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Research

Fine-scale empirical data on niche divergence and homeolog expression patterns in an allopolyploid and its diploid progenitor species

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Summary

- Polyploidization is pervasive in plants, but little is known about the niche divergence of wild allopolyploids (species that harbor polyploid genomes originating from different diploid species) relative to their diploid progenitor species and the gene expression patterns that may underlie such ecological divergence. We conducted a fine-scale empirical study on habitat and gene expression of an allopolyploid and its diploid progenitors.
- We quantified soil properties and light availability of habitats of an allotetraploid *Cardamine flexuosa* and its diploid progenitors *Cardamine amara* and *Cardamine hirsuta* in two seasons. We analyzed expression patterns of genes and homeologs (homeologous gene copies in allopolyploids) using RNA sequencing.
- We detected niche divergence between the allopolyploid and its diploid progenitors along water availability gradient at a fine scale: the diploids in opposite extremes and the allopolyploid in a broader range between diploids, with limited overlap with diploids at both ends. Most of the genes whose homeolog expression ratio changed among habitats in *C. flexuosa* varied spatially and temporally.
- These findings provide empirical evidence for niche divergence between an allopolyploid and its diploid progenitor species at a fine scale and suggest that divergent expression patterns of homeologs in an allopolyploid may underlie its persistence in diverse habitats.

Introduction

Polyploidization is pervasive in plants (Otto & Whitton, 2000) and is one of the major mechanisms of speciation (Ramsey & Schemske, 1998; Soltis & Soltis, 2009). In allopolyploids (species with multiple genome sets from different diploid species), differential expression of homeologs (potentially redundant copies of genes derived from the progenitor species) has been speculated to reflect selective use of the homeologs in responding to various environmental conditions (Madlung, 2013; Yoo *et al.*, 2013), in addition to higher evolvability due to redundancy (Douglas *et al.*, 2015; Paape *et al.*, 2018). This, in turn, might enable allopolyploids to thrive in a range of environments. Consistent with this idea, manipulative experiments in laboratories demonstrated homeolog expression ratio changes in response to environmental factors such as temperature, water availability, and metal concentration (Liu & Adams, 2007; Dong & Adams, 2011; Akama *et al.*, 2014; Liu *et al.*, 2015; Paape *et al.*, 2016; but see Combes *et al.*, 2013). Recent empirical studies show that a small proportion (*c.* 1%) of all genes exhibits a ratio change, many of which are known to be involved in response to the specific conditions that were manipulated in the experiment (Akama *et al.*, 2014; Paape *et al.*, 2016). These results suggest that allopolyploids selectively express the homeologs relevant for an appropriate response to the environment. Although the expression patterns in the laboratory and in naturally fluctuating environments, or *in*

natura, may be distinct (Shimizu *et al.*, 2011; Yamasaki *et al.*, 2017; Song *et al.*, 2018), little is known about environment-dependent changes in the homeolog expression ratio of allopolyploids in natural habitats and about the divergence of expression patterns from progenitors.

Ecological differentiation enables allopolyploids to establish and persist amid well-established and possibly locally adapted diploid progenitor species, otherwise they would be excluded by competition (Hardin, 1960). Newly emerged polyploids may be prepared to exploit novel habitats, as they inherited divergent genomes of the diploid progenitor species (Levin, 2003; Paape *et al.*, 2020). This leads to an expectation that allopolyploids and their progenitor species occupy different habitats (niche divergence). Niche divergence between an allopolyploid and its progenitor species has been extensively studied using ecological niche modeling, where species geographic distributions are modeled in relation to large-scale climatic and topographic variables (Wake *et al.*, 2009). There is support for niche divergence, with intermediate and broad niche detected for allopolyploids, however, at the same time, several studies also report niche overlap between allopolyploids and their progenitors (Soltis *et al.*, 2010; McIntyre, 2012; te Beest *et al.*, 2012; Glennon *et al.*, 2014; D. E. Soltis *et al.*, 2014; P. S. Soltis *et al.*, 2014; Marchant *et al.*, 2016; Huynh *et al.*, 2020; López–Alvarez *et al.*, 2020). A possible explanation for niche overlap is the spatial scale of a study. Ecological niche modeling approaches typically evaluate environmental factors at a resolution of > 1 km^2. Multiple environmental factors are known to vary at fine spatial scale, including water availability, light availability, and soil properties (Denney *et al.*, 2020). These factors have been associated with niche divergence (Silvertown, 2004) and not yet incorporated in large-scale climatic modeling (www.worldclim.org). Under this circumstance, fine-scale empirical studies would have a better ability to detect niche divergence at fine scale. Furthermore, water availability varies not only spatially but also temporally (Denney *et al.*, 2020). Data from multiple time points should enable the evaluation of temporal variation in habitats as well as differential gene expression (Aubin-Horth & Renn, 2009; Aikawa *et al.*, 2010; Richards *et al.*, 2012; Alvarez *et al.*, 2015).

The genus *Cardamine* of Brassicaceae has long been a model to study ecological polyploid speciation (Howard, 1948; Hussein, 1948; Shimizu-Inatsugi *et al.*, 2017; Akiyama *et al.*, 2020; Sun *et al.*, 2020). The allotetraploid *Cardamine flexuosa* in Europe derived from diploid progenitor species *Cardamine amara* (genome size 217–273 Mb) and *Cardamine hirsuta* (genome size 198–225 Mb) (Johnston *et al.*, 2005; Mandáková *et al.*, 2013; Mandáková *et al.*, 2014; Gan *et al.*, 2016; in-house measurement) offers a promising system with which to study the niche divergence and associated homeolog expression of allopolyploids in comparison with the diploid progenitor species. *Cardamine flexuosa* is estimated to have emerged between 10 000 ka and 1 Ma (Mandáková *et al.*, 2014). The three species are common in central Europe (Carlsen *et al.*, 2009), and their distributions largely overlap at the scale of 5 km^2 in Switzerland (Supporting Information Fig. S1; Lauber *et al.*, 2012). However, anecdotal reports suggest niche divergence among species, as *C. hirsuta* plants are typically observed on roadsides and in ditches, *C. amara* on river banks and in wet woodlands, and *C. flexuosa* along forest roads (Urbanska & Landolt, 1978; Koch *et al.*, 2003; Lihová *et al.*, 2006; Grime *et al.*, 2007; Lauber *et al.*, 2012; Tedder *et al.*, 2015). In a manipulative laboratory experiment where the three species underwent drought, submergence, or fluctuation of the two, *C. hirsuta* performed the best in drought and worst in submergence, the opposite was the case for *C. amara*, and *C. flexuosa* performed similