Abstract
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 and trnQ–rps16). Sequence data were evaluated with parsimony and Bayesian analyses. Divergence time estimates were carried out with the software package BEAST. We also performed isozyme, cytological, morphological and biogeographic studies. 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 autoploid species of multiple origin, whereas the Bulgarian endemic C. thracica (SC; 2n = 32) is allopolyploid and emerged from interspecific hybridization 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.

Keywords: biogeography, Capsella, cpDNA, isozymes, ITS, phylogeny age estimation

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Introduction
Wild relatives of the model organism Arabidopsis are increasingly in focus of contemporary evolutionary research programmes (Mitchell-Olds 2001; Koch et al. 2003; Hurka et al. 2005; Franzke et al. 2011). From all wild relatives of Arabidopsis currently used as study objects, Capsella is the most closely related genus. Molecular systematic studies confirm that both genera belong to the same 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 & 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 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 et al. 2005; Slotte et al. 2006; St. Onge 2010), but, so far, no convincing hypothesis has been put forward. This has lead to controversy regarding, for example, phylogenetic relationships, mode of
speciation, biogeographic origin and age estimations of the genus and its species.

Species delimitation is difficult and controversial because of the enormous morphological variation within the genus. Chater (1993) list in Flora Europaea four Capsella species, which are commonly mostly accepted: C. grandiflora (Fauché & Chauv.) Boiss., C. rubella Reuter, C. bursa-pastoris (L.) Medik., including C. thraica 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. thraica have never been the subject of experimental work, obviously due to the fact that no seed material was available. We included both taxa in our study and have, for the first time, explored the biosystematics and phylogenetics of these taxa.

The aim of this study was to reveal phylogenetic and biogeographic patterns within the genus Capsella covering all currently accepted taxa (Chater 1993). We analysed the nuclear internal transcribed spacers ITS1 and ITS2 including the 5.8 S 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 \(\text{trn}L – \text{trn}F\) intergenic spacer region and a highly variable cpDNA region, the \(\text{trn}Q – \text{rps}16\) intergenic spacer, as well as two regions more or less intermediate in their levels of variation \((\text{trn}H – \text{psb}A\) intergenic spacer, \(\text{rps}16\) intron). We also performed isozyme analyses to study the genetic variation between and within species. The investigations were complemented by morphological, cytological and biogeographic studies. In the light of all the data presented in this study, it is obvious that C. orientalis and C. thraica hold a key position in our endeavours towards understanding the evolutionary history of the genus Capsella.

**Material and methods**

**Origin of plant material**

Seeds from Capsella orientalis were collected from single plants randomly taken from natural populations. The origin of the seed material is given in Table 1. Plants were cultivated from seeds either under greenhouse conditions or in the experimental garden of the Osnabrück University Botanical Garden and were used for phenotypic character analyses, cytology and isozyme studies. Herbarium specimens used for DNA sequencing and corresponding GenBank accession numbers are given in Table 2. Additional Capsella specimens were sequenced for ITS, and ITS sequences were also retrieved from GenBank, the origin or GenBank accession numbers of which are as follows: C. grandiflora: OSBU (Osnabrück University Herbarium) 12499; accession from seed genebank Gatersleben/Germany; sequence AM905718.1; C. rubella: OSBU 20858; C. orientalis: OSBU 10587; C. bursa-pastoris: OSBU 17229; OSBU 12500; sequences DQ310530.1; AF055196.1; AFI28110; AFI28111.1; Neslia paniculata: sequence AF137576.

**Geographical distribution of Capsella orientalis**

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. The following herbaria have been examined: ALTB (Altai State University, Barnaul, Russia); KW (Kholodny Institute of Botany, Kiev, Ukraine); LE (Komarov Botanical Institute, St. Petersburg, Russia); MHA (Moscow Main Botanical Garden, Russia); MW (Moscow State University, Russia); NS (Central Siberian Botanical Garden, Novosibirsk, Russia); OSBU (Botany Dept., University of Osnabrück, Germany); SVER (Institute of Plant and Animal Ecology, Jekaterinburg, Russia); TK (Tomsk State University, Russia); and without acronym: Pavlodar Pedagogical Institute (Pavlodar, Pavlodarskaya oblast, Kazakhstan).

**Cytology and flow cytometry**

Young flower buds were fixed overnight in Carnoy solution (acetic acid/ethanol = 1:3) at 4 °C, washed three times with ethanol (70%) and finally stored in ethanol (70%) at minus 20 °C. For preparation, the buds were washed twice with distilled water and three times with citrate buffer (pH 4.8). The material was digested with a pectolytic enzyme mix (cellulase, pectolyase, cysteolicase), and the buds were squeezed on glass slides with acetic acid, warmed to 50 °C and subsequently cooled with Carnoy solution and dried. Selected chromosome spreads of (pro)metaphase chromosomes of pollen mother cells were stained with 1–2 µg/mL DAPI (Roth, Karlsruhe), mounted in Vectashield and photographed at 1000-fold magnification using the Olympus BX-61 epifluorescence microscope system equipped with a Zeiss AxioCam HR CCD camera. To slow down bleaching of the fluorescence dye, a drop of DABCO solution (Roth, Karlsruhe, Germany) was applied. Pictures were viewed and processed with the photoshop software. At least five chromosome figures per slide and accession were analysed.

Flow cytometry was used to determine the relative DNA amount. Fresh leaf material was harvested, and c. 0.5 cm² leaf material was chopped with a sharp razor blade in a DAPI solution and filtered into a sample tube. Subsequent flow cytometry was performed on a Partec Ploidy Analyser-I (Partec, Münster, Germany).
Table 1 Origin of Capsella orientalis seed samples

<table>
<thead>
<tr>
<th>Pop. no.</th>
<th>Country of origin, locality, habitat</th>
<th>Coordinates</th>
<th>Collector/remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1718</td>
<td>MN; Bayan-Olgii Aymag; eastern end of lake Hoton Nuur, weed in lawn, mixed stand with C. bursa-pastoris</td>
<td>48° 35' N</td>
<td>H. Hurka, B. Neuffer;</td>
</tr>
<tr>
<td>1719</td>
<td>MN; Bayan-Olgii Aymag; between lakes Hoton Nuur and Horgon Nuur, sheep paddock</td>
<td>48° 35' N</td>
<td>B. Neuffer, H. Hurka;</td>
</tr>
<tr>
<td>1938</td>
<td>RU; Siberia, Altai Kraj; city of Barnaul, ruderal, mixed stand with C. bursa-pastoris</td>
<td>53° 20' N</td>
<td>D.A. German;</td>
</tr>
<tr>
<td>1939</td>
<td>KZ; Pavlodarskaya Oblast, Pavlodar, 400 km north-north-east from Astana, ruderal in lawn</td>
<td>52° 16' N</td>
<td>D.A. German;</td>
</tr>
<tr>
<td>1940</td>
<td>KZ; Pavlodarskaya Oblast, 300 km east of Astana, near Bayanaul, ruderal in steppe country</td>
<td>50° 47' N</td>
<td>D.A. German;</td>
</tr>
<tr>
<td>1941</td>
<td>KZ; Vostocho-Kazakhstanskaya Oblast, 750 km east of Astana; northern foothills of Kalbinskiy Mt. Range, 15 km south of village Gagarino, steppe slopes</td>
<td>49° 59' N</td>
<td>S.V. Smirnov;</td>
</tr>
<tr>
<td>1978</td>
<td>RU; Siberia, Altai Kraj; Tretjakovsk raion, river valley Beresovja, at the Gilevskoe water reservoir, ruderal in steppe country</td>
<td>51° 06' N</td>
<td>D.A. German, N. Friesen</td>
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<tr>
<td>1979</td>
<td>RU; Siberia, Altai Kraj; Loktevsk raion, village Gilevo, ruderal in village</td>
<td>51° 07' N</td>
<td>D.A. German, N. Friesen;</td>
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<tr>
<td>1980</td>
<td>RU; Siberia, Altai Kraj; Loktevsk raion, river valley Tushkanchikha, western slopes of mountain range, steppe slopes</td>
<td>51° 10' N</td>
<td>D.A. German, N. Friesen;</td>
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<tr>
<td>1981</td>
<td>RU; Siberia, Altai Kraj; Loktevsk raion, village Ut'syanka, ruderal in village</td>
<td>51° 08' N</td>
<td>D.A. German, N. Friesen;</td>
</tr>
<tr>
<td>1982</td>
<td>RU; Siberia, Altai Kraj; Rubzovsk raion, city of Rubzovsk, ruderal</td>
<td>51° 30' N</td>
<td>D.A. German, N. Friesen;</td>
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<tr>
<td>1983</td>
<td>RU; Siberia, Altai Kraj; Smeinogorsk raion, Kolyvanskoe Lake, ruderal in steppe country</td>
<td>51° 22' N</td>
<td>D.A. German, N. Friesen;</td>
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<tr>
<td>1984</td>
<td>RU; Siberia, Altai Kraj; city centrum of Barnaul, ruderal</td>
<td>53° 21' N</td>
<td>D.A. German;</td>
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<tr>
<td>1985</td>
<td>RU; Siberia, Altai Kraj; city of Barnaul, north-western part, ruderal</td>
<td>53° 21' N</td>
<td>D.A. German;</td>
</tr>
<tr>
<td>2005</td>
<td>CN; Xinjiang, Dzungaria, 485 km north of Urumchi, Mongolian Altai, Fuha county, ruderal</td>
<td>48° 05' N</td>
<td>D.A. German et al.;</td>
</tr>
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<td>2006</td>
<td>CN; Xinjiang, Dzungaria, 390 km northwest of Urumchi; Jeminay county, Saur, valley of Tastykarasu, 55 km south-east of Jeminay, rocky steppe slopes</td>
<td>47° 09' N</td>
<td>D.A. German et al.;</td>
</tr>
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<td>2007</td>
<td>CN; Xinjiang, Dzungaria, 410 km northwest of Urumchi; Jeminay county, Saur, 30 km south of Jeminay, meadow steppe, roadside</td>
<td>47° 14' N</td>
<td>D.A. German et al.;</td>
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<tr>
<td>2008</td>
<td>CN; Xinjiang, Dzungaria, 400 km northeast of Urumchi; Qinghe county, 40 km east of Qinghe, Mongolian Altai, valley of Tsagan-gol, 15 km northeast of Dunfyn; ruderal at local forest station</td>
<td>46° 37' N</td>
<td>D.A. German et al.;</td>
</tr>
</tbody>
</table>

Pop. no. refers to the Capsella seed collection hold at the Botany Dept. of the University of Osnabrück; country codes: CN, China; KZ, Kazakhstan; MN, Mongolia; RU, Russia; samples are individual seed samples except for pop. 1941. ALTB: Herbarium Altai State University, Barnaul, Russia; OSBU: Herbarium Botany Dept., University Osnabrück, Germany.

Petroselinum crispum was used as an internal standard (2C-value of absolute DNA amount 4.46 pg, Yoyoka et al. 2000); 1C-value of absolute DNA amount for C. rubella 0.22 pg (2C = 0.44 pg) and 1C-value of absolute DNA amount for C. bursa-pastoris 0.4 pg (2C = 0.8 pg), Lysak et al. 2009).

Isozyme analyses

Isozyme investigations of Capsella orientalis and of C. thraica were carried out with progeny raised from the provenances listed in Table 1 or Table 2, respectively. Rosette leaves of single plants, and c. 10 weeks old, were harvested and stored at –80 °C. Electrophoresis was performed in a continuous system on vertical polyacrylamide gel slabs. The following enzyme systems were assayed: aspartate aminotransferase (AAT; EC 2.6.1.1), glutamate dehydrogenase (GDH; EC 1.4.1.4) and leucine aminopeptidase (LAP; 3.4.11.1). Buffer systems and other experimental details are given in Hurka et al. (1989) for AAT, in Hurka & Düring (1994) for GDH and in Neuffer & Hurka (1999) for LAP. The

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### Table 2 Provenances of *Capsella* and *Neslia* specimens used for DNA sequencing and GenBank accession numbers

