http://biology.num.edu.mn
http://mjbs.100zero.org/
Volume 12(1-2), 2014

Mongolian Journal of Biological Sciences

ISSN 1684-3908 (print edition)
MJBS
ISSN 2225-4994 (online edition)

Original Article

Evolutionary History of the Genus *Capsella* (Brassicaceae) - *Capsella orientalis*, New for Mongolia

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Abstract

Key words: Sheperd's purse, flow cytometry, leaf morphology, flowering time, pollen tube growth, crossing experiments, phylogenetic age estimation, biogeography

Article information:
Received: 04 Oct. 2013
Accepted: 12 Sept. 2014
Published: 15 Apr. 2015

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To elucidate the evolutionary history of the genus *Capsella*, we included the hitherto poorly known species *C. orientalis* and *C. thracica* into our studies together with *C. grandiflora*, *C. rubella*, and *C. bursa-pastoris*. We sequenced the ITS, and four loci of noncoding cpDNA regions (trnL – F, rps16, trnH – psbA, trnQ – rps16). In common garden field experiments *C. orientalis* turned out as early flowering with a specific leaf type. The crossing ability of the species was tested in pollen germination experiments. *Capsella orientalis* (self-compatible, SC; 2n = 16) forms a clade (eastern lineage) with C. *bursa-pastoris* (SC; 2n = 32), which is a sister clade (western lineage) to *C. grandiflora* (self-incompatible, SI; 2n = 16) and *C. rubella* (SC; 2n = 16). *Capsella bursa-pastoris* is an autopolyploid species of multiple origin, whereas the Bulgarian endemic *C. thracica* (SC; 2n = 32) is allopolyploid and emerged from interspecific hybridisation between *C. bursa-pastoris* and *C. grandiflora*. The common ancestor of the two lineages was diploid and SI, and its distribution ranged from eastern Europe to central Asia, predominantly confined to steppe like habitats. Biogeographic dynamics during the Pleistocene caused geographic and genetic subdivisions within the common ancestor giving rise to the two extant lineages. *Capsella orientalis* is verified at several positions in western Mongolia.

Cite this paper as: Neuffer, B., Hurka, H., Friesen, N., German, D. A. & Franzke, A., 2014. Evolutionary History of the Genus *Capsella* (Brassicaceae) - *Capsella orientalis*, New for Mongolia. *Mong. J. Biol. Sci*., 12(1-2): 3-18.

Introduction

Molecular systematic studies confirm that the genus *Capsella* belongs to the tribe, Camelineae (Al-Shehbaz *et al*., 2006; Bailey *et al*., 2006; German *et al*., 2009; Warwick *et al*., 2010). Scientific research is focusing its attention increasingly on *Capsella* addressing such key issues as speciation, adaptation, mating systems, and evolutionary developmental biology of plant form (Hurka & Neuffer, 1997; Foxe *et al*., 2009; Guo *et al*., 2009; Paetsch *et al*., 2010; Neuffer, 2011; Sicard *et al*., 2011, Theißen, 2011). Additionally, sequencing of the *Capsella rubella* genome is currently being carried out by the Joint Genome Institute, United States Dept. of Energy.

Many attempts to elucidate the evolutionary history and biology of the genus *Capsella* in which one of the most widespread flowering plants on earth (*C. bursa-pastoris*) is included (Coquillat, 1951), have already been undertaken (e.g. Shull, 1929; Hurka & Neuffer, 1997; Ceplitis & Lascoux, 2005; Slotte *et al*., 2006; St. Onge, 2010). This has lead to controversy regarding, e.g. phylogenetic relationships, mode of speciation, biogeographic origin and age estimations of the genus and its species. Hurka *et al*. (2012) recently formulated a new hypothesis which is referred here.

Species delimination is difficult and controversial due to the enormous morphological variation within the genus. Tutin *et al*. (1993) list in Flora Europaea seven *Capsella* species: commonly accepted are *C. grandiflora* (Fauché & Chaub.) Boiss., *C. rubella* Reuter, *C. bursa-pastoris* (L.) Medik. including *C. thracica* Velen. as a subspecies, and *C. orientalis* Klokov. *Capsella grandiflora* and *C. rubella* are diploid (2n = 2x = 16), and *C. bursa-pastoris* is tetraploid (2n = 4x = 32).

Interestingly, *Capsella orientalis* and *C. thracica* for a long time have been excluded as subject of experimental work, obviously due to the fact that no seed material was available. We included both taxa exploring the biosystematics and phylogenetics of these (Hurka *et al*., 2012). With all probability *Capsella heegeri* Solms-Laub. with its characteristic ellipsoidal fruits is extinguished for decades. *Capsella gracilis* Gren. is a sterile hybrid between *C. bursa-pastoris* and *C. rubella* often observed in mixed populations in the overlapping distribution area. Here we reveal biological, phylogenetic and biogeographic patterns within the genus *Capsella* covering all currently accepted taxa (Tutin *et al*., 1993).

We analysed the nuclear internal transcribed spacers ITS1 and ITS2 including the 5.8S gene, together with four different noncoding regions of the chloroplast genome. Shaw *et al*. (2007) provided an index of the relative levels of cpDNA variability. From among that list we chose the less variable trnL -trnF intergenic spacer region and a highly variable cpDNA region, the trnQ -rps16 intergenic spacer, as well as two regions more or less intermediate in their levels of variation (trnH -psbA intergenic spacer, rps16 intron) (Hurka *et al*., 2012).

The investigations were complemented by morphological, cytological, and biogeographic studies. Our main questions are:

- What is the biogeography of the genus *Capsella*?

- And how and where was the origin of the polyploid and highly successful colonizer *C. bursa-pastoris*?

In the light of all the data presented in this study, it is obvious that *C. orientalis* and *C. thracica* hold a key position in our endeavours towards understanding the evolutionary history of the genus *Capsella*.

Material and Methods

Information about the origin of seed and herbarium material as well as GenBank accession numbers of DNA sequence analyses is given in Hurka *et al*. (2012). The geographical distribution of *C. orientalis* was established through literature surveys (Ebel, 2002; German & Ebel, 2009), our own field collections, and by investigating herbarium collections (Fig. 1; Hurka *et al*., 2012). Cytological studies, flowcytometry, and sequencing analyses of nuclear and plastidic DNA as well as the phylogenetic analyses follow the instructions of Hurka *et al*. (2012).

Our findings resulted in divergence time estimates done with the software package BEAST v1.4.8 (Drummond & Rambaut, 2007) based on ITS sequences (ITS1 and 2 regions combined, 5.8S gene region excluded). No intraspecific ITS variation was detected between 5 provenances of *Capsella grandiflora*; 3 of *C. rubella*; 4 of *C. orientalis*, and 9 of *C. bursa-pastoris* (Hurka *et al*., 2012). In a common garden field experiment we planted 1088 individuals of wild populations of four *Capsella* species in the experimental field of the Botanical Garden, Osnabrück (Table 1). We collected one rosette leaf of each individual for determining the leaf type after Shull (1909) and recorded the onset of flowering in days from sowing to breaking the first flower bud. In crossing experiments plants were grown in an unheated greenhouse.

