ECOLOGICAL AND MOLECULAR CHARACTERISATION OF A NATURALLY OCCURRING FLORAL HOMEOTIC VARIANT OF *CAPSELLA BURSA-PASTORIS* (L.) MEDIK.

Dissertation

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Introduction

Natural variation in flower morphology within the Brassicaceae

Striking feature of angiosperm diversity is the vast number of variations in flower morphology. The fundamental flower architecture of most eudicotyledons is characterized by a restricted number of floral organs: these are perianth organs (calyx, corolla) which enclose male (stamen) and/or female (carpel) reproductive organs. Within the mustard family (Brassicaceae), interpretation of the floral structure is somewhat controversial but the general floral ground plan is highly conserved throughout the family (Endress 1992). Referring to the molecular model plant *Arabidopsis thaliana* (L.) Heynh., it usually consists of four sepals and four petals, six stamens (2 + 4) and two fused carpels, all arranged in concentric rings (whorls). Although diverse explanations for the occurrence of four medial stamens and two shorter lateral ones are discussed, these two "rings" of stamens are treated as a single whorl of male reproductive organs in this study, following Meyerowitz *et al*. (1989). However, it is the occurrence of two "rings" of stamens that creates the disymmetric flower shape typical for the whole family and which is discussed as the result of a *dédoublement* of the inner male organs (de Candolle 1821). Apart from the conserved 'brassicaceous' floral ground plan, variations in flower morphology occur in 15 out of 350 genera (5%) within the family (Endress 1992). Such modifications include the transition from disymmetrical to monosymmetrical flowers in some genera (e.g. *Iberis*, *Teesdalia*), lack of petals (e.g. *Rorippa*, *Lepidium*, *Cardamine*, *Capsella*), increased number of carpels and even unisexual flowers are reported (for review see Endress 1992; Appel & Al-Shehbaz 2003). Apparently flower modifications are rare and often restricted to single taxa. The occurrence of unisexual flowers is only known from four out of ~3500 species within the family and increased number of stamens (24) is solely reported for one taxon, *Megacarpea polyandra* (Al-Shehbaz 1986). Nevertheless, alterations in the characteristic number of floral organs are reported more often and the species-rich genus *Lepidium* is of outstanding importance considering deviations from the floral ground plan (Bowman 1999). Within ~175 species, petals are lacking in at least 25% and more than one half show a reduced number of stamens (Al-Shehbaz 1986). Such complex changes in flower morphology are assumed to be genetically controlled due to the generally conserved ground plan throughout the family (Bowman 1999). Quite a few morphological changes like flower size and shape as well as colour and scent are the result of natural selection. Another kind of variation, namely the transformation of floral organs into another category

of floral organs is nowadays discussed in the context of non-gradualistic evolution (Theißen 2006). The role of such homeotic alterations in the origin and radiation of angiosperm flowers has been intensively studied but is still somewhat controversial (for reviews, see Ronse de Craene 2003; Theißen & Melzer 2007). Thus, analyses of naturally occurring variation in flower morphology, especially within the Brassicaceae might shed light on the evolutionary relevance of such novelties.

Evolutionary developmental genetics of floral organ identity

The current knowledge of mechanisms controlling flower development was exceedingly enhanced by studies in *Arabidopsis thaliana*, the most prominent member of the mustard family. Analyses of mutants which display alterations in the identity of floral organs, so called homeotic mutants, have led to the postulation of the ABC model in the early 1990s (Coen & Meyerowitz 1991). This model postulates, that identity of floral organs is specified by the activity of three classes of genes A, B, and C (e.g. Krizek & Fletscher 2005). Most of these floral organ identity genes encode putative transcription factors of the MADS-domain protein family, and their overlapping expression pattern is realized in distinct spatial boundaries within a single flower. In wild-type *A. thaliana* flowers, activity of these genes leads to a dissected floral primordium and results in the arrangement of floral organs in four concentric whorls: sepals are established in the outer whorl by class A gene activity and petals though overlapping activity of class A + B genes in the second whorl. Reproductive organs like stamens are defined by class B + C activity (whorl 3) and carpels in the fourth whorl by class C genes (Figure 1a & c). In *A. thaliana*, class A genes are represented by *APETALA1* (*AP1*) and *APETALA2* (*AP2*), class B genes by *APETALA3* (*AP3*) and *PISTILLATA* (*PI*), and the class C gene by *AGAMOUS* (*AG*) (for reviews about MADS-box genes in plants see Theißen *et al*. 2000; Krizek & Fletscher 2005). This basic floral ground plan is applicable to most angiosperm flowers. Due to some shortcomings of the initial ABC model (Theißen 2001), it was stepwise expanded for two further functions: the D function is involved in ovule development (Colombo *et al*. 1995), whereas activity of class E genes (Pelaz *et al*. 2001) is required for the specification of floral organs in whorls 2-4 in combination with the ABC genes. Even two decades since the initial ABC model was postulated, *A. thaliana* is still the main focus of "evo-devo" research, but certainly a single species may not serve as a comprehensive model to unravel all aspects of ecology and evolution (Tonsor *et al*. 2004). Apart from the analysis of induced homeotic mutants in model plants, there is growing interest to employ the achieved knowledge (e.g. flower

development) from studies in *A. thaliana* on wild relatives (Mitchell-Olds 2001). In line with this, the occurrence of a floral homeotic variant in natural populations of *Capsella bursa-pastoris* (L.) Medik. might represent a promising model for evolutionary studies in a closely related species (Hintz *et al*. 2006; Nutt *et al*. 2006). In this variant all petals are transformed into additional stamens, a homeotic change which may be explained by a modified ABC model (Figure 1b & c).

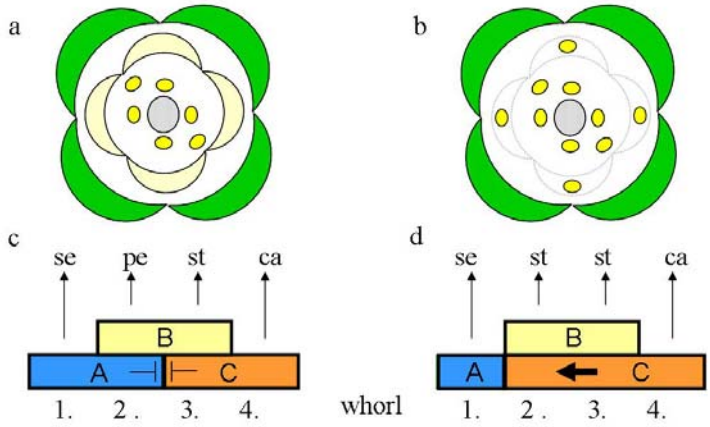

Figure 1: Schematic illustration of floral components in (**a**) wild-type and (**b**) decandric flowers of *Capsella bursa-pastoris*. Floral organs are arranged in four concentric whorls: The outer whorl is formed by sepals (se; green), followed by the petal whorl (pe; pale yellow). In the inner two whorls stamens (st; bright yellow) and carpels (ca; shaded) are established. The outlined formation of wild-type flowers is in accordance with the initial ABC model (**c**) whereas in the homeotic variant (**d**) the C function is ectopically shifted into the second floral whorl leading to additional stamens.

Recent insights in the genus *Capsella*

Based on a recent study from Beilstein *et al*. (2006), a new concept of tribes in the Brassicaceae was introduced by Al-Shehbaz (2006) and generally confirmed in a latest Brassicaceae phylogeny (Franzke *et al*. 2009). In these studies three major lineages (I-III) were confirmed and the results clearly contradict the former association of *Capsella* to the tribe Lepidieae (Hayek 1911; Schulz 1936; Janchen 1946), which was mainly set up due to morphological-based phylogenies like fruit shape (e.g. angustiseptat *vs*. latiseptat). Further studies with a more specific focus on several sub-lineages within the family revealed that the genus *Capsella* is closely related to the *Arabidopsis thaliana*-lineage (Koch *et al*. 1999; Koch *et al*. 2001; O'Kane & Al-Shehbaz 2003), both belonging to the tribe Camelineae (Al-Shehbaz 2006). This was currently confirmed in a comprehensive Brassicaceae ITS phylogeny including all tribes (German *et al*.; in press). In early approaches of phylogenetic reconstructions, the solely use of morphological traits certainly has led to

inflating species numbers, but not all of these taxa sustained when molecular studies were assessed. In fact the number of species in quite a few genera strikingly decreased when molecular studies were associated (for review see Al-Shehbaz 2006). Such a trend was apparent within the genus *Capsella* as well: in the beginning of the 20th century, Almquist (1907, 1923) distinguished about 200 taxa of shepherd's purse, *C. bursa-pastoris* by fruit characters. At the same time Shull (1923, 1929) began to strengthen the taxonomy within the genus and summarized ten species. Until to date the phylogeny of *Capsella* is not entirely interpreted but a convenient species concept was given by Hurka & Neuffer (1997) considering the diploid species *C. grandiflora* (Fauché & Chaub.) Boiss and *C. rubella* Reut. as well as the tetraploid *C. bursa-pastoris*. Recently, the speciation of the two closely related diploids has been estimated suggesting a divergence in the past 25000-35000 years (Foxe *et al*. 2009; Guo *et al*. 2009), whereas the origin of the tetraploid *C. bursa-pastoris* is still unresolved. The scenario of auto-polyploidization within *C. grandiflora* was favored by Hurka & Neuffer (1997), but the authors did not exclude an allopolyploid origin of *C. bursa-pastoris*. Indeed, the reported disomic inheritance of investigated allozymes favours an allotetraploid origin (Hurka *et al*. 1989). In a recent study (Slotte *et al*. 2006) a novel approach was conducted to resolve the relationship of the three species which are approved so far. In the latter study, cpDNA sequences as well as nuclear gene sequences were combined and revealed that neither *C. grandiflora* nor *C. rubella* is likely to be a maternal parent. Additionally, the authors argued that *C. bursa-pastoris* is not an allopolyploid of the two diploids or an autopolyploid of *C. grandiflora*. The identified shared alleles between *C. bursa-pastoris* and *C. rubella* are discussed as the result of postpolyploidization hybridization and introgression (Slotte *et al*. 2008). However, even with regard to molecular studies the polyploid origin of *C. bursa-pastoris* stays an open subject for further analysis and might remain unresolved unless further species (*Capsella* taxa or closest relatives) are identified and embedded in phylogenetic reconstructions. The diploid *C. grandiflora*, however, is certainly the ancestral taxon within the genus since breakdown of self-incompatibility (*C. rubella*, *C. bursa-pastoris*) and polyploidization (*C. bursa-pastoris*) are commonly derived traits.

Apart from ploidy level, the mentioned *Capsella* species are differentiated in mating system, flower shape and habitat distribution (Hurka & Neuffer 1997): cross-fertilization is required in the self-incompatible (SI) *C. grandiflora*, whereas self-compatibility (SC) has evolved in *C. rubella* and *C. bursa-pastoris*. In the latter species, the breakdown of SI-system and polyploidization has led to enormous colonization ability in almost all man-

made habitats world-wide (Neuffer & Hurka 1999). In contrast, *C. grandiflora* is restricted to Greece, Albania and northern Italy and *C. rubella* naturally occurs in the Mediterranean region, the Middle East and was introduced by European settlers to America, Australia as well as New Zeeland (Hurka & Neuffer 1997). In 1984, the genus *Capsella* was already predicted as a model for evolutionary studies (Hurka 1984) and since decades, a large amount of data has been accumulated for wild populations of *Capsella* species (e.g. Baskin & Baskin 1989; Hurka & Neuffer 1997; Neuffer & Hurka 1999; Hawes *et al*. 2005; Paetsch *et al*. 2006). Especially *C. bursa-pastoris* has been intensively studied for genetic differentiation (Neuffer 1986; Neuffer & Hurka 1997) and adaptations in flowering time (e.g. Neuffer & Hurka 1986; Neuffer & Bartelheim 1989; Neuffer & Hurka 1999; Linde *et al*. 2001; Ceplitis *et al*. 2005; Slotte *et al*. 2007). Allowing for broad ecological adaptations and the close relationship to the molecular model plant *Arabidopsis thaliana*, the genus *Capsella* in fact represent a beneficial model in evolutionary studies.

Capsella apetala revised - case studies in a floral homeotic variant

Polyploidization, breakdown of self-incompatibility and reduction or total loss of petals are common within the Brassicaceae (Hurka *et al*. 2005) and represent evolutionary tendencies which might be involved in speciation processes. In this context, the persistent occurrence of a floral variant of *C. bursa-pastoris* in natural populations might be of great evolutionary relevance. This *Capsella* variant has already been described from different locations throughout Europe in the literature of the early 19th century: In Prague, Opiz (1821) observed a shepherd's purse, showing an altered floral morphology with ten instead of six stamens as a consequence of transformed petals (Figure 2b). Due to its ten stamens, the author named this phenotype 'decandric' and considered the variant as a new species, called *Capsella apetala* Opiz. To that time the taxon was additionally reported from populations in Vienna (Trattinnick 1821), Braunschweig (Wiegmann 1823), surroundings of Frankfurt/Main (Becker 1828) and it was also mentioned in the *Flora Berolinensis* by Schlechtendal (1823) as well as in the *Flora von Westfalen* (Beckhaus 1893). Almquist (1923) described twelve variants of *C. bursa-pastoris* from northern Europe (mainly Sweden) showing apetalous flowers, but only one was characterized by ten stamens (*C. bursa-pastoris* (L.) *litoralis* f. *coronopus* E. AT). In his essay about '*Staminale Pseudapetalie*', Murbeck (1918) reported additional locations with apetalous individuals of shepherd's purse in Berlin (Germany), Sweden (Sköfde, Norrköping) and another one from North America (South Dakota, Deadwood).

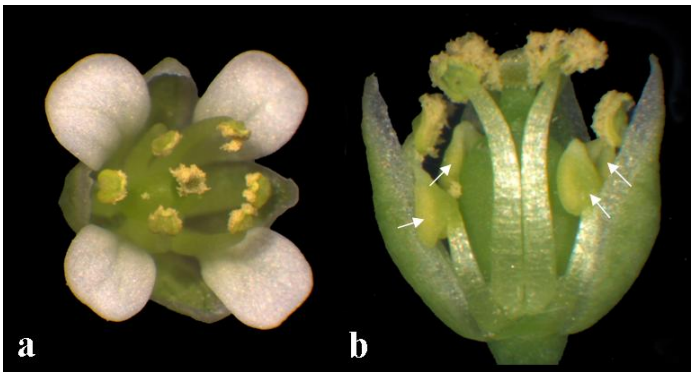

Figure 2: Single *Capsella bursa-pastoris* flowers of: (**a**) wild-type with four showy petals and six stamens and (**b**) the *Spe* variant with petals transformed into additional stamens (marked with arrows).

Recently, the decandric variant has been treated as a floral homeotic mutant and preliminary evidences suggest that a single locus might be affected (Nutt *et al*. 2006; Hameister *et al*., unpubl. data). This locus was termed '*Stamenoid petals*' (*Spe*). Both, *Spe* and decandric will be used synonymously for the phenotypic description. Applying the ABC model (Figure 1b & d) which explains floral organ identity (outlined above), the transformation of petals into stamens in the homeotic variant might be the consequence of ectopic expression of a class C gene that may suppress the expression of class A genes in the second whorl (Hintz *et al*. 2006; Nutt *et al*. 2006). This scenario is supported by studies in transgenic *A. thaliana*, in which also stamenoid petals were observed when the class C gene *AGAMOUS* or a closely related gene was affected (Mizukami & Ma 1992; Jack *et al*. 1997; Pinyopich *et al*. 2003).

To date only a few natural habitats of the decandric variant are known. These include populations in vineyards (Reichert 1998) or ruderal hillsides (Nutt *et al*. 2006) in Germany, and ruderal provenances in the surroundings of Vienna (H Hurka pers. communication; S Hameister).

Gau-Odernheim

To our knowledge, the most established German population of *C. bursa-pastoris* in which the homeotic variant coexists with wild-type plants is located in intensively managed vineyards near Gau-Odernheim, ~25km southwest of Mainz (Rhinehessen). The surroundings of Gau-Odernheim are one of the warmest and driest regions in Germany. Due to these conditions, Rhinehessen became the most important wine-growing region in the country visible in an intensively managed landscape. Conspicuous landmark is the 'Petersberg' with an altitude of 246 m; adjacent hills are 'Kreuzberg' (211 m), 'Lieberg'

(188 m), and 'Neuberg' (173 m). In this hillsides east of Gau-Odernheim, the soil is set up by clay or loess and highly calcareous. Within vineyards, wild-type shepherd's purse is one of the predominant species in the area with at least tens of thousands of individuals. The decandric variant is occurring with a frequency of ~10% referred to wild-types abundance. *Stellaria media* (L.) Vill., *Taraxacum officinale* L., *Senecio vulgaris* L. and *Lamium purpureum* L. are further species in the associated flora. The given soil and climate conditions enable thermophilic species to colonize the region, remarkably apparent in the largest population of *Tulipa sylvestris* L. in the north of the Alps.

Warburg

A second German population of the decandric *C. bursa-pastoris* is located in Warburg (North-Rhine Westphalia, Germany). In contrast to Gau-Odernheim, this sampling site is of low individual number due to the limited extension on basalt subsoil on the hilltop of the extinct volcano 'Desenberg' (343 m). The occurrence of decandric *C. bursa-pastoris* is entirely restricted to the hilltop within a range of 200m², whereas the wild-type occurs more frequently, and is also observed on hillsides and at lower parts of the 'Desenberg'. Interestingly, the non-natural occurrence of *Paronychia kapela* (Hacq.) Kerner (native in Southern Europe) indicates a warm and dry local climate comparable with Gau-Odernheim.

Single individual records

For three additional provenances of the decandric variant of *C. bursa-pastoris* only single individuals were recorded so far. During a field trip in July 1997 seeds from *C. bursa-pastoris* plants were collected in a ruderal population on a train platform in Petrozavodsk (Russia). In subsequent analysis, the progeny of 15 collected plants revealed one decandric individual. A second one showed intermediate organs in the second floral whorl. During an excursion to Lower Austria (August 2005), two more sampling sites were discovered. A single apetalous individual was identified in Haugsdorf (H Hurka) and two further individuals with decandric morphology were collected in Vienna-Hütteldorf (S Hameister).

Thesis objectives and chapter outline

The overall aim of this thesis is the ecological as well as molecular characterization of a naturally occurring floral homeotic variant of common shepherd's purse, *Capsella bursa-pastoris*. So far, only little is known about the establishment of evolutionary novelties in wild populations. To promote the understanding of mechanisms which allow the maintenance of such novelties, the genetic differentiation was analyzed among known decandric populations and within one large sympatric population (Gau-Odernheim). In a comparative approach among floral variants we evaluated ecological adaptive traits such as the onset of flowering and life history traits. Furthermore, we proved heredity of the novel phenotype and intended the chromosomal localization of the assumed single locus *Spe*. The impact of such minor genetical modifications in a single or just a few loci has already been reported (Doebley *et al*. 1995; Comes 1998; Bradshaw & Schemske 2003). Thus, the *Capsella* variant might to some extent promote the controversy about non-gradual evolution. Since changes in flower morphology are often accompanied by (pollinator-mediated) selection, the effect of selection on floral display in the selfing *C. bursa-pastoris* will be discussed. Finally, this thesis may contribute to the question whether the floral homeotic variant will fall back into oblivion or whether it may represent a promising model for evolutionary studies (Theißen 2006).

In order to prove the evolutionary significance of the floral variant, ecological consequences of the homeotic change were analyzed under greenhouse as well as field conditions and the localization of the assumed single locus was initiated by a marker-assisted mapping approach. All these aspects will be thoroughly examined in four chapters, beginning with an ecological characterization and going up to genetic analyses for a molecular characterization.

In **chapter 1,** the occurrence of decandric phenotypes in four geographically distant populations throughout Europe was analyzed to obtain hints for a single or multiple origin of the novel phenotype. AFLP loci and allozymes of aspartate aminotransferase (AAT) were used as molecular markers. Clustering methods (principal co-ordinate analysis, neighbour-joining) have been performed to elucidate patterns of genetic differentiation among the different provenances. The results will be discussed in the light of convergent evolution, as this principle is common in floral traits (e.g. flower colour, size, scents). The natural occurrence of the floral homeotic mutant within a wild-type population composed

of tens of thousands individuals, offers the unique opportunity to elucidate the significance of homeotic mutants with respect to population structure and ecological differentiation. This aspect was focus of **chapter 2.** Here, genetic differentiation and flowering time variation of the decandric variant and wild-type plants were analyzed in a sympatric population in Gau-Odernheim. Again, AFLPs and allozymes of aspartate aminotransferase (AAT) have been used as molecular markers. Genetic variation was estimated by analysis of molecular variance (AMOVA) and principle co-ordinate analysis has been performed to elucidate the population structure. Assignment of individuals into clusters has been carried out using Structure and AFLPOP. A greenhouse experiment was conducted to investigate a possible differentiation in the onset of flowering among both variants.
In the first two chapters, special focus was pointed at the following aspects:

- Is the occurrence of the stamenoid phenotype in geographically distinct populations of *C. bursa-pastoris* the result of convergence?
- Does the population structure of a broad sympatric population of decandric and wild-type individuals reflect any genetic differentiation among variants?
- Are there mechanisms which might cause reproductive isolation among both variants?

In **chapter 3**, further mechanisms were evaluated which might enable the decandric variant to establish and maintain within a wild-type population of *C. bursa-pastoris*. Performing a progeny approach, life-history traits such as fitness components and flowering time of both floral variants were assessed in two common garden field experiments. In addition to it, the potential pollinator assemblage of shepherd's purse was surveyed by collecting flower visitors during field work in the natural habitat Gau-Odernheim. This chapter may answer the following questions:

- Are there differences in fitness components detectable among floral variants in a common garden field experiment?
- Does the field experiment provide insights into different levels of gene flow within or among floral variants?
- How is outcrossing managed in this highly selfing species?

In the **chapter 4**, the inheritance of the decandric phenotype was analyzed in a F2 mapping population. Marker-assisted mapping was performed to localize the assumed single locus.

A cross-species linkage to the genome of *A. thaliana* was intended to constrain candidate genes. The major focus of this chapter can be summarized in the following questions:

- Is the phenotype heritable and does the mode of inheritance corresponds to early reports of the variant?
- Can the assumed single locus be identified in a genetic mapping approach?
- May a cross-linkage to the genome of the molecular model plant provide hints to rule out candidate genes?

The combination of ecological as well as molecular studies may contribute to improve the understanding of evolutionary processes in (sympatric) plant populations in general. The thesis may furthermore represent the first attempt to elucidate the evolutionary relevance of homeotic novelties and there maintenance in wild populations.

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CHAPTER 1


REPEATED EVOLUTION OF A FLORAL TRAIT IN *CAPSELLA BURSA-PASTORIS* - MULTIPLE ORIGINS OF A HOMEOTIC VARIANT IN NATURAL POPULATIONS

Abstract

Background and Aims: Striking feature of angiosperm diversity is a huge number of variations in corolla morphology. Apart from differences in colour and size, more complex novelties are common, such as changes in corolla symmetry and the identity and number of floral organs. Within the Brassicaceae, the disymmetric flower structure is highly conserved but floral alterations are also observed within the family: for instance, a variant of the self-compatible *Capsella bursa-pastoris*, in which all petals are transformed into additional stamens. The present study may contribute to elucidate whether the occurrence of this variant in several provenances throughout Europe is a consequence of multiple origins.