<table>
<thead>
<tr>
<th>Species</th>
<th>Country of origin, locality, coordinates</th>
<th>Voucher</th>
<th>GenBank accession numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. grandiflora</em></td>
<td>GR; Prov. Ioannina, Metsovo; N 39° 46′, E 21° 10′</td>
<td>OSBU 7339</td>
<td>FR773701, FR822325, FR822324, FR82264, FR822352, FR822334</td>
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<tr>
<td><em>C. grandiflora</em></td>
<td>IT; Prov. Brescia, Pilzone/Lago Iseo; N 45° 41′, E 10° 05′</td>
<td>OSBU 18615</td>
<td>FR773702, FR822324, FR822235, FR822353, FR822335</td>
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<tr>
<td><em>C. rubella</em></td>
<td>CL; Región Biobio, near Concepción; S 36° 50′, W 73° 03′</td>
<td>OSBU 7334</td>
<td>FR773704, FR822322, FR822236, FR822350, FR822336</td>
</tr>
<tr>
<td><em>C. rubella</em></td>
<td>IT; Prov. Foggia, Mte. Gargano, Foresta Umbra; N 41° 49′, E 15° 59′</td>
<td>OSBU 20857</td>
<td>FR773703, FR822323, FR822363, FR822351, FR822337</td>
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<tr>
<td><em>C. bursa-pastoris</em></td>
<td>DE; North Rhine-Westphalia, north of Muenster; N 52° 19′, E 0° 56′</td>
<td>OSBU 14439</td>
<td>FR773707, FR822329, FR822338, FR822345, FR822341</td>
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<tr>
<td><em>C. bursa-pastoris</em></td>
<td>RU; Novosibirskaya Oblast, near Novosibirsk, N 52° 20′, E 82° 54′</td>
<td>OSBU 12815</td>
<td>FR773706, FR822330, FR822356, FR822346, FR822342</td>
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<tr>
<td><em>C. bursa-pastoris</em></td>
<td>TR; Prov. Antalya, Taurus Mts., Bey Daglari massif, N 36° 52′, E 30° 15′</td>
<td>OSBU 18590</td>
<td>FR773705, FR822331, FR822337, FR822344, FR822343</td>
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<tr>
<td><em>C. orientalis</em></td>
<td>KZ; Pavlodarskaya Oblast, Pavlodar; N 52° 16′, E 76° 57′</td>
<td>OSBU 18248</td>
<td>FR773709, FR822327, FR822360, FR822347, FR822339</td>
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<td><em>C. orientalis</em></td>
<td>KZ; Pavlodarskaya Oblast, near Bayanaul; N 50° 47′, E 75° 41′</td>
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<td>FR773710, FR822326, FR822361, FR822348, FR822340</td>
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<td><em>C. orientalis</em></td>
<td>MN; Bayan-Olgii Aymag; Lake Hoton Nuur, N 48° 35′, E 88° 26′</td>
<td>OSBU 10588</td>
<td>FR773708, FR822328, FR822359, FR822349, FR822338</td>
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<tr>
<td><em>C. thracica</em></td>
<td>BG; Sozopol, c. 20 km south-east from Burgas, N 42° 25′, E 27° 42′</td>
<td>OSBU 20859</td>
<td>HE575237, HE575238, HE575239, HE575240, HE575241</td>
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<td><em>C. thracica</em></td>
<td>BG; Sozopol, c. 20 km south-east from Burgas, N 42° 26′, E 27° 42′</td>
<td>OSBU 20860</td>
<td>HE575242, HE575243, HE575244, HE575245, HE575246</td>
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<td><em>C. thracica</em></td>
<td>BG; Thracian Plain, Kurtovo Konare, N 42°05′, E 24°30′</td>
<td>OSBU 20875</td>
<td>HE575227, HE575228, HE575229, HE575230, HE575231</td>
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<td><em>N. paniculata</em></td>
<td>DE; Bavaria, Frankonian mountain region; N 50° 06′, E 11° 01′</td>
<td>OSBU 6887</td>
<td>HE575225, HE575226, HE575227, HE575228, HE575229</td>
</tr>
</tbody>
</table>

OSBU, Herbarium of the Botany Dept. of the University of Osnabrück, Germany; country codes: BG, Bulgaria; CL, Chile; DE, Germany; GR, Greece; IT, Italy; KZ, Kazakhstan; MN, Mongolia; RU, Russia; TR, Turkey.
genetics of these enzyme systems in Capsella has been deciphered in the above-cited literature, and the previous nomenclature of the enzyme loci and their iso-enzymes was adopted in this study. Isozyme data for the species C. grandiflora, C. rubella and C. bursa-pastoris either were previously published or are presented here for the first time.

DNA sequencing

The nuclear ribosomal internal transcribed spacers ITS1 and ITS2 including the 5.8 S region as well as four non-coding regions of the chloroplast genome have been analysed. Genomic DNA was sampled from herbarium specimens listed in Table 2 using the ‘InnuPREPP Plant DNA kit’ (Analytic Jena AG) according to the instructions of the manufacturer and was used directly in PCR amplifications.

Amplification and sequencing primers for ITS are given in German et al. (2009). Primers for the chloroplast regions were as follows: for the trnQ-rrps16 region described in Shaw et al. (2007), for rps16 intron described in Oxelman et al. (1997), for trnL-trnF described in Taberlet et al. (1991) and for trnH-psbA described in Kress et al. (2005). Products of the cycle sequencing reactions were run on an ABI 377XL automated sequencer. Forward and reverse sequences from each individual were manually edited in CHROMAS Lite 2.1 (Technesylum Pty Ltd) and combined in single consensus sequences. The sequences of all samples were aligned with CLUSTAL X (Thompson et al. 1997) and subsequently corrected manually in MEGA 5 (Tamura et al. 2011).

To test for multiple ITS copies within individuals of C. thracica, we also cloned PCR amplicons using the TOPOTA Cloning® kit (Invitrogen) according to the instructions of the manufacturer. The DNA of 16 clones was isolated with NucleoSpin plasmid kit (Macherey Nagel, Düren, Germany) according to the instructions of the manufacturer and prepared for sequencing. Sequencing was performed on ABI 377XL automatic sequencer with universal M13 forward and reverse primers.

Phylogenetic analyses

Neslia paniculata (L.) Desv. has been chosen as an outgroup based on the analyses of Bailey et al. (2006) and Couvreur et al. (2010). Parsimony analysis was performed with PAUP* 4.0b10 (Swofford 2002) using heuristic searches with TBR and 100 random addition sequence replicates. Bootstrap support (BS; Felsenstein 1985) was estimated with 100 bootstrap replicates, each with 100 random addition sequence searches. Bayesian analyses were implemented with MrBayes 3.1.23 (Ronquist & Huelsenbeck 2003). Sequence evolution models were evaluated using the Akaike Information Criterion (AIC) with the aid of Modeltest 3.7 (Posada & Crandall 1998). Two independent runs each of eight chains, 10 million generations, sampling every 100 trees. 25% of initial trees were discarded as burn-in. The remaining 28 000 trees were combined into a single data set and a majority-rule consensus tree obtained. Bayesian posterior probabilities were calculated for that tree in MrBayes 3.1.23.

Divergence time estimates in Capsella

Divergence time estimates were carried out with the software package BEAST v1.4.8 (Drummond & Rambaut 2007) based on ITS sequences (ITS1 and ITS2 regions combined, 5.8 S gene region excluded). No intraspecific ITS variation was detected between five provenances of Capsella grandiflora; three of C. rubella; four of C. orientalis; and nine of C. bursa-pastoris (see chapter Origin of plant material). Therefore, for the BEAST analysis, the ITS data matrix was reduced to four taxon sequences. Branch length was calibrated using a mean published ITS substitution rate for herbaceous annual/perennial angiosperms of $4.13 \times 10^{-9}$ substitutions/site/year (Kay et al. 2006) under the GTR + I + G substitution model, the uncorrelated lognormal relaxed clock approach, the Birth-Death speciation process performing a chain length of 100 000 000. Stationarity of the MCMC chain and the effective sampling size (ESS) of each parameter were examined in Tracer v1.4.1 (Drummond & Rambaut 2007, available from http://beast.bio.ed.ac.uk/Tracer), and each ESS was above 1000.

Results

Morphology, cytology and geographical distribution of Capsella orientalis and Capsella thracica

Capsella orientalis. Capsella orientalis is morphologically very close to C. bursa-pastoris and often confused with it. Chromosome counts of $2n = 16$ for C. orientalis are cited by Dorofeyev (2002) but without reference. Krasnoborov et al. (1980) reported $2n = 16$ for ‘C. bursa-pastoris’, a count that was probably based on C. orientalis and not on C. bursa-pastoris. Our data unambiguously prove diplody for C. orientalis with $2n = 16$ (Fig. 1). 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. 1). 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. 1). *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. 2). 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*. *Capsella thracica* is a Bulgarian endemic (Fig. 2) 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

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**Fig. 1** 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 flow cytometry, standard: *Petroselinum crispum*; *n* = number of measured individuals.

**Fig. 2** 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.

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flow cytometry (Fig. 1) and is predominantly selfing as revealed by isozyme progeny analyses.