Controlled pollinations were performed by withdrawing single mature anthers from fully-opened flowers with a forceps, and by rubbing the pollen sacs onto the papillar cells of the stigmatic surface. Stigmas were completely covered with pollen grains. To avoid uncontrolled self-pollination in the self-compatible species flowers

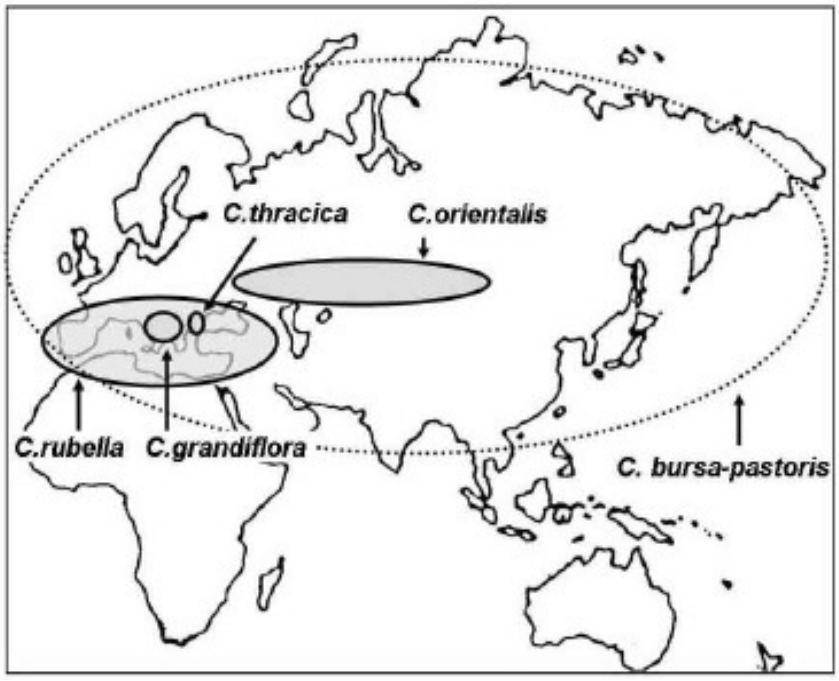

Figure 1. Outline distribution map of *Capsella* species. *Capsella grandiflora*: western Balkan, northern Italy; *C. rubella*: circum Mediterranean; *C. orientalis*: eastern Europe to central Asia; *C. thracica*: Bulgaria. Putative native range of *C. bursa-pastoris* is shown by dotted line. The worldwide distribution of *C. bursa-pastoris* and colonized regions of *C. rubella* in the New World and Australasia are not indicated (see Hurka *et al*., 2012).

Table 1. Provenances of the investigated *Capsella* populations in the common garden field experiment (onset of flowering and leaf types).

Pop. Nr.	Provenance	Coordinates	Elevation above sea level	Species
698	Alpi Apuani, Italy	44°02'N, 10°18'E	1180 m	*C. rubella*
910	Doukades, Greece	39°41'N, 19°44'E	150 m	*C. grandiflora*
918	Pantokrator, Greece	39°45'N, 19°52'E	910 m	*C. grandiflora*
921	Paleokatritsa, Greece	39°40'N, 19°42'E	50 m	*C. grandiflora*
925	Joannina, Greece	39°40'N, 20°51'E	450 m	*C. grandiflora C. rubella C. bursa-past.*
928	Metsovo, Greece	39°46'N, 21°10'E	1150 m	*C. grandiflora*
933	Katara pass, Greece	39°48'N, 21°11'E	1500 m	*C. grandiflora C. bursa-past.*
934	Metsovo, Greece	39°46'N, 21°10'E	1350 m	*C. grandiflora*
935	Sokraki, Korfu, Greece	39°43'N, 19°48'E	500 m	*C. grandiflora*
936	Pantokrator, Greece	39°45'N, 19°52'E	760 m	*C. grandiflora*
984	Mallorca, Spain	39°30'N, 03°00'W	500 m	*C. rubella*
1215	Teneriffa, Spain	28°29'N, 16°19'W	10 m	*C. rubella*
1377	Buenos Aires, Argentina	34°40'S, 56°30'W	10 m	*C. rubella*
1482	Perth, Australia	31°56'S, 115°50'E	25 m	*C. rubella*
1938	Barnaul, Russia	53°20'N, 83°45'E	150 m	*C. orientalis*
1939	Pawlodar, Russia	52°16'N, 76°57'E	120 m	*C. orientalis*
1940	Bayanaul, Russia	50°47'N, 75°41'E	460 m	*C. orientalis*
1949-1963	Gau-Odernheim, Germany	49°47'N, 12°10'E	200 m	*C. bursa-past*. wt, spe, int

were emasculated on the first day of flower opening, when anthers are still closed due to protogyny (Hurka *et al*., 1976, Neuffer & Paetsch, 2013). Development of pollen was measured by revealing the appearance of the pollen tubes within tissues of the pistils through visualization of callose plugs, whose formation serve as a sensitive indicator of relative pollen tube growth rates (Snow & Spira, 1991). Germination and further development of pollen was inferred by staining pollen tubes following the method described by Kho and Baer (1968), and Neuffer and Paetsch (2013).

Results

Geographical distribution, karyological analyses and flowcytometry of *Capsella* species

Capsella orientalis is morphologically very close to *C. bursa-pastoris* and often confused with it. Our data unambiguously prove diploidy for *C. orientalis* with 2n = 16 (Fig. 1; Hurka *et al*., 2012). Thus, in addition to morphological details, the most important difference between *C. orientalis* and *C. bursa-pastoris* is the ploidy level: *C. orientalis* is diploid with 2n = 2x = 16, and *C. bursa-pastoris* is tetraploid with 2n = 4x = 32 (Fig. 2; Hurka *et al*., 2012). Flowcytometry suggests that, despite equal chromosome numbers, the relative DNA content between *C. orientalis* and the other diploid species, *C. grandiflora* and *C. rubella*, is somewhat different between the three diploid species (Fig. 2; Hurka *et al*., 2012). *Capsella orientalis* is fully self-compatible, as proven by our own greenhouse and field experiments.

Our literature and herbarium survey revealed that *C. orientalis* has a much wider distribution area than hitherto reported (Fig. 1; Hurka *et al*., 2012). It ranges from the middle Ukraine through the southern part of European Russia, the South Urals, northern Kazakhstan, south-west Siberia up to western Mongolia and north-western China (Xinjiang region). This distribution coincides noticeably with the middle and western part of the Eurasian steppe belt which stretches from south-eastern Europe to north-eastern China.

Capsella thracica is a Bulgarian endemic (Fig. 1) and, like *C. orientalis*, morphologically very close to *C. bursa-pastoris*. The main feature differentiating this species from *C. bursa-pastoris* is the elongated style. Just like *Capsella bursa-pastoris*, *C. thracica* is tetraploid as has been revealed by chromosome counts and flowcytometry (Fig. 2), and is predominantly selfing (Hurka *et al*., 2012).

