The Evolutionary History of the Arabidopsis arenosa Complex: Diverse Tetraploids Mask the Western Carpathian Center of Species and Genetic Diversity

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Abstract

The Arabidopsis arenosa complex is closely related to the model plant Arabidopsis thaliana. Species and subspecies in the complex are mainly biennial, predominantly outcrossing, herbaceous, and with a distribution range covering most parts of latitudes and the eastern reaches of Europe. In this study we present the first comprehensive evolutionary history of the A. arenosa species complex, covering its natural range, by using chromosome counts, nuclear AFLP data, and a maternally inherited marker from the chloroplast genome [trnL intron (trnl) and trnL/F intergenic spacer (trnL/F-IGS) of trnRNeu and trnRL-Fone, respectively]. We unravel the broad-scale cytogeographic and phylogeographic patterns of diploids and tetraploids. Diploid cytotypes were exclusively found on the Balkan Peninsula and in the Carpathians while tetraploid cytotypes were found throughout the remaining distribution range of the A. arenosa complex. Three centers of genetic diversity were identified: the Balkan Peninsula, the Carpathians, and the unglaciated Eastern and Southeastern Alps. All three could have served as long-term refugia during Pleistocene climate oscillations. We hypothesize that the Western Carpathians were and still are the cradle of speciation within the A. arenosa complex due to the high species number and genetic diversity and the concurrence of both cytotypes there.

Citation: Schmickl R, Paule J, Klein J, Marhold K, Koch MA (2012) The Evolutionary History of the Arabidopsis arenosa Complex: Diverse Tetraploids Mask the Western Carpathian Center of Species and Genetic Diversity. PLoS ONE 7(8): e42691. doi:10.1371/journal.pone.0042691

Competing Interests: The authors have declared that no competing interests exist.

Introduction

Pleistocene climatic fluctuations strongly shape the evolutionary history of new species (e.g., grasshoppers [1]) and the distribution of genetic variants at the population level within species (e.g., Arabidopsis [2] and hominins [3]). In numerous studies of the European flora and fauna, these aspects of Pleistocene climatic oscillations have been investigated, but mainly confined to a single species. In this study we consider a species complex of wild relatives of the model plant Arabidopsis thaliana (L.) Heynh. with various taxa and cytotypes to investigate the hypothesis that past and ongoing gene flow between taxa and ploidal levels contributes to the ability of populations to adapt and survive in rapidly changing environments, particularly during the Pleistocene [4], [5].

The Arabidopsis arenosa species complex is one of three major species complexes within the genus Arabidopsis [6]–[10], formerly treated as Cardaminopsis [6], [7]. Arabidopsis arenosa and its segregates might not only represent the most ancestral species complex compared to the other two major species lineages, namely Arabidopsis lyrata (L.) O’Kane & Al-Shehbaz and A. halleri (L.) O’Kane & Al-Shehbaz [9], but the various taxa also harbor greater genetic diversity than any other Arabidopsis species and show a remarkable broad spectrum of ecological adaptations from high alpine regions in the High Tatras to sand dune vegetation in Scandinavia [9], [10]. A taxonomic overview of the various taxa of the A. arenosa species complex, including ploidal level and geographic distribution, is provided in Table 1. Arabidopsis arenosa is a colline, montane, and subalpine species complex with a mainly Central European distribution range including parts of the Alps and Carpathians. Only a few studies have been attempted to unravel the evolutionary history of the A. arenosa complex [9], [10]. Several studies focused on the natural hybrid A. sueca (Fr.) Norrl., which is of allopolyploid origin with the maternal parent A. thaliana and a paternal parent from the A. arenosa species complex [11], [12], confirmed also by artificial crosses [13]. Polyploidisation, mainly tetraploidisation, is frequent in several taxa of the A. arenosa complex [14], indicating repeated independent polyploidisation events. Intrigression, the stable integration of genetic material from one species into another through repeated backcrossing, was observed between members of the A. arenosa and A. lyrata complexes [5], [15]. According to different authors, the Arabidopsis arenosa complex comprises several taxa at various taxonomic levels. The complex has been treated as one species [A. arenosa (L.) Lawalrée] with two subspecies of partly overlapping distribution ranges in Central Europe [16]: the mainly tetraploid subsp. arenosa (2n = 16/32), also occurring in northern Europe, growing mainly on siliceous bedrock and sandy soil, and the tetraploid subsp. borbasii (Zapat.)
Table 1. Taxonomy, ploidal level, and geographic distribution of the various taxa of the Arabidopsis arenosa species complex (for details refer to the text).