Methods: The evolutionary relationship between four known provenances with decandric individuals was examined using molecular markers. Principal-coordinate and neighbor-joining analyses were performed based on AFLP and allozyme studies.

Key Results: The molecular marker-based analyses revealed a clustering of the floral variant into provenance-specific populations rather than a flower-type dependent allocation. Three groups were obtained: two German populations were convincingly separated and another group includes individuals from Russia and Lower Austria. We did not provide any support for a multiple origin within one well-established population in Germany, but it appeared that in this habitat both floral phenotypes are highly differentiated.

Conclusions: The revealed separation into well-defined populations in accordance with their geographical origin provided substantial evidence for a repeated evolution of the decandric phenotype, independently in the considered habitats. We hypothesize, that the parallel evolution and the establishment of the decandric variant in at least one of the analysed population is mainly driven by predominant self-pollination of *C. bursa-pastoris*.

Introduction

The common architecture of most angiosperm flowers is characterized by the perianth enclosing reproductive organs. These floral organs are arranged in four concentric rings (whorls) and the precise development of organ identity is specified by three classes of regulatory genes (Krizek & Fletcher 2005). The current knowledge of mechanisms controlling flower development was exceedingly enhanced by studies in *Arabidopsis thaliana* (L.) Heynh., a member of the mustard family (Brassicaceae). Throughout this family, the disymmetric flower structure is highly conserved (Endress 1992). It usually consists of four sepals and four petals, six stamens (four medial and two shorter lateral ones) and two fused carpels. Apart from this conserved floral ground plan, variations in flower morphology occur within the family. The transition to monosymmetric flowers is rare (*Iberis*, *Teesdalia*, *Calepina*), whereas alterations in the characteristic number of floral organs are more common like reported for the genus *Lepidium* (Bowman 1999) and also in *Cardamine* (Hegi 1986).

In the genus *Capsella*, another pattern of floral variation has been described in the literature of the 19th century from different locations throughout Europe: In Prague, Opiz (1821) observed a variant of shepherd's purse, *Capsella bursa-pastoris* (L.) Medik., showing a altered floral morphology with ten instead of six stamens as a consequence of transformed petals. Due to its ten stamens, the author named this phenotype 'decandric' and considered the variant as a new species, called '*Capsella apetala*' Opiz. This taxon was reported from Vienna (Trattinnick 1821), Braunschweig (Wiegmann 1823) and surroundings of Frankfurt/Main (Becker 1828) and is listed in the *Flora Berolinensis* by Schlechtendal (1823) and also by Beckhaus in the '*Flora von Westfalen*' (1893). In the beginning of the 20th century, Almquist (1907, 1923) distinguished about 200 taxa of *C. bursa-pastoris* from northern Europe (mainly Sweden), amongst twelve variants showing apetalous flowers. Only one of them was characterized by ten stamens (*C. bursa-pastoris* (L.) *litoralis* f. *coronopus* E. AT). In his essay about '*Staminale Pseudapetalie*', Murbeck (1918) reported additional locations with apetalous individuals of shepherd's purse in Berlin (Germany), Sweden (Sköfde, Norrköping) and another one from North America (South Dakota, Deadwood).

Heredity of the decandric phenotype is known from earlier reports (Opiz 1821) and in addition to wild-type and decandric plants, individuals with intermediate organs in the second floral whorl are described in the literature (Opiz 1854; Dahlgren 1919). Such

"heterozygotes" in floral organ morphology in the petal whorl were also observed in progenies from our field collections and indicate occasional crossings among the two variants (i.e. wild-type and decandric). The heritability of the novel phenotype and its natural occurrence in stable populations suggest an evolutionary relevance of this variant (Theißen 2006). The formerly known *C. apetala* was recently stated as a floral homeotic mutant and the assumed single locus was termed '*Stamenoid petals*' (*Spe*; Nutt *et al*. 2006). The modified phenotype may be explained by a homeotic transformation of petals into stamens (Hintz *et al*. 2006; Nutt *et al*. 2006) applying the knowledge about how regulatory genes control floral organ identity in *A. thaliana*. Control of organ identity by homeotic genes is explained by the ABC model, postulated by Coen & Meyerowitz (1991). The model is applicable to a wide range of plant species, and ectopic expression of a class C gene in the second floral whorl was considered to describe the homeotic transformation of petals into stamens (Hintz *et al*. 2006; Nutt *et al*. 2006). Changes in expression patterns of these control genes are one factor for changes in morphology and may even lead to evolutionary novelties (Theißen 2000).

However, to date the decandric *C. bursa-pastoris* is no longer treated as an independent taxon and only a few populations of this variant are currently known. One broad population was discovered in the southwest of Mainz (Germany; Reichert 1998). A second German habitat was identified in Warburg, Westphalia (Nutt *et al*. 2006). Additionally, single individuals were recorded from the surroundings of Vienna (Lower Austria) and also in offspring from field collections in Petrozavodsk (Russia). The occurrence of homeotic *C. bursa-pastoris* in geographically distant populations throughout Europe introduces the question whether the origin of this floral phenotype is due to long-distance dispersal, fragmentation of formerly connected habitats or the result of repeated evolution. Amplified fragment length polymorphisms (AFLPs) and allozyme analyses of the aspartate aminotransferase (AAT) have been carried out to elucidate the evolutionary relationship between these locations. The origin of the modified floral phenotype in *C. bursa-pastoris* will be discussed in the context of convergence *versus* parallelism. Allowing for the knowledge about artificially induced homeotic mutants in the closely related *A. thaliana*, possible candidate genes are considered which might be involved in the molecular basis of the decandric *C. bursa-pastoris*.

Methods

Plant material

Common shepherd's purse, *Capsella bursa-pastoris*, is an annual weed occurring in all man-made habitats and the loss of self-incompatibility was accompanied with polyploidisation in this species (Hurka & Neuffer 1997). Although polyploid, chromosomal reduction in meiosis behaves like in diploid species since disomic inheritance was reported, which suggests an allotetraploid origin (Hurka *et al*. 1989). Polyploidisation has certain led to high colonization ability and a broad range of phenotypic variability (Hurka & Neuffer 1991).

Four known geographically distant populations of *C. bursa-pastoris* were included harbouring individuals with wild-type and decandric floral phenotypes (Figure 1; Table 1). An individual-rich population of *C. bursa-pastoris* in which the decandric variant coexists with wild-type plants is located in intensively managed vineyards in hillsides close to Gau-Odernheim about 25 km southwest of Mainz (Rhinehessen, Germany).

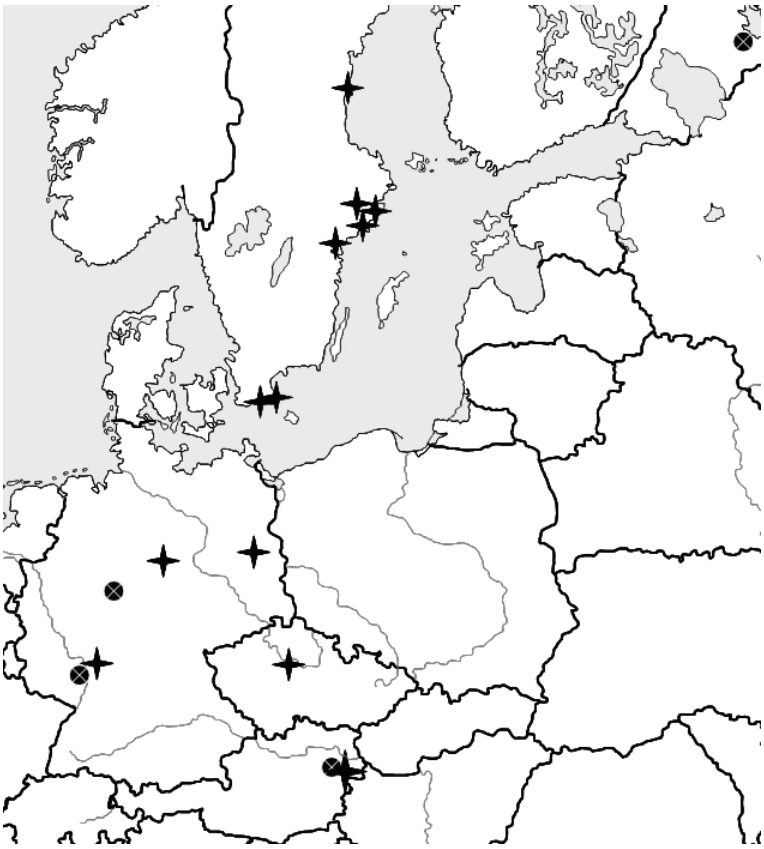

Figure 1: Geographic distribution of decandric *C. bursa-pastoris* throughout Europe including four known extant populations [⊗] and mentioned locations in early literature reports [✦].

In Gau-Odernheim, wild-type shepherd's purse is one of the predominant species in vineyards with at least tens of thousands of individuals. The abundance of the decandric variant is approximately 10% of the total population size compared with the occurrence of wild-type plants. A second population of the decandric *C. bursa-pastoris* is located in Warburg (Westphalia, Germany). In contrast to Gau-Odernheim, this population is of low individual number due to the limited extension on basalt subsoil on the hilltop of the 'Desenberg'. The occurrence of less than 20 decandric *C. bursa-pastoris* is entirely restricted to the hilltop within an area of 200m², whereas the wild-type occurs more frequently, and is also observed on hillsides and at lower parts of the 'Desenberg'. Only single individuals were available for three additional provenances. These are ruderal populations in Petrozavodsk (Russia), in Haugsdorf and Vienna-Hütteldorf (both in Lower Austria).

Seed collection and sampled individuals

In May 2005 seeds of both floral variants of *C. bursa-pastoris* were collected in vineyards close to Gau-Odernheim. Due to the broad habitat range, the population was divided into 15 sub-sites (Pop.-No 1949-1964). Seed collection in the Warburg population (Pop.-No. 1965) was done in June 2005 and 2006. During a field trip in July 1997 seeds from 15 *C. bursa-pastoris* plants were collected on a train platform in Petrozavodsk (Russia). In subsequent analysis, the progeny revealed one decandric individual (1528/2) while a second one showed an intermediate floral phenotype (1528/5). On a field trip to Lower Austria in August 2005, two additional locations were discovered. A single individual was recorded in Haugsdorf (Lower Austria, Pop.-No.1966; H Hurka pers. communication) and two further individuals with decandric morphology were collected in Vienna-Hütteldorf (Austria, Pop.-No. 1967).

Offspring from field collections were cultivated in a greenhouse under controlled conditions (12h illumination / day: min 14°C - max 30°C; night: min 10°C). In total, 34 decandric individuals were applied for the analyses (Table 1). In addition to decandric individuals, 29 wild-type samples were included (63 samples in total). Due to the large Gau-Odernheim population, only a subset of individuals (23 decandric / 21 wild-type plants) was chosen by chance for the present study. Two progenies of a single decandric individual from Russia were considered. Among three decandric samples from Lower Austria, one individual (1966/1; Haugsdorf) was excluded from all further analyses since the petals were apparently not transformed but completely lacking.

Table 1: Collection and sampling data for the decandric *Capsella bursa-pastoris* from Europe.
(*= two progenies from one parental individual; **= apparently a different mutation since no additional stamens were observed but petals totally lacking).

Provenance	Pop.-ID	Location	Habitat	Collection *Spe* : Wt	Sampling *Spe* : Wt
Gau-Odernheim; Rhinehessen; Germany	1949-1963	N49°47' E08°12'	vineyards, field margin, fallow	200 : 179	23 : 21
Warburg; NRW; Germany	1965	N51°30' E09°12'	grassland	18 : 22	7 : 6
Petrozavodsk; Karelia; Russia	1528	N61°23' E34°28'	trailway, ruderal	1 : 14	2* : 2
Haugsdorf; Austria	1966	N48°43' E16°03'	roadside	1 : -	excluded**
Hütteldorf; Vienna; Austria	1967	N48°12' E16°11'	roadside	2 : -	2:-

Molecular markers: *AFLPs and allozymes*

Genomic DNA was isolated from fresh leaves with Invisorb® Spin Plant Kit (Invitek, Berlin, Germany) according to the manufacturer's manual. AFLP procedure (Vos *et al*. 1995) followed the AFLP™ Plant Mapping Protocol (Applied Biosystems) with one modification: Restriction of genomic DNA (*Eco*RI, *Mse*I) and ligation of double-stranded adaptors were conducted within a single reaction. AFLP™ Ligation & Preselective Amplification Module was used (Applied Biosystems). Selective amplification conditions were 1.5µl 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, Muenster, Germany). Cycle parameters were in accordance with the AFLP™ Plant Mapping Protocol. Primer tests revealed that *Eco*RI-ACA/*Mse*I-CAC, *Eco*RI-AAG/*Mse*I-CAC, *Eco*RI-ACC/*Mse*I-CTA are informative and therefore were selected for our approach. Fragments were electrophoretically separated on an ABI Prism™ 377 sequencer (Applied Biosystems) with GeneScan-500 Rox as internal standard. GeneScan 3.1 and Genotyper 2.1 (Applied Biosystems) were used for editing raw data and determining fragment size, respectively. Electropherograms were manually evaluated for presence (1) or absence (0) of fragments.

Like AFLP data, the allozymes have thus been analysed as dominant markers, comparable to the approaches for polyploid plant species (e.g. Bleeker & Hurka 2001). To study allozyme patterns of the aspartate aminotransferase (AAT; EC 2.6.1.1), 0.7 g of rosette leaves from ten weeks old plants were harvested and immediately frozen in -80°C. Extracts were prepared on ice in 1.4 ml chilled extraction buffer (0.160 M tris, 0.107 M glycin, pH

8.0). For native electrophoresis 50 µl samples were separated on 7.5% polyacrylamide gels. More detailed information about experimental procedures and nomenclature of AAT allozymes are given in Hurka *et al*. (1989).

Data analysis

For all individuals, genotypes of three duplicated *Aat* loci (locus *1A/B, 2A/B, 3A/B*) were determined. The percentage of polymorphic AFLP loci was calculated using AFLPsurv 1.0 (Vekemans 2000). AFLP fingerprints and allozyme data were combined to generate a 0/1 matrix. This matrix was used for principal co-ordinate analysis (PCO) and neighbor-joining analysis. To display the genetic distance between the analysed populations, PCO was performed in MVSP 3.13 (Kovach Computing System) using Nei & Li coefficient for binary data. For insights on individual level, neighbor-joining was conducted in Treecon 1.3b (van de Peer 1994). A wild-type individual of *C. bursa-pastoris* from USA (Reno, Nevada; progeny of 740/6) was used as outgroup. Wild-type plants from Gau-Odernheim and Warburg were included for all analyses.

Results

Allozyme variability

For 34 decandric samples, genotypes of three duplicated *Aat* loci (locus *1A/B, 2A/B, 3A/B*) were determined. In total, six different multilocus genotypes were detected. In Gau-Odernheim as well as Warburg, genotype II (1111 1144 1155) was the most common genotype with a frequency of 74% and 57%, respectively. Both individuals from Vienna-Hütteldorf revealed a single genotype (1111 1111 1155) and the two progenies of the Russian ancestor also showed just one genotype (1111 1111 1122).

The AFLP analysis yielded 101 reliable AFLP fragments. Among the analysed populations, 76 fragments (75.2%) were polymorphic. In decandric individuals the percentage of polymorphic loci (*PLP*) varied from 36.5% in Warburg to 68.3% in Gau-Odernheim, whereas the *PLP* in the two Russian individuals was only 3.2%. The two samples from Vienna-Hütteldorf were not polymorphic in AFLP loci.

Genetic differentiation

A combined 0/1-matrix of AFLPs and allozyme data was generated to elucidate the single or multiple origin of the decandric variant. Figure 2 shows the results of a principal co-

ordinates analysis based on the combined data set. The first two axes accounted for 29.4% of the total variation and separated the four analysed provenances into distinct clusters. This differentiation was primarily explained by axis 1 (16.3%) and further promoted by the second axis (13.1%) which additionally displayed a separation within Gau-Odernheim population into two groups. In the first group decandric and wild-type individuals occur, whereas the second group was entirely set up by decandric samples. A few individuals were placed in-between these two groups. Wild-type and *Spe* individuals from Warburg population formed another distinct cluster, whereas the decandric *C. bursa-pastoris* from Vienna-Hütteldorf groups within the Russian cluster.

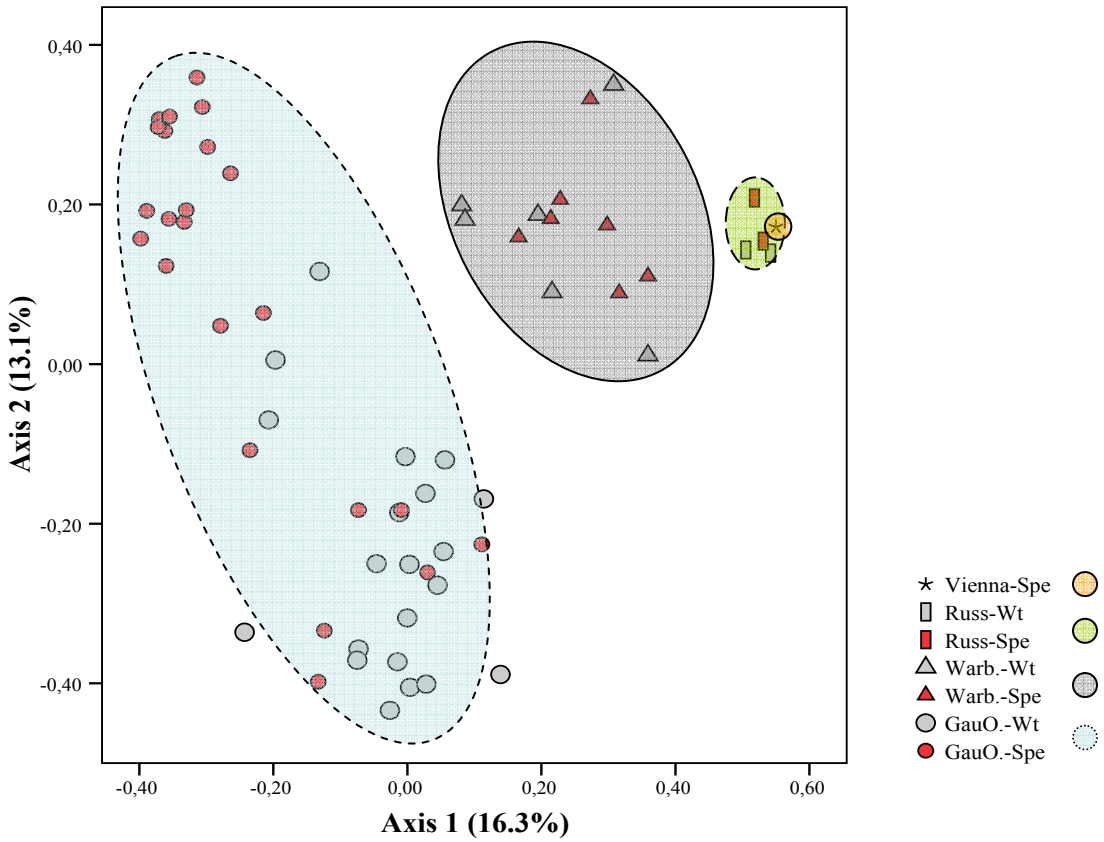

Figure 2: Principal co-ordinate analysis (PCO) based on pairwise genetic distances inferred from a distance matrix using Nei & Li's coefficient. Analysis was performed for a combined dataset including 101 AFLP markers and eight *Aat* loci of four distinct *C. bursa-pastoris* populations harbouring both floral phenotypes. The first axis separates the analysed provenances into distinct clusters (16.3% of the total variation).

In the neighbor-joining distance analysis (Figure 3), decandric individuals clustered clearly within their source location and did not found a monophyletic cluster referring to flower morphology. A wild-type individual from USA as outgroup provided convincing bootstrap support for a differentiation into a German cluster (Warburg and Gau-Odernheim) and a opposing Russian/Lower Austrian cluster. The latter populations are again separated with

high support, whereas the biphyletic origin of Warburg and Gau-Odernheim was less convincing. However, the topology within the German cluster is more representative, due to the higher number of sampled individuals sampled.

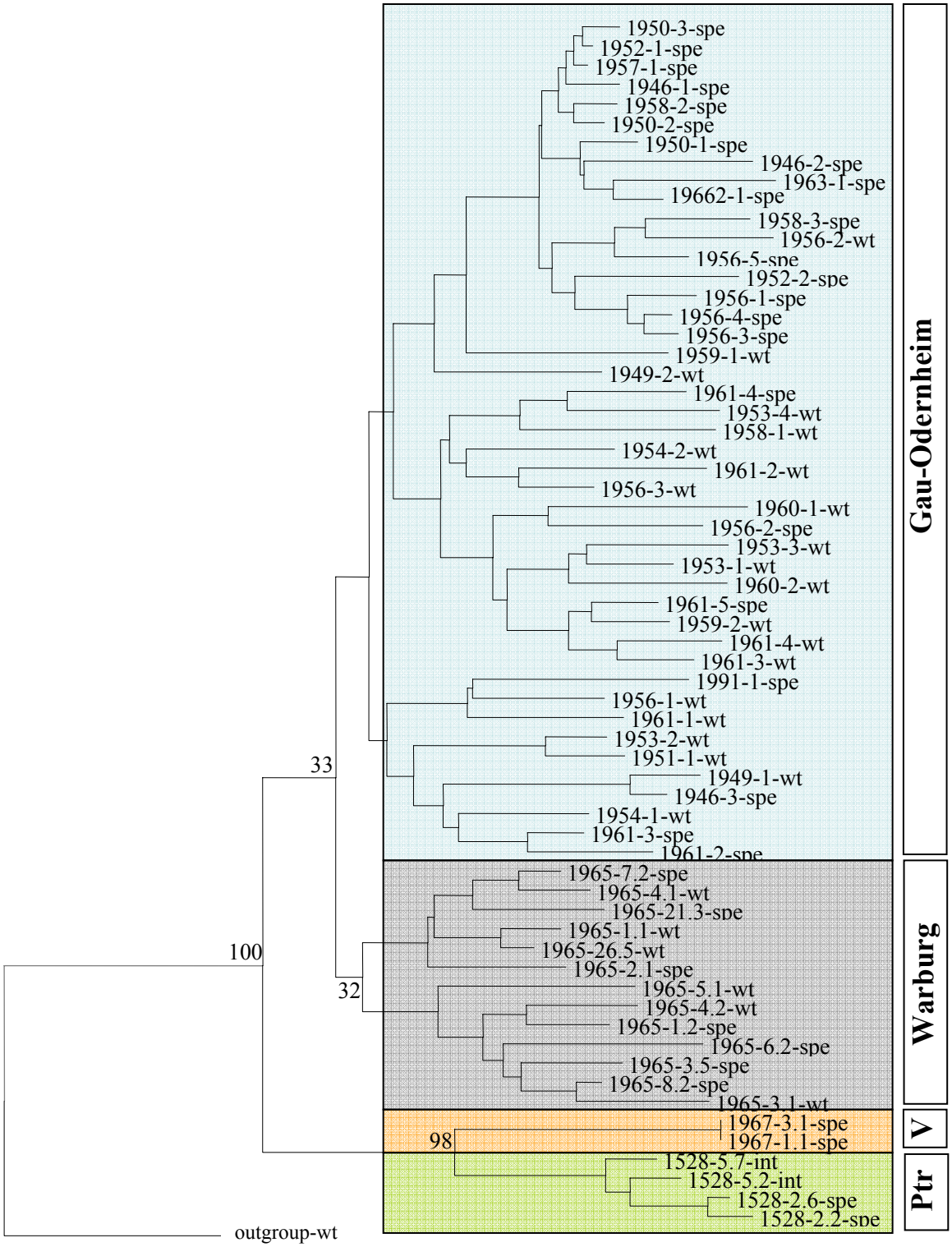

Figure 3: Cluster analysis (neighbor-joining) based on 101 AFLP and eight *Aat* loci detected in four geographical distinct *C. bursa-pastoris* populations in which the decandric phenotype occurs. Decandric individuals clearly cluster within wild-types, suggesting a multiple independent origin of the floral variant. Bootstrap value are denoted for major clades (V = Vienna, Ptr = Petrozavodsk; Karelia).

Discussion

Multiple origin of the decandric variant

The detected genetic differentiation reflects a structuring into biogeographic clusters rather than a flower-type dependent assignment. Both clustering approaches (PCO, NJ) revealed well separated populations in accordance with their geographical origin. This finding suggests a repeated evolution of the decandric flower shape, independently in the considered habitats. So far, the detected genetic differentiations among included populations indicate a four-fold independent origin of decandric phenotypes within wild-type populations. The maintenance of multilocus genotypes for more than 100 years in new colonized regions is reported for *Capsella bursa-pastoris* (Neuffer & Hurka 1999), which is mainly driven by a high degree of self-pollination. Apart from the preservation of an introduced genotype also the maintenance of a single spontaneous mutation within a wild-type population may be facilitated by this mechanism. The occurrence of the decandric phenotype in several locations might be an example for convergent evolution like reported for other variations in flower morphology. For instance the evolution of petal colour (Whittall *et al*. 2006), heterostyly (Perez *et al*. 2003) or floral scents (Andersson *et al*. 2002) are other floral traits for which convergent evolution is discussed. Such alterations in corolla characteristics are often the result of pollinator-mediated selection as reported in Ree (2005), a principle commonly considered as a driving force in the evolution of plants (Schemske & Bradshaw 1999). In selfing species this mechanism might be less crucial as the selection pressure for the attraction of pollinators is nearly abolished. But Gomez *et al*. (2006) reported the adaptive significance of variations in flower symmetry in a natural population of *Erysimum mediohispanicum* Polatschek (Brassicaceae), a self-compatible species which requires cross-fertilization for full seed set. Within the genus *Capsella*, the transition to self-compatibility (SC) is accompanied by a decrease in corolla size (Paetsch *et al*. 2006), which is also established in other Brassicaceae such as *Lepidium* (Lee *et al*. 2000), *Leavenworthia* (Anderson & Busch 2005), *Cardamine* as well as *Rorippa* (Bleeker 2007). In selfing species the demand on pollinators is almost abolished and the reduction in flower size is discussed as a consequence of weakened selective force for corolla function (Rollins 1963; Barrett 2002). In *C. bursa-pastoris*, selection on corolla function is nearly identical in all habitats due to predominant selfing (Hurka & Neuffer 1997). Additionally, *C. bursa-pastoris* prefers open-soil habitats in which selection pressure is less interfering (Bosbach 1982). Convergent evolution in general is assumed to be the result of identical selection forces in comparable environments (Stebbins 1974). This might be applicable for

the repeated evolution of decandric flowers in *C. bursa-pastoris*, although the included provenances may differ in environmental parameters with regard to their geographical position or man-made disturbance (Table 1). Adopting the brief synopsis given in Bowman (2006), convergent evolution refers to the independent evolution of similar traits based on different developmental genes, whereas in parallelism the same genetical changes cause the origin of similar traits. Considering that convergence is referred to if similar morphological novelties have independently evolved in different species, the repeated evolution of the decandric phenotype in *C. bursa-pastoris* in separated localities might be more likely the result of parallelism.

Molecular origin of decandric flowers in Capsella

The occurrence of individuals with intermediate organs in the second floral whorl suggests a co-dominant mode of inheritance, like already suggested by Dahlgren's (1919) crossing experiments with wild-type and decandric plants. It was recently confirmed in a segregating F2 population and a subsequent linkage analysis identified a single locus which might have caused the altered phenotype of the decandric *C. bursa-pastoris* (Hameister *et al*.; unpublished data). Allowing for the recent knowledge about how (many) regulatory genes are involved in the precise development of floral organs several candidate genes are feasible to explain the *Spe* phenotype (Nutt *et al*. 2006). Adopting the ABC model which predicts the control of floral organ identity by homeotic genes (Coen & Meyerowitz 1991), altered expression patterns of involved genes is the most likely scenario to explain the modified flower shape. The ABC model is particularly based on studies in *A. thaliana*. In artificially induced homeotic *Arabidopsis* mutants, comparable phenotypes like the decandric *C. bursa-pastoris* are reported, also showing stamenoid petals in the second floral whorl. For instance, Mizukami & Ma (1992) described this phenotype in transgenic *Arabidopsis* in case that the floral organ identity class C gene, *AGAMOUS* (*AG*), is ectopically expressed in the second floral whorl. In wild-type *A. thaliana*, this homeotic gene is essential for the development of stamens and carpels. Another line of evidence is due to a study from Jack *et al*. (1997): Misexpression of *AG* in the second floral whorl under the control of *APETALA3* promoter also led to stamenoid petals. Taking into account that *C. bursa-pastoris* is one of the closest wild relatives of the model plant *A. thaliana* (Al-Shehbaz *et al*. 2006), this is certainly the most probable candidate gene. Additionally, altered gene expression of *AG*-paralogues, *SHATTERPROOF1* and *2* (*SHP1*; *SHP2*), also caused stamenoid organs in the petal whorl

(Favaro *et al*. 2003). However, most of these mutants showed pleiotropic effects, which in contrast were not detected in the decandric variant. Thus the regulation of floral organ identity might differ between *C. bursa-pastoris* and *A. thaliana* (Nutt *et al*. 2006). Even within *C. bursa-pastoris*, a different molecular basis of the decandric variant in the sampled populations can not be excluded, due to the number of candidate genes described so far.

Conclusion

Although only single individuals were recorded for two of the sampled populations (Russia, Austria), it is reasonable, that the occurrence of the floral homeotic variant of *C. bursa-pastoris* is a result of repeated evolution. Assuming a single origin of the decandric variant, the geographically distant populations might be explained by long-distance dispersal or fragmentation of formerly connected habitats. In fact, in early literature reports the decandric variant was described to occur frequently in the reported habitats (Opiz 1821; Trattinnick 1821), while to date the floral variant is apparently almost extinct. The obtained clustering, however, corresponds to the geographical origin of sampled populations and thus a single origin appears to be an unlikely scenario. Allowing for the number of possible candidate genes, the molecular genetic base may differ among the considered locations. Thus, we can not distinguish between convergence and parallelism so far. Neither the individual-based clustering approach (neighbour joining) nor the intensively studied genetic differentiation of wild-type and decandric samples from Gau-Odernheim (Hameister *et al*. 2009) provided evidences for a multiple origin of the variant within a single location. In contrast, the latter study revealed a striking flower-type dependent differentiation whereas no fine-scaled spatial discrimination into sub-populations was identified. The study also exposed that predominant selfing, an initial barrier of gene flow, is further enhanced by a shifted onset of flowering among variants. Due to this temporal flowering time differentiation, both flower types may be treated as isolated subpopulation. Reproductive isolation among variants is one key factor for the maintenance of the decandric phenotype within the wild-type population, in addition to anthropogenic disturbance by mechanical processing in vineyards of Gau-Odernheim. The extraordinary abundance and the persistence for at least two decades (Reichert 1998) suggest that the floral variant is at least well-established within this population. As the homeotic transformation of petals into stamens has obviously no negative effect in fitness, the floral variant might have the potential to found an evolutionary novelty like proposed

by Theißen (2000). With regard to the remarkable molecular and morphological differentiation, the decandric variant might (again) be treated as an independent taxon, resurrecting *Capsella apetala* from its early reports. Since no ecological adaptation to a certain (yet unknown) niche or any selective benefit was identified for the decandric variant, we hypothesize that the persistence of *C. apetala* is most likely the result of genetic hitch-hiking.

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Chapter 2

POPULATION STRUCTURE AND PREZYGOTIC ISOLATION WITHIN A SYMPATRIC POPULATION OF A NATURAL FLORAL HOMEOTIC VARIANT AND WILD-TYPE OF *CAPSELLA BURSA-PASTORIS* (BRASSICACEAE)

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 analyzed 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. AFLP 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 analyzed population, the *Spe* variant is flowering 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 serve as a model for the analysis of the evolutionary significance of homeotic mutants in wild populations.

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; Hurka & Neuffer 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 ca. 200 years ago (Opiz 1821) and considered as an independent species named *C. apetala* Opiz. Flowers of the plants observed by Opiz (1821) were characterized by ten instead of six stamens (decandric), indicating that petals are not fully lost but transformed into additional stamens. 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 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), where 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, e.g. the origin of maize (Doebley *et al*. 1995), the loss of ray floret in *Senecio* (Comes 1998) and flower color 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 and wild-type *C. bursa-pastoris*. A major question regarding this coexistence is, how the homeotic variant could be maintained within a wild-type population. High rates of self-fertilization in *C. bursa-pastoris* undoubtably facilitate prezygotic isolation. Outcrossing rates are low in *C. bursa-pastoris* and vary between 0 - 20% (Shull 1929; Hurka & Neuffer 1997). In this context, the altered flower morphology of the decandric variant might in fact 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 favor 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 to explain the 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 composed of tens of thousands individuals, 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 (AFLPs) and the allozyme aspartate aminotransferase (AAT) have been used as molecular markers. Differences in the onset of flowering among both variants have been analyzed in a greenhouse experiment. The significance of the sympatric occurrence of *Spe* and wild-type is discussed in an evolutionary context.

Methods

Plant material

The studied population is located in intensively cultivated vineyards close to Gau-Odernheim, ca. 25 km southwest of Mainz (Rhinehessen, Germany; Reichert 1998). The sampling site is characterized by a tremendous abundance of *C. bursa-pastoris* as it is the predominant species in single rows of vine plantation. 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 (12h 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.

AFLP analysis

Genomic DNA was isolated from fresh leaves (100 mg) using the Invisorb® Spin Plant Kit (Invitek, Berlin, Germany). 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 (5U respective 1 U per reaction) were used to digest DNA. For ligation and amplification, kits available from ABI were used. For selective amplification, 5µl 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, Münster, Germany) 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 done 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; Figure 1).

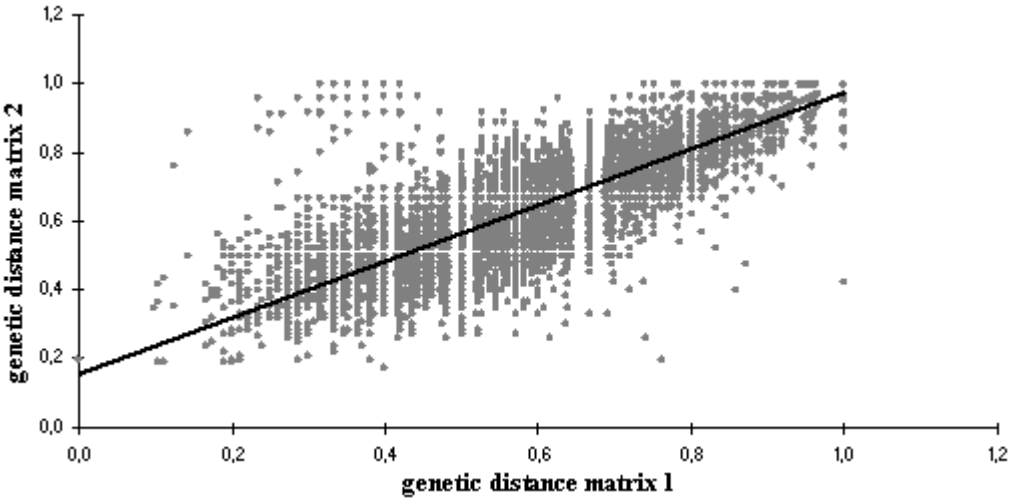

Figure 1: Relationship between two genetic distance matrices inferred from AFLP (0/1) scoring of bands, carried out from two collaborators independently. The correlation between pairwise genetic distances was investigated by Mantel test. The genetic distance for both raw data matrices was calculated using the Nei & Li coefficient for binary data (MVSP 3.13). The conformity of matrices is highly significant (R^2=0.73; $p < 0.001$).

Allozyme studies

The allozyme aspartate aminotransferase (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 ten-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). For native electrophoresis, 50 µl samples were separated on 7.5% polyacrylamide gels (19:1 acrylamide:bisacrylamide). Overnight staining was done 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 intended 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. The allozyme data have thus been analyzed as dominant markers, comparable to the approaches for polyploid plant species (e.g. Bleeker & Hurka 2001). Calculation of genetic distance and principal co-ordinate analysis (PCO) was performed in MVSP 3.13 (Kovach Computing System). The Nearest Neighbour clustering method was applied using the Nei & 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 1992). The Re-allocation procedure in AFLPOP 1.1 (Duchesne & Bernatchez 2002) was used to analyze 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 originally been developed for analyzing 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 expand 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 ten *Spe* individuals (family) were cultivated in a greenhouse under the same conditions as described above. Five individuals per family were analyzed 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, ten different multilocus genotypes were detected. While all ten 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 *C. 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).

Table 1: Frequency (in percentage) of allozyme genotypes for aspartate aminotransferase (AAT) in wild-type (*Wt*) and mutant (*Spe*) phenotypes. 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.

geno -type	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	.

In the AFLP analysis, three primer combinations yielded a total of 81 reliable bands (AFLP loci), 47 (58%) of them were polymorphic within the analyzed population. A Mantel-test revealed high consistency between two genetic distance matrices, generated independently by one of the authors (SH) and a former colleague ($R^2 = 0.73$; $p < 0.001$, see Figure 1). 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 $I^* = 0.374$ (±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.

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
		***				***	***	

Figure 2 shows the results of a principal co-ordinate 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 (Figure 2).

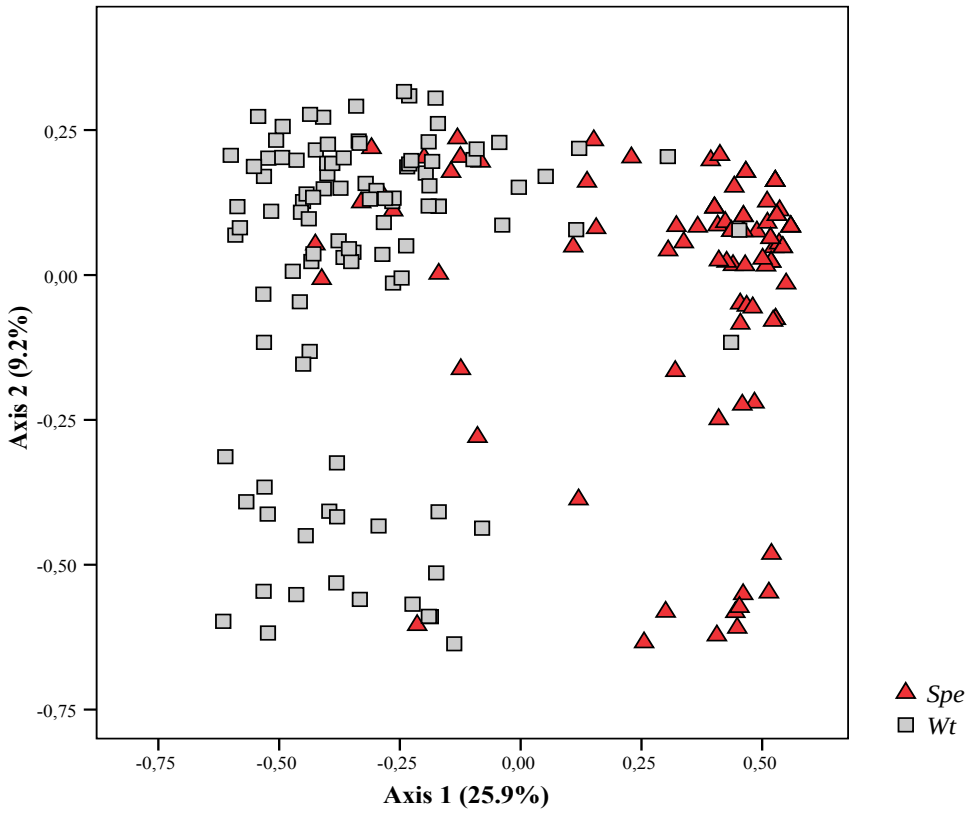

Figure 2: Principal co-ordinate analysis (PCO) based on pairwise genetic distances inferred from a distance matrix using Nei & Li's coefficient. Analysis was performed for a combined dataset including 47 AFLP markers and eight allozymes of two *C. bursa-pastoris* floral phenotypes which occur sympatrically.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

The result of the AMOVA confirmed a considerable differentiation among the two subsamples within the Gau-Odernheim populations: 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 and within floral types (16.5%) was lower than variation among floral types (27.4%).

Table 3: Analysis of molecular variance (AMOVA) among and within two floral variants (*Spe*, wild-type) occurring 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	

Two different approaches were employed in order to further analyze 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 78.4% of the *Spe* individuals were successfully re-allocated with log likelihood differences > 2 (Table 4).

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 8.6% in AFLPOP respective 2.1% in Structure were not allocated with a significant posterior 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

Only 3.9% of the wild-type individuals and 15.9% 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 ten independent runs for $K = 1$-16 using the procedure described in Evanno *et al*. (2005). Adopting the transformation of calculated ln Pr(X | *K*) into Δ*K*, 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 valuable characterization (Figure 3). 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.

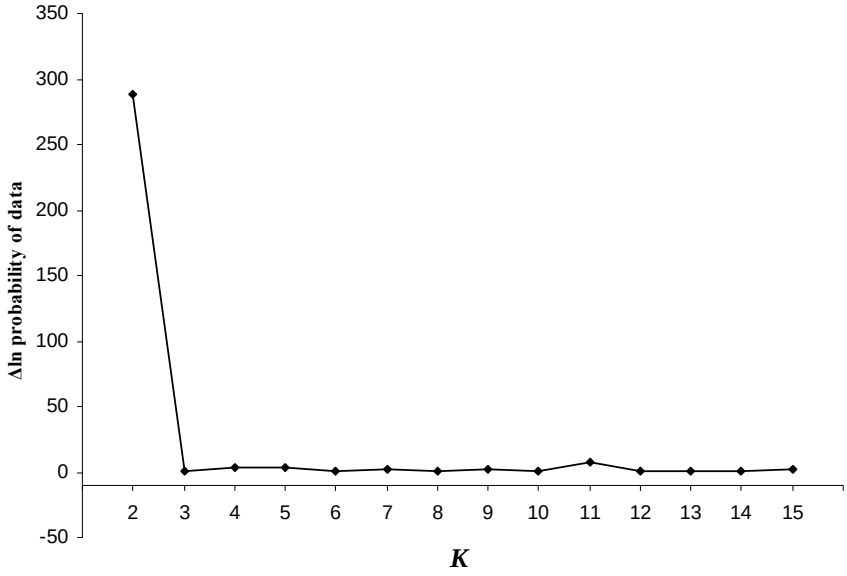

Figure 3: Graphic display for the true number of cluster *K* estimated in Structure analyses for wild-type (n = 103) and the *Spe* variant (n = 88). The second order rate of change (Δ*K*) was calculated as described in Evanno *et al*. (2005). Results exposed the highest probability at *K* = 2 indicating two clusters as the most likely population structure.

Flowering time

The onset of flowering has been analyzed 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 detected between both variants, as the onset of flowering was significantly later in *Spe* compared to 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 (Figure 4).

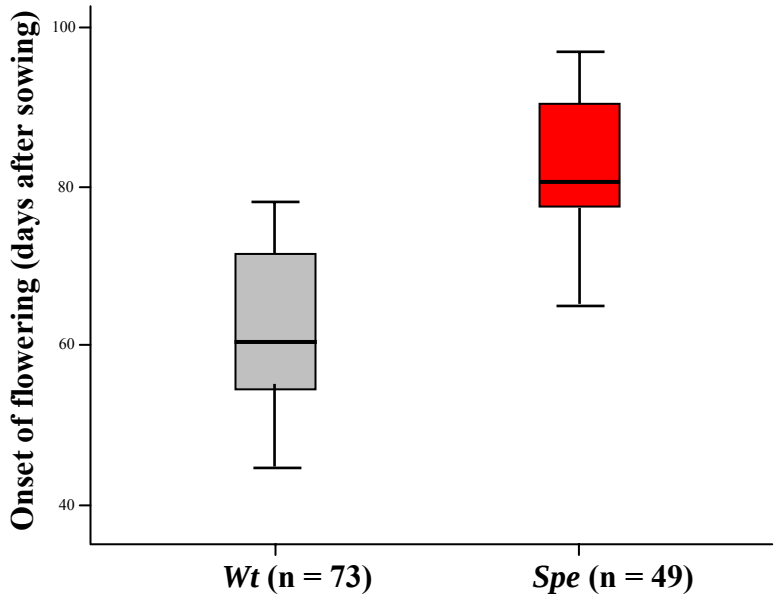

Figure 4: Boxplot of the number of days until flowering in two *C. bursa-pastoris* floral phenotypes which occur sympatrically. Family mean scores of wild-type (*Wt*) and '*Stamenoid petals*' (*Spe*) are separated in their onset of flowering significantly ($p < 0.001$).

Discussion

AFLP and allozyme data provided evidence for a genetic differentiation within the Gau-Odernheim population, which coincides with the phenotypic discrimination of *C. 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 (Figure 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 twelve

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 (Figure 5).

Figure 5: Impact of the mechanical proccesings in the 'natural', yet man-made habitate in vineyards of Gau-Odernheim. In 2005 (02. May) a single row of wine cultivation was characterized by a tremendous abundance of both floral variants of *C. bursa-pastoris*. In the following year 2006 (03. May) no flowering individual was recorded due to massive disturbance through rotary tilling in this row. In 2007 (28. April) the same row was mowed an no flowering indivudal was surveyed. All processing lead to occasional extinction but also facilitate dispersal of seeds or plant material within adjacent cultivation destricts.

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 (Figure 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. In this context, a field experiment may be useful to estimate relative outcrossing rates within wild-type and *Spe* respectively, *versus* 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 favor 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 intra-population 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 (Ziermann *et al*.; 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. However, these mutations exposed a shift to early flowering rather than to late flowering as in the *C. bursa-pastoris* variant (Borner *et al*. 2000; Yu *et al*. 2002; Michaels *et al*. 2003). *Arabidopsis* mutants which show ectopic expression of *AGAMOUS*, the most probable candidate gene to resolve the *Spe* phenotype, flower early (Mizukami & Ma 1997; Koornneef 1998; Simpson 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 QTL analysis do not provide evidence for a linkage of *Spe* and flowering time (Hameister *et al*. 2009, unpublished data). Isolation of candidate genes and analyses of expression patterns (in-situ hybridization) are underway (G Theißen; pers. 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) and be 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 in low number of

individuals. In the proximity of the Gau-Odernheim vineyards, no further population was discovered so far. But a floristic survey from the early nineteenth 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 nineteenth century (Trattinnick 1821; Opiz 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 shortened by the existence of additional populations. In fact, based on molecular analysis for extant populations (Hameister *et al.*; unpub. results), we argue for multiple independent origins of the *Spe* variant as 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. *C. 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.

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CHAPTER 3

ESTABLISHMENT OF A NATURAL FLORAL VARIANT OF SHEPHERD'S PURSE IN THE WILD: ANALYSIS OF LIFE-HISTORY TRAITS IN *CAPSELLA APETALA* OPIZ

Abstracts

Variation in flower morphology played a key role in the recent understanding of flower developmental genetics. Most of the studies are based on artificially modified alterations in floral architecture. The exploration of naturally occurring variation, e.g. flower shape, may be of great benefit to further proceed in evo-devo research and contribute to evaluate the evolutionary significance of such taxonomic novelties. In this context, we analyzed life-history traits including fitness components and flowering time, of two floral variants of *Capsella bursa-pastoris*: the homeotic variant '*Stamenoid petals*' (*Spe*) with petals replaced by additional stamen was compared with the wild-type *C. bursa-pastoris*. Both occur sympatrically in a highly disturbed vineyard. The fitness evaluation of these variants referred to as the fruits per plant (female fitness) and the number of seeds per fruits (male fitness), revealed significantly different investment strategies which are almost counterbalanced in the overall reproductive fitness (seeds per plant). Wild-type donates more pollen for cross-fertilization, since floral visits are more common in this variant. Furthermore, both variants are separated in the onset of flowering ($p < 0.001$) exposing that *Spe* is a late flowering ecotype. Based on our outcome, we conclude that the maintenance of the decandric variant within a broad wild-type population is driven by complementary mechanisms including high rates of self-fertilization, ecological differentiation as well as beneficial anthropogenic disturbance in vineyards. In fact, the floral variant might have the potential to establish an evolutionary novelty. Taking into account that fitness in *Spe* is apparently not reduced despite its drastic floral aberration, the variant may serve as a model to study an early stage of speciation in a non-gradualistic manner, adopting Goldschmidt's concept of “hopeful monsters”.

Introduction

Progress in evolutionary developmental biology (evo-devo) in higher plants mainly focused on three angiosperm species: *Antirrhinum majus* L., *Arabidopsis thaliana* (L.) Heynh. and *Petunia hybrida* L. Analyses of mutants which display alterations in the identity of floral organs, so called homeotic mutants, have led to the postulation of the ABC model in the early 1990s (Coen & Meyerowitz). This model postulates, that the identity of floral organs is specified by the activity of three classes of genes A, B, and C (for review see e.g. Krizek & Fletscher, 2005). Due to some shortcomings, this model was stepwise expanded for a function D (Colombo *et al*. 1995) and function E (Pelaz *et al*. 2002). Even two decades since the initial ABC model has been postulated, *A. thaliana* is still key focus of evo-devo research, but this species may not serve as a comprehensive model to unravel all aspects of ecology and evolution (Tonsor *et al*. 2004). Apart from the analysis of induced homeotic mutants, there is growing interest to employ the achieved knowledge of flower development from studies in model plants (e.g. *A. thaliana*) on variability in wild populations of closely related species (Mitchell-Olds 2001). Studies of naturally occurring variation, like floral alterations, may contribute to improve the understanding about the origin of novel traits and expose their evolutionary significance in natural populations.

So far, rather nothing is known about genetic differentiation and the establishment of natural homeotic mutants in nature. With regard to the controversy about the evolutionary relevance of such mutants in wild populations, one homeotic variant of shepherd's purse, *Capsella bursa-pastoris* (L.) Medik., might represent a promising model (Hintz *et al*. 2006; Nutt *et al*. 2006). The variant is characterized by an increased number of stamens (ten), as a consequence of homeotic transformed petals and the modified phenotype was termed decandric (Opiz 1821). Heredity of this trait was already mentioned in previous reports (Opiz 1821; Schlechtendahl 1823; Dahlgren 1919). Recent crossing experiments and a linkage map analysis (Hameister *et al*.; unpublished data) suggests that the decandric phenotype is most likely caused by a single co-dominant inherited locus named '*Stamenoid Petals*' (*Spe*; Nutt *et al*. 2006). Decandric flowers in *C. bursa-pastoris* were reported for the first time almost 200 years ago in quite a few locations throughout Europe (Opiz 1821; Trattinnick 1821; Wiegmann 1823; Becker 1828). Lately, it was resurrected from oblivion since a population was discovered in vineyards in Southwest Germany (Reichert 1998). In his observation, Reichert (1998) found that the number and distribution of decandric plants

is quite stable. More recent field surveys in four successive years (2005-2008) revealed that, *C. bursa-pastoris* is one of the predominant species (Figure 1) in single rows of vine plantation with tens of thousands of individuals (Hameister, unpublished data), and both variants of *C. bursa-pastoris* co-exist in high frequencies. The population covers an area of at least 12km² and is severely affected by dynamical processes due to cultivation in the intensively managed vineyards.

Figure 1: The Gau-Odernheim habitat is characterized by intensive anthropogenic disturbance due to mechanical processing in vineyards leading occasionally to a kind of "monoculture" of *Capsella bursa-pastoris*. This is one key factor for maintenance of the sympatric occurrence of both floral variants of shepherd's purse.

Allowing for this coexistence of *Spe* and wild-type *C. bursa-pastoris* for at least 20 years in a natural population, it is reasonable to consider mechanisms which may facilitate the persistence of the mutant in the wild. In fact, the homeotic replacement of petals causes additional pollen producing stamens. Thus, it is questionable if this floral novelty is of any selective advantage or more likely less competitive compared with wild-type plants. Although, the decandric flower shape did not affect the disymmetry, which is typical for Brassicaceae, the lack of petals (function) might result in a shift in the flower visitor assemblage. As a consequence, this may lead to strengthened rates of self-fertilization within the *Spe* sub-population compared with wild-type. In addition, there is evidence that both phenotypic subgroups are ecologically differentiated according to a shifted onset of flowering leading to prezygotic reproductive isolation (Hameister *et al*. 2009). In contrast to the assumed negative impact on reproductive success and long-term survival of

homeotic mutants, the persistence for at least two decades in the wild might indicate that *Spe* is apparently competitive enough under local conditions. This may supported the concept of 'hopeful monsters', founded by Richard Goldschmidt in 1940, who postulated that mutants might yield adaptive novelties in case they are not attended by a drastic injury of fitness (for review see Dietrich 2000). In line with this, the natural occurrence of the *bicalyx* variant of *Clarkia concinna* Fisch. & Mey. (Ford & Gottlieb 1992) and the peloric *Linaria vulgaris* (L.) Mill. (Cubas *et al*. 1999) are two well-known examples which confirmed that morphological novelties based on minor genetical changes are able to establish in wild populations.

Here, we evaluated mechanisms which might enable the decandric variant to establish and maintain within a wild-type population of *C. bursa-pastoris*. Progenies of field collections were used in field experiments. The first approach is performed to detect possible differences in reproductive fitness among *Spe* and wild-type plants. A second experiment is intended to shed light on the relative hybridization rate among floral phenotypes using enzyme aspartate aminotransferase (AAT) as amolecular marker to detect heterozygotes in the F1 progeny. Finally, the predicted role of the *Spe* variant as a model for non-gradual evolution (Theißen 2006, 2009) will be will be discussed.

Methods

Plant material

The self-compatible *C. bursa-pastoris* is an annual to winter-annual polyploid weed, which is (pre-)adapted to a wide range of ecological niches. To evaluate reproductive fitness of the decandric variant compared with wild-type *C. bursa-pastoris*, a population in which both floral variants coexist was analyzed. This population is located in vineyards in the surroundings of Gau-Odernheim in southwest Germany (Rhinehessen). Because of its broad extension, the population was subdivided into 15 sampling sites (Pop.-No.: 1949-1963). Out of these, ten sub-sites were considered for analyses.

For the estimation of hybridization rates among floral variants, selfed progenies of two *C. bursa-pastoris* individuals with both floral phenotypes were cultivated. The wild-type individual 740/6/1/2 originated from Reno (Nevada, USA) and the decandric individual 1948-spe/2/4/5 from an additional sampling location in which *Spe* was reported (Warburg, Germany; Nutt *et al*. 2006).

Fitness evaluation under field conditions

In this approach, selfed progenies of plants from Gau-Odernheim were cultivated in a randomized common garden field experiment (12 May - 15 July, 2007) in the Botanical Garden of the University of Osnabrueck, Lower Saxony (Germany). Sowing was done in an unheated not artificially illuminated greenhouse. For each out of ten sampling sites, ten individuals were considered and both floral variants applied in equal shares. In total, 92 individuals were analyzed with regard to the following traits:

For a rough estimate of fitness, the total number of *fruits per plant* was counted at the end of the vegetation period. *Seeds per fruit* were averaged on ten mature fruits of each individual when fruits were counted and the total *amount of seeds* was extrapolated from both measurements. Additionally, the *onset of flowering* was recorded (days after sowing) as opening of the first flower bud was indicated. The *plant height* (cm) of the main inflorescence axis was measured at the end of its flowering period.

Data analysis

Mean values, standard deviation (s), range (r) and coefficients of variation (cV) were calculated for wild-type and decandric individuals. Student's t-test was used to assess whether means of the two morphological clusters (i.e. wild-type and *Spe*) are statistically different in the analyzed parameters. As a prerequisite for t-test, the normal distribution was proven by Kolmogorov-Smirnov test. All analyses were performed with SPSS 15.0.

Hybridization among variants

Randomized plant cultivation in a second field experiment was also performed in the Botanical Garden of Osnabrueck. This cultivation aimed to investigate the rate of hybridization among floral phenotypes. In total, 80 individuals were cultivated in five plots (A-E). In every plot, eight individuals of each flower phenotype were planted in square by a distance of 0.25 m. All plants were vernalized for three weeks to assure synchronized flowering. The considered parental individuals (wild-type and *Spe*) differ in the AAT genotype: wild-type from USA showed AAT = 2244 1111 3355 and the *Spe* mutant from Warburg AAT = 1111 1144 1155. This enzyme system was used to detect heterozygous individuals in the F1 generation. Therefore, 60 mature fruits of ten central plants per flower type (two per plot) were collected at the end of the flowering period. From each considered central plant (family), 60 progenies were intended to be screened with respective to the AAT genotype (leading to 1200 individuals). Cultivation of F1 was carried out in a climate

chamber (10°C/20°C, 12h photoperiod). After ten weeks of growth 0.7 g of rosette leaves were cut and stored in -80°C until preparation. Plant material from F1 growing was ground on ice. Extracts were stored in -28°C until processing

In addition to this experimental approach, progenies from field collections in Gau-Odernheim were also screened for AAT genotypes to unravel potential gene flow events in the natural habitat. Therefore, 28 progenies (families) with ten individuals each were cultivated under controlled greenhouse conditions (12h light / day: min 14°C - max 30°C; night: min 10°C). For wild-type, 13 families were intended and 15 for *Spe*, respectively (280 individuals in total). Plant material (leaves) of this cultivation was processed as described above.

AAT genotyping

For native gel electrophoresis 50 µl samples were loaded on 7.5% polyacrylamide gels. After 0.5h of pre-run at 35 mA, electrophoresis was performed at 4°C for 3.5h at constant amperage of 70 mA following basically Stegemann (1979). Overnight staining of enzyme was done according to Wendel & Weeden (1989). Further experimental details for AAT analysis and genetics of this isozyme system are given in Hurka *et al*. (1989). Nomenclature of enzyme loci and allozymes is in accordance with literature. In case of complete self-fertilization in the parental generation, the offspring would represent inbred lines. Consequently, the occurrence of more than a single AAT genotype in the analyzed progenies (within family heterozygosity), indicates crossings events among individuals with varying AAT genotypes (e.g. among floral variants).

Survey of flower visitors

A survey of the potential pollinator assemblage of shepherd's purse was intended by collecting flower visitors during field work in the natural habitat in Gau-Odernheim. Floral visitors observed on *C. bursa-pastoris* inflorescences were captured by net in May of three successive years (2006-2008). Sampling was carried out from 11:00 till 14:00 o'clock on two following days for 30 min at 2-5 sites. The flower type was denoted for individual plants on which insects were collected. Specimens were frozen until determination. For qualitative evaluation of potential pollinator assemblage of *C. bursa-pastoris*, specimens were identified to genus level. Determination of wild bees from all collections was carried out in cooperation with the Department of Ecology, University of Osnabrueck (N. Exeler).

Results

Fitness evaluation

The Kolmogorov-Smirnov test showed that all morphological traits correspond to the assumption of a normal distribution, which allows subsequent analysis of mean values and linear relationships. Under field conditions, the wild-type (n = 48) and decandric (n = 44) variant of *C. bursa-pastoris* revealed evident differences in mean scores of three measured traits. These differences are statistically significant (Table 1). Wild-type plants (1314.2) exhibited significantly more *fruits*/*plant* compared to the *Spe* sample (894.1). In contrast, the *Spe* variant provided the larger amount of *seeds*/*fruit* (24.6) than wild type (21.2). Extrapolating the data of both measurements, wild-type individuals showed sparsely more seeds in total than the decandric variant but this tendency was proven with low statistical assurance (p = 0.046*).

Table 1: Evaluation of fitness traits in a comparative approach of wild-type (*Wt*) and decandric (*Spe*) individuals in a field experiment. Under local conditions in Osnabrueck, both floral variants are separated in the onset of flowering, whereas differences in reproductive fitness (fruits/plant; seed/fruit) are almost compensated in the amount of total seeds (std = standard deviation, cV = coefficient of variance).

					Wt *vs*. *Spe*		
Trait	Type	N	Mean (± std)	cV (%)	T	df	p
Onset flowering	Wt	44	55.9 (± 7.23)	12.9			
	Spe	48	67.0 (± 7.24)	10.8	7.39	89	0.000***
Plant height (cm)	Wt	48	54.4 (± 17.5)	32.2			
	Spe	44	55.4 (± 15.8)	28.5	0.29	87	n.s.
Fruits / plant	Wt	48	1314.2 (±648.2)	49.3			
	Spe	44	894.1 (±629)	70.4	-3.15	90	0.002**
Seed / fruits	Wt	48	21.2 (±4,7)	22.2			
	Spe	44	24.6 (±6,7)	27.2	2.83	76	0.006**
Total seeds	Wt	48	28518.5 (±15999.1)	56.1			
	Spe	44	21633.9 (±16576.7)	76.6	-2.02	89	0.046*

Another significant differentiation was detected for the *onset of flowering*. Wild-type plants started to flower at an average of 55.9 days after sowing, while the homeotic mutant altered to reproductive lifecycle at an average of 67.1 days. A t-test (Figure 2) confirmed that the temporal separation of 11 days in the onset of flowering between both variants is highly significant (p < 0.001***). No statistical differentiation was obtained for the morphological trait *plant height*. The Pearson correlation analysis was performed, to provide evidence whether there is any linear dependence between the measured traits. As

both floral variants are most likely separated into two subpopulations, the correlation analysis was carried out for each floral phenotype independently. In both variants, the total number of fruits (*seeds*/*plant*) was positively correlated with the *plant height* and showed an inverse correlation with the *onset of flowering* (Table 2).

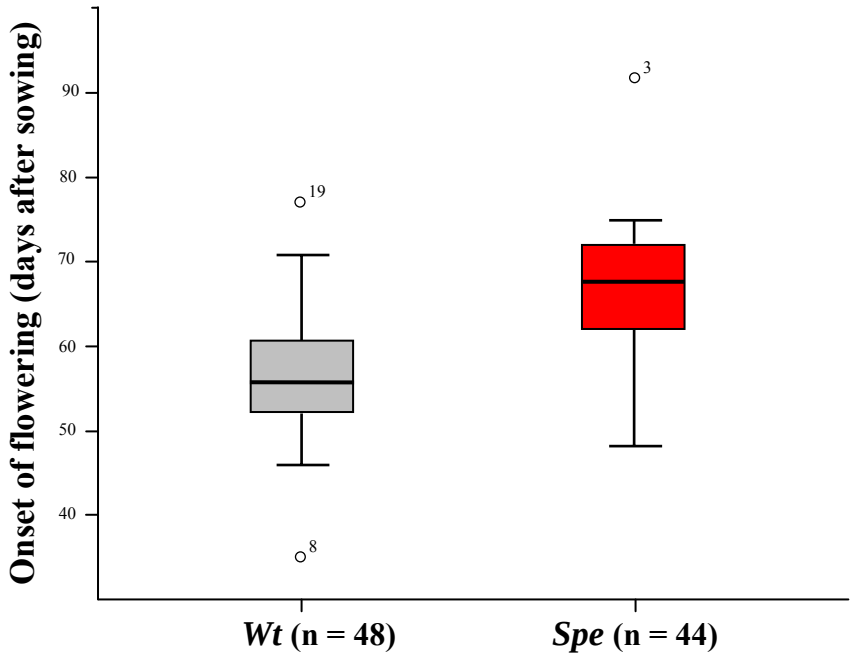

Figure 2: Differentiation in the onset of flowering of two floral variants of *C. bursa-pastoris* under local conditions in a common garden field experiment in Osnabrueck. Wild-type (*Wt*) and decandric variant (*Spe*) are significantly separated in their onset of flowering ($p < 0.001$***). The shifted flowering phenology enhances non-random mating between wild-type and the decandric *C. bursa-pastoris*.

Hybridization among variants

The detection of hybridization events was carried out through analyses of field collections (280 progenies out of 28 families) and in a designed field experiment (681 progenies out of 17 families). Divergent sampling sizes in the latter study resulted from either low germination capacity or hampered survival of seedlings. Both studies exposed striking differences in the outcrossing behavior in the wild-type and decandric sub-sample based on the identified AAT genotype patterns.

Table 2: Pearson correlation coefficient for fitness components in the decandric (*Spe*) and wild-type (*Wt*) sub-sample (dflower = days till onset of flowering; ns = not significant; * $P \leq 0.5$; ** $P < 0.01$; *** $P < 0.001$).

Spe (n =44)	dflower	plant height	fruits / plant	seeds / fruit	total seeds
dflower	.	.	.	.	.
plant height	-0.294 ns	.	.	.	.
fruits / plant	-0.463 **	0.631 **	.	.	.
seeds / fruit	0.173 ns	0.045 ns	-0.092 ns	.	.
total seeds	-0.342 *	0.545 **	0.883 **	0.341 *	.

Wt (n = 48)	dflower	plant height	fruits / plant	seeds / fruit	total seeds
dflower	.	.	.	.	.
plant height	-0.78 ns	.	.	.	.
fruits / plant	-0.356 *	0.569 **	.	.	.
seeds / fruit	-0.122 ns	0.094 ns	0.227 ns	.	
total seeds	-0.290 *	0.491 **	0.907 **	0.573 **	.

The data from the field collections revealed, that the within-family heterogeneity of AAT genotypes was higher in wild-type families than in *Spe*. Within more than every second family, at least two genotypes were identified, whereas in the decandric sub-sample only three out of 15 families revealed more than one genotype per family (Table 3). Within wild-type families, the degree of heterozygosity ranges from 9.09% to 63.64% and in *Spe* 9.09% to 18.18%, respectively. Referring to the total sample size, a relative rate of heterozygosity in the wild-type sample was 18.2%, whereas only 2.42% genotypes in *Spe* are a consequence of outcrossing events. Since the results from the field collection are not able to identify differences in real crossing events among both variants, the field experiment was performed additionally.

Table 3: The allozyme aspartat aminotransferase (AAT) was used as a molecular marker to assess genetic variability in two experiments. Progenies (families) from field collection were surveyed to detect heterozygosity in AAT genotypes in the offspring cultivated in a greenhouse. A field experiment was established using one parental plant per floral type with a known AAT genotype and screened for variability in the offspring indicating outcrossing events.

	Field collection		Garden experiment	
	Wt	*Spe*	*Wt*	*Spe*
Families (individuals)	13 (130)	15 (150)	8 (362)	9 (304)
Families (individuals) variable in AAT genotype	7 (26)	3 (4)	1 (4)	4 (11)
AAT variability within flower type	20.0%	2.67%	1.09%	3.49%
Rate of AAT variability within population	9.29%	1.43%	0.59%	1.62%

Heterozygotes were easily detectable in the progeny due to known homozygote parental AAT genotypes. Taking into consideration, that for each floral variant only one AAT genotype was applied, crossings within variants could not be identified. In the field

experiment, 15 heterozygotes were detected in a total of 681 individuals. This corresponds to a relative outcrossing rate of 2.2% among floral variants. In addition, differences in the pattern of pollen donation among variants can be elucidated in the analyses since parental genotypes and flower-types can be allocated to the sampled offspring (Figure 3). Conspicuously, the decandric sub-sample is more often recipient of pollen from wild-type plants than *vice versa*. Out of 15 detected heterozygotes, eleven were identified in the progenies from a decandric parent. These individuals belong to four out of nine analyzed families of the decandric *C. bursa-pastoris*. The remaining four heterozygous samples are offspring from the only one out of eight wild-type families (Table 3).

Flower visitors

Qualitative field collections revealed unexpected high diversity of "large" insects, visiting flowers of shepherd's purse. Specimens of three successive years (67 in total) were assigned to Hymenoptera, Diptera or Coleoptera. Survey of smaller insects (e.g. thrips) was not intended. Among field records, wild bees (Apidae) are the most frequent visitors (40.3%) of shepherd's purse inflorescences followed by hoverflies, Syrphidae (29.9%). Within wild bees, ten species from three genera were detected. In contrast, only three species from three genera were recorded in hoverflies, *Sphaerophoria scripta* with a dominant frequency (25.8%). Additionally, further taxa from the Diptera and Coleoptera were determined (Table 4). With respect to a phenotype-specific analysis of visiting insects, a tentative differentiation was indicated for wild-type *versus* decandric individuals (Figure 3). Almost two-third (64.2%) of the specimens (n = 43) was collected on wild-type plants, including 21 wild bees (48.8%). In contrast, among 24 insects captured on the *Spe* variant (35.8%), only six wild bees were recorded.

Table 4: Floral visitors recorded on two floral variants of *C. bursa-pastoris*. Collection of insects was performed in the natural habitat Gau-Odernheim during flowering season.

Flower type	Order	Family	Genus	Ind.
Spe	Diptera	Bibionidae	*Bibio*	2
		Sacrophagidae	-	1
		Syrphidae	*Melanostoma*	1
			Spaerophoria	6
			Platycheirus	1
		Tachinidae	*Tachina*	1
	Coleoptera	Cantharidae	*Cantharis*	1
		Crysomelidae	*Phyllotreta*	1
		undetermined	-	3
	Hymenoptera	Apidae	*Andrena*	1
			Halictus	3
			Lasioglossum	2
Wild-type	Diptera	Anthomyiidae	*Anthomyiinae*	2
		Bibionidae	*Bibiodes*	2
		Conopidae	*Tecophora*	1
		Platystomatidae	*Platystoma*	3
		Syrphidae	*Sphaerophoria*	10
			Platycheirus	1
		Tachinidae	*Tachina*	1
	Coleoptera	Cantharidae	*Cantharis*	1
		undetermined	-	1
	Hymenoptera	Apidae	*Andrena*	9
			Halictus	4
			Lasioglossum	8

Discussion

The establishment of evolutionary novelties in natural populations is strikingly dependent on the ability to compete under local field conditions compared to the progenitor taxon. In this context, the sympatric occurrence of the homeotic mutant *Spe* and wild-type plants of *Capsella bursa-pastoris* for decades is a first hint that this floral variant might not be hampered under the natural conditions in Gau-Odernheim. With respect to the high frequency of *C. bursa-pastoris*, mechanisms are crucial to realize the maintenance of possibly just a single initial *Spe* individual within the vast amount of wild-type plants.

Based on the outcome of the present study, we exposed that the persistence of *Spe C. bursa-pastoris* within a wild-type population is accomplished in complementary means. Under the given conditions in a common garden field experiment, both floral variants showed different strategies for the investment in the progeny but the overall reproductive

fitness (*seeds*/*plant*) is almost counterbalanced. Although this is certainly a rough estimate of fitness, the results at least indicate that the reproductive success of *Spe* is not negatively affected under the given conditions in the field experiment. This might correspond to the situation in Gau-Odernheim as well, since both variants co-exist for decades. Apart from compensated fitness, further isolation mechanisms among variants are essential for sustainability and differentiation: key factors are the predominant selfing and ecotypic variation of shepherd's purse yielding in a high colonizing ability (Hurka & Neuffer 1997). Such high rates of selfing may act as an initial barrier of gene flow (Levin 1971). In addition to it, the detected divergence in the onset of flowering also hampers genetic admixture among floral variants like reported for differences in flowering phenology in *Brassica rapa* L. (Weis & Kossler 2004). Both mechanisms clearly evoke prezygotic isolation and may lead to a further differentiation of the two phenotypes. Genetic analysis of the population structure in Gau-Odernheim revealed that both variants are well separated into distinct clusters, which reflect a flower-type dependent assignment (Hameister *et al*. 2009). In the latter study the onset of flowering of the decandric variant was also delayed compared with wild-type, which is consistent with results of the present investigation.

Combining the results of genetical and ecological differentiation obtained in these studies, it is most likely, that the two morphological variants in Gau-Odernheim may be treated as independent subpopulations. For the establishment of these subpopulations further mechanisms which facilitate a divergent evolution must be considered. A different level of cross-pollination among both variants might be one feature, due to the fact that *C. bursa-pastoris* is proterogynous which generally favors outcrossing (Hurka *et al*. 1976) and rates of cross-pollination are known to vary (Hurka & Neuffer 1997). Although pollinator attraction might be a life trait of minor importance in the self-compatible *C. bursa-pastoris*, quite a few bee species are known to trigger shepherd's purse as food source (Westrich 1989). Our qualitative recordings of the potential pollinator assemblage in Gau-Odernheim uncovered an unexpected diversity of floral visitors. Wild bees from the genera *Andrena*, *Halictus* and *Lasioglossum* and hoverflies (Syrphidae) were the most frequent visiting insects. This is in accordance with a former observation in Gau-Odernheim (Reichert 1998) and results from a common garden experiment (Ziermann *et al*. 2009, unpublished data). In line with this, a quite similar species assemblage was reported for the closely related and predominantly selfing *A. thaliana* (Hoffmann *et al*. 2003).

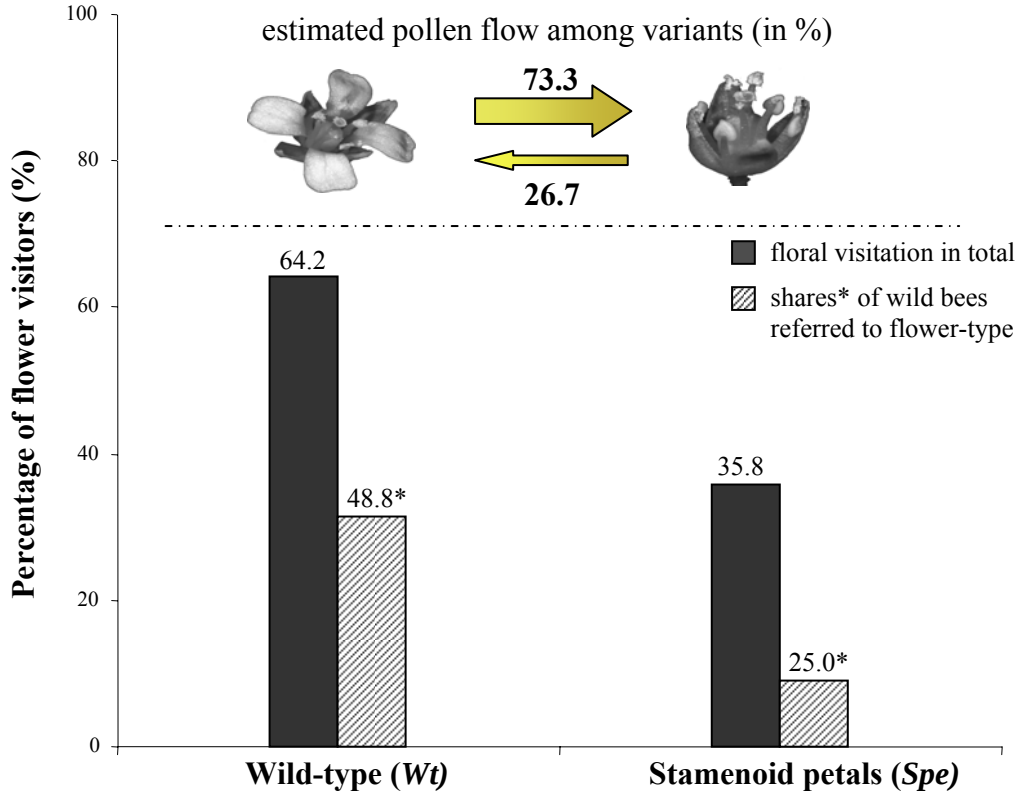

Figure 3: Percentage of flower visitors recorded on the two floral phenotypes of *C. bursa-pastoris* and indicated direction of pollen flow among variants. **Bar plot**: Field surveys revealed that insect visits are twice as often in wild-type (*Wt*) than in decandric plants (*Spe*). Effective pollinators like wild bees are more frequent on wild-type inflorescences. **Illustration:** Genetic analyses in progenies from a common garden field experiment exposed an exceedingly low crossing rate among variants (2.2%). Interestingly, wild-type is pollen donor in almost three-quarter (73.3%) of the estimated crossing events.

Some of the insects might visit *C. bursa-pastoris* flowers at random, but the detected species include effective pollinators like apoide hymenopterans and hoverflies, which indicates the potential impact on cross-fertilization. The common garden experiment by Ziermann *et al*. (2009) revealed flower-type specific differences in the assemblage of floral visitors. In general, visiting insects were observed twice as often on wild-types plants. Relative ratios of the observed visitors exposed that bees were recorded more often on wild-type whereas hoverflies and beetles preferred *Spe* plants rather than wild-type. A shifted frequency and assemblage of visiting insects might affect patterns of cross-pollination among floral variants and contribute to the differentiation into subpopulations.

According to Holsinger (2000), changes in flower morphology may cause differences in the quantity of pollen donation for cross-fertilization like reported for outcrossing *versus* selfing taxa of *Mimulus* (Scrophulariaceae; Ritland 1991). This might be applicable in our wild-type against decandric flower-type comparison: as more floral visitors were observed on wild-type inflorescences, it is reasonable that cross-fertilization is higher in wild-type

plants than in the decandric mutants. Indeed, genotyping of AAT allozymes in progenies from field collections supports this hypothesis (Table 3). While 80% of *Spe* families were monomorphic in the AAT genotype, more than one half of the wild-type families were variable in the progeny. We assume that this different rate of heterozygosity is caused by alterations in the frequencies of insect visitations. The common garden field experiment indicated that the decandric *C. bursa-pastoris* is more often recipient of wild-type pollen than *vice versa*. Thus, *Spe* rarely contributes pollen for cross-fertilization. This might be the consequence of reduced attraction due to the altered corolla architecture and benefits our assumption that outcrossing is more frequent in wild-type plants. Although not measured quantitatively yet, we hypothesize that showy petals and floral scents in wild-type promote attraction of pollinators even at a distance, while insect visits on *Spe* inflorescences may occur occasionally as a by-product of wild-type attraction.

Apart from biotic factors, the anthropogenic influence in the intensively managed vineyards is highly relevant. The cultivation process, mainly plowing and mowing, starts in spring when shepherd's purse begins to flower in this region. The agricultural processing affects *C. bursa-pastoris* in multiple ways: plowing could resurrect seeds from the soil seed bank and enhance the genetic diversity (Bosbach & Hurka 1981), whereas mowing might restrict the temporal extent of the period favorable for flowering, i.e. plants will be cut before ripening. Due to the mucilaginous seed layer (Hurka & Haase 1982), the mechanical processing certainly promotes seed dispersal within vineyards. In-between single rows of wine cultivation, this occasionally leads to a kind of monoculture of *C. bursa-pastoris* (Figure 1) like reported for *Diplotaxis erucoides* (L.) DC in vineyards of Sicilia (Bernhardt 1986). Due to the parcelling of vineyard properties to different owners, spreading of plant or seed material in adjacent vineyards is also presumable within the region.

With respect to the evolutionary relevance of homeotic alterations in natural population, the persistence of the floral variant of *C. bursa-pastoris* will be discussed in the light of selection. Gau-Odernheim is a highly disturbed habitat in which selection pressure is reduced (Bosbach & Hurka 1981). This might be the prevailing requisite for the maintenance of the decandric variant. In addition to it, reproductive assurance and small effective population size, which is frequently observed in selfing taxa (Pollak 1987), was possibly the driving force for the establishment of *Spe* within the wild-type population. According to Levin (1971) autogamy by itself can be treated as a reproductive isolation

barrier in flowering plants, which is promoted by a shift in the onset of flowering here. The extent of flowering time differentiation on local adaptation has recently been shown for populations of *Mimulus guttatus* DC (Scrophulariaceae; Hall & Willis 2006). Further studies in this taxon revealed that plants with an annual life-cycle tend to flower early in the season (van Kleunen 2007) and that floral visits of pollinators are strikingly dependent on flower size (Martin 2004). In the opposite, alterations in the lower corolla lip (Arathi & Kerry 2004) or a general decrease of the corolla size are often followed by increasing selfing rates in *Mimulus* (Ritland & Ritland 1989). Consequently, pollinator-mediated selection of flower morphology is certainly highly relevant in an entomophilous species like the intensively studied *M. guttatus*. A strong selection for flower shape was also reported in *Erysimum mediohispanicum* Polatschek (Brassicaceae), a species which requires cross-pollination for fully seed set (Gomez *et al*., 2006). In contrast, low selection on flower size and shape is reported for *Raphanus raphanistrum* L., another related but self-incompatible Brassicaceae (Connor *et al*. 1996).

Flower morphology in the predominant selfing *C. bursa-pastoris* is a trait which might be not under selection. With regard to former reports about a causal relation of increased flower numbers or flower size and the amount of floral visitations (Conner & Rush 1996; and literature cited therein), this should be considered in our study. Apparently, the corolla of the decandric mutant is smaller compared with the wild-type. In addition to it, the number of fruits is negatively correlated with the onset of flowering in both variants (Table 2). Consequently, the increased number of fruits, respective flowers, in wild-type is due to the early onset of flowering. We suggest that this increased number of flowers is sufficient to enhance attractiveness of the wild-type inflorescence, leading to more floral visits of potential pollinators. As a result, outcrossing events might be more frequent in wild-type than in *Spe*. Pollinator-mediated selection, however, is almost neglectable in a self-compatible species, taking into account that flower visitors also facilitate selfing of adjacent flowers in one individual (geitonogamy).

Since morphological alterations like *Spe* are discussed as a result of macroevolution, our intra-population model provides the opportunity to survey a (macro)evolutionary novelty attended by continuous micro-evolutionary adaptation. The study clearly exposed, that the analyzed fitness components counterbalance each other leading to a comparable reproductive fitness which indicates the promising potential of the decandric *C. bursa-pastoris*. In fact, it involves the concept of 'hopeful monsters' founded by Richard

Goldschmidt. Thus far, the evolutionary relevance of homeotic mutants is still discussed controversially (Theißen 2006). Based on our outcome, the *Spe* variant serves as a recent example for the persistence of morphological novelties in natural populations, in line with well known objectives like *Linaria* (Cubas *et al*. 1999) or *Clarkia* (Ford & Gottlieb 1992). For the origin of the *Spe* variant in Gau-Odernheim, two scenarios are feasible: A spontaneous mutation within the wild-type population or the introduction of seeds (respective plant material) from another habitat. In this context, it might be interesting to discuss the origin of the flowering time differentiation. In case the variant originated from a wild-type in Gau-Odernheim, it is likely that the ancestor was adapted to late flowering. Until now, we provided no evidences that the assumed single allele responsible for the homeotic mutation has also caused the shift to late flowering. In the second scenario, the introduced genotype (e.g. *via* seeds) might have been pre-adapted to a certain ecosystem in which late flowering was advantageous. Both, pre-adaptation as well as ecotypic differentiations in the onset of flowering time are known for *C. bursa-pastoris* (Hurka & Neuffer 1997; Neuffer & Hurka 1986; Linde *et al*. 2001). Apart from selfing, the flowering time differentiation might be the key factor for divergent evolution of both variants. The shifted flowering time might indicate a differentiation in the lifecycle strategies (van Kleunen 2007). Apart from increased pollen donation in *Spe* due to the enhanced male function (additional stamens, higher male fitness), there are no further evidences for an assumed tendency to wind pollination as argued in Nutt *et al*. (2006). Although anemophily is reported for Brassicaceae, e.g. *Pringlea antiscorbutica* R. Br. ex Hook. (Al-Shebaz 1984) or *Hormathophylla spinosa* (L.) Kuepfer (Gomez & Zamora 1996), key adaptations to wind pollination like altered pollen structure or stigma surface are missing in *C. bursa-pastoris*.

In conclusion, the observed maintenance of the floral variant *Spe* is accomplished by interacting mechanisms involving (1) high rates of self-fertilization in C. *bursa-pastoris* in general, (2) anthropogenic disturbance facilitating (seed) dispersal (3) differentiation in flowering time leading to (3) morphotype specific mating. Additionally, altered patterns of (4) outcrossing and (5) compensated reproductive fitness were identified. Long-time monitoring of artificial populations “inoculated” with wild-type and *Spe*, respectively, may be useful to elucidate further variations in adaptive traits which might enable the decandric variant to colonize different ecological niches than wild-type.

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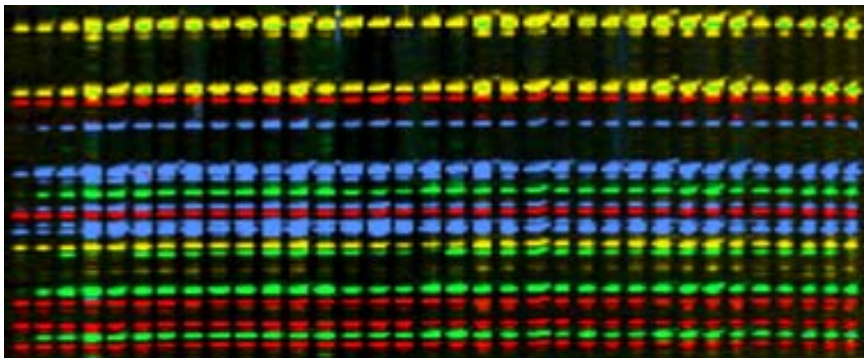
CHAPTER 4



IDENTIFICATION OF A SINGLE LOCUS INVOLVED IN THE ORIGIN OF '*STAMENOID PETALS*' IN A NATURAL HOMEOTIC VARIANT OF SHEPHERD'S PURSE - MAPPING A FLORAL TRAIT IN CAPSELLA BURSA-PASTORIS (BRASSICACEAE)

Abstracts

In order to promote the understanding of the origin of morphological novelties, the natural occurrence of a floral homeotic variant of *Capsella bursa-pastoris* might represent a valuable model for evolutionary biology. In this variant all petals are replaced by additional stamens. The resulting phenotype is heritable and the homeotic change might be explained by ectopic expression of a class C floral organ identity gene. To unravel the chromosomal localization of the putative single locus *Stamenoid petals* (*Spe*), linkage map analysis was carried out using molecular markers (AFLPs, RAPDs). To constrain assumed candidate genes, a relation of the generated linkage map to the genome of *Arabidopsis thaliana* was intended. The final map includes 15 linkage groups and the floral trait was integrated on linkage group 12 (CBP12) including six AFLP markers. Out of these, five markers were successfully sequenced and revealed sequence identities with chromosome IV of the *A. thaliana* genome. Interestingly, *AGAMOUS* is located on this chromosome, the only class C floral organ identity gene in the *A. thaliana* genome, which is compatible with the assumption that *Spe* is an allele of *AGAMOUS* rather than a regulator of that gene. In QTL analyses, none of the considered quantitative traits such as flowering time and plant height was associated to the corresponding linkage group of the *Spe* flower-type.

Introduction

Throughout the mustard family (Brassicaceae), the conserved floral ground plan is a common feature. However, alterations in organ number and identity are also observed within the family. For instance, variations in the characteristic number of floral organs are occasionally reported, especially within the genus *Lepidium* (Bowman 1999). In contrast to alterations in organ number, a naturally occurring floral variant of *Capsella bursa-pastoris* (L.) Medik., is characterized by replacement of petals by additional stamens (Figure 1b), whereas the number of floral organs is not affected. Due to the changed floral morphology, the resulting phenotype was described as 'decandric' (referring to the 6 + 4 = 10 stamens) almost 200 years ago (Opiz 1821). At that time, the decandric form of *C. bursa-pastoris* was reported from several European habitats (Opiz 1821; Trattinnick 1821; Becker 1828). Nowadays, still a few populations are known at natural habitats, such as vineyards (Reichert 1998) or ruderal hillsides (Nutt *et al*. 2006) in Germany, and ruderal provenances in the surroundings of Vienna (H Hurka, pers. communication). Recently, the decandric variant has been treated as a floral mutant, termed *Stamenoid petals* (*Spe*), based on preliminary evidence that a single locus is affected (Nutt *et al*. 2006).

The analysis of modified morphologies of floral organs in *Arabidopsis thaliana* (L.) Heynh. has been a powerful tool to investigate the genetic control of precise organ development. Since Coen & Meyerowitz (1991) have proposed the ABC model, constant progress has increased the knowledge about how regulatory genes control the development of floral organs. If changes in such regulatory genes cause a shift of floral organ identity, these alterations can be interpreted as a homeotic transformation.

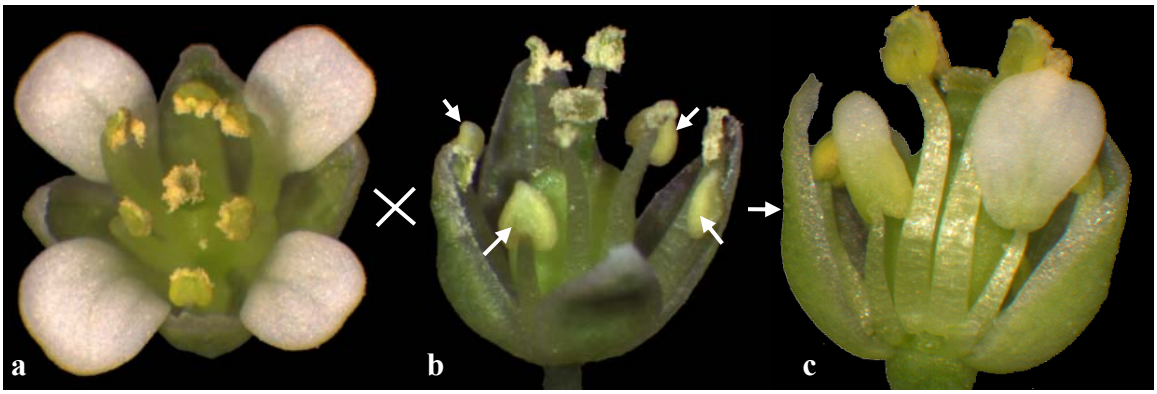


Figure 1. Differentiation in flower morphology of *Capsella bursa-pastoris* individuals observed in a mapping population for linkage analysis. **a** wild-type parent from USA (pollen recipient) **b** *Spe* variant (pollen donor) with petals replaced by stamens (marked with white arrows) **c** intermediate flower shape in the segregating F2 population.

The role of such homeotic alterations in the origin and radiation of angiosperm flowers has been intensively discussed but is still somewhat controversial (for reviews, see Ronse de Craene 2003; Theißen 2006; Theißen & Melzer 2007). In *A. thaliana*, floral homeotic mutants are subdivided into three classes A, B, C, according to the ABC function of the established model. For each class, genes were identified which are involved in the genetic control of floral organ formation. Most of these floral organ identity genes encode putative transcription factors of the MADS-domain protein family, and their overlapping expression pattern is realized in distinct spatial boundaries within a single flower. In wild-type *A. thaliana* flowers, activity of these genes leads to a patterned floral primordium and results in the arrangement of floral organs in four concentric rings (or whorls). In the model plant *A. thaliana*, sepals are established in the outer whorl (class A gene activity), followed by petals (class A + B gene) in the second whorl and the reproductive organs like stamens in the third whorl are specified by activity of class B + C genes and carpels in the fourth whorl by class C genes (for review see e.g. Theißen 2001, Krizek & Fletcher 2005). Although this model was stepwise extended to a function D (Colombo *et al*. 1995) and a function E (Pelaz *et al*. 2000), the initial ABC model comprises sufficient information to set up hypothesis for explaining the genetic basis of the floral variant of *C. bursa-pastoris*. Applying the ABC model to this mutant, the transformation of petals into stamens might be the consequence of ectopic expression of a class C gene that may suppress the expression of class A genes in the second whorl (Hintz *et al*. 2006; Nutt *et al*. 2006). Indeed, phenotypes with stamenoid petals are known in transgenic *A. thaliana* involving ectopic expression of the class C gene *AGAMOUS* (*AG*; Mizukami & Ma 1992; Jack *et al*. 1997) or closely related *AG*-clade genes like *SHATTERPROOF1* and *2* (*SHP1* and *SHP2*; Pinyopich *et al*. 2003). Hence, *AG* or any other member of the *AG*-clade might be affected and therefore are the most valuable candidate genes to elucidate the corresponding phenotype with stamenoid petals in the second floral whorl. With regard to the origin of morphological novelties in the wild, the persistent occurrence of the floral variant of *C. bursa-pastoris* in natural populations might be of great evolutionary relevance. In fact, evolutionary tendencies such as polyploidization, breakdown of self-incompatibility and reduction or total loss of petals are common within the Brassicaceae (Hurka *et al*. 2005) and might be involved in speciation processes. Allowing for the change in mating system in *Capsella* (Hurka & Neuffer 1997), the switch to self-compatibility (SC) coincides with a decrease in corolla size (Paetsch *et al*. 2006). In this context, the total abandonment of petals (function) in the decandric variant might accomplish the evolution to self-

fertilization since selection pressure for floral attractants is almost lost in SC species. Hence, the *Spe* variant may represent a promising model system for evolutionary biology (Theißen 2006).