Phylogenetic analyses

ITS sequence data. Direct sequencing of the ITS PCR products produced unambiguous sequences, with the exception of *Capsella thracica* accessions. In *C. thracica*-12, we

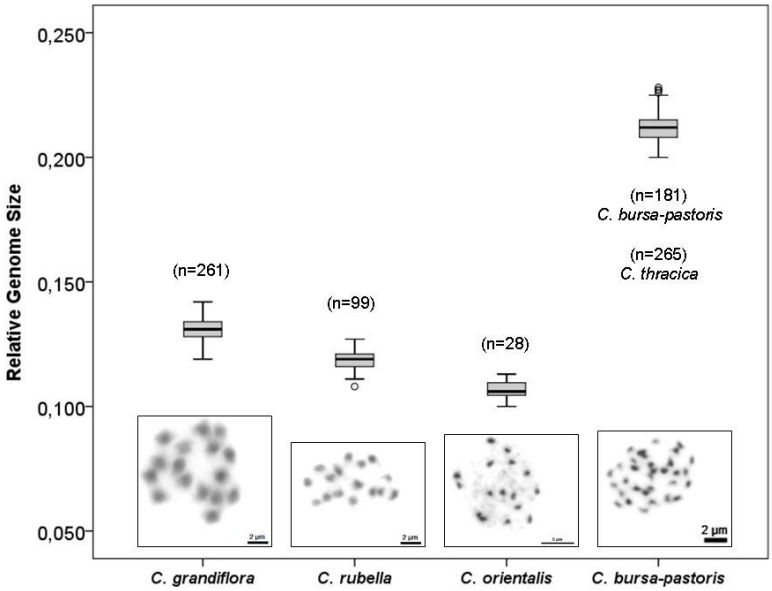

Figure 2. Figuration of chromosomes and relative DNA amount of *Capsella* species: Chromosome pictures are from metaphase plates from pollen mother cells. Relative DNA amount revealed by flowcytometry, standard: *Petroselinum crispum*; n = number of measured individuals (see Hurka *et al*., 2012).

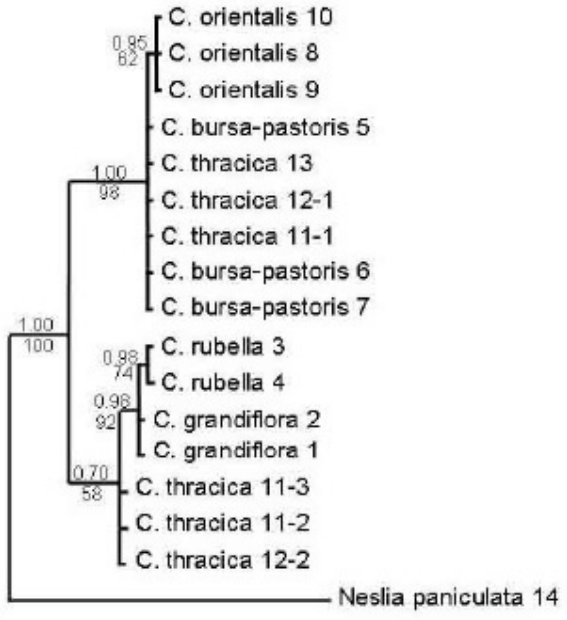

Figure 3. Phylogenetic tree for *Capsella* species based on ITS: Bayesian posterior probabilities above branches, bootstrap support over 50% below branches. For *C. thracica* 13 only the original sequence with two peaks at positions 122-126 was included in the analyses (see Hurka *et al*., 2012)

obtained different sequences using forward and reverse primers. The forward primer resulted in a sequence almost identical to *C. grandiflora*, and the reverse primer in a sequence identical to *C. bursa-pastoris*/*C. orientalis*. The two other *C. thracica* accessions, no. 11 and 13, displayed at ITS sequence positions 122 – 126, two identical peaks which can be translated as RWWW (R = A and G; W = A and T), showing that *C. thracica* has at least two different copies of rDNA in its genome. To confirm this, we cloned ITS PCR products of accession *C. thracica*-11. In the 16 sequenced clones, 14 sequences were identical with *C. bursa-pastoris*, and two sequences almost identical to *C. grandiflora*; in *C. thracica* one nucleotide was missing in a poly-T-motif. These additional copies were included in the analyses (Hurka *et al*., 2012).

The alignment of combined ITS1 and ITS2 sequences, including the 5.8-S gene, generated a matrix of 640 characters, of which 10 were parsimony informative. For the Bayesian analyses, the substitution model K80 was chosen by AIC in Modeltest 3.7. Unweighted parsimony analysis of the 19 sequences resulted in a single most parsimonious tree of 60 steps (CI = 1.000; Fig. 3; Hurka *et al*., 2012).

Capsella bursa-pastoris and *C. orientalis* formed a clade supported by 98% bootstrap value and 1.00 Bayesian posterior probabilities. This clade is a sister group to the clade consisting of *C. grandiflora* and *C. rubella* (88% bootstrap support, 0.74 Bayesian posterior probabilities) (Fig. 3; Hurka *et al*., 2012). Within the two sister clades, *C. orientalis* is resolved from *C. bursa-pastoris* by 62% bootstrap support and 0.95 Bayesian posterior probabilities, and *C. rubella* from *C. grandiflora* by 74% bootstrap and 0.98 Bayesian probabilities. The *C. thracica* accessions analysed displayed two different ITS sequence types, one from the *C. grandiflora*/*C. rubella* lineage, and one from the *C. bursa-pastoris*/*C. orientalis* lineage (Fig. 3; Hurka *et al*., 2012).

CpDNA sequence data. Phylogenetic analyses were conducted separately with each cpDNA region sequenced. The alignments generated matrices of 855 characters for the rps16 intron with 8 (0.93%) parsimony informative characters; 366 characters for the trnHpsbA region with 10 (2.73%) parsimony informative characters; 469 characters for the trnQ-rps16 region with 13 (2.77%) parsimony informative characters and 756 characters for the trnL-trnF region with 101 (13.35%) parsimony informative characters (Hurka *et al*., 2012).

The trnL-F spacer region in *Capsella* displayed noticeable length variations caused by varying numbers of up to six repeats of 70 to 80 bp length. The repeats are characterised by a recurrent motif of ca. 10 bp (GCTTTTTTTG), occasionally modified by single nucleotide and indel polymorphism. Excluding the gaps in the total alignment of 756 characters, trnL-F intergenic spacer length was 720 bp in *Capsella grandiflora* and *C. rubella*, and 703 bp in *C. bursa-pastoris*, *C. thracica*, and *C. orientalis* accessions 8 and 10, whereas *C. orientalis* 9 had a length of only 562 bp due to complete or part loss of three out of the six repeats (Hurka *et al*., 2012).

Following Koch *et al*. (2005, 2007), we interpret the repeats as trnF pseudogenes which, according to the above mentioned authors, cause extensive length variation of the trnL-F regions in many Brassicaceae. We removed the region with the varying repeats (pseudogenes) from the total trnL-F alignment. The discarded fragment had a length of 432 characters (alignment positions 310 to 742) leaving a trnL-F alignment of 322 characters which was implemented in the

phylogenetic analysis.