| Taxon                                         | Ploidal level | Distribution range                                      |
|-----------------------------------------------|---------------|--------------------------------------------------------|
| Arabidopsis arenosa (L.) Lawallée             | 2n = 16/32    | Central and Western Europe, Scandinavia (lower altitudes) |
| subsp. arenosa                               | 2n = 16/32    | Carpathians                                             |
| subsp. arenosa var. intermedia (Kovats) Hayek | 2n = 32       | Southeastern Austrian Alps                              |
| subsp. borbasi (Zapalowicz) O’Kane & Al-Shehbaz | 2n = 32   | Central and Western Europe (mountain ranges, higher altitudes) |
| Arabidopsis carpatica, nom. prov.            | 2n = 16       | Carpathians (limestone)                                 |
| Arabidopsis croatica (Schott) O’Kane & Al-Shehbaz | 2n = 16/32 | Bosnia, Croatia                                         |
| Arabidopsis neglecta (Schultes) O’Kane & Al-Shehbaz | 2n = 16/32 | Carpathians (alpine ranges)                             |
| subsp. neglecta                               | 2n = 32       | Carpathians (alpine ranges, only occasionally in lower altitudes) |
| subsp. robusta, nom. prov.                   | 2n = 32       | Carpathians (mountain ranges, middle to subalpine altitudes) |
| Arabidopsis nitida, nom. prov.               | 2n = 16       | Carpathians                                             |
| Arabidopsis petrogena (A. Kern) V.I. Dorof.   | 2n = 16       | Carpathians                                             |
| subsp. petrogena                              | 2n = 32       | Carpathians                                             |
| subsp. exoleta, nom. prov.                   | 2n = 16       | Carpathians                                             |

Several taxa are awaiting taxonomic recognition (indicated with nom. prov.).

O’Kane & Al-Shehbaz (2n = 32), growing predominantly on calcareous bedrock and additionally found in the Carpathians. Diploid A. neglecta (Schult.) O’Kane & Al-Shehbaz (2n = 16) was described mainly from the Carpathians and rarely from the Alps, but its occurrence in the Alps is doubtful, since in the Alps this taxon has been introduced as Cardaminopsis arenosa var. intermedia (Kovats) Hayek [17]. Based on morphological and karyological data, several additional, mainly diploid Carpathian taxa at the species and subspecies level have been proposed, which were at that time attributed to the genus Cardaminopsis [14], [18]. These names were, however, never validly published and kept as nomina provisoria (nom. prov.) [19], pending ongoing studies aimed at clarifying their exact taxonomic status: Arabidopsis carpatica, nom. prov. (2n = 16), A. nitida, nom. prov. (2n = 16), A. petrogena (A. Kern) V.I. Dorof. subsp. petrogena (2n = 16), and A. petrogena subsp. exoleta, nom. prov. (2n = 32). In general, taxonomic concepts in the A. arenosa species complex are strongly debated [10].

There is an increasing interest in A. arenosa as a model system for adaptation to calcareous versus siliceous bedrocks (Koch and Widmer, ongoing studies; Bombelis et al., ongoing studies), or character trait research such as shade-tolerance (Bombelis et al., ongoing studies). Additionally, A. arenosa is interesting in terms of hybrid speciation, as it is the paternal parent of the natural allopolyploid A. suecica. Studies on the genomic consequences of hybridization are underway, and a first assembly of the A. arenosa genome is available (wiki.bioinformatics.ucdavis.edu/index.php/ Arabidopsis_arenosa_whole_genome_assembly). Working with A. arenosa as a model system needs careful consideration of the evolutionary history of the taxa one is investigating, particularly the distribution of natural genetic variation within and among taxa.

The following two aspects are the focus of our research: Unravelling the broad-scale cyto geographic and phylogeographic patterns of diploids and tetraploids: We are particularly interested in contact zones of populations with different or mixed ploidal levels, as they can indicate ongoing species differentiation. The second is detecting centers of genetic diversity: In the northern hemisphere late Quaternary climate oscillations, especially the last glacial maximum (LGM), about 26,500 to 19,000–20,000 years ago, had the most severe influence on present-day distribution and diversity of plant taxa. Arabidopsis arenosa is distributed both in regions that remained largely unglaciated during Pleistocene climate oscillations and in areas formerly covered by glaciers, making it well-suited for comparative studies of evolution in changing environments.

Materials and Methods

Plant material

The accession list is provided in Table S1. Geographic distribution of single accessions is shown in Fig. 1 A. Ploidy information was obtained from 214 accessions (126 populations, one to eleven individuals with five flowers each), AFLP data from 336 accessions (275 populations, one to seven individuals each), and plastid trn L/F sequence data from 365 accessions (260 populations, one to eleven individuals each) (Table S1).

Mitotic chromosome preparations

Ploidy was determined both in this and a previous publication [5] and additionally from herbarium vouchers from the Herbarium of the Natural History Museum Vienna on which Polatschek had indicated chromosome numbers. The respective source of ploidy determination is recorded in Table S1. Cytological methods and light microscopy were applied according to Schmickl et al. [5].

DNA isolation and amplified fragment length polymorphisms (AFLPs)

Total DNA was obtained from dried leaf material and extracted according to a CTA B protocol [20] with modifications according to previous studies [4].

AFLP analysis was performed using a standard protocol [21] with the following modifications: Approximately 200–500 ng DNA was digested and ligated in a 15 µl reaction mix containing T4 ligase buffer and ATP solution (Bioline, USA), 50 mM NaCl, 0.75 µg BSA, 1.5 U T4 ligase (Bioline, USA), 1 U MseI and 5 U EcoRI (New England Biolabs, USA), 0.37 µM EcoRI adapter and 3.67 µM MseI adapter. The reaction mix was incubated for 3 h at 37°C, followed by an inactivation step for 10 minutes at 65°C.
The restriction-ligation product was subsequently diluted tenfold. In the pre-selective PCR 2.5 μl of the diluted restriction-ligation product was used in a total reaction volume of 12.5 μl containing PCR buffer II [Applied Biosystems (ABI), USA], 2 mM MgCl₂, 0.5 mM dNTP mix, 0.08 μM EcoRI-A primer (5′-GAC TGC GTA CCA ATT CA-3′), 0.2 μM MseI-C primer (5′-GAT GAG TCC TGA GTA AC-3′), and 0.2 μU AmpliTaq Gold (ABI). The reactions were held at 72°C for 2 min followed by 20 cycles of 94°C for 20 s, 56°C for 30 s, and 72°C for 2 min with a final 30 s extension at 60°C. The pre-selective PCR product was visualized on a 1.5% agarose gel and diluted tenfold. For selective PCR we used 2.5 μl of the diluted pre-selective PCR product as template in a total reaction volume of 12.5 μl. The PCR mix contained 1× GoldTaq buffer (ABI), 2.5 mM MgCl₂, 0.8 mM dNTP mix, 0.08 μM EcoRI fluorescence labelled primer, 0.2 μM Mse primer [EcoRI-AGG(TET)/MseI-CTC, EcoRI-AAC(6-FAM)/MseI-CTG, EcoRI-AAG(HEX)/MseI-CAC], and 0.5 U AmpliTaq Gold (ABI). The reactions were held at 95°C for 5 min followed by 13 cycles of 94°C for 30 s, 56°C–56°C (–0.7°C per cycle) for 1 min, and 72°C for 1 min, followed by 23 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 1 min, and 72°C for 1.5 min with a final 8 min extension at 72°C.

Three differentially fluorescence labelled PCR products of the same sample were multiplexed and diluted, and the fragments were electrophoretically separated on a MegaBase 500 sequencer together with an ET-ROX 550 size standard (Amersham Biosciences, USA). For each run a total of 48 samples were analyzed, including one standard sample, one negative control, one repeat within the runs, and several other replicates (altogether 6.5%), as recommended by Bonin et al. [22]. Raw data were visualized and the fragments in the range of 60–513 bp manually scored using GeneMarker version 1.9 (SoftGenetics, USA). Processed data were exported as a presence/absence matrix.

**TrnL/F amplification and sequencing**

For the cpDNA markers trnL intron and trnL/F intergenic spacer (trnL/F-IGS), primers, PCR cycling scheme, purification of the amplified fragment, cycle sequencing, and sequencing on a MegaBace 500 sequencer followed the protocol of Schmickl et al. [4]. Amplified sequences of trnL/F-IGS included the complete trnL/F-IGS and the first 18 bases of the trnF gene.

**AFLP genetic diversity statistics and Principal Component Analysis (PCA)**

Several statistical parameters were computed using the R script AFLPdat [23], R 2.9.2 environment [24] for geographic and taxonomic groups: proportion of variable markers (FP) and Nei's gene diversity (Hₑ [25]). The following seven geographic regions were considered: (1) Balkan Peninsula (Balk), (2) Carpathians (Carp), (3) unglaciated Eastern Southeastern Alps (UnglaESEA), (4) glaciated Eastern Alps (GlaEA), (5) glaciated Western Alps (GlaWA), (6) unglaciated Central Europe (UnglaCentrEur), and (7) glaciated northern Europe (GlaNEur). These regions are illustrated in Figure 1. Regarding taxonomy, six entities were distinguished following Mesiček [14], [18] and Kolnik [19]: *A. arenosa* subsp. *arenosa*, *A. arenosa* subsp. *borbasii*, *A. carpatica*, *A. neglecta*, *A. nitida*, and *A. petrogena*. In order to analyze and display the similarity among the AFLP genotypes, a Principal Component Analysis (PCA) was performed using MVSP version 3.1 (Kovach Computing Services, UK). Pairwise Euclidean distance was applied as distance measure and, alternatively, both Jaccard and simple match coefficient [26].
Plastid trnL/F sequence definition, network analysis, genetic diversity statistics, and map reconstruction

