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

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**Abstract**

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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.

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Introduction

Molecular systematic studies confirm that the genus *Capsella* belongs to the tribe, Camelinae (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 & Neiffer, 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 delimitation 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 region excluded). No intraspecific ITS variation was detected between 5 provenances (*Capsella grandiflora*; 3 of *C. rubella*; 4 of *C. bursa-pastoris*; 4 of *C. orientalis*; and 9 of *Capsella grandiflora*; 3 of *C. rubella*; 4 of *C. orientalis*, and 9 of *Capsella 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

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Figure 1. Outline distribution map of Capsella species. Capsella grandiflора: 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).

<table>
<thead>
<tr>
<th>Pop. Nr.</th>
<th>Provenance</th>
<th>Coordinates</th>
<th>Elevation above sea level</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>698</td>
<td>Alpi Apuani, Italy</td>
<td>44°02’N, 10°18’E</td>
<td>1180 m</td>
<td>C. rubella</td>
</tr>
<tr>
<td>910</td>
<td>Doukades, Greece</td>
<td>39°41’N, 19°44’E</td>
<td>150 m</td>
<td>C. grandiflора</td>
</tr>
<tr>
<td>918</td>
<td>Pantokrator, Greece</td>
<td>39°45’N, 19°52’E</td>
<td>910 m</td>
<td>C. grandiflора</td>
</tr>
<tr>
<td>921</td>
<td>Paleokatritsa, Greece</td>
<td>39°40’N, 19°42’E</td>
<td>50 m</td>
<td>C. grandiflора</td>
</tr>
<tr>
<td>925</td>
<td>Joannina, Greece</td>
<td>39°40’N, 20°51’E</td>
<td>450 m</td>
<td>C. grandiflора C. rubella</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C. bursa-past.</td>
</tr>
<tr>
<td>928</td>
<td>Metsovo, Greece</td>
<td>39°46’N, 21°10’E</td>
<td>1150 m</td>
<td>C. grandiflора</td>
</tr>
<tr>
<td>933</td>
<td>Katara pass, Greece</td>
<td>39°48’N, 21°11’E</td>
<td>1500 m</td>
<td>C. grandiflора C. bursa-past.</td>
</tr>
<tr>
<td>934</td>
<td>Metsovo, Greece</td>
<td>39°46’N, 21°10’E</td>
<td>1350 m</td>
<td>C. grandiflора</td>
</tr>
<tr>
<td>935</td>
<td>Sokraki, Korfu, Greece</td>
<td>39°43’N, 19°48’E</td>
<td>500 m</td>
<td>C. grandiflора</td>
</tr>
<tr>
<td>936</td>
<td>Pantokrator, Greece</td>
<td>39°45’N, 19°52’E</td>
<td>760 m</td>
<td>C. grandiflора</td>
</tr>
<tr>
<td>984</td>
<td>Mallorca, Spain</td>
<td>39°30’N, 03°00’W</td>
<td>500 m</td>
<td>C. rubella</td>
</tr>
<tr>
<td>1215</td>
<td>Teneriffа, Spain</td>
<td>28°29’N, 16°19’W</td>
<td>10 m</td>
<td>C. rubella</td>
</tr>
<tr>
<td>1377</td>
<td>Buenos Aires, Argentina</td>
<td>34°40’S, 56°30’W</td>
<td>10 m</td>
<td>C. rubella</td>
</tr>
<tr>
<td>1482</td>
<td>Perth, Australia</td>
<td>31°56’S, 115°50’E</td>
<td>25 m</td>
<td>C. rubella</td>
</tr>
<tr>
<td>1938</td>
<td>Barnaul, Russia</td>
<td>53°20’N, 83°45’E</td>
<td>150 m</td>
<td>C. orientalis</td>
</tr>
<tr>
<td>1939</td>
<td>Pawlodar, Russia</td>
<td>52°16’N, 76°57’E</td>
<td>120 m</td>
<td>C. orientalis</td>
</tr>
<tr>
<td>1940</td>
<td>Bayanaul, Russia</td>
<td>50°47’N, 75°41’E</td>
<td>460 m</td>
<td>C. orientalis</td>
</tr>
<tr>
<td>1949-1963</td>
<td>Gau-Odernheim, Germany</td>
<td>49°47’N, 12°10’E</td>
<td>200 m</td>
<td>C. bursa-past. wt, spe, int</td>
</tr>
</tbody>
</table>
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 flow cytometry 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). Flow cytometry 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 flow cytometry (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...
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*/*C. orientalis*. The two other *C. thracica* accessions, no. 11 and 13, displayed at ITS sequence positions 122 – 126 was included in the analyses (see 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) (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 trnH-psbA 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 (GCTTTTTTG), 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. 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 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 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
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 straightforward. 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
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 (Bleecker 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., 2006, 2010).