The primary aim of this study is the chromosomal localization of the putative *Spe* locus in a co-segregation analysis using a mapping population. The generated linkage map is based on AFLP loci, applying the advantage of reproducibility, genome-wide spread and high density of informative characters. Additionally, RAPD markers were included to prove consistency with existing *C. bursa-pastoris* linkage maps (i.e. Linde *et al*. 2001). To constrain assumed candidate genes, a cross linkage to the genome of *A. thaliana* was carried out. The close relationship to *A. thaliana* may facilitate the studies, particularly because of high genome co-linearity (Acarkan *et al*. 2000; Boivin *et al*. 2004; Koch & Kiefer 2005). To achieve a cross-species comparison, segregating AFLP traits were isolated, cloned and sequenced. This technique was used for molecular markers forming the linkage group of the putative *Spe* locus. Furthermore, genetic analyses of quantitative traits (QTLs) were embedded, with special regard to the onset of flowering, since *C. bursa-pastoris* is known for variation in this adaptive trait (e.g. Neuffer & Bartelheim 1989; Neuffer & Hurka 1999; Slotte *et al*. 2007) and in addition, a shift in flowering time is frequently reported in artificial homeotic mutants (Borner *et al*. 2000; Yu *et al*. 2002; Michaels *et al*. 2003).

Methods

Plant material, growth conditions

To generate a mapping population, a single inbred plant of the homeotic mutant (1948-*Spe*/2/4/5 Warburg, Germany) as pollen donor was crossed with a selfed wild-type individual as recipient (740/6/1/2; Reno, NV, USA). Since parental genotypes are clearly distinguishable in the isozyme aspartate aminotransferase (AAT; EC 2.6.1.1), the crossing achievement was confirmed *via* AAT analysis in F1 individuals. The mapping population includes 155 F2 individuals and was cultivated under controlled climate chamber conditions (12h illumination; night 10°C - day 20°C; ca. 55% air humidity). This population was analyzed for the segregation of molecular markers and phenotypic traits, with special regard to the segregation of the floral morphology and a differentiation in the onset of flowering.

Phenotypic traits

The mode of inheritance for the decandric phenotype was recorded for ten flowers per plant of the F2 population. The morphological trait 'leaf-type locus *B*' (dissection of the leaflets to the midrib) was stated in the adult ontogenetic stage of the plants. This phenotype is a single locus trait showing dominant inheritance (Shull 1909). Further phenotypic variation was analyzed allowing for possible pleiotropic effects of the homeotic change within the *Spe* variant (e.g. flowering time). The following traits were consulted for QTL analysis: The 'onset of flowering' was recorded in days after sowing. The 'height at onset of flowering' (cm) of the main inflorescence axis was measured and 'plant height' (cm) recorded at the end of its flowering period. The total 'number of fruits' per plant was counted when plant height was measured, likewise the 'number of branches'.

Molecular markers

For marker studies, genomic DNA was isolated from fresh leaves (100 mg) with Invisorb® Spin Plant Kit (Invitek, Berlin, Germany) according to the manufacturer's manual. DNA concentration was quantified and quality assessed by gel electrophoresis (0.8% agarose).

AFLPs

The AFLP procedure (Vos *et al*. 1995) followed the AFLP™ Plant Mapping Protocol (Applied Biosystems) with minor modifications. Restriction of genomic DNA (0.3-0.4 µg) was done with *Eco*RI (5 U) and *Mse*I (1 U) in a single reaction with the ligation of double-stranded adaptors to generated fragments (2 h at 37°C). AFLP™ Ligation & Preselective Amplification Module from Applied Biosystems was used. The following selective amplification conditions were chosen: 1.5µl 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, Muenster, Germany). Cycle parameters were in accordance with the AFLP™ Plant Mapping Protocol. Amplified products were separated by gel electrophoresis 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 with Genotyper 2.1 (Applied Biosystems). The evaluation for presence (1) or absence (0) of parental markers was done manually by electropherograms.

RAPDs

RAPD markers were included on the basis of existing linkage maps (Linde *et al*. 2001). Amplification conditions included 3.0 µl of template DNA (1:10 dilution of total genomic DNA; 0.3-0.4 µg), 0.5 µM primer, 2 mM $MgCl_2$, 0.1 U Biotherm™ *Taq*-Polymerase (GeneCraft, Muenster, Germany). Cycle parameters were 4 min initial denaturation at 94°C, 45 cycles of 30 sec at 94°C denaturation, annealing at 36°C for 30 sec with a ramping of 0.4°C/sec, elongation at 72°C for 1 min and final elongation at 72°C for 4 min. PCR products were separated on 1.5% agarose gels. The electrophoresis ran for 3 h at 120 V. Ethidium bromide was used for gel staining after electrophoresis, bands were visualized by an UV transilluminator and scored for presence (1) or absence (0) of parental markers.

Data analysis, Map construction, QTL

Inheritance of the floral phenotype and segregation patterns of molecular marker loci (AFLPs, RAPDs) were tested for deviation from the expected 3:1 or 1:2:1 ratio for dominant and co-dominant markers using χ^2-test. These calculations were performed in SPSS 15.0.

Mapmaker/EXP 3.0 software (Lander *et al*. 1987; Lincoln *et al*. 1992a) was used for linkage map construction using a minimum LOD score of 4.5 and a maximum distance of 40.0 cM between two loci. The Kosambi mapping function was used for calculating the map distances. QTL-analysis (interval mapping) was applied for traits showing normal distribution with Mapmaker/QTL 1.1 (Paterson *et al*. 1988; Lincoln *et al*. 1992b).

Scar markers

Segregating AFLP markers which found a linkage group with the phenotypic flower trait *Spe* were isolated, cloned and sequenced. This procedure was mainly in accordance with previous reports to establish 'sequence characterized amplified region' markers (SCAR; Paran & Michelmore 1993; Linde *et al*. 2004). Products from selective amplification of a certain primer combination in which the considered AFLP band is present in the one parent were loaded to the gel with ten replications. Additionally, two lanes were loaded with amplification products of the other parent in which the trait is absent. Immediately when the desired band appeared in the electropherogram preview of the collection software (Applied Biosystems), the gel run was cancelled and the position of the laser marked on the glass plate. Lanes where indicated, and the ten bands cut out in five fractions from the

gel. Fractions were briefly washed in 500 µl dist. H_20 for 10 min. After transferring into 200 µl distilled H_2O, shaking and incubated at 60°C for 30 min, the DNA was eluted overnight at room temperature. For subsequent re-amplification with the selective primer combination 2 µl of the DNA dilution was used and ran on the sequencer for a second time. The sample with the lowest number of additional bands was applied for final selective PCR. The obtained products were purified using the NucleoSpin® Extract II kit (Machery & Nagel, Düren). These fragments were cloned into the pCR 2.1 vector (TA cloning kit; Invitrogen) according to the manufacturer's instructions. Ten separate clones were picked and sequenced in forward directions using universal M13 primer. Sequencing of samples was performed on an ABI 377er using Big Dye^{TM} Terminator sequencing kit (Applied Biosystems).

Results

Inheritance of Spe and quantitative traits

Inheritance of the mutant phenotype *Spe* was verified by ten flowers per individual of the F2 population. Results indicated a co-dominant inheritance pattern proven by χ^2-test. Floral phenotypes were distinguishable for 146 F2 individuals: Out of these, 33 wild-type plants were detected, while further 81 plants revealed intermediate organs (between petals and stamen; see Figure 1C) in the second floral whorl. The remaining 31 individuals were classified as *Spe* mutants. Thus, statistically the observed frequencies (0.9: 2.22: 0.85) do not differ from the expected (χ^2=0.503) and consequently fit the required Mendelian ratio.

In addition, the morphological single locus marker 'leaf-type locus *B*' is a dominant trait (Shull 1909) and also revealed the expected ratio (2.9: 1.1) in our analysis. Five phenotypic traits were considered for a genome wide QTL scan (Table 1). Three traits produced four QTL and were located on two linkage groups (Figure 2). QTL were assigned within the chromosomal map with a LOD score >3.5. The explained phenotypic variation ranged from 14.0% for 'plant height' (CBP09) to 62.1% for 'height at onset of flowering' (CBP14; Figure 2). Both traits were linked to the 'leaf-type locus *B*' on CBP09. The only QTL for the 'onset of flowering' was also related to this position. None of the considered traits was linked with the identified locus *Spe* in this analysis.

Table 1. Phenotypic traits of parental plants and the F2 mapping population considered for QTL analysis. Data for parent individuals and F2 are not comparable due to different growth conditions (asterisks indicate normally distributed data; sd = standard deviation).

		wild-type	***Spe* variant**	**F2**
Onset of flowering*	mean (±sd)	77.58 (±4.89)	80.67 (±4.83)	69.01 (±10.02)
	range	70.0-87.0	72.0 - 88.0	51.0 - 105.0
Number of branches	mean (±sd)	4.92 (±1.68)	5.58 (±2.47)	3.71 (±1.19)
	range	3.0-7.0	2.0 - 8.0	0.0 - 7.0
Height at flowering (cm)*	mean (±sd)	5.0 (±2.38)	7.67 (±2.47)	6.11 (±3.11)
	range	2.0 - 8.0	5.0 – 13.0	2.0 - 16.0
Number of fruits	mean (±sd)	349.0 (±108.1)	347.92 (±90.23)	212.56 (±59.3)
	range	171.0 - 512.0	197.0 – 497.0	56.0 – 422.0
Plant height (cm)*	mean (±sd)	70.04 (±4.59)	64.17 (±10.23)	67.9 (±6.13)
	range	61.5 - 76.5	46.0 - 75.5	49.5 - 81.5

Linkage analysis

For linkage analysis, 27 AFLP primer combinations were applied and yielded 102 consistent polymorphic characters between the parental plants (wild-type: 740/6/1/2; *Spe* 1948-*Spe*/2/4/5). In the F2 population, segregation of 87 AFLP loci was in accordance with the expected Mendelian ratio of 3:1. Additionally, 34 RAPD primers were screened. Out of these, seven were polymorphic between the two parents and produced further 13 markers. Seven RAPD markers segregated in the requested ratio and were deployed.

In total, 96 markers were considered in the linkage analysis to set up a chromosomal map. Fourteen AFLP and two RAPD markers were not assigned to any linkage group. Consequently, the final map consists of 80 markers (73 AFLPs; five RAPD, two morphological). At a LOD score of 4.5, all remaining markers were assigned into 15 linkage groups. The number of markers per linkage group ranged from two to twelve (5.3 on average). The distance between two markers varied from 1.0 cM to 32.9 cM. The average distance between two loci was 7.6 cM and the total length of the map was 612.1 cM (Kosambi function). From the considered markers, 18 AFLP loci were associated in eight marker assemblages, i.e. were linked with a distance <1 cM. These associated markers were treated as a single locus within the final map (denoted with asterisks in Figure 2).

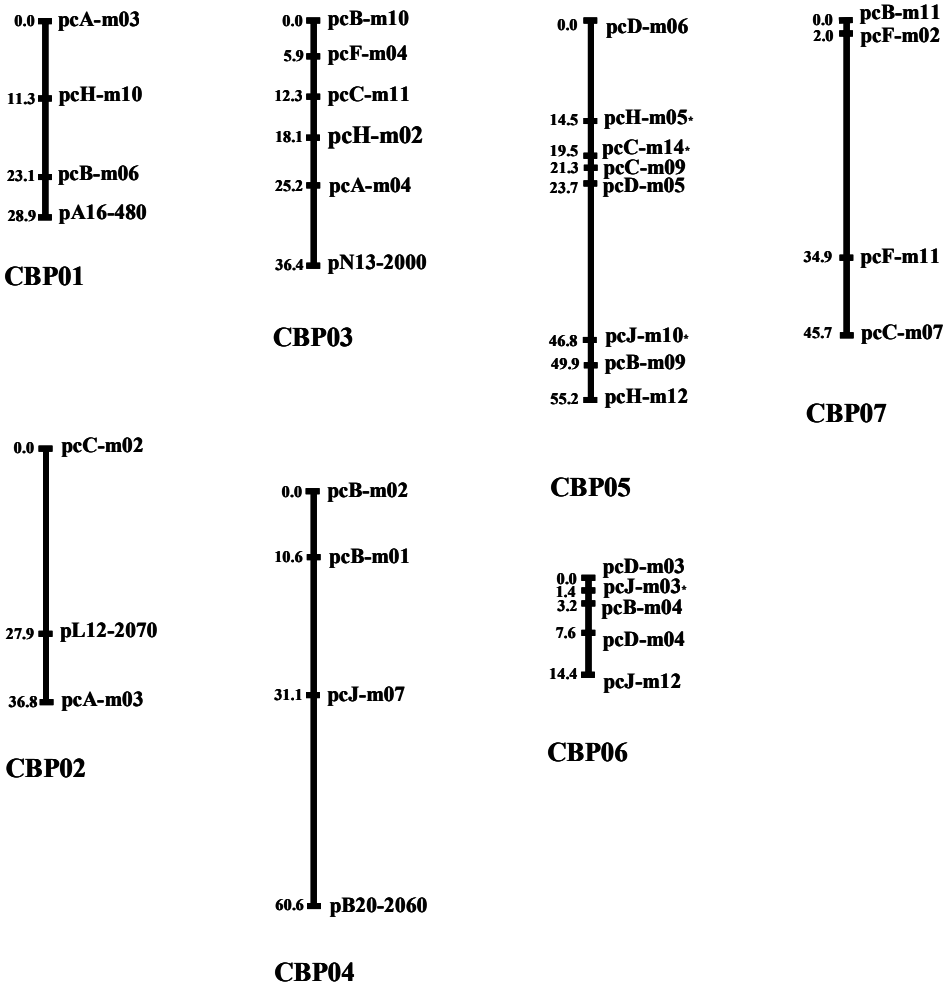
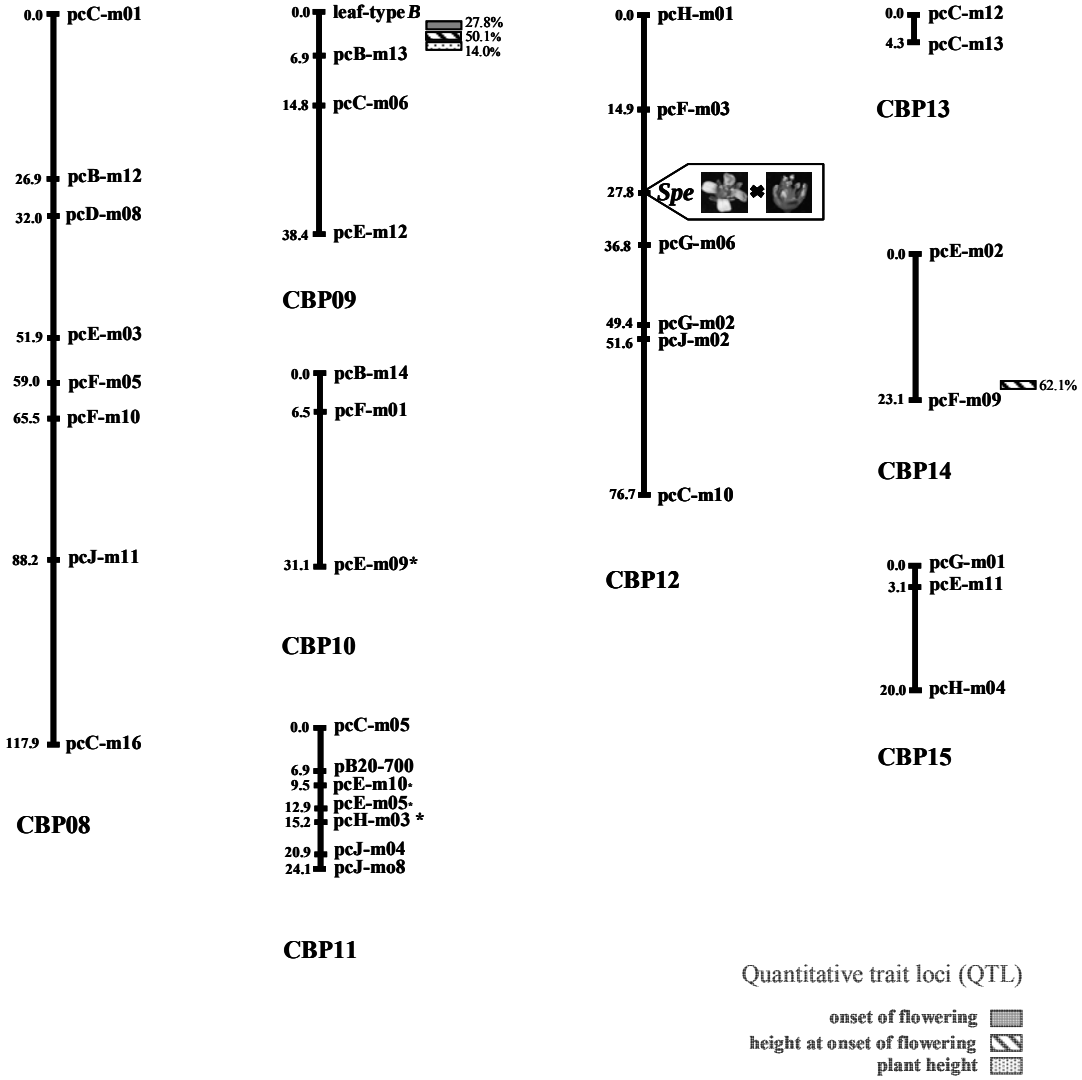


Figure 2. Achieved linkage map of *Capsella bursa-pastoris* based on 78 molecular marker loci. 15 linkage groups (CBP01-15) were detected. Distances between markers are displayed in centi Morgan (cM). A single locus *Spe* for morphological trait 'flower-type' is integrated into the final map on CBP12, associated with six AFLP markers. QTL for three phenotypic traits are placed in their approximate position to the closest molecular marker. Percentage values indicate the explained phenotypic variation. Asterisks indicate maker assemblages.

Scar study

The obtained chromosomal map revealed that the morphological trait *Spe* is mapped on linkage group CBP12 (Figure 2 & 3). Within this linkage group, the flower-type marker is co-segregating with six molecular markers, covering a region of 76.7 cM (12.4% of the total map). The two AFLP markers embedding the unravelled *Spe* locus span a distance of 21.9 cM (pcG-m06; 9.0 cM & pcF-m03; 12.9 cM).



To relate CBP12 of the *C. bursa-pastoris* genetic map to parts of the *A. thaliana* genome, the corresponding six AFLP markers forming this group were sequenced according to the SCAR technique described in Linde *et al*. (2004). Obtained *C. bursa-pastoris* sequences were compared with the genome of *A. thaliana* to identify possible co-linearity between both genomes on a minor scale. Sequence similarity search was done using database information (NCBI; discontiguous megablast for cross-species comparison). Lengths of the analyzed sequences varied between 96-269 bp and were almost entirely in accordance with the expected fragment sizes determined in AFLP analysis (Table 2). Only sequences of marker pcF-m03 exceeded the expected length for 6-11%. Also, the sequence of this

marker lacked the *Eco*RI AFLP adaptor sequence. However, all remaining markers were either accurate in length and *Eco*RI / *Mse*I adaptor sequences were identified in the isolated fragments. The artificial adaptors (15bp each) were excluded from Blastn search.

The sequences of three out of six AFLPs of the linkage group CBP12, exclusively matched to regions on chromosome IV in *A. thaliana* (Figure 3). The maximal base pair identity ranges from 83% in pcC-m09 to 87% in pcG-m06 and 94% in pcH-m01, respectively. Marker pcJ-m02 showed supporting hits on chromosome IV (80% bp identity) but also matched to sequences located on chromosome V. AFLP trait pcG-m02 shows high sequence similarity with a region on chromosome I (92% base pair identity) and chromosome V (82% base pair identity). Only two SCAR markers show partly hits within protein coding regions in the *A. thaliana* genome: marker pcG-m06 is similar to a transcription factor (WRKY DNA-binding protein 53; At4g23810) and AFLP loci pcG-m02 revealed sequence similarity to a protein which is similar to a COP1-Interactive Protein 1 (CIP1; At5t41790) and additionally to a hypothetical protein (At1g64330).

Table 2. SCAR marker results for linkage group CBP12. Five out of six AFLP loci were successfully sequence-characterized (* artificial adaptor sequences were excluded from Blast search).

Marker label	**AFLP fragment**	**Blasted sequence***	***A. thal.* chromoso**	**bp identity**	**cover**	**gene**	***A. thaliana* coordinates**
pcC-m09	169 bp	136 bp	chrom IV	83%	96%	-	17284601-17284722
pcF-m03	243 bp	251 bp	-	-	-	-	-
pcG-m02	159 bp	127 bp	chrom I	92%	99%	At1g64330	23877127-23877251
			chrom V	82%	100%	At5g41790	16749134-16749222
pcG-m06	96 bp	63 bp	chrom IV	88%	98%	At4g23810	12393476-12393539
pcH-m01	99 bp	69 bp	chrom IV	95%	61%	-	16178739-16178780
pcJ-m02	136 bp	106 bp	chrom V	80%	96%	-	9212615-9212694
			(IV)				5491868-5491947

Discussion

In this study, we obtained substantial support for a co-dominant inheritance of the *Spe* phenotype from Warburg population and determined the chromosomal localization of the putative single locus causing the homeotic phenotype in *C. bursa-pastoris*. Five out of six AFLP loci forming the linkage group including the putative *Spe* locus were successfully sequence characterized and revealed sequence similarities to the *A. thaliana* genome. No analyzed quantitative trait was closely linked to this locus.

Comparison of Capsella linkage maps, QTL

The heredity of the decandric phenotype was already mentioned in early reports (Opiz 1821; Schlechtendahl 1823; Dahlgren 1919). Dahlgren's crossing experiments of wild-types with decandric *C. bursa-pastoris* revealed an approximately 1:2:1 segregation of plants with stamenoid, intermediate and petal organs in the second floral whorl. These different kinds of organ morphology in the second whorl were also observed in our study (Figure 1A-C). Segregation patterns of the analyzed cross of a homeotic mutant and wild-type confirmed these reports and results from further crossing experiments (Nutt *et al*. 2006). These findings suggest a co-dominant inheritance of *Spe* by a single locus (or a few closely linked loci) and facilitated our aim to use a molecular marker-assisted mapping approach for the chromosomal localization of the putative locus. Consequently, the phenotypic trait was successfully integrated into the AFLP-based chromosomal map, located on linkage group CBP12 (Figure 2). The final map consists of 15 linkage groups spanning a total length of 612.1 cM. Allowing for recent *Capsella* maps, our map length might be an underestimation: a genetic map of *C. bursa-pastoris* with a length of 1064.4 cM was presented by Linde *et al*. (2001) and another map of an inter-specific crossing of the diploids *C. grandiflora* × *C. rubella* was 582.1 cM in length (Boivin *et al*. 2004). In line with this, the 1C DNA amount of the tetraploid *C. bursa-pastoris* is nearly doubled compared with the diploid *C. rubella* (Lysak *et al*. 2009).

