.

Borner R, Kampmann G, Chandler J, Gleißner R, Wisman E, Apel K, Melzer S (2000) A MADS domain gene involved in the transition to flowering in *Arabidopsis*. *The Plant Journal*, **24**, 591–599.

Bosbach K, Hurka H (1981) Biosystematic studies on *Capsella bursa-pastoris* (Brassicaceae): enzyme polymorphisms in natural populations. *Plant Systematics and Evolution*, **137**, 73–94.

Bradshaw HD, Schemske DW (2003) Allele substitution at a flower colour locus produces a pollinator shift in monkey flowers. *Nature*, **426**, 176–178.

Bradshaw HD Jr, Wilbert SM, Otto KG, Schemske DW (1995) Genetic mapping of floral traits associated with reproductive isolation in monkeyflowers (*Mimulus*). *Nature*, **376**, 762–765.

Bradshaw HD Jr, Otto KG, Frewen BE, McKay JK, Schemske DW (1998) Quantitative trait loci affecting differences in floral morphology between two species of monkeyflower (*Mimulus*). *Genetics*, **149**, 367–382.

Coen ES, Meyerowitz EM (1991) The war of the whorls: genetic interactions controlling flower development. *Nature*, **353**, 31–37.

Comes HP (1998) Major gene effects during weed evolution: phenotypic characters cosegregate with alleles at the ray floret locus in *Senecio vulgaris* L. (Asteraceae). *Journal of Heredity*, **89**, 54–61.

Doebley J, Stec A, Gustus C (1995) *Teosinte branched 1* and the origin of maize: evidence for epistasis and the evolution of dominance. *Genetics*, **141**, 333–346.

van Doorn WG (1997) Effects of pollination on floral attraction and longevity. *Journal of Experimental Botany*, **48**, 1615–1622.

Duchesne P, Bernatchez L (2002) AFLPOP: a computer program for simulated and real population allocation based on AFLP data. *Molecular Ecology Notes*, **3**, 380–383.

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software structure: a simulation study. *Molecular Ecology*, **14**, 2611–2620.

Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics*, **86**, 991–1000.

Hall MC, Willis JH (2006) Divergent selection on flowering time contributes to local adaptation in *Mimulus guttatus* populations. *Evolution*, **60**, 2466–2477.

Hawes C, Begg GS, Squire GR, Iannetta PPM (2005) Individuals as the basic accounting unit in studies of ecosystem function: functional diversity in shepherd's purse, *Capsella*. *Oikos*, **109**, 521–534.

Hintz M, Bartholmes C, Nutt P, Ziermann J, Hameister S, Neuffer B, Theißen G (2006) Catching a 'hopeful monster': sheperd's purse (*Capsella bursa-pastoris*) as a model system to study the evolution of flower development. *Journal of Experimental Botany*, **57**, 3531–3542.

Hurka H, Neuffer B (1991) Colonising success in plants: genetic variation and phenotypic plasticity in life-history traits in *Capsella bursa-pastoris*. In: *Modern Ecology: Basic and Applied Aspects* (eds Esser G, Overdieck D), pp. 77–96. Elsevier, Amsterdam, The Netherlands.

Hurka H, Neuffer B (1997) Evolutionary processes in the genus *Capsella* (Brassicaceae). *Plant Systematics and Evolution*, **206**, 295–316.

Hurka H, Freudner S, Brown AHD, Plantholt U (1989) Aspartate aminotransferase isozymes in the genus *Capsella* (Brassicaceae): subcellular location, gene duplication, and polymorphism. *Biochemical Genetics*, **27**, 77–90.

Husband BC, Schemske DW (2000) Ecological mechanisms of reproductive isolation between diploid and tetraploid *Chamerion angustifolium*. *Journal of Ecology*, **88**, 689–701.

Jack T, Sieburth L, Meyerowitz EM (1997) Targeted misexpression of *AGAMOUS* in whorl 2 of *Arabidopsis* flowers. *The Plant Journal*, **11**, 825–839.

Koornneef M, Alonso-Blanco C, Peeters AJM, Soppe W (1998) Genetic control of flowering time in *Arabidopsis*. *Annual Review of Plant Physiology and Plant Molecular Biology*, **49**, 345–370.

Lewontin RC (1972) The apportionment of human diversity. *Evolutionary Biology*, **6**, 381–398.

Linde M, Diel S, Neuffer B (2001) Flowering ecotypes of *Capsella bursa-pastoris* (L.) Medik. (Brassicaceae) analysed by a cosegregation of phenotypic characters (QTL) and molecular markers. *Annals of Botany*, **87**, 91–99.

Mahy G, Vekemans X, Jacquemart A-L (1999) Patterns of allozymic variation within *Calluna vulgaris* populations at seed bank and adult stages. *Heredity*, **82**, 432–440.

Martin NH, Willis JH (2007) Ecological divergence associated with mating system causes nearly complete reproductive isolation between sympatric *Mimulus* species. *Evolution*, **61**, 68–82.