Since the phylogenetic trees for the single four cpDNA regions did not produce contradictory results (trees not shown), we combined the cpDNA sequences, generating a combined matrix of 2012 characters, of which 34 (1.7%) were parsimony informative (Hurka *et al*., 2012). Parsimony analysis resulted in a single most parsimonious tree of 132 steps (CI = 0.992). For the Bayesian analysis, the substitution model TIM+I was selected by AIC in Modeltest 3.7. The resulting phylogenetic tree (Fig. 4, Hurka et al., 2012) reflects the main features: The sister group relationship between the clade *C. bursa-pastoris*/*C. orientalis*/*C. thracica* on the one side and the clade *C. grandiflora*/*C. rubella* on the other is supported by high significance values. There are subgroups within the two clades, e.g. one *C. orientalis* accession clustered with *C. bursa-pastoris*, and there is also clustering between the *C. bursa-pastoris* accessions (Hurka *et al*., 2012). The subgroups in the combined DNA data set mirror corresponding variation in the trnQ-rps16 and trnH-psbA intergenic spacer regions, known to be highly variable noncoding cp DNA regions (Shaw *et al*., 2007).

Relaxed clock estimates using BEAST and a published ITS substitution rate for herbaceous/perennial angiosperms resulted in a crown age of

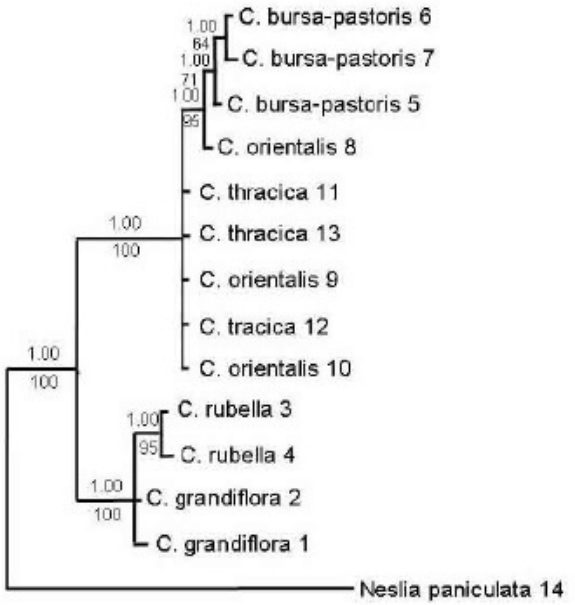

Figure 4. Phylogenetic tree for *Capsella* species based on a combined cpDNA data set: trnL -trnF, rps16, trnH -psbA, trnQ -rps16 regions. Bayesian posterior probabilities above branches, bootstrap support below branches (see Hurka *et al*., 2012).

the genus *Capsella* of 3.18 myr (Hurka *et al*., 2012). The split between *C. rubella* and *C. grandiflora* was dated 0.86 myr, and the divergence time of *C. bursa-pastoris* and *C. orientalis* was estimated at 0.87 myr (Hurka *et al*., 2012).

Leaf morphology

The highly variable leaf morphology in *Capsella* can be explained by two Mendelian genes, A and B, each with two alleles (Shull, 1909). The dominant A allele results in an elongation of the primary lobes, the dominant B allele divides the leaf to the midrib. This leads to four major phenotypes whose homozygous genotypes (Shull, 1909) for diploid species are given in brackets: heteris (AABB), rhomboidea (aaBB), tenuis (AAbb), and simplex (aabb) (Fig. 5).

One well-developed leaf of each individual, usually between the 8th and 15th rosette leaf, was deposited in the Herbarium of the University of Osnabrück (OSBU, Index Herbariorum). The leaf type of C. orientalis in this field experiment was not scorable to the Shull-system (Fig. 5).

Beginning of flowering

In a common garden field experiment in the botanical garden of Osnabrück four *Capsella* species have been grown together (Table 1). In this field experiment the diploid selfincompatible *Capsella grandiflora* generally was the earliest flowering species (Fig. 6). For *C. bursa-pastoris* we observed very early and late flowering ecotypes. *Capsella bursa-pastoris* originated from mixed populations occurring together with *C. grandiflora* and *C. rubella* in Greece (Table 1). The span from the first to the last individual beginning with flowering is quite similar to *C. grandiflora* occurring at the same places. This might be the same flowering adaptation to the specific places in Greece.

Capsella rubella is not able to flower that early but the time span is completely within *C. grandiflora* and *C. bursa-pastoris*. *Capsella bursa-pastoris* wt, spe and int depend on individuals with different flower morphology growing together within one big population in central Germany (Hameister *et al*., 2009): wt means ‘wildtype’ and corresponds to the normal flower type, spe corresponds to a specific flower morphology with stamens instead of petals, and int is an intermediate possibly hybrid status. Both types, wt and spe, occur sympatrically in