Spe in context of evolutionary developmental biology

Applying the ABC model, the development of stamen identity in the third floral whorl depends on class C gene activity. Thus, ectopic expression of a class C gene in the second floral whorl, possibly followed by the repression of class A gene activity, is a reasonable scenario to explain the molecular basis leading to the *Spe* phenotype in *C. bursa-pastoris*

(Nutt *et al*. 2006). In *A. thaliana* mutants, *Spe*-like phenotypes are known to be the result of ectopic expression of the class C gene *AG* (Mizukami & Ma 1992; Jack *et al*. 1997), but also *AG*-like genes (*SHP1* & *SHP2*) are identified to cause stamenoid petals in *A. thaliana* (Pinyopich *et al*. 2003). These artificial mutants also showed transformation in other floral organs, except the transgenic *A. thaliana* in which *AG* is under control of the *AP3* promoter (Jack *et al*. 1997). Apart from a class C gene itself, *trans*-acting regulators of *AG* like *APETALA2* (*AP2*; Drews *et al*. 1991) or *CURLY LEAF* (*CLF*; Goodrich *et al*. 1997) might represent further candidate genes involved in the development of stamenoid organs instead of petals. Indeed, *AP2* is known to cause such alterations in *A. thaliana* (Bowman *et al*. 1991) and also a stamenoid variant of *Brassica napus* is reported in which *CLF* might be affected (Fray *et al*. 1997). However, vegetative leaves in the *B. napus* variant showed pleiotropic effects, and in *A. thaliana ap2*-mutants, carpelloid sepals were observed in the outer floral whorl. Due to the fact that no further morphological effects are observed in case of the *Spe* variant, *AP2* or *CLF* are certainly not very promising candidates to explain the decandric *C. bursa-pastoris*. Taken together, the extension of *AG* expression into the second floral whorl is still the most feasible explanation for the origin of decandric flowers in *C. bursa-pastoris*. In-situ hybridization studies of *Spe* expression, co-segregation of *Spe* with an *AG* orthologue and DNA sequence analysis of this locus, all involving another population (Gau-Odernheim) of the *Spe* variant, make it most likely that one of the two *AG* genes in the tetraploid genome of *C. bursa-pastoris* represents the *Spe* locus (P. Nutt, J. Ziermann & G. Theißen, unpublished data). Consequently, this makes the scenario of a mutation in a *trans*-acting negative regulator unlikely. However, since a binding site of a regulation factor might be affect in the assumed *AG* allele, further investigations will include protein-binding studies (G Theißen, personal communication).

In *A. thaliana* mutants, the ectopic expression of *AG* is also accompanied by a shift to early flowering (Mizukami & Ma 1992; Goodrich *et al*. 1997). In the analyzed crossing both parents did not differ significantly in the onset of flowering. Following the classification given in Linde *et al*. (2001) for the population 740 from mountain regions in the Sierra Nevada, USA, (used as wild-type parent here) the mutant parent from Warburg must be rated as a late flowering ecotype. The differentiation in flowering time is frequently reported in *C. bursa-pastoris* (e.g. Neuffer & Bartelheim 1989; Neuffer & Hurka 1999; Slotte *et al*. 2007) and alterations in this trait might be given in the floral homeotic variant *per se*. But a shift in flowering time might also be a pleiotropic effect of altered expression

of the involved gene(s) leading to the *Spe* morphology. To unravel a possible linkage of the homeotic phenotype and an altered onset of flowering in the decandric variant, a QTL analysis was carried out including four additional traits (Table 3). Neither the onset of flowering nor any other quantitative trait was associated with the detected single flower-type locus *Spe* on CBP12.

Table 3. Identified QTL controlling phenotypic variation in the analyzed crossing (genetic models to explain effects for QTL: add. = additive; reces. = recessive).

trait	Linkage group	LOD score	Genetic model	Variance explained	approx. pos.	Interval / cM
dayflow	CBP09	8.51	addi.	27.8 %	leaf-type	pcB-m13 - leaf-type B / 7.2
heiflow	CBP09	7.87	reces.	50.1 %	leaf-type	pcB-m13 - leaf-type B / 7.2
	CBP14	9.09	reces.	62.1 %	pcF-m09	pcE-m02 - pcF-m09 / 23.1
planthei	CBP09	3.57	addi.	14.0 %	leaf-type	pcB-m13 - leaftype B / 7.2

Thus we hypothesize, that the shift to late flowering in the *Spe* variant of *C. bursa-pastoris* may have originated more likely in a wild-type adapted to a certain (i.e. late) flowering time, than be associated to the homeotic change causing the aberrant flower morphology. This conclusion is supported by the reported shift to early flowering in homeotic *A. thaliana* mutants (Borner *et al*. 2000; Yu *et al*. 2002; Michaels *et al*. 2003). As we can not exclude that the *Spe* variant from different provenances throughout Europe may differ in the molecular origin, the assumed independence of homeotic alteration and shift in flowering time might only be validated for the *Spe* mutant from Warburg. But, in fact, studies in the natural sympatric population of *Spe* and wild-type plants in Gau-Odernheim also revealed that the decandric variant is late flowering compared with wild-type (Hameister *et al*. 2009). Thus, if altered expression of *AG* or any related *AG*-clade gene has caused the *Spe* phenotype, this mutation may not affect the flowering time of *C. bursa-pastoris* in the analyzed populations.

Linkage mapping as a tool to detect candidate genes

Former comparative linkage map analysis confirmed high genome co-linearity between *A. thaliana* and *Capsella* (Boivin *et al*. 2004; Koch & Kiefer 2005). Taking into account that n = 8 is the assumed ancestral karyotype in Brassicaceae, this indicates a lot of chromosomal rearrangements allowing for the exceedingly variable number of chromosomes within the family. For instance, linkage maps comparisons of *A. thaliana* and *Brassica* species revealed 26 large chromosomal rearrangements between *B. oleracea* (Kowalski *et al*. 1994) and even 90 in comparison to *B. nigra* (Lagercrantz *et al*. 1998).

Focussing on the *Arabidopsis* - *Capsella* lineage, Boivin *et al*. (2004) identified 14 large rearrangements in a linkage map comparison. So far, comparative mapping is a valuable tool to elucidate chromosome evolution in the Brassicaceae and high genome co-linearity of closely related species allows to utilize the genome information from *A. thaliana*, e.g. to identify candidate genes (Kuittinen *et al*. 2004).

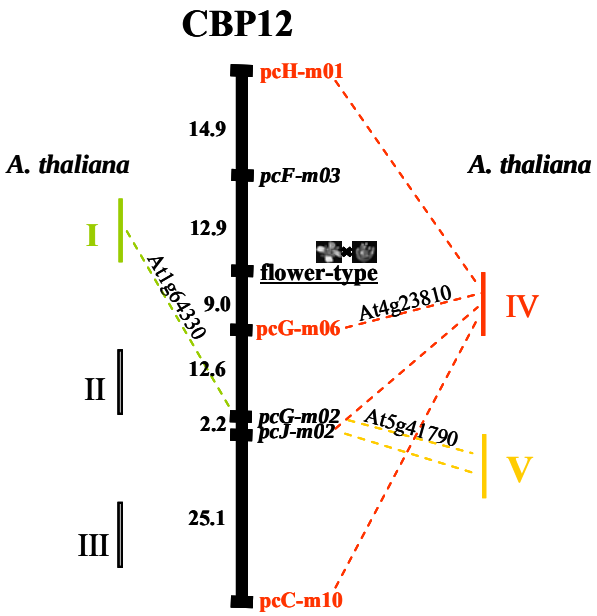


Figure 3. Sequence-characterized AFLP traits associated with the putative flower-type locus on linkage group CBP12. Three sequence-characterized AFLP markers (pcH-m01, pcG-m06, pcC-m10) exclusively match on chromosome IV of *A. thaliana* in a NCBI discontiguous megablast search (83%-94% bp identity). A further marker (pcJ-m02) revealed a supporting hit on chromosome IV.

For a convincing comparative approach to *A. thaliana*, the establishment of reliable sequence-characterized or gene-based molecular markers is crucial and then linkage mapping and QTL analyses are suitable to constrain candidate genes involved in phenotypic variation. As there is some information about sequence- (or gene) based markers in *Capsella*, some of these characters were screened, but polymorphisms between parental individuals, a prerequisite for linkage mapping, were barely detectable in our crossing. Thus we isolated, cloned and sequenced AFLP traits which form the corresponding linkage group CBP12 of the putative *Spe* locus. Sequence characterization of five AFLPs was successful. This technique might be a valuable tool in a cross-species comparison for species closely related to *A. thaliana*, at least on a small scale. However, sequences of three markers show suitable hits on *A. thaliana* chromosome IV. This might be a tentative hint to consider candidate genes located on this genome region, which is in accordance with the fact that the most probable candidate gene *AG* is indeed located on chromosome IV in *A. thaliana*.

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Conclusion

Stamenoid petals in the second floral whorl are reported in quite a few studies in artificial homeotic mutants of *A. thaliana*. Nearly all of these studies unravelled pleiotropic effects in other floral traits (Bowman *et al*. 1991; Mizukami & Ma 1992; Pinyopich *et al*. 2003; Drews *et al*. 1991; Goodrich *et al*. 1997). The only phenotype comparable to the decandric *C. bursa-pastoris* was described in a highly artificial approach (Jack *et al*. 1997) which might be unlikely to arise and establish in wild populations. An inevitable requirement for the establishment of evolutionary novelties in natural populations is the heritability of the novel trait, a fact which is often unknown in floral homeotic variants (e.g. Murbeck 1918; Meyerowitz *et al*. 1989; Ronse De Crane 2003). The heritability of the decandric phenotype in *C. bursa-pastoris* was previously mentioned (Opiz 1821; Schlechtendahl 1823; Dahlgren 1919) and confirmed in the present study. In a linkage analysis (**chapter 4**) a single locus, *Spe*, was identified which is most likely an allele of the class C floral organ identity gene *AGAMOUS* and might cause the homeotic phenotype in *C. bursa-pastoris*. The intended cross-species linkage to genomic sequences of *A. thaliana* basically failed since appropriate molecular markers (e.g. sequence-based markers, microsatellites) were not available. The density of AFLP loci in the linkage map was low, leading to large relative distances between molecular markers and the flower-type locus *Spe*. To reliably constrain the number of candidate genes, linkage of co-segregating AFLPs and *Spe* locus should be closely linked; taking into account that 1 centi Morgan still corresponds to approx. 250 kB in *A. thaliana* (Lukowitz *et al*. 2000). Although marker-assisted mapping and QTL analyses are considered as valuable tools to identify candidate genes (Kuittinen *et al*. 2004) these techniques might be less useful in far related taxa. In fact, the regulation of floral organ identity might differ among the close relatives *C. bursa-pastoris* and *A. thaliana* (Nutt *et al*. 2006) and even within *C. bursa-pastoris* the molecular genetic basis of *Spe* might be different among populations (e.g. Gau-Odernheim *vs*. Warburg, etc.). With regard to the disjunct distribution pattern of *Spe*, the question arises whether the occurrence of decandric phenotypes in populations of *C. bursa-pastoris* is a result of single or multiple origin. Based on the detected genetic differentiation in **chapter 1**, it is reasonable that the novel flower shape has evolved several times independently. While the independent evolution of similar traits based on different molecular mechanisms (e.g. different genes) refers to convergence, similar novel traits based on identical genetic changes might be the result of parallelism (Bowman 2006). Taking into account that

several candidate genes are known in *A. thaliana* and the molecular genetic base leading to *Spe* is not fully resolved yet, it remains uncertain whether the repeated evolution of this trait is due to parallelism. Since a single allele (e.g. of *AGAMOUS*) is hypothesized to cause stamenoid petals in *C. bursa-pastoris*, parallelism is likely and the variant might represent a current example for the impact of minor genetical changes involved in evolutionary processes. In fact, the identified population structure in the sympatric population in Gau-Odernheim clearly reflects a genetic differentiation into two sub-populations which corresponds to the phenotypic discrimination of flower-types (**chapter 2**). This differentiation suggests that the evolutionary novelty *Spe* has established itself as an isolated entity within a wild-type population. The restricted genetic variability in *Spe* is most likely the result of a rather young origin either within the population or by a recent introduction and indicates a recent bottleneck. Although the influence of anthropogenic disturbance is unambiguous in Gau-Odernheim, field management in general might influence both variants similarly. The persistence of maybe just one initial *Spe* individual (genotype) in this tremendous wild-type population is accomplished in complementary means: As a prerequisite for its maintenance, the reproductive success of *Spe* is not negatively affected compared with wild-type plants (**chapter 3).** In addition, high percentage of selfing represents a highly important barrier of gene flow (Levin 1971) since *C. bursa-pastoris* is known for predominant selfing (Hurka & Neuffer 1997). The detected variation in the onset of flowering among variants (**chapter 2 + 3**) may act as an accessory factor for the establishment of separated subpopulations through reduced gene flow. The remaining overlap in flowering period may explain rare but occasional admixture among the two variants as intermediates were also identified in genetic studies (**chapter 2**). Such events of interbreeding among floral variants are less surprisingly due to the fact that detected flower visitors include effective pollinator species like wild-bees and hoverflies (**chapter 3**). Reasonable, floral visits can not be disregarded and treated as "just by chance" but rather indicate the impact of pollinator attraction for cross-fertilization even in self-compatible species. As a consequence of lost attraction outcrossing might be reduced in the decandric population explaining the finding of low genetic variability in *Spe* apart from the assumed recent origin (**chapter 2 + 4**).

It remains an open question whether the occurrence of decandric individuals within wild-type populations of *C. bursa-pastoris* is due to local origin or introduction of seeds/plants. In this context, it is remarkable that most of the known habitats (either extinct or extant)

are located in warm and dry climates: Vienna and Prague are characterized by continental climate (warm-dry vegetation period) and Berlin is located in a merging zone of maritime respective continental climate. The studied populations in Gau-Odernheim and Warburg are also characterized by such warm and dry local climate conditions which enable thermophilic plant species to persist in these locations. Surprisingly, in *Spe* the most frequent genotype of aspartate aminotransferase allozymes (AAT; **chapter 2**) corresponds to the predominant AAT genotype from the Mediterranean (Mediterranean multilocus genotype; Neuffer & Hurka 1999). Plants from the Mediterranean tend to flower early, which contrasts an assumable hint for an introduction of decandric *C. bursa-pastoris* individual(s) from a Mediterranean population. Additionally, no stamenoid phenotype was recognized in the huge number of the sampled individuals from Southern Europe. However, the supposed repeated evolution of the *Spe* phenotype in *C. bursa-pastoris* suggests the local origin within populations. The occurrence in warm and dry regions could imply that the *Spe* variant of *C. bursa-pastoris* might favour such climate conditions. In line with this, the beginning of the 19th century was the ending of a temporary warm period (Glaser 2001) and to that time the decandric variant was observed in high frequencies (Opiz 1821; Trattinnick 1821; Becker 1828). Nowadays the floral variant is apparently almost extinct but the missing corolla is still listed as a trait in determination keys (Rothmaler 19. Auflage 2005; Schmeil-Fitschen 94. Auflage 2009), whereas nothing is mentioned about the occurrence of additional stamens. Taking into account that German floras do not distinguish this variant as an independent taxon any longer it might be overlooked in floristic studies. The detection of further locations is a necessary future objective in order to elucidate whether the molecular basis for the evolution of decandric flowers in *C. bursa-pastoris* is different among populations. This will also include the increase of sampled individuals/populations (e.g. Vienna, Warburg); for instance a population in the surroundings of Brünn (Czech Republic) was recently observed by D. German (pers. communication) and additional individuals were collected in Vienna (S. Hameister).

Evolutionary significance of the *Spe* variant

The occurrence of natural variation in flower morphology of *C. bursa-pastoris* is certainly of evolutionary importance and the decandric variant provides the opportunity to study an ongoing evolutionary process in a natural population. Since the *Spe* variant was postulated as a model for non-gradual evolution (Theißen 2006) and decandric and wild-type plants

occur sympatrically, two highly contentious topics are conjoined by this novelty. In the context of such an implied saltational sympatric speciation process, the question remains open whether the occurrence of additional stamens in decandric flowers is advantageous or whether the novel phenotype is selective neutral, maybe just a genetic hitch-hike? Thus it will be discussed whether there is any line of evidence that the origin of the decandric variant within wild-type populations corresponds to recent concepts of speciation processes.

The origin of new species occurs in a spatial context (Levin 1993) and for historical reasons, models of speciation were postulated by the geographical distribution of populations (Schluter 2001). Due to the degree of geographical separation, theoretical models are distinguished as allopatric (entirely separated entities), parapatric (adjacent entities) and sympatric speciation (overlapping or identical distribution). Until today, quite a large number of empirical studies have confirmed the prevalent occurrence of allopatric speciation in accordance with Mayr's (1954) prediction, whereas sympatric speciation is reported less frequently (reviewed in Turelli *et al*. 2001). However, either within or among populations, isolation mechanisms are a major requirement for divergent evolution including both prezygotic as well as postzygotic isolation. Reproductive isolation is given in geographically separated populations in principle, but the evolution of new taxa in close or direct proximity involves complex processes which are crucial to prevent interbreeding among the derived novelty and its progenitor taxon. In the context of speciation caused by reproductive barriers, hybridization and polyploidization are two well-established mechanisms. According to Levin (2000), both are examples how sympatric speciation may occur. Other postulation suggests that sympatric speciation might be the result of competition for resources which was supported by several empirical studies (Dieckmann & Doebeli 1999; and literature cited therein). Further studies account for the impact of sexual selection (Turner & Burrows 1995; Higashi *et al*. 1999). Especially in zoological studies, non-random choice of mates, assortative mating, is also reported to be involved in the origin of descendant taxa in sympatric populations. Both, sexual selection and assortative mating might be more relevant factors for divergent evolution in animal populations. In sympatric plant populations, however, the differentiation in flowering phenology may be treated as a driving force for disruptive selection since the impact for premating isolation due to variation in this trait has frequently been reported (Stam 1983; Petit *et al*. 1997; Husband & Schemske 2000; Martin & Willis 2007). Particularly in self-incompatible zoogamous species, in which pollinators as well as flowering mates are required for sexual

reproduction, precise timing of transition to reproductive stage is crucial (Riihimäki & Savolainen 2004). To some extent, a shifted flowering phenology can be considered as a sort of assortative mating in plant species (Weis & Kossler 2004). In the present study, the shifted onset of flowering facilitates morphotype-specific mating (wild-type → wild-type *vs*. *Spe* → *Spe*), which in fact might correspond to assortative mating in animal populations. Although one might argue that this is of minor relevance in a highly selfing taxon, it certainly promotes the maintenance of two genetically differentiated sub-populations within one habitat. Seasonal differences in flowering time create temporal isolation which may contribute to the disruptive evolution within plant populations (Savolainen *et al*. 2006; Wendt *et al*. 2002). Regardless its occurrence in animal or plant populations, sympatric speciation initiates with a first genetic polymorphism within a population attended by reproductive isolation. In a final step, disruptive selection may lead to the origin of new species. Adopting this brief outline of sympatric speciation to the studied decandric variant, at least some aspects conform to this general concept. The homeotic change to stamenoid petals may certainly represent the initial genetic polymorphism. Reproductive isolation is realized by predominant selfing and further strengthened through flowering time differentiation. But is there any line of evidence for current selection on the novel flower trait in one of the analyzed populations of decandric *C. bursa-pastoris*?

With regard to the homeotic changed flower morphology in *Spe*, pollinator-mediated selection might be another driving force as it represents a common process in the evolution of flower morphology (Bradshaw & Schemske 2003; Gomez *et al*. 2006; Anderson & Busch 2006). Interestingly, in a closely related but outcrossing Brassicaceae, *Arabidopsis lyrata* (L.) O'Kane & Al-Shehbaz, pollinator-mediated selection was recently reported for both traits flower size and flowering time (Sandring & Ågren 2009). In the self-compatible *C. bursa-pastoris* this mode of selection might be less important, taking into account that insect visitations also facilitate pollination of adjacent flowers within one individual (geitonogamy). Thus, flower morphology in the predominant selfing *C. bursa-pastoris* might be a trait which is not selected against at all. In contrast, field management processes are known to select for plant traits in natural populations (Hawes 2005). As discussed in **chapter 2 + 3**, the anthropogenic disturbance in vineyards certainly was one major force for the establishment of the *Spe* variant in the huge wild-type population. Especially dispersal is facilitated by mechanical processing, not even in single rows of wine-growing

but moreover in the whole region due to parcelling of vineyard properties to different owners. Nevertheless, selection pressure is less interfering in open-soil habitats (Bosbach & Hurka 1981) like the intensively managed vineyards in Gau-Odernheim which is another advantageous incident for the persistence of decandric plants. Apart from beneficial influences, field management in Gau-Odernheim may affect one variant more than the other. For instance, mowing in-between rows of wine cultivation might limit the temporal extent of the period favorable for flowering, i.e. plants will be cut before withering. In line with this, studies in *Mimulus* revealed that plants with an annual life-cycle tend to flower early in the season (van Kleunen 2007). Interestingly, a tendency to winter-annual life-cycle was observed in the *Spe* subpopulation when seeds from field collection were sown in a heated greenhouse in early autumn (September 2005; diploma thesis F Buschermöhle). In this cultivation, more than 70% of the analyzed wild-type plants (n = 414) started to flower within 80 days, whereas one-third of decandric individuals (n = 294) did not alter to reproductive life-cycle within 200 days. Allowing for field experiments including different flowering ecotypes of *Arabidopsis thaliana*, the classification into distinct classes of life-histories (winter-annual or rapid-cycling) might be less useful (Wilczek *et al*. 2009). Thus, field observations are required to prove whether dynamic transitions of life-cycle strategies exist among variants in Gau-Odernheim or whether *Spe* represents a winter-annual ecotype. A general tendency to winter-annual life-cycle, however, might be another consequence of the cultivation process. Mowing in vineyards starts in early May when *C. bursa-pastoris* starts to bloom in the region. This might be treated as positive selection for extreme early respective late flowering ecotypes and might affect the decandric subpopulation more since the onset of flowering is delayed in this variant (**chapter 2**). In this context, it would be relevant to study whether there is any variation in germination behaviour among variants that may support the assumption of altered life-cycle strategies. Contrary to the benefits of anthropogenic disturbance in vineyards, the decandric population in Warburg is threatened to become extinct by a modified field management practice. When the population was discovered, both floral variants occurred on the hilltop as well as on pastures on hillside of the nature reserve 'Desenberg' (Nutt *et al*. 2006). Unfortunately, sheep grazing, the only meadow processing is now conducted later in the season than formerly (P Nutt, pers. communication). Due to the shifted management of meadows, grass species are certainly favoured. This will suppress species adapted to open-soils and *C. bursa-pastoris* becomes less competitive. A direct consequence was surveyed in four successive years (2005-2008): the distribution of wild-type individuals of

shepherd's purse persists on trails and limited trampling areas on the hilltop (approx. ~300-400) and the decandric variant is now entirely restricted to single spots of open-soil close to the "Desenburg ruin" on top of the hill (not exceeding 25 individuals).

Apart from ecological and molecular characterization of the decandric variant, one major intention was to improve the understanding of an evolutionary significance of homeotic variants in natural populations. Since no flowering time QTL was associated to the *Spe* locus and flowering ecotypes are known in *C. bursa-pastoris* (Neuffer & Hurka 1986; Linde *et al*. 2001), it is feasible to assume that the shift to late flowering in the *Spe* variant is not a pleiotropic effect of the homeotic mutation. Since the altered flowering phenology might not be linked to the homeotic change, reduced pollinator attraction is the only ecological consequence identified in decandric populations so far. Due to this loss-of-traits (petal function), it is reasonable to ask whether the decandric variant will sustain its predicted role as an evolutionary novelty (Theißen 2006) or whether it will fall back into oblivion.