**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. bursapastoris/C. orientalis*. The two other *C. thracica* accessions, no. 11 and 13, displayed at ITS sequence positions 122–126, two identical peaks that 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. bursapastoris* 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.

The alignment of combined ITS1 and ITS2 sequences, including the 5.8S gene of the taxa listed in Table 2, 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). Capsella bursapastoris 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 the reverse primer in a sequence identical to *C. bursapastoris*. The two sister clades, bootstrap support and 0.98 Bayesian posterior probabilities) (Fig. 3). Within the two sister clades, *C. orientalis* is resolved from *C. bursapastoris* 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 (Table 2) displayed two different ITS sequence types, one from the *C. grandiflora/C. rubella* lineage and one from the *C. bursapastoris/C. orientalis* lineage (Fig. 3).

**CpDNA sequence data.** Phylogenetic analyses were conducted separately with each cpDNA region sequenced. The alignments generated matrices of 855 characters for the *rps*16 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.

The *trnL-F* spacer region in *Capsella* displayed noticeable length variations caused by varying numbers of up to six repeats of 70–80 bp length. The repeats are characterized by a recurrent motif of *c. 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. bursapastoris*, *C. thracica* and *C. orientalis* accessions 8 and 10, whereas *C. orientalis* 9 had a length of only 562 bp because of complete or partial loss of three of the six repeats. 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 varying repeats (pseudogenes) from the total *trnL-F* alignment. The discarded fragment had a length of 432 characters (alignment positions 310–742) leaving a *trnL-F* alignment of 322 characters, which was implemented in the phylogenetic analysis.

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As 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. 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) 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, for example, one *C. orientalis* accession clustered with *C. bursa-pastoris*, and there is also clustering between the *C. bursa-pastoris* accessions. 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 cpDNA regions (Shaw et al. 2007).

**Divergence time estimates with BEAST**

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 (95% HPD, 0.58 to 6.98 myr; HPD, highest posterior density intervals, is equivalent to confidence intervals). The split between *C. rubella* and *C. grandiflora* was dated 0.86 myr (95% HPD, 0.015–2.45 myr), and the divergence time of *C. bursa-pastoris* and *C. orientalis* was estimated at 0.87 myr (95% HPD, 0.006–2.44 myr).

**Isozyme analyses**

Whereas allozyme frequencies within *C. grandiflora*, *C. rubella* and *C. bursa-pastoris* have been intensively studied (Hurka & Neuffer 1997; Neuffer & Hoffrogge 2000; Neuffer & Hurka 1999; Neuffer et al. 1999; Neuffer 2011; Neuffer & Hurka, unpublished), isozyme data for *Capsella orientalis* and *C. thracica* are documented here for the first time. *Capsella grandiflora* and *C. bursa-pastoris* share most of their allozymes, but the two alleles *Aat1*-4 and *Aat3*-5, rather common in *C. bursa-pastoris*, have not been recorded for *C. grandiflora* and thus appear unique for *C. bursa-pastoris* (Fig. 5). All *C. orientalis* plants that we have analysed so far (123 individuals from 16 populations from Siberia, Kazakhstan, Mongolia and China, Table 1) were nearly monomorphic regarding the isozyme loci analysed. Only at the *Aat2* locus did we find two alleles, *Aat2*-1 and *Aat2*-7 (Fig. 5). The frequency of *Aat2*-1 was *f* = 0.77 and that of *Aat2*-7 was *f* = 0.29. Four heterozygotes between *Aat2*-1 and *Aat2*-7 have been detected so far. All alleles found in *C. orientalis* have also been recorded for the diploid *C. grandiflora* and the tetraploid *C. bursa-pastoris*, but *C. orientalis* displayed only a fraction of the allele spectrum discovered in the latter two species (Fig. 5). All allozymes recorded for *C. thracica* are also found in *C. bursa-pastoris*, and no private alleles for *C. thracica* have been detected so far.

**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* (Fig. 3 and 4).

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. The phylogenetic position of the tetraploid *C. thracica* is discussed later.