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

<table>
<thead>
<tr>
<th>Crossings</th>
<th>Start of germination after pollination</th>
<th>Last pollen tube reached ovule</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C. grandiflora</strong> (selfing)</td>
<td>no success</td>
<td>no success</td>
</tr>
<tr>
<td><strong>C. grandiflora</strong> (outcrossing)</td>
<td>150 min</td>
<td>at least 255 min</td>
</tr>
<tr>
<td><strong>C. rubella</strong> (selfing)</td>
<td>15 min</td>
<td>60 min</td>
</tr>
<tr>
<td><strong>C. orientalis</strong> (selfing)</td>
<td>15 min</td>
<td>90 min</td>
</tr>
<tr>
<td><strong>C. bursa-pastoris</strong> wt (selfing)</td>
<td>60 min</td>
<td>120 min</td>
</tr>
<tr>
<td><strong>C. bursa-pastoris</strong> spe (selfing)</td>
<td>45 min</td>
<td>45 min</td>
</tr>
<tr>
<td><strong>C. bursa-pastoris</strong> wt x spe</td>
<td>60 min</td>
<td>240 min</td>
</tr>
<tr>
<td><strong>C. rubella</strong> x <strong>C. orientalis</strong></td>
<td>180 min</td>
<td>300 min</td>
</tr>
<tr>
<td><strong>C. orientalis</strong> x <strong>C. rubella</strong></td>
<td>60 min</td>
<td>120 min</td>
</tr>
</tbody>
</table>
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).

Polyplody 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 autoploids 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 al., 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 \textit{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 \textit{C. orientalis} and \textit{C. bursa-pastoris} was most probably diploid and self-incompatible (SI). The shift from SI to SC in \textit{C. bursa-pastoris} might have coincided with the polyploidisation process leading to the extant tetraploid \textit{C. bursa-pastoris}. Although the multiple origins of \textit{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 \textit{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 \textit{Capsella bursa-pastoris} would then be another prime example of colonisation success of a polyploid plant species. Middle to late Pleistocene origin of tetraploid \textit{C. bursa-pastoris} is also in line with fossil records. Macrofossils (seeds) of \textit{Capsella} have been reported from the Interglacial deposits at Ilford, Essex, England, and have been identified as \textit{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 (Hoxnian = 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 \textit{Capsella} in western Europe, and \textit{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 \textit{C. bursa-pastoris} is eastern Europe/western to central Asia. (i) The main distribution area of \textit{C. orientalis}, the sister species of \textit{C. bursa-pastoris}, is eastern Europe (Transvolga) through North Kazakhstan to south-west Siberia, north-west China and western Mongolia (Hurka et al., 2012).

\textbf{Capsella thracica}. \textit{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 \textit{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 \textit{C. bursa-pastoris} is the presence of an elongated style in \textit{C. thracica}. We included \textit{C. thracica} in our studies, and although details of this will be given elsewhere, we report on some of the main features here.

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

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

\textbf{Leaf type, flowering and crossing ability}

Leaves of the genus \textit{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 adaptive 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 hybrideise 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).

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