Michaels SD, Ditta G, Gustafson-Brown C, Pelaz S, Yanofsky M, Amasino RM (2003) *AGL24* acts as a promoter of flowering in

Arabidopsis and is positively regulated by vernalization. *The Plant Journal*, **33**, 867–874.
Mizukami Y, Ma H (1997) Determination of *Arabidopsis* floral meristem identity by *AGAMOUS*. *The Plant Cell*, **9**, 393–408.
Morris AB, Baucom RS, Cruzan MB (2002) Stratified analysis of the soil seed bank in the cedar glade endemic *Astragalus bibullatus*: evidence for historical changes in genetic structure. *American Journal of Botany*, **89**, 29–36.
Nei M (1973) Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Sciences, USA*, **70**, 3321–3323.
Nei M, Li WH (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proceedings of the National Academy of Sciences, USA*, **76**, 5269–5273.
Neuffer B, Hurka H (1986) Variation of development time until flowering in natural populations of *Capsella bursa-pastoris* (Cruciferae). *Plant Systematics and Evolution*, **152**, 277–296.
Neuffer B, Hurka H (1999) Colonization history and introduction dynamics of *Capsella bursa-pastoris* (Brassicaceae) in North America: isozymes and quantitative traits. *Molecular Ecology*, **8**, 1667–1681.
Nutt P, Ziermann J, Hintz M, Neuffer B, Theißen G (2006) *Capsella* as a model system to study the evolutionary relevance of floral homeotic mutants. *Plant Systematics and Evolution*, **259**, 217–235.
Opiz PM (1821) 2. *Capsella apetala Opiz. Eine neue merkwürdige Pflanze. Flora* Nr. 28, oder: Botanische Zeitung, Regensburg, Germany.
Peakall R, Smouse PE (2006) GenAlEx 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*, **6**, 288–295.
Pichersky E, Gershenzon J (2002) The formation and function of plant volatiles: perfumes for pollinator attraction and defense. *Current Opinion in Plant Biology*, **5**, 237–243.
Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. *Genetics*, **155**, 945–959.
Reichert H (1998) Eine kronblattlose Sippe des Hirtentäschels (*Capsella bursa-pastoris*) seit Jahren bestandsbildend bei Gau-Odernheim/Rheinhessen. *Hessische Floristische Rundbriefe*, **47**, 53–55.
Sandring S, Riihimäki M-A, Savolainen O, Ågren J (2007) Selection on flowering time and floral display in an alpine and a lowland population of *Arabidopsis lyrata*. *Journal of Evolutionary Biology*, **20**, 558–567.
Savolainen V, Anstett M-C, Lexer C, Hutton I, Clarkson JJ, Norup MV, Powell MP, Springate D, Salamin N, Baker WJ (2006) Sympatric speciation in palms on an oceanic island. *Nature*, **441**, 210–213.
Shull GH (1929) Species hybridization among old and new species of shepherd's purse. *Proceedings of the International Congress of Plant Sciences*, **1**, 837–888.
Simpson GG, Dean C (2002) *Arabidopsis*, the rosetta stone of flowering time? *Science*, **296**, 285–289.
Stam P (1983) The evolution of reproductive isolation in closely adjacent populations through differential flowering time. *Heredity*, **50**, 105–118.
Stinchcombe JR, Weinig C, Ungerer M (2004) A latitudinal cline in flowering time in *Arabidopsis thaliana* modulated by the flowering time gene *FRIGIDA*. *Proceedings of the National Academy of Sciences, USA*, **101**, 4712–4717.
Theißen G (2000) Evolutionary developmental genetics of floral symmetry: the revealing power of Linnaeus' monstrous flower. *Bioessays*, **22**, 209–213.
Theißen G (2006) The proper place of hopeful monsters in evolutionary biology. *Theory in Biosciences*, **124**, 349–369.
Trattinnick L (1821) Botanische Bemerkungen. *Flora*, **46**, 622.
Vekemans X (2002) *AFLP-SURV*, Version 1.0. Laboratoire de Génétique et Ecologie Végétale. Université Libre de Bruxelles, Brussels, Belgium.
Vos P, Hogers R, Bleeker M *et al*. (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research*, **23**, 4407–4414.
Wendel FJ, Weeden NF (1989) Visualization and interpretation of plant isozymes. In: *Isozymes in Plant Biology* (eds Soltis DE, Soltis PS), pp. 5–45. Dioscorides Press, Portland, Oregon.
Wendt T, Canela MBF, Klein DE, Rios RI (2002) Selfing facilitates reproductive isolation among three sympatric species of *Pitcairnia* (Bromeliaceae). *Plant Systematics and Evolution*, **232**, 201–212.
Yeh FC, Yang R-C, Boyle TBJ, Ye Z-H, Mao JX (1997) *POPGENE, the User-friendly Shareware for Population Genetic Analysis*. Molecular Biology and Biotechnology Centre, University of Alberta, Alberta, Edmonton, Canada.
Yu H, Xu Y, Ling Tan E, Kumar PP (2002) *AGAMOUS-LIKE 24*, a dosage-dependent mediator of the flowering signals. *Proceedings of the National Academy of Sciences, USA*, **99**, 16336–16341.

S. Hameister works on the ecological and molecular characterisation of natural occurring variation in the genus *Capsella*. The presented study was part of his PhD thesis. B. Neuffer works on molecular systematics and evolutionary processes within the Brassicaceae with special focus on the genus *Capsella*. W. Bleeker works on molecular systematics and biogeography of the Brassicaceae. He is currently concentrating on the impact of hybridisation among native and non-native plant populations.

Supporting information

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

Fig. S1 Relationship between two genetic distance matrices inferred from AFLP (0/1) scoring of bands, carried out from two collaborators independently.

Fig. S2 Graphic display for the true number of cluster *K* estimated in Structure analyses for wild-type (n=103) and the *Spe* variant (n=88).

Table S1 Matrix 0-1

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.