**Divergence time estimates**

Published time estimates for Brassicaceae ‘lineage I’, to which *Arabidopsis* and *Capsella* belong (Beilstein et al.
Fig. 5 Presence/absence allozyme profiles of *Capsella* species: isozyme loci are given at the head of the diagrams. Rf values refer to an internal standard allozyme band set at value 100. Individuals examined: *C. orientalis* $n = 123$ of 16 populations; *C. thracica* $n = 30$ of 3 populations; *C. grandiflora*, *C. rubella* $n > 1000$ for each of the species and *C. bursa-pastoris* $n > 20\,000$ covering the entire species ranges.
2006), are 19–13 myr (Koch et al. 2000, 2001), 19.0–8.0–0.5 myr (Franzke et al. 2009), 36.1–27.3–18.2 (Couvreur et al. 2010) and 42.8–35.6–28.5 myr (Beilstein et al. 2010). The age of the tribe Camelineae, which includes Arabidopsis and Capsella, is estimated to be 17.9–13.0–8.0 myr (Beilstein et al. 2010). The split between the Arabidopsis lineage and its sister clade that includes Capsella is estimated at 14.6–10–5.7 myr (Koch et al. 2000), and separation of Arabidopsis and Capsella is dated 9.8–6.2 myr by Acıarkan et al. (2000). Divergence between Arabidopsis thaliana and its close relatives is estimated at 9.0–5.0–3.1 myr by Koch et al. (2000), whereas Osowsky et al. (2010) advocate the separation of Arabidopsis thaliana (self-compatible) from A. lyrata (self-incompatible) 18 myr ago. Such a high age, in connection with the assumption that A. thaliana probably has been self-sterile since its separation from A. lyrata (Wright et al. 2002), appears to contrast with the statement of Tang et al. (2007) that selfing in A. thaliana most likely evolved a ‘million years ago or more’. Thus, age estimates published for Arabidopsis and its close relative Capsella vary considerably, and it is well known that molecular date estimates may be full of substantial errors (Graur & Martin 2004; Welch & Bromham 2005; Pulquério & Nichols 2007). Nevertheless, lacking old Capsella fossils, we used published ITS substitution rates to provide rough estimates for dating divergences within the genus. Given the large range of the 95% highest posterior density intervals (HPD, equivalent of confidence intervals) of our analysis, we do not want to over-interpret our dating estimates. 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.

Mode, time and place of origin of Capsella species

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 hybridizations. Thus, autopolyploidy is synonymous with the intraspecific mode of origin and allopolyploidy with the interspecific mode of origin.

Capsella grandiflora and Capsella rubella. Capsella grandiflora is diploid and self-incompatible (SI) because of a sporophytic self-incompatibility system (Paetsch et al. 2006). 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 earlier, 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. 2), we hypothesize that the place of origin for both species was the western part of a former larger distribution area of the most recent common ancestor as will be discussed below (Fig. 6).

The diploid, predominantly selfing, C. rubella is a derivative of the C. grandiflora-like most recent common ancestor (MRCA).
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). Capsella rubella harvested only a fraction of the allozyme diversity of C. grandiflora (Fig. 3), which in connection with the findings of Guo et al. (2009) of only 1 or 2 alleles at most loci argues for a single origin. 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 c. 25 000–50 000 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 biogeographic scenarios for Brassicaceae taxa (Bleecker et al. 2002; Franck et al. 2004; Mummenhoff et al. 2004). The place of origin of C. rubella was presumably the eastern Mediterranean region. Subsequently, C. rubella extended its range, colonized 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. 2010).

Capsella orientalis and Capsella bursa-pastoris. Capsella orientalis is, as is C. rubella, a diploid and predominantly selfing species (SC) with very low allozyme variability (Fig. 5). However, the distribution areas of the two diploid species appear to be mutually exclusive (Fig. 2), and the phylogenetic roots of the two species are different as clearly shown by ITS and cpDNA data (Figs 3 and 4).

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. 2) 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 2 and 6). The DNA variation detected in C. orientalis and C. bursa-pastoris (Fig. 4) might argue for multiple origins of both species.

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 and 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), high isozyme polymorphism (Fig. 5), 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 (Alquist 1907, 1921). Presence/absence data on allozymes reveal that C. grandiflora and C. bursa-pastoris share most of their allozymes (Fig. 5). As there is no progenitor-derivative relationship between the two species (Figs 3 and 4), we interpret the concurrence of the allozymes, which are low mutation markers, in these two species as an ancient polymorphism inherited from the most recent common ancestor. It is highly unlikely that the shared allozymes are because of convergence.