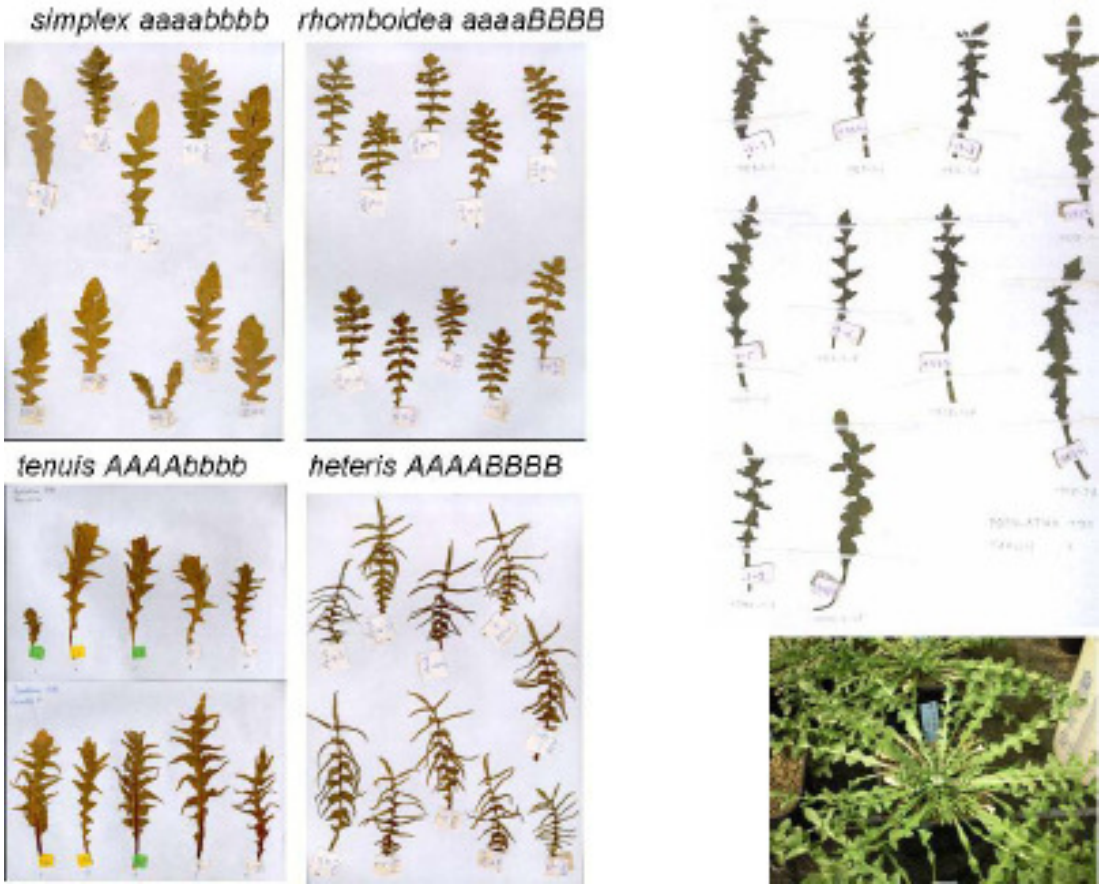

Figure 5. Four leaf types after Shull (1909, reviewed in Neuffer, 1989) shown in monomorphic progenies of *Capsella bursa-pastoris* on the left side. The dominant A-allele results in an elongation of the primary lobes, the dominant B-allele divides the leaf to the midrib. On the right the leaf type of *C. orientalis*, not scorable with the Shull-system.

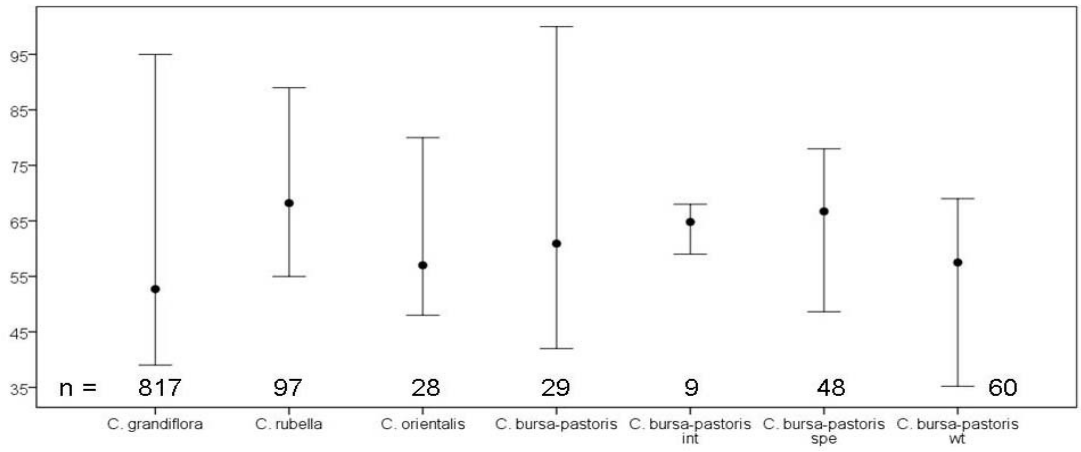

Figure 6. Onset of flowering in days after sowing of four *Capsella* species within one common garden field experiment. The whole range from the first to the last individual beginning with flowering is shown, ● shows the arithmetic mean, n = number of individuals.

the vineyards of Gau-Odernheim for decades of years. *C. orientalis* is beside *C. rubella* the other diploid selfcompatible species and performs a summer annual early flowering type.

Crossing experiments and pollen tube growth

The crossing ability within and between species were tested by pollen germination experiments and as far as possible by seed set (Table 2). In Fig. 7

Table 2. Ability for crossing between Capsella species so far known. (+/-) = not all crossings successfull (see Paetsch *et al*., 2010); n = pollen germinated and pollen tubes reached ovules, but experiment was not verified by seed set.

Pollen donor Mother plant	*C. grandiflora*	*C. rubella*	*C. orientalis*	*C. bursa-pastoris*
C. grandiflora	yes	yes (+/-)	yes	yes
C. rubella	yes (+/-)	yes	yes (n)	yes (sterile)
C. orientalis	yes (n)	yes (n)	yes	not known
C. bursa-pastoris	yes (n)	yes (sterile)	not known	yes

pollen of one *C. grandiflora* individual germinated well on another *C. grandiflora* individual and the pollen tube is strictly growing to the ovules. Using pollen of *C. rubella* for crossing with *C. grandiflora* leads not in all to success. Several *C. grandiflora* selfincompatibility alleles seem to refuse *C. rubella* pollen. This situation is quite similar for *C. orientalis*.

Furthermore the guidance of the pollen tube is not as straight forward. Sometimes the pollen did not grow directly to the ovules. At least for the selfers we observed that self pollen is germinating always much quicker than outcrossing pollen (Table 3). So the pollen of another plant is in concurrence with the self pollen and is not able to reach the ovules in time.

Discussion

Molecular phylogeny of the genus *Capsella*

Two lineages within *Capsella*. The principle finding of our phylogenetic studies is evidence of