Plastid trnL/F sequences were defined as i) haplotypes and ii) supraphylogenetic groups following our previous studies (e.g., [4]). Haplotypes (i) are characterized by varying (in sequence and structure) trnL/F pseudogenes in the 3’-region of the trnL/F-IGS close to the functional trnL gene; Haplotypes belonging to one supraphylogenetic (ii) share the same base order throughout the whole sequence except for the psuedogene-rich region, where they vary in both length and base content. Mutation rate within the psuedogene-rich region is about 10 to 20 times higher than within the non-coding spacer and intron regions [27]. Therefore, our cpDNA dataset is based on trnL/F supraphylogenetic only. Supraphylogenetic differ from each other by single point mutations and/or indels. Newly defined trnL/F haplotypes were assigned to GenBank numbers [FJ477684-FJ477690, FJ477705-FJ477716] (Table S1). The network was constructed using TCS version 1.21 [28] using the statistical parsimony algorithm [29]. Single gaps (except polyT stretches) were coded as single additional binary characters. Genetic diversity statistics were performed with Arlequin version 3.11 [30]: Genetic diversity was estimated as effective genetic diversity according to Gregorius (V_e) [31], nucleotide diversity π and Nei’s unbiased gene diversity H_E.

In order to visualize the geographical data, ArcView version 8.2 (ESRI, USA) was used. The maximum extent of the ice sheets during the LGM was taken from Ehlers and Gibbard [32].

Results

Chromosome counts identify diploids exclusive to the Balkan Peninsula and the Carpathian Mountains

Two ploidal levels, diploid and tetraploid, were observed within our sampling (Fig. 1B). Diploids were exclusively found in southeastern and eastern Europe on the Balkan Peninsula, in northern Hungary, and in the Carpathians. In contrast, tetraploids have a large distribution range and occur from the Julian Alps (Slovenia) in the south, the Western Carpathians (Slovakia) in the east, France and Belgium in the west, and Scandinavia in the north. Several regions were reported as areas of recent, mainly anthropogenically influenced colonisation after 1980 [33] [Belgium, Finland, France, Great Britain, Greenland], frequently along railway tracks. The Balkan Peninsula and the Western Carpathians were the only regions where both diploid and tetraploid populations were found. Populations of mixed ploidal levels were not observed, but can not be completely ruled out, as only a limited number of populations from the Eastern Alps (n = 28) and the Western Carpathians (n = 9) were analyzed with more than one individual per population.

AFLP data indicate similar values for gene diversity throughout Europe and demonstrate high gene diversity of a widespread tetraploid

Diversity statistics, based on AFLP data, showed similar values for gene diversity throughout the whole distribution range of the A. arenosa species complex (Table 2), ranging from H_E = 0.133 (GlaNEur) to H_E = 0.159 (UnglaESEAlps). The proportion of variable markers differed more strongly between geographic regions, ranging from FP = 0.411 (Balk) to FP = 0.906 (UnglaESEAlps). However, the proportion of variable markers is biased with respect to sample size (Balk: n = 9, UnglaESEAlps: n = 114) and, therefore, not a valid measurement of genetic diversity. Genetic diversity patterns in the A. arenosa complex need additional consideration in terms of taxonomy, as for certain regions, e.g., Carp, numerous taxa are described, and in other regions, e.g., GlaNEur, only one taxon is found. Gene diversity of the different taxa ranged from H_E = 0.157 (A. arenosa subsp. arenosa) and H_E = 0.155 (A. arenosa subsp. borbasii) to H_E = 0.138 (A. carpatica and A. petrogena) and H_E = 0.125 (A. neglecta). The proportion of variable markers is, again, highly correlated with sample size (A. neglecta: n = 6, FP = 0.296; A. arenosa subsp. arenosa: n = 277, FP = 0.905) and so should be treated with caution.