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 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/C. rubella lineage and the C. orientalis/C. 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 C. bursa-pastoris. However, it should be kept in mind that signals indicating the hybrid nature of a species may be eradicated with time.

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 polyploidization process leading to the extant tetraploid C. bursa-pastoris. Although the multiple origin of C. bursa-pastoris may imply origin not only at different places but also at different times, we nevertheless argue that polyploidization occurred in the Middle/Late Pleistocene times. 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 7000–17,000 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 and 430,000 years ago (Pleistocene). Tetraploid Capsella bursa-pastoris would then be another prime example of colonization success of a polyploid plant species. A 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, 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 c. 125,000–110,000 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 colonized western Europe in the middle Pleistocene. This does not contradict or deny postglacial anthropogenic introduction.

Based on several arguments, we hypothesize 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 southwest Siberia, northwest China and western Mongolia. Allozyme Aat2-7 that had a considerably high frequency of f = 0.29 in C. orientalis was also detected in C. bursa-pastoris, but only in accessions from eastern Europe (Russia: Moscow region, Voronezh/Don, Astrakhan, Teberda/Caucasus) and central Asia (Kirgistan: Tian Shan and Pamir Alai). (ii) Some alleles were unique for C. bursa-pastoris including the very common alleles Aat1-4 and Aat3-5 (Fig. 5). It is unlikely that we missed these alleles in C. grandiflora because of under-sampling, because we sampled C. grandiflora throughout its distribution area intensively but could find no evidence of these alleles. It would appear that these allozymes private for C. bursa-pastoris were also acquired from the most recent common ancestor, postulating that the allozymes concerned had an eastern distribution within the common ancestor’s distribution area. Alternatively, they might have been lost in C. grandiflora because of bottleneck effects.

Capsella thracica. Capsella thracica has been described by Velenovsky (1893) from Bulgaria. It is sometimes given species rank (e.g. Chater 1964) and sometimes treated as a subspecies of C. bursa-pastoris (Chater 1993), a view also adopted by Ancèv (2007). It is a Bulgarian endemic reported from the Thracian lowlands, Black Sea coast and the Rhodopes Mts. (Ancèv 2007). The main feature discriminating this species from C. bursa-pastoris is the presence of an elongated style in C. thracica. To date, no chromosome numbers have been documented, neither are detailed studies concerning that taxon available. 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. 1) and shares its cpDNA regions with C. bursa-pastoris (Fig. 4). The ITS sequences of the C. thracica accessions analysed (Table 2), however, are characterized by two different copies, one from C. bursa-pastoris and one from C. grandiflora/C. rubella (Fig. 3), 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 (Neuffer, unpublished). Interspecific hybridization 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 hypothesized ‘diploid’ C. bursa-pastoris may have fused.

Evolutionary history of the genus Capsella, conclusions

Based on our results and present knowledge, we hypothesize the following scenario outlined in Fig. 6. 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 2, 3 and 4). 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 *Clusia 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 (see Fig. 3 and the Discussion chapter). 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 hybridization zones, supports the view that *C. bursa-pastoris* colonized Europe from Asia. A similar scenario has been demonstrated for *Arabidopsis thaliana* (Sharbel et al. 2000). The time estimate for the origin of the *Capsella* species is, therefore, compatible with the historical biogeographic events outlined earlier.

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.

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H.H. is especially interested in the evolution of Brassicaceae and in its biogeography with a focus on the Florogenesis of Eurasia. N.F. works on phytogeography of Amaryllidaceae (genera Allium and Galanthus), Ranunculaceae and Brassicaceae with molecular and cytological methods as well as DNA taxonomy and barcoding. D.G. is interested in taxonomy, systematics, phylogeny and phylogeography of Cruciferae of Asia. A.F.’s research deals with molecular systematics, phylogeny and biogeography of the Brassicaceae. B.N. is working on speciation processes and evolution of the mating system of Brassicaceae.

Data accessibility

1 DNA sequences: Genbank accessions FR773701–FR773711; FR822322–FR822365; HE575225–HE575244 (see Table 2).
2. Final DNA sequence assembly: alignments are provided as supporting information.

3. Sample locations: for Capsella orientalis see Table 1, and for the specimens used for DNA sequencing Table 2.

Supporting information

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

Appendix S1. ITS sequences.
Appendix S2. cpDNA sequences.
Appendix S3. cp DNA alignment.

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