In three case studies, the evolutionary importance of homeotic alterations in flower morphology has recently been outlined in the light of flower evolution and pollination biology in general (Ronse de Crane 2003). In this study, the transformation of stamens into petals is assumed to promote insect pollination while replacement of petals by stamens is assumed to be related to wind pollination. The latter scenario is interesting to discuss for the decandric *C. bursa-pastoris*. Apart from increased pollen donation due to additional stamens, no further key adaptations for anemophily like altered pollen structure or stigma surface are noticeable in the *Spe* variant. Thus, the occurrence of "super-male" flowers in *C. bursa-pastoris* must not be miss-interpreted as an evolutionary tendency to wind pollination as previously suggested (Nutt *et al*. 2006). In fact, assuming wind pollination is contrary to the obtained results as anemogamy enhances outcrossing. The present study suggests that outcrossing is reduced in the homeotic variant compared with wild-type, confirmed by the finding that genetic diversity was strikingly low and floral visits by potential pollinators are rare compared with wild-type. As the decandric subpopulation was founded most likely by just a single individual, different patterns of outcrossing among or within variants (interbreeding) might be an auxiliary barrier of gene flow and crucial for the maintenance of *Spe*. The recognition of self *versus* foreign pollen grains and subsequent rejection is generally known as another strong barrier of interspecific crossings in hermaphrodite plants (Bomblies & Weigel 2007). This is a wide-spread principle tied to the breakdown of self-incompatibility and involved in the origin of new species. As

discussed already, the impact of cross-fertilization might be less important in a highly selfing taxa. But preliminary tests revealed that, growth of pollen tubes in artificially self-fertilized *C. bursa-pastoris* is obviously faster in the decandric variety than in analyzed wild-type plants (exam thesis; S Bicker 2007). Based on this finding, an experiment is intended to elucidate and confirm possible differences in the recognition of self *versus* foreign pollen among both *Capsella* variants. However, the formation of stamenoid petals causes a reduction in flower size, which is generally discussed as a consequence of weakened selective force for corolla function (Rollins 1963; Barrett 2002). Such a reduction of the corolla size is often followed by increasing selfing rates (Ritland & Ritland 1989). In line with this, the transition to self-compatibility (SC) coincides with a decrease in corolla size in the genus *Capsella* (Hurka & Neuffer 1997). Thus, the total loss of petal function in the decandric variant might accomplish the evolution to self-fertilization within the genus.

Taking together, although thoroughly studied from the field to the laboratory, neither a remarkably advantageous nor any hampering affect of the homeotic transformation in flowers of *C. bursa-pastoris* was identified so far. Contrary, the persistence for decades of the decandric variant in at least the vineyards of Gau-Odernheim is most likely solely driven by intrinsic characteristics of shepherd's purse: high rates of selfing accompanied by ecotypic variation in flowering time (Hurka & Neuffer 1997). As a consequence of the predominant selfing the whole genome might be considered to be fixed and a new character may easily sustain within populations, in case it is not deleterious like stamenoid petals in the second floral whorl. Referring to high levels of self-pollination in *C. bursa-pastoris*, the affected (reduced) pollinator attraction due to the altered flower shape in *Spe* might be regarded as a drop in a bucket. Nevertheless it represents another feature enhancing the initial barrier of gene flow among variants and maintains/contributes to the genetic differentiation into two sub-populations. This significant flower-type dependent population structure in Gau-Odernheim may justify its predicted role in the light of evolution, since *Capsella* variants are easy to distinguish in the field and molecular analysis. However, assuming a sympatric speciation process (either in Gau-Odernheim or any other provenance) remains a contentious issue, likewise the benefits for the understanding of non-gradual evolution. To further enlighten these prospects, several subsequent analyses are inevitable. First of all the molecular genetic base has to be entirely resolved, including the question for different mechanisms among populations. Second,

studies about the fitness of F1 hybrids among variants must be included in future objectives since selection against hybrids (inbreeding depression) is a strong isolation barrier. If any hint for disruptive selection will be unravelled (e.g. in experiments under controlled conditions), this might support the hypothesis of an ongoing speciation process and then the decandric variant may represent a comprehensive model for both, ecology and evolution. But what is the final conclusion with regard to the evaluation of the significance of a homeotic alteration like stamenoid petals?

This comprehensive study including molecular as well as ecological analyses provided substantial evidence that the *Spe* variant, formerly known as *Capsella apetala*, in fact represents a recent model for the persistence of morphological novelties in natural populations. Thus, it corresponds to classical examples like the origin of peloric *Linaria* (Cubas *et al*. 1999) and the homeotic *Clarkia* variant (Ford & Gottlieb 1992). With respect to the obtained results, "*Capsella apetala*" represents a feasible model for non-gradualistic evolution Theißen (2006). Allowing for the hypothesis that a single locus *Spe* has caused the homeotic change, its predicted contributes to the context of saltational evolution of novel traits was renewed in a recent review (Theißen 2009). The impact of such minor genetical modifications has already been shown, e.g. the origin of maize (Doebley *et al*. 1995), the loss of ray floret in *Senecio* (Comes 1998) and flower color variants in *Mimulus* (Bradshaw & Schemske 2003). Taking into account that the homeotic variant was not negatively affected in fitness, the concept of 'hopeful monster' founded by Richard Goldschmidt (1940) may be adopted as suggested by Theißen (2006). This concept contrasts the origin of new species through natural selection as supposed by Darwin (1859) but involves the impact of "macromutations" for the spontaneous arise of evolutionary novelties adapted to a certain niche. Such 'hopeful monsters' were not confirmed in empirical studies so far (Mayr 2001). In contrast to the ongoing controversy about the relevance of *C. apetala* in saltational evolution (Theißen 2009), it might be more important, that the sympatric population in Gau-Odernheim provides the opportunity to survey a (macro)evolutionary novelty in association of continuous micro-evolutionary adaptation. However, as far as no ecological adaptation to a certain niche is traceable, *C. apetala* might be strikingly dependent on beneficial local conditions and it is reasonable to assume that the persistence of decandric flowers in *C. bursa-pastoris* might be more likely the result of genetic hitch-hiking with adaptive traits.

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Summary

The impact of homeotic alterations on the origin of evolutionary novelties is still a highly controversial subject in plant sciences. One reason for the ongoing controversy might be, that most of the studies were primary intended to unravel underlying genetical mechanisms. A lot of progress was achieved about how regulatory genes control precise development of floral organs. This research was mainly based on studies in artificial homeotic mutants of molecular model plants. The exploration of naturally occurring homeotic variants, however, remained under-represented. To date, there is growing interest to investigate natural variation like homeotic changes, in order to fully understand the role of such morphological modifications in evolutionary processes. Moreover, homeotic mutants are discussed in the light of non-gradual evolution. Such saltational origin of new taxonomic entities may occur within ancestral (wild-type) populations, thus involving sympatric speciation as another contentious issue in plant research. Therefore, the analysis of homeotic variants in wild plant populations might be of great benefit to evaluate the evolutionary consequences of such taxonomic novelties. The discovery of a floral homeotic mutant of shepherd's purse, *Capsella bursa-pastoris* (L.) Medik., occurring naturally within wild-type populations, offers the unique opportunity to elucidate the evolutionary significance of homeotic mutants.

In this thesis, the combination of ecological as well as molecular characterization of a natural floral variant of *C. bursa-pastoris* may improve the understanding of evolutionary processes in (sympatric) plant populations in general. The thesis may furthermore represent one of the first attempts to elucidate the evolutionary relevance of homeotic novelties and their maintenance in wild populations.

Reproductive isolation of a derived entity and its progenitor is crucial for sustainability of novel traits. In geographically separated populations, isolation is realized in a spatial context while in sympatric populations the isolation among novel taxon and progenitor may establish through ecological divergence. In the presented case study of floral variants of *C. bursa-pastoris*, predominant self-pollination is a first barrier of gene flow and might be regarded as a fixation of the whole genome. Nevertheless, the persistence of maybe just one initial mutant individual may be threatened by quite a few circumstances: the novel entity might be drastically hampered in survival, or be less competitive under local conditions compared with the wild-type, and also extinction might happen just by chance. Thus, apart from selfing as an initial reproductive barrier, further mechanisms are

necessary to facilitate the persistence of a new taxon in wild populations. The derived floral phenotype of *C. bursa-pastoris* is characterized by the homeotic transformation of petals into additional stamens. The variant was called 'decandric' or '*Stamenoid Petals*' and is currently known from several locations throughout Europe. These provenances vary strikingly in the abundance of the variant and also in environmental features. The only population revealing a high frequency of both, mutant and wild-type individuals is located in intensively managed vineyards. Three remaining provenances are rather ruderal sites and only single individuals were recorded so far.

Genetic differentiation

The occurrence of decandric phenotypes in these geographically isolated populations was analyzed to achieve hints for a single or multiple origin of the novel phenotype. Genetical studies clearly suggested a repeated evolution of the novel flower morphology, independently in the considered habitats. Thus, the decandric variant is a feasible example for convergent evolution of floral traits. Since little is known about the establishment of evolutionary novelties within local wild-type populations, the broad population located in cultivated vineyards was thoroughly studied with respect to population structure. Based on fingerprint markers, the genetic analyses provided substantial evidence, that the two floral variants are well-defined into flower-type dependent sub-samples within this population. A high degree of self-fertilization in *C. bursa-pastoris* was certainly the major driving force for this genetic differentiation since it represents as a strong barrier of gene flow. In case that the genetic differentiation of wild-type and variant is further promoted by ecological divergence in adaptive traits, the population may become an interesting model for sympatric speciation.

Ecological differentiation

The successful establishment in at least one location suggests that the homeotic variant is not drastically affected in fitness. This hypothesis was proven by the evaluation of ecologically adaptive traits (fitness traits) in a comparative approach among both variants in a field experiment. Interestingly, the two flower-types pursue different strategies for the investment in the succeeding generation. The overall reproductive fitness, however, was counterbalanced under local conditions in the field experiment. Additionally, both variants revealed an ecological differentiation in the onset of flowering. Under greenhouse conditions as well as in a field experiment, the onset of flowering was significantly delayed

in the homeotic variant. This detected flowering time differentiation certainly enhances reproductive isolation among variants which was generally caused by a high degree of selfing in general.

Molecular characterization

Finally, the analysis of a generated F2 mapping population (derived from a cross of wild-type and homeotic variant), revealed a co-dominant inheritance of a single locus (*Spe*) which might be involved in the molecular origin of the novel flower shape. The marker-assisted mapping approach exposed the chromosomal localization of this single locus *Spe* in a genetic map. The intended cross-species linkage to the genome of the closely related molecular model plant *Arabidopsis thaliana*, stuck in an early stage of methodical establishment due to the lack of appropriate molecular markers. Therefore, the number of considered candidate genes which might be involved in the molecular origin of the homeotic variant could not be constrained. As a first tentative hint, the linkage group including the *Spe* locus showed sequence similarities with chromosome IV of the *A. thaliana* genome. Interestingly, *AGAMOUS* is located on this chromosome, the only class C floral organ identity gene in the *A. thaliana* genome, which is compatible with the assumption that the locus *Spe* is an allele of *AGAMOUS* rather than a regulator of that gene.

Evolutionary significance

Based on the outcome of genetic and flowering time analyses, it is reasonable to interpret both flower variants as separated sub-populations and the homeotic variant may be treated as an established entity within a wild-type population. The analysis of quantitative traits (QTL), including flowering time, indicated that this shifted flowering time in the floral mutant is not linked to the homeotic change. Therefore an indicated ecological separation among variants is most likely not directly associated with the homeotic change in decandric variant.

The comprehensive study of ecological and molecular aspects contributed to evaluate the evolutionary significance of a homeotic variant in wild populations of *C. bursa-pastoris*. At least in one population, the floral homeotic variant may be treated as an established taxonomic entity and proved the predicted role as a model for evolutionary objectives. Consequently, the homeotic *C. bursa-pastoris* may represent a feasible example for non-gradual evolution as previously suggested. The establishment of such novelties might still

depend on local adaptation and micro-evolutionary mechanisms. This in turn, involves natural selection for a driving force for the origin of new species as postulated by Charles Darwin. Yet, the origin of decandric flowers in *C. bursa-pastoris* might only represent a non-gradual step in an evolutionary process in general, assisted by additional micro-evolutionary factors. To conclude, this thesis supports the controversy that homeosis is a thinkable scenario for the origin of evolutionary novelties. But the frequency of this principle in wild populations remains an open question for future studies.

Zusammenfassung

Die Bedeutung von homöotischen Veränderungen für die Entstehung neuer Arten ist immer noch ein kontroverses Thema in Pflanzenwissenschaften. Eine mögliche Erklärung für die anhaltende Diskussion liegt vermutlich darin, dass viele der durchgeführten Studien in erster Linie bemüht waren grundsätzliche genetische Mechanismen aufzuklären. Ein großer Fortschritt wurde im Bereich der Entwicklungsgenetik erzielt, mit deren Hilfe heutzutage die genetisch-kontrollierte Entwicklung von Blütenorganen detailliert erklärt werden kann. Der Großteil der hierfür durchgeführten Studien wurde mit Hilfe von künstlich erzeugten homöotischen Mutanten an Modellpflanzen erreicht. Die Untersuchung natürlich vorkommender Blütenvarianten blieb dagegen weitestgehend unberührt. Das Interesse an natürlicher Variation rückt derzeitig immer mehr in den Fokus aktueller Forschung, nicht zuletzt um die Bedeutung von morphologischen Änderungen im Zusammenhang mit evolutionären Prozessen besser zu verstehen. Gelegentlich werden homöotische Mutanten sogar als Beispiele für nicht-graduelle Evolution diskutiert. Solch eine sprunghafte Entstehung von taxonomischen Neuheiten kann auch innerhalb der (wild-typischen) Abstammungs-Population stattfinden. In diesem Fall können abrupte morphologische Änderungen einen möglichen Mechanismus für sympatrische Artbildung darstellen.

Eine einzigartige Chance die evolutionäre Bedeutung von homöotischen Varianten am Modell zu studieren, bietet die Entdeckung einer natürlich vorkommenden homöotischen Blütenvariante des gewöhnlichen Hirtentäschels, *Capsella bursa-pastoris* (L.) Medik. Ziel der Arbeit ist die ökologische und molekulare Charakterisierung dieser Blütenvariante des Hirtentäschels, um das Verständnis von evolutiven Abläufen in (sympatrischen)

Pflanzenpopulationen zu erweitern. Diese Arbeit ist eine der ersten Studien, welche neben der molekularen Erforschung von homöotischen Neuheiten auch deren Etablierung in wilden Populationen untersucht.

Reproduktive Isolation zwischen einer neu entstandenen Variante und dessen Vorfahr ist eine unumgängliche Bedingung für den Erhalt des neuen Taxons. In geografisch getrennten Populationen wird die Isolierung durch den räumlichen Zusammenhang sichergestellt, während in sympatrischen Populationen die Isolation zwischen abgeleiteter Form und Stammform in der Regel durch eine ökologische Trennung entsteht. Im Falle der Blütenvarianten des Hirtentäschels, stellt das hohe Maß an Selbstbefruchtung eine erste Genfluss-Barriere dar, so dass das gesamte Genom als fixiert betrachtet werden kann. Dennoch, der Erhalt eines einzigen mutierten Individuums wird gefährdet durch eine Reihe von äußeren Gegebenheiten: zum Beispiel erschweren/verhindern drastisch reduzierte Überlebenschancen, geringere Wettkampffähigkeit unter lokalen Bedingungen oder zufälliges Aussterben eine Etablierung in der Natur. Neben der Selbstbefruchtung muss es demzufolge weitere Mechanismen geben, welche die Persistenz eines neuen Taxons in natürlichen Populationen ermöglichen. Der veränderte Blütentyp in *C. bursa-pastoris* ist gekennzeichnet durch die homöotische Transformation von Blütenblättern in zusätzliche Staubblätter. Bezeichnet wird dieser Phänotyp als dekandrisch bzw. 'Stamenoide Petalen' und ist zurzeit von vier Standorten innerhalb Europas bekannt. Diese Standorte unterscheiden sich deutlich in der Häufigkeit der Variante sowie in äußeren Faktoren. Nur eine Population in intensiv bewirtschafteten Weinbergen zeichnet sich durch ein hohes Auftreten der Mutante aus. Drei weitere Herkünfte sind ruderale Standorte in denen in der Regel nur Einzel-Individuen nachgewiesen wurden. An allen Standorten dominiert der Wildtyp.

Genetische Charakterisierung

Das Vorkommen des dekandrischen Phänotyps in vier geografisch isolierten Populationen wurde im Hinblick auf einmalige oder mehrfach-unabhängige Entstehung analysiert. Die genetischen Untersuchungen sprechen für eine wiederholte Entstehung in den einzelnen Habitaten, so dass die unabhängige Evolution des dekandrischen Phänotyps ein aktuelles Beispiel für Konvergenz verkörpert. Da bislang nur wenig über die Etablierung von homöotischen Neuheiten in natürlichen Populationen bekannt ist, wurde die individuenreiche Population in kultivierten Weinbergen als Modell für Populationsstruktur und ökologischer Differenzierung herangezogen. Auf Grundlage von molekularen

Markerstudien (*fingerprints*) ergab die genetische Analyse eindeutige Beweise, dass sich innerhalb der Weinberg-Population beide Varianten in deutliche Blütentyp-Gruppen einteilen lassen. Ein generell hohes Maß an Selbstbefruchtung in *C. bursa-pastoris* ruft eine erste Genfluss-Barriere zwischen den Varianten hervor und stellt einen wirksamen Mechanismus dar, der diese eindeutige genetische Differenzierung erklären kann. Sollten zusätzliche ökologische Faktoren diese genetische Differenzierung verstärken, bekäme die Population insgesamt einen Modellcharakter für empirische Untersuchungen von sympatrischer Artbildung.

Ökologische Differenzierung

Die erfolgreiche Etablierung in mindestens einem Standort spricht dafür, dass die Fitness der Variante nicht negativ beeinträchtigt ist. Diese Hypothese wurde durch die Analyse von adaptiven Merkmalen (Fitness-Parameter) in einer vergleichenden Studie zwischen den zwei Blütenvarianten analysiert. Auffällig ist, dass beide Typen unterschiedliche Strategien bei der Investierung in die nachfolgende Generation verfolgen, die generelle reproduktive Fitness insgesamt aber ausgeglichen war. Eine ökologische Trennung der beiden Varianten wurde dagegen für den Zeitpunkt des Blühbeginns nachgewiesen. Sowohl unter Gewächshaus- als auch unter Freilandbedingungen wurde ein späteres Aufblühen der dekandrischen Form beobachtet. Diese zeitliche Trennung im Wechsel von vegetativer zu reproduktiver Phase war in beiden Studien signifikant. Der gefundene Unterschied im Blühbeginn ist ein wichtiger Faktor, der die reproduktive Isolation durch vorwiegende Selbstbefruchtung verstärkt.

Molekulare Charakterisierung

Die Untersuchungen der F2 Generation einer künstlichen Kreuzung zwischen Wildtyp und dekandrischer Variante (Kartierungs-Population) ergaben einen co-dominant vererbten Locus (*Spe*), der möglicherweise den molekularen Ursprung des neuen Blütentyps darstellt. Eine Kopplungsgruppen-Analyse ergab die chromosomale Lokalisation dieses einzelnen Locus *Spe* in einer Genkarte. Die Bestrebungen eines art-übergreifenden Vergleichs mit dem Genom der molekularen Modellpflanze *Arabidopsis thaliana*, befindet sich in der methodischen Etablierung, da bislang keine geeigneten molekularen Marker zur Verfügung stehen. Aus diesem Grund konnte auch die Zahl der in Frage kommender Kandidatengene nicht eingeschränkt werden. Ein erster Hinweis ergibt sich allerdings aus der Tatsache, dass die Kopplungsgruppe, welche den *Spe* Locus einschließt, DNA-Sequenz

Übereinstimmungen zum Chromosom IV des *A. thaliana* Genoms aufweist. Interessanterweise ist auf diesem Chromosom das Blütenorganidentitätsgen *AGAMOUS* lokalisiert, das einzige Gen welches vereinbar ist mit der Annahme, dass der *Spe* Phänotyp durch ein verändertes Allel und nicht durch einen Regulator dieses Gens hervorgerufen wird.

Evolutionäre Signifikanz

Im Bezug auf die beobachtete genetische und auch ökologische Differenzierung, können beide Varianten als eigenständige Subpopulationen interpretiert werden. Die umfassende Studie von Ökologie und Genetik der natürlichen Blütenvariante von *C. bursa-pastoris* konnte dazu beitragen, die evolutionäre Bedeutung homöotisch entstandener Neuheiten in wilden Populationen an einem Fallbeispiel zu bewerten. In mindestens einer der analysierten Population kann die dekandrische Blütenvariante als eine etablierte taxonmische Einheit innerhalb einer wildtypischen Population angesehen werden. Aufgrund der molekularen Ergebnisse entspricht die homöotische Hirtentäschel-Variante dem vermuteten Modellcharakter für Evolutionsstudien und bestätigt die Hypothese eines Beispiels für nicht-graduell verlaufende Evolution. Die Etablierung solcher morphologischen Neuerungen in natürlichen Populationen kann aber immer noch auf lokale Anpassung und mikroevolutiven Abläufe beruhen. Das wiederum erfordert das Einbeziehen von Artbildungsprozessen auf Ebene von natürlicher Selektion wie in den Ursprüngen der Evolutionsforschung durch Charles Darwin postuliert. Im Kontext der Bedeutung von homöotischen Varianten, deuten die Ergebnisse einer Analyse quantitativer Merkmale (QTL) an, dass der verzögerte Blühbeginn in der dekandrischen Form des Hirtentäschels nicht an die homöotische Mutation selbst gekoppelt ist. Aus diesem Blickwinkel betrachtet, stellt die genetische Entstehung von dekandrischen Blüten in *C. bursa-pastoris* nur einen nicht-graduellen Schritt in einem Evolutionsprozess dar. Die Etablierung dagegen wird von zusätzlichen mikroevolutiven Vorgängen getragen. Schlussendlich erhärtet die durchgeführte Studie die bislang kontroverse Sicht von homöotische Veränderungen als möglichen Mechanismus für die Entstehung von evolutionären Neuerungen. Für zukünftige Untersuchungen ergibt sich daraus die Frage nach der Häufigkeit dieses Prinzips in der freien Natur.

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Erklärung über die Eigenständigkeit der erbrachten wissenschaftlichen Leistung

Gem. § 8 Abs. 2 Buchstabe b der Promotionsordnung der Fachbereiche Biologie/Chemie, Mathematik/Informatik und Physik der Universität Osnabrück

Ich erkläre hiermit, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe der Quelle gekennzeichnet. Bei der Bearbeitung meines Forschungsthemas haben mich die nachfolgend aufgeführten Personen in der jeweils angegebenen Weise unentgeltlich unterstützt.

1. Bei der Vorbereitung von Studien sowie der Datenauswertung und Verfassung von Manuskripten stand mir apl. Prof. Dr. Barbara Neuffer als Betreuer zur Seite.
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Weitere Personen waren an der inhaltlichen und materiellen Erstellung der vorliegenden Arbeit nicht beteiligt. Insbesondere habe ich hierfür nicht die entgeltliche Hilfe von Vermittlungs- bzw. Beratungsdiensten (Promotionsberater oder andere Personen) in Anspruch genommen. Niemand hat von mir unmittelbar oder mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen.

Die Arbeit wurde weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

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Ort, Datum Unterschrift