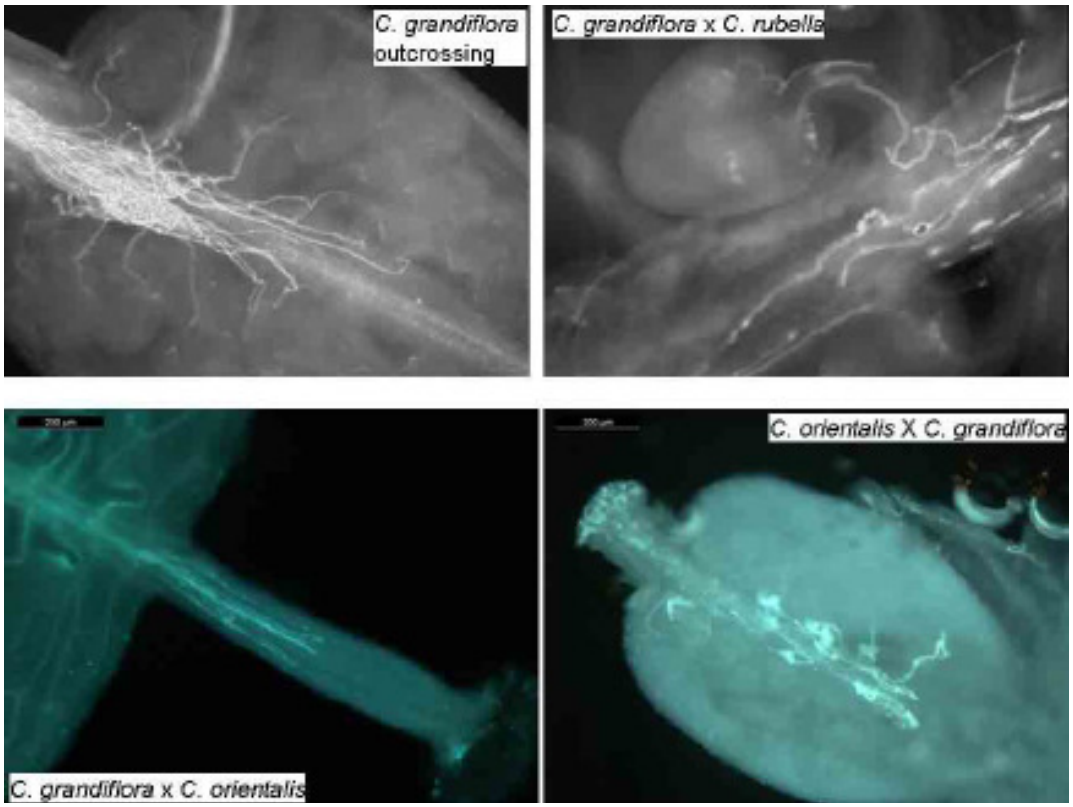

Figure 7. Pollen tube growth in selfing and outcrossing experiments of three *Capsella* species Above left: *Capsella grandiflora* pollen germinated only in crossing experiments, pollen tube grew straight to the ovules. Above right: Pollen germinated in crossing experiments with *C. rubella* as the pollen donor; pollen tube did not grow straight to the ovules. Below left: Pollen tube growth in an interspecific crossing experiment with *C. orientalis* as pollen donor. Below right: Pollen tube growth in an interspecific crossing experiment with *C. grandiflora* as pollen donor. (Fluorescence-microscope)

Table 3. Pollen germination and speed of pollen tube growth in selfing and crossing experiments of *Capsella species*.

Crossings	Start of germination after pollination	Last pollen tube reached ovule
C. grandiflora (selfing)	no success	no success
C. grandiflora (outcrossing)	150 min	at least 255 min
C. rubella (selfing)	15 min	60 min
C. orientalis (selfing)	15 min	90 min
C. bursa-pastoris wt (selfing)	60 min	120 min
C. bursa-pastoris spe (selfing)	45 min	45 min
C. bursa-pastoris wt x spe	60 min	240 min
C. rubella x *C. orientalis*	180 min	300 min
C. orientalis x *C. rubella*	60 min	120 min

two extant groups within the genus *Capsella*. The two diploid species *C. grandiflora* and *C. rubella* are a sister clade to a clade consisting of the diploid *C. orientalis* and the tetraploid *C. bursa-pastoris* (Figs. 3-4; Hurka *et al*., 2012). In these taxa, no intraspecific variation of the nuclear ribosomal ITS region was detected (Fig. 3), in contrast to the noncoding cpDNA (Fig. 4) analysed (Hurka *et al*., 2012). The phylogenetic position of the tetraploid *C. thracica* is discussed below.

Our main conclusion from our dating analysis is that the genus *Capsella* is of pre-Pleistocene origin and that diversification within the genus which lead to its extant members most likely occurred during Pleistocene times. Thus, our date estimates are within the range of most published age estimates on *Capsella* and its close relatives (Hurka *et al*., 2012). To avoid confusion of terminology, and in accordance with the recent relevant literature (Ramsey & Schemske, 2002; Soltis *et al*., 2007), we have used the term autopolyploidy to denote origin of a polyploid taxon within or between populations of a single species, whereas allopolyploids are derived from interspecific hybridisations.

***Capsella grandiflora* and *C. rubella*.** *Capsella grandiflora* is diploid and self-incompatible (SI) due to a sporophytic self-incompatibility system (Paetsch *et al*., 2006, 2010). Although the majority of extant *Capsella* species are self-compatible (SC), self-incompatibility should surely be regarded as the ancestral character state (e.g. Sherman-Broyles & Nasrallah, 2008). As stated above, we conclude from our dating estimates that *C. grandiflora* and *C. rubella* are of Pleistocene age. Based on the present day distribution of *C. grandiflora* and its sister taxon *C. rubella* (Fig. 1; Hurka *et al*., 2012), we hypothesise that the place of origin for both species was the western part of a former larger distribution area of the most recent common ancestor (Fig. 8; Hurka et al., 2012).

The diploid, predominantly selfing, *C. rubella* is a derivative of the *C. grandiflora*-like most recent common ancestor (diploid and SI) of the western lineage. Associated with this speciation process was the transition from SI to SC (Hurka & Neuffer, 1997; Foxe *et al*., 2009; Guo *et al*., 2009; Hurka *et al*., 2012). Foxe *et al*. (2009) and Guo *et al*. (2009) estimated that the two species, *C. grandiflora* and *C. rubella* separated very recently, from less than 25,000 (Foxe *et al*., 2009) to 30,000 to 50,000 years ago (Guo *et al*., 2009). A Pleistocene origin of *C. rubella* and *C. grandiflora* is also indicated by our dating estimates (0.015 -) 0.86 (- 2.45) myr. A young age of ca. 25,000 to 50,000 years as advocated by Foxe *et al*. (2009) and Guo *et al*. (2009) (transition from Pleistocene to Holocene) would imply unprecedented high ITS substitution rates, whereas the ITS substitution rates used in our analysis are in line with other accepted Quaternary ITS-based biographic scenarios for Brassicaceae taxa (Bleeker *et al*., 2002; Mummenhoff *et al*., 2004; Franzke *et al*., 2004).

The place of origin of *C. rubella* was presumably the eastern Mediterranean region. Subsequently, *C. rubella* extended its range, colonised all Mediterranean countries, and spread later with European colonists to North and South America and Australasia (Neuffer & Hurka, 1999; Neuffer *et al*., 1999; Paetsch *et al*.,

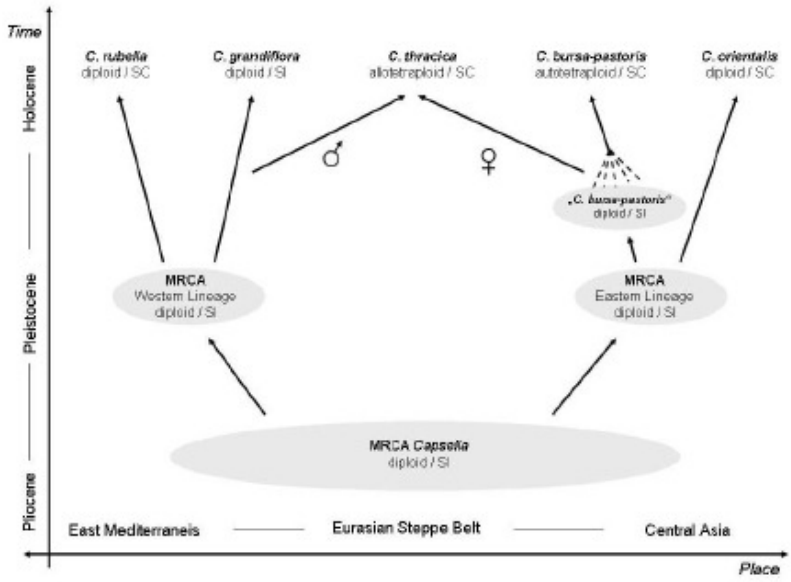

Figure 8. Outline of the evolutionary history of the genus *Capsella*. Broken lines indicate multiple origins of *C. bursa-pastoris* (Hurka *et al*., 2012).

2010; Hurka *et al*., 2012).

***Capsella orientalis* and *C. bursa-pastoris*.** The distribution areas of the two diploid species *Capsella orientalis* and *C. rubella* appear to be mutually exclusive (Fig. 1; Hurka *et al*., 2012), and the phylogenetic roots of the two species are different as clearly shown by ITS and cpDNA data (Figs. 3-4; Hurka *et al*., 2012).

The split between the sister species *C. orientalis* and the tetraploid self-compatible *C. bursa-pastoris* was estimated by us to be (0.006-) 0.87 (-2.44) myr ago (Pleistocene), which is the same as has been estimated for the split between *C. grandiflora* and *C. rubella*. The present day distribution area of *C. orientalis* (Fig. 1; Hurka *et al*., 2012) suggests that the species split between *C. orientalis* and *C. bursa-pastoris* has occurred in the more eastern parts of the Eurasian distribution belt (Figs. 1, 8; Hurka *et al*., 2012).

The DNA variation detected in *C. orientalis* and *C. bursa-pastoris* (Fig. 4) might argue for multiple origins of both species (Hurka *et al.*, 2012). Our present data on nuclear and chloroplast DNA variation demonstrate that *C. bursa-pastoris* is not, as was argued earlier, a derivative species of *C. grandiflora* (Figs. 3, 4) (Hurka & Neuffer, 1997, Slotte *et al*., 2006, 2008; St. Onge, 2010), nor does this uphold an argument in favour of single origin (Slotte *et al*., 2006, 2008). Instead, cpDNA variation data (Fig. 4; Hurka *et al*., 2012), high isozyme polymorphism (Hurka *et al*., 2012), as well as RAPD (Neuffer, 1996) and AFLP data (Hameister *et al*., 2009) support the assumption of multiple origin of *C. bursa-pastoris*, as does the enormous morphological polymorphism (Almquist, 1907, 1921).

Polyploidy in *Capsella bursa-pastoris*. There is no clear evidence for an allopolyploid origin of the tetraploid *C. bursa-pastoris*. Attributes of *C. bursa-pastoris*, like disomic inheritance, shown for allozymes (Hurka *et al*., 1989; Hurka & Düring, 1994; Neuffer & Hurka, 1999) and morphological characters (Shull, 1929), and 'fixed heterozygosity' (true-breeding multiple banded isozyme patterns, Hurka *et al*., 1989; Hurka & Düring, 1994), may argue for allopolyploid origin. However, it is well known that autopolyploids often behave cytologically like allopolyploids (Ramsey & Schemske, 2002). Allopolyploids should retain a degree of hybrid character of their genomes (Ramsey & Schemske, 2002) which could not as yet be demonstrated for *C. bursa-pastoris*.

The occasional findings of *C. rubella* nuclear haplotypes in *C. bursa-pastoris* in southern Europe where the *C. grandiflora*/*rubella*-lineage and the *C. orientalis*/*bursa-pastoris*-lineage are sympatric, are probably due to introgression (Slotte *et al*., 2006, 2008). This interpretation is supported by the lack of such haplotypes in *C. bursa-pastoris* from China, where neither *C. grandiflora* nor *C. rubella* occur (Slotte *et a*l., 2008). In agreement with previous studies (Hurka & Neuffer, 1997; Slotte *et al*., 2006,

2008; St. Onge, 2010), we thus again argue for an autopolyploid origin of *C. bursa-pastoris*. However, it should be kept in mind that signals indicating the hybrid nature of a species may be eradicated with time (Hurka *et al*., 2012).

The ancestor that gave rise to *C. orientalis* and *C. bursa-pastoris* was most probably diploid and self-incompatible (SI). The shift from SI to SC in *C. bursa-pastoris* might have coincided with the polyploidisation process leading to the extant tetraploid *C. bursa-pastoris*. Although the multiple origins of *C. bursa-pastoris* may not only imply origin at different places but also at different times, we nevertheless argue that polyploidisation occurred in the Middle/Late Pleistocene times (Hurka *et al*., 2012). Such a scenario is in accordance with recent coalescence analyses.

Based on microsatellite data, the most recent common ancestor for the chloroplast genome of *C. bursa-pastoris* has been estimated at 7000 to 17,000 years ago by Ceplitis *et al*. (2005) (late Pleistocene to Holocene), whereas Slotte *et al*. (2006), basing their estimate on cpDNA sequence data, date this occurrence between 43,000 to 430,000 years ago (Pleistocene). Tetraploid *Capsella bursa-pastoris* would then be another prime example of colonisation success of a polyploid plant species. Middle to late Pleistocene origin of tetraploid *C. bursa-pastoris* is also in line with fossil records. Macrofossils (seeds) of *Capsella* have been reported from the Interglacial deposits at Ilford, Essex, England, and have been identified as *C. bursa-pastoris* (West *et al*., 1964). The sediments are deemed to be Ipswichian (Eemian of continental Europe), and thus correlate with MIS (Marine Isotope Stage) 5e (Shackleton *et al*., 2003). More recently, however, it has been argued that the Ilford deposits belong to the penultimate Interglacial complex (Hoxne = Holstein Interglacial) and correlate to MIS 7 (Turner, 2000). Estimations for the duration of MIS 5e are ca. 125,000 to 110,000 years BP (late Pleistocene), and for MIS 7 from 245,000 to 185,000 years BP (middle Pleistocene). In any case, there is evidence of a pre-(last) glacial occurrence of *Capsella* in western Europe, and *Capsella* might already have colonised western Europe in the middle Pleistocene. This does not contradict or deny postglacial anthropogenic introduction (Hurka *et al*., 2012). Based on several arguments, we hypothesise that the place of origin of *C. bursa-pastoris* is eastern Europe/western to central Asia. (i) The main distribution area of *C. orientalis*, the sister species of *C. bursa-pastoris*, is eastern Europe (Transvolga) through North Kazakhstan to south-west Siberia, north-west China and western Mongolia (Hurka *et al*., 2012).

Capsella thracica. *Capsella thracica* has been described by Velenovsky (1893) from Bulgaria. It is sometimes given species rank (e.g. Tutin *et al*., 1964), and sometimes treated as a subspecies of *C. bursa-pastoris* (Tutin *et al*., 1993), a view also adopted by Ančev (2007). It is a Bulgarian endemic reported from the Thracian lowlands, Black Sea coast, and the Rhodopes Mts. (Ančev, 2007). The main feature discriminating this species from *C. bursa-pastoris* is the presence of an elongated style in *C. thracica*. We included *C. thracica* in our studies, and although details of this will be given elsewhere, we report on some of the main features here.

Capsella thracica is tetraploid as revealed by its genome size (Fig. 2; Hurka *et al*., 2012), and shares its cpDNA regions with *C. bursa-pastoris* (Fig. 4; Hurka *et al*., 2012). The ITS sequences of the *C. thracica* accessions analysed, however, are characterised by two different copies, one from *C. bursa-pastoris* and one from *C. grandiflora*/*C. rubella* (Fig. 3; Hurka *et al*., 2012) indicating a hybrid origin of *C. thracica*. The place of origin of *C. thracica* would appear to be Bulgaria. We argue that the pollen recipient parent species was *C. bursa-pastoris*, as indicated by cpDNA sequences, and the pollen donator was *C. grandiflora* or its progenitor, indicated by the ITS sequences and the length of the style - only *C. grandiflora* and *C. thracica* have an elongated style (Hurka *et al*., 2012, Neuffer & Paetsch 2013; Neuffer, unpublished data).