PCA according to regions (Fig. 2A) resulted in overlapping groups of AFLP genotypes from nearly all regions, except for accessions from parts of Carp and UnglaESEAlps. Groups of AFLP genotypes according to taxonomy also largely overlapped (Fig. 2B). Widespread A. arenosa subsp. arenosa but also subsp. borbasii formed large groups of AFLP genotypes in comparison to A. carpatica, A. neglecta, and A. petrogena (A. nitida was omitted, as it was represented by one accession only). This finding underlines the high genetic plasticity of the tetraploids (A. arenosa subsp. arenosa and subsp. borbasii) in contrast to the mainly diploids [A. carpatica (exclusively diploid), A. neglecta (predominantly diploid), A. petrogena (partially diploid)]. PCA according to ploidal levels (Fig. 2C) revealed two partly overlapping clusters of diploids and tetraploids, but due to many accessions without ploidal level estimates the two clusters could actually be more strongly intermingled.

The Balkan Peninsula, the Carpathians and the unglaciated Eastern and Southeastern Alps are the three centers of chloroplast sequence diversity of the A. arenosa species complex

Based on trnL/F sequence data, we detected three centers of genetic diversity of the A. arenosa complex (Table 2). The unglaciated Eastern and Southeastern Alps displayed highest effective diversity according to Gregorius (V_e = 4.14), highest nucleotide diversity (π = 0.483%), and highest Nei’s gene diversity (H_E = 0.764) within the whole dataset. The Balkan Peninsula was detected as the second center of genetic diversity (V_e = 2.94, π = 0.395%, H_E = 0.733). The third center of genetic diversity, the Carpathians, was characterized by gene diversity values similar to those of the Balkan Peninsula (V_e = 3.49, π = 0.352%, H_E = 0.719). In contrast to these three genetically highly diverse regions, which remained largely unglaciated during Pleistocene climate oscillations, formerly glaciated regions showed reduced values of effective genetic diversity according to Gregorius, nucleotide and Nei’s gene diversity: The part of the Eastern Alps formerly covered by glaciers was characterized by V_e = 2.23, π = 0.310%, and H_E = 0.561. The formerly glaciated Western Alps showed strongly reduced values (V_e = 1.24, π = 0.109%, H_E = 0.205). Glaciated northern Europe was characterized by V_e = 1.81, π = 0.315%, and H_E = 0.491. Although Central Europe remained largely unglaciated during Pleistocene climate oscillations, genetic diversity was also reduced (V_e = 1.64, π = 0.241%, H_E = 0.403). In contrast to AFLP data, effective genetic diversity according to Gregorius, nucleotide and Nei’s gene diversity of diploid A. carpatica (V_e = 3.71, π = 0.514%, H_E = 0.751) exceeded that of tetraploid A. arenosa subsp. arenosa (V_e = 3.48, π = 0.410%, H_E = 0.714).

In all geographic regions, except GlaWAps, unique supraphylogenotypes were found, which occurred in one region only (Figs. 1C, 3A). These unique types are nearly all derived from the “core” supraphylogenotypes A, B, and E: AY (Balk); AE, AZ, BA, BE, P, Y (Carp); F, O, Z (UnglaESEAlps); AW, AX, I, M (GlaESEAlps); BB, BC (UnglaCarCentrEur); W (GlaNEur). In contrast to the supraphylogenotypes, which were shared between regions (Fig. 3A) and also between taxa (Fig. 3B; A, B, C, E, L, Q, U), these regionally unique supraphylogenotypes were found in exclusively one taxon,
Table 2. (a) Regional genetic differentiation and (b) genetic differentiation according to taxonomy, based on AFLP and chloroplast DNA sequence data (trnL/F suprahaplotypes).

| (a) Geographic region | AFLPs | AFLPs | AFLPs | trnL/F | trnL/F | trnL/F | trnL/F |
|-----------------------|-------|-------|-------|--------|--------|--------|--------|
|                       | n     | Nei's gene diversity ($H_d$) | Proportion of variable markers (FP) | n     | $V_a$ | Nucleotide diversity ($\pi \times 10^{-2}$) | Nei's gene diversity ($H_d$) |
| Balk                  | 9     | 0.144 | 0.411 | 10    | 2.94  | 0.395/±0.254 | 0.733/±0.101 |
| Carp                  | 88    | 0.144 | 0.885 | 107   | 3.49  | 0.352/±0.208 | 0.719/±0.030 |
| UnglaESEAlps          | 114   | 0.159 | 0.066 | 132   | 4.14  | 0.483/±0.217 | 0.764/±0.024 |
| GlaEAlps              | 50    | 0.143 | 0.740 | 51    | 2.23  | 0.310/±0.190 | 0.561/±0.076 |
| GlaWAlps              | 32    | 0.144 | 0.661 | 19    | 1.24  | 0.109/±0.090 | 0.205/±0.119 |
| UnglaCentrEur         | 46    | 0.151 | 0.773 | 35    | 1.64  | 0.241/±0.157 | 0.403/±0.102 |
| GlaNEur               | 17    | 0.133 | 0.480 | 11    | 1.81  | 0.315/±0.209 | 0.491/±0.175 |