Interspecific hybridisation by fusion of an unreduced diploid *C. grandiflora* (or progenitor) pollen with a normally reduced egg cell of the autotetraploid *C. bursa-pastoris* would lead to the allotetraploid *C. thracica*. Alternatively, an unreduced pollen gamete of *C. grandiflora* (or progenitor) and an unreduced egg cell of hypothesised "diploid" *C. bursa-pastoris* (see above) may have fused (Hurka *et al*., 2012).

Leaf type, flowering and crossing ability

Leaves of the genus *Capsella* can be classified in four types which are encoded by two loci and each locus by two alleles. Sometimes alleles

have a specific distribution pattern which seems to evidence an adaptic value like Neuffer & Bartelheim (1989, *Capsella bursa-pastoris*) showed for the occurrence of the B-allele along an altitudinal gradient in the alps. In other studies no geographic distribution pattern was obvious (Neuffer, 2011; *Capsella bursa-pastoris*). The adaptive value of these leaf types after Shull (1909), therefore is questionable. However, the distribution pattern might be the result of colonization history.

The classification of leaves failed in the case of *C. orientalis*. This might rely on the field conditions of the common garden field experiment. The field experiment allows the comparability between the species, but at the place of origin under steppe climatic condition the leaf type could differ. Differences of leaf types under varying climatic condition sometimes are tremendous, mostly but not always for *C. bursa-pastoris* the classification is stable (Neuffer, 1989). This new leaf type of *C. orientalis* might contribute to another leaf lobe factor which sustained in *C. orientalis* or possibly is a new evolutionary character.

Flowering in short living species of the genus *Capsella* is under high selective pressure (reviewed in Neuffer *et al*., 2011). Variability of *C. grandiflora* and *C. bursa-pastoris* is highest (Fig. 6) in this dataset. *Capsella rubella* is not able to begin with flowering as early as these other two species. This is in accordance with earlier findings about *C. rubella* and *C. bursa-pastoris* populations from the regions surrounding the Mediterranean Sea, the overlapping distribution area of these two species (Neuffer & Eschner, 1995; Neuffer & Hoffrogge, 2000).

Capsella bursa-pastoris wt, spe and int correspond with different flower morphology of one very large population in the centre of Germany. Wt (wild type) is an earlier flowering ecotype with normal flowers. Spe (stamenoid petals) is a later flowering ecotype with stamens instead of petals (see Hameister *et al*., 2009). Both populations occur sympatrically in this habitat. The different flowering of both types is interpreted as an isolation factor. However, sometimes both types hybridise which is morphologically observable by intermediate petal types (int). This is the first time we can show flowering date of *C. orientalis*. The early flowering summer annual type is perfectly adapted to steppe climatic regions in Middle Asia. All our so far collected *Capsella* specimens of western Mongolia have been determined as *C. orientalis*. Our findings showed no mixed populations. Nevertheless pollen germination experiments showed that *C. orientalis* is still crossable on the diploid level with *C. grandiflora* and *C. rubella*. So far we have no information whether these crossings can result in a fertile seed. Furthermore we have no results about crossability with C. *bursa-pastoris* which is the only species with a probably overlapping distribution area. If these two species stay side by side, and crossing would be possible, then even in the case of postzygotic breakdown in the F2-generation a backcross to one parental species could lead to introgression.

Evolutionary history of the genus *Capsella*, conclusions

Based on our results and present knowledge, we hypothesise the following scenario outlined in Fig. 8 (Hurka *et al*., 2012). The genus *Capsella* is of Eurasian origin and comprises two evolutionary lineages, the western lineage (*C. grandiflora*, *C. rubella*), and the eastern lineage (*C. bursa-pastoris*, *C. orientalis*, see Figs. 1, 3-4, Hurka *et al*., 2012). Their common ancestor was diploid and self-incompatible, and its distribution ranged from Eastern Europe to western or even central Asia, predominantly confined to Mediterranean and steppe like climates. Such a continuous steppe belt from central Asia to south-eastern Europe formed, at the latest, at the end of the Pliocene, 2.5 – 1.6 million years ago (Kamelin, 1998; Velichko, 1999).

Several climatic macrocycles with glacial and interglacial phases during the Pleistocene are associated with latitudinal range shifts of the steppe belt. The steppe belt also faced significant longitudinal splits during the ice ages (for more detailed discussion, see Franzke *et al*., 2004). These biogeographic dynamics caused geographic and genetic subdivisions within the common ancestor into an eastern and a western lineage, as has also been demonstrated for the Brassicacean Eurasian steppe plant *Clausia aprica* (Franzke *et al*., 2004), and for many other organisms (Hewitt, 2001, 2004). The eastern lineage gave rise to *C. bursa-pastoris* and *C. orientalis*, whereas, in the western part of the common ancestor's distribution belt, populations gave rise to *C. grandiflora* and *C. rubella*. The current areal of *C. grandiflora* might be regarded as a relict areal. Later, range

expansions of *C. bursa-pastoris* to the West led to contact zones with the western lineage species. This facilitated introgression of western lineage genetic material into the eastern genomes (Slotte *et al*., 2006, 2008) on the one side, and led to hybrid speciation on the other, giving rise to the allotetraploid species *C. thracica* in Bulgaria (Fig. 3; Hurka *et al*., 2012).

The place of the hybrid zones in Bulgaria, which is the south-western boundary of the Eurasian steppe belt, indicates that *C. grandiflora* or its progenitor once had a wider range than today, which is in line with our hypothesis of a relict areal of *C. grandiflora*. Also, the location of the secondary contact zones in middle and western Europe, as indicated by the introgression and hybridisation zones, supports the view that *C. bursa-pastoris* colonised Europe from Asia. The time estimate for the origin of the *Capsella* species is, therefore, compatible with the historical biogeographic events outlined above (Hurka *et al*., 2012). The inclusion of the so far "missing link" species *C. orientalis* and *C. thracica* into our phylogenetic and biogeographic concept will greatly expand the possibilities of using *Capsella* as a model plant genus (Hurka *et al*., 2012).

Acknowledgements

The authors wish to thank Ulrike Coja, Claudia Gieshoidt, and Rudi Grupe for technical assistance in sequencing and cultivation of plants; Sara Mayland-Quellhorst and Carina Titel for chromosome counting and flowcytometry analyses. We thank Jennifer Kiel and Lydia Schmitz for performing the common garden field experiment and the staff of the Botanical Garden Osnabrück for their help. We thank Melanie Paetsch and Sabine Wegener for crossing experiments and pollen tube growth studies. We are thankful to Lucille Schmieding for correcting the English text. Financial support by the Deutsche Forschungsgemeinschaft, DFG and by the Deutscher Akademischer Austauschdienst, DAAD is greatly acknowledged.

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