| (b) Taxon | AFLPs | AFLPs | AFLPs | trnL/F | trnL/F | trnL/F | trnL/F |
|-----------|-------|-------|-------|--------|--------|--------|--------|
| A. arenosa subsp. arenosa | 277   | 0.157 | 0.985 | 282   | 3.48  | 0.410/±0.235 | 0.714/±0.023 |
| A. arenosa subsp. borbasii | 20    | 0.155 | 0.622 | 14    | 1.34  | 0.111/±0.093 | 0.275/±0.148 |
| A. carpatica, nom. prov. | 29    | 0.138 | 0.651 | 37    | 3.71  | 0.514/±0.293 | 0.751/±0.054 |
| A. neglecta            | 6     | 0.125 | 0.296 | 6     | 2.00  | 0.470/±0.319 | 0.600/±0.215 |
| A. petrogena            | 23    | 0.138 | 0.602 | 24    | 2.42  | 0.241/±0.159 | 0.612/±0.089 |

Sample size (n), Nei’s gene diversity ($H_d$), proportion of variable markers (FP), and nucleotide diversity ($\pi$) with standard deviation are provided. For trnL/F suprahaplotypes effective genetic diversity according to Gregorius ($V_a$) is additionally displayed. The following seven geographic regions were considered: (1) Balkan Peninsula (Balk), (2) Carpathians (Carp), (3) unglaciated Eastern and Southeastern Alps (UnglaESEAlps), (4) glaciated Eastern Alps (GlaEAlps), (5) glaciated Western Alps (GlaWAlps), (6) unglaciated Central Europe (UnglaCentrEur), and (7) glaciated northern Europe (GlaNEur). Arabidopsis arenosa var. intermedia is integrated within A. arenosa subsp. arenosa. Arabidopsis nitida was omitted from the analyses, as it was represented by one (AFLPs) and three (trnL/F suprahaplotypes) accession(s) only.

doi:10.1371/journal.pone.0042691.t002
except P and Y (Fig. 3B). Regarding ploidal levels (Fig. 3C), diploids and tetraploids shared numerous suprahaplotypes (A, AV, B, C, E, L, U) but also had unique ones (diploids: AY, BA, BE, P, Y; tetraploids: AU, AW, AX, AZ, BB, BC, D, Q).

Discussion

The Western Carpathian Mountains are the cradle of speciation within the *A. arenosa* complex

Speciation is often accompanied by polyploidisation [34]. Within the *A. arenosa* complex two ploidal levels, diploid and tetraploid, were observed. Contact zones of these two cytotypes were localized in the northwestern part of the Balkan Peninsula and in the Western Carpathians, and, consequently, at least one independent polyploidisation event can be assumed for each of these regions. Regarding the Western Carpathians, we observed a mosaic pattern of diploids and tetraploids, which is in congruence with Mešícˇek [14] (see also [19]). In the contact zones of diploids and tetraploids in the Western Carpathians we found no populations with mixed ploidal levels. This finding can be discussed with respect to the origin of the tetraploids: Populations of mixed ploidal levels could either be the result of a relatively recent autopolyploidisation event or the result of secondary contact of formerly allopatric populations with different ploidy levels. The genus *Melampodium* L., in particular the white-rayed species complex with mainly diploids and tetraploids, is an example for postglacial formation of polyploids via autopolyploidisation. Intrapopulational cytotype mixture was reported, but emphasized as rare [35]. In contrast, secondary contact of formerly allopatric populations with different ploidy is the explanation for populations of mixed ploidal levels (diploids and tetraploids or tetraploids and hexaploids) in the *Knautia arvensis* agg. [36]. Therefore, we conclude, that the lack of mixed ploidy populations and the lack of uneven ploidy and aneuploidy in the *A. arenosa* complex excludes recent polyploidisation events as well as secondary contact zones. We assume ancient polyploidisation, probably dated several glacial cycles ago. Slightly different ecological adaptations of diploids and tetraploids might have favored such a distinct pattern of diploid and tetraploid populations. Diploid *A. neglecta* subsp. *neglecta*, for example, is found on siliceous substrates, mostly in high alpine habitats above the tree line, where it grows along mountain streams. The tetraploid subspecies *robusta* (previously recognized as *Cardaminopsis neglecta* subsp. *robusta* [18], but never validated by publication), corresponds to a taxon, which, although it also occurs on siliceous substrates, is found in different mountain ranges and at lower altitudes compared to typical subspecies (mostly around or below the tree line [Kolnı´k, unpubl. data]). This is comparable to *Senecio*

![Figure 2. Principal Component Analysis of AFLP data from the Arabidopsis arenosa species complex.](http://www.plosone.org/article.fulltext/42691/f002)

![Figure 3. Chloroplast DNA trnL/F suprahaplotype networks of the Arabidopsis arenosa species complex.](http://www.plosone.org/article.fulltext/42691/f003)
Evolutionary History of Arabidopsis arenosa

Long-term evolution in two glacial refugia: the Carpathians and the unglaciated Eastern/Southeastern Alps

In numerous studies of both plant and animal species three classical LGM refugia were reported, based on the fossil record [38] and species and genetic diversity [39]: the Balkan Peninsula, the Iberian Peninsula, and the Appenin. Out of these three Pleistocene refugia, the Balkan Peninsula was emphasized as the most important refugium, especially for tree species [40], but also for upper and lower montane taxa of especially eastern European regions, exclusively tetraploid regions (UnglaCentrEur). In all these four glaciers (GlaEAlps, GlaWAlps, GlaNEur) in comparison to the Western Carpathians, the Carpathians as diploid (subsp. robustus) [18; Mesić, unpubl. data]. Arabidopsis hetersogena is exclusively described from the Carpathians as diploid (subsp. petrogena) and as tetraploid (subsp. exoleta [18; Mesić, unpubl. data]). Arabidopsis nitida is the third unique taxon of the Carpathians. And within broadly defined A. borbasi a Carpathian subspecies (subsp. carpatica, referred to as A. carpatica, nom. prov. [19], and as C. borbasi subsp. carpatica [18]) is discussed. However, taxonomy of this highly diverse species complex needs to be revised in the near future.

Parallel evolution in the Eastern Alps and the Western Carpathians

According to mNL/F sequence data, the unglaciated Eastern/Southeastern Alps and the Carpathians formed two distinct genetic groups: the Alps characterized by suprahaplotype B and the Carpathians by suprahaplotype L. Although other suprahaplotypes, e.g., A and E, were shared between these two mountain ranges, we assume strong barriers to gene flow between the Alps and Carpathians, due to the Pannonian Basin, which constituted a lowland barrier for montane to subalpine taxa since the Holocene warming. Long-term genetic isolation between the Alps and Carpathians was also proposed for several other plant species, such as Campanula alpina [44]. Additional studies from Ranunculus glacialis L. [51] and Rosa pendulina L. [52] support this view.

However, an alternative explanation for genetic differentiation between A. arenosa populations from the Alps and Carpathians has to be considered. Based on our karyologic data, independent colonisation of both mountain ranges from the Balkan Peninsula can be hypothesized: Compared to all other regions, exclusively diploid cytotypes (except for one accession in the northern part) were found on the Balkan Peninsula. Diploid A. arenosa could have migrated northwards and experienced multiple polyplodisation events, especially in the Carpathians, where taxa of both diploid and tetraploid cytotype are described. The Alps were probably colonised by (a) tetraploid cytotype(s) of A. arenosa, which could have originated in the Julian Alps, where only tetraploids were found. The Balkan Peninsula as the ancient refuge area for the A. arenosa species complex is additionally supported by the occurrence of an Arabidopsis species endemic to the Balkan Peninsula, Arabidopsis cretica. This endemic is closely related to the A. arenosa complex, based on ITS sequence data (internal transcribed spacer region of nuclear encoded ribosomal DNA) [9], [10].

Taxa in refuge areas like the Eastern Alps and the Western Carpathians probably underwent long-term adaptational processes, which could result in adaptation to the same ecological niche in the two mountain ranges in parallel. Indeed, we found one example for parallel evolution in the Eastern Alps and the Western Carpathians: Diploid A. neglecta grows on siliceous bedrock along mountain streams in alpine habitats of the High Tatras (Slovakia), a similar ecological niche in the Eastern Alps (Wolzer Tauern, Styria) is occupied by a tetraploid taxon corresponding to Cardaminopsis arenosa var. intermedia (Kovats) Hayek [17]. Besides sharing similar ecological demands, the two taxa are discussed to be morphologically more similar to each other than to any other member of the A. arenosa complex. However, AFLP data did not confirm that the vicariant populations from the Eastern Alps and the High Tatras represent a single species (Fig. 2B). This needs further experimental confirmation based on a broader sampling of both taxa. Interestingly, two other species from the Brassicaceae co-occur in the same or neighbouring alpine regions on siliceous bedrock along mountain streams: hexaploid Cochlearia tatrae Borbás in the High Tatras (Slovakia) and diploid Cochlearia excelsa Zahlbr. ex Fritsch in the Eastern Alps (Seckauer Tauern, Styria). In parallel to A. neglecta and C. arenosa var. intermedia, these two Cochlearia species evolved independently and most likely within the last approximately 100,000 years [53], [54]. Parallel evolution of species pairs implies effective reproductive isolation between the two mountain ranges and limited multiple immigration.

Tetraploid A. arenosa subsp. arenosa is a highly genetically diverse taxon

Based on both AFLP and chloroplast sequence data, genetic diversity was not strongly reduced in regions formerly covered by glaciers (GlaEAlps, GlaWAlps, GlaNNeur) in comparison to formerly unglaciated regions (UnglaCentrEur). In all these four regions, exclusively tetraploid A. arenosa subsp. arenosa and subsp. borbasii were found. Local, periglacial survival could serve as one explanation for the high genetic diversity of especially A. arenosa subsp. arenosa. In Central Europe, numerous populations are restricted to relict habitats on exposed rocks in low mountain ranges, e.g., the Black Forest, the Eifel, the Elbe Sandstone

carniolicae Willd. with an altitudinal, ecological gradient composed of mainly diploid and hexaploid populations in the Eastern Alps [37]. Additionally, the high genetic diversity, based on plastid mNL/F, in the Western Carpathians is an indicator for past and also ongoing speciation within the A. arenosa complex. Not only the high genetic diversity but also the high number of species and subspecies underline the Western Carpathians as a cradle of A. arenosa speciation. Numerous proposed taxa are unique for the Carpathians: A. neglecta is considered to comprise two subspecies, one diploid (subsp. neglecta) and one tetraploid (subsp. robustus) [18; Mesić, unpubl. data]. Arabidopsis hetersogena is exclusively described from the Carpathians as diploid (subsp. petrogena) and as tetraploid (subsp. exoleta [18; Mesić, unpubl. data]). Arabidopsis nitida is the third unique taxon of the Carpathians. And within broadly defined A. borbasi a Carpathian subspecies (subsp. carpatica, referred to as A. carpatica, nom. prov. [19], and as C. borbasi subsp. carpatica [18]) is discussed. However, taxonomy of this highly diverse species complex needs to be revised in the near future.
Mountains, the Harz Mountains, and the Swabian Mountains, where they often co-occur with Pleistocene relic species such as *Dianthus gratianopolitanus*. [Vill. [Koch, unpubl. data]. A second explanation could be the lack of genetic bottlenecks and the maintenance of large effective population sizes during postglacial migration into formerly glaciated regions, probably enhanced by the plant’s biennial life cycle. In a third alternative, gene flow between different taxa and/or ploidal levels could account for the high genetic diversity in especially tetraploid *Artemisia* subsp. *arnosa*, probably before this taxon migrated from the Eastern Alps and Western Carpathians, its putative refuge areas, to Central Europe. Gene flow between different taxa of the *Artemisia* species complex is documented by numerous hybrids, including triploid ones, reported from the Western Carpathians [19]. Gene flow between different ploidal levels was recently described for *A. lyrata* [15]; Based on an isolation with migration model analysis [55], [56], gene flow from diploids to tetraploids and vice versa was hypothesized. We assume that all three factors could have contributed to the high genetic diversity of tetraploid *A. arnosa*, which could have resulted in its ability to be a successful colonizer on various different substrate types (e.g., limestone, sandstone, granite, basalt) in various different habitat types (“natural” sites on rocks, gravel, and sand; anthropogenically influenced sites such as railway tracks and rural areas).

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### Supporting Information

**Table S1** Information about accession details and experimental results: taxonomic unit, taxon name on herbarium voucher, herbarium/herbarium voucher no., accession no., geographic region, latitude/longitude, locality, collector/date of collection, ploidal level/publication or other source, AFLPs, trnL intron type, trnL intron GenBank no., trnL/F-IGS type, trnL/F-IGS GenBank no., trnL intron+trnL/F-IGS type, trnL intron+trnL/F-IGS supra-haplotype. Accessions, for which AFLP data were obtained, are marked (see AFLPs).

### Acknowledgments

We thank the curator of the Herbarium of the Natural History Museum Vienna, Ernst Vitek, for providing plant material, Adolf Polatschek for providing various chromosome counts and sharing his knowledge during the course of the project, Susanne Ball and Michaela Wernisch for laboratory assistance, Martin Kolník for locality data, Jurgen Ehlers and Phil Gibbard for shapes of the LGM, and Graham Muir for critically reading the manuscript.

### Author Contributions

Conceived and designed the experiments: MAK RS. Performed the experiments: RS JP JK. Analyzed the data: RS JP JK MAK. Wrote the paper: RS MAK JP JK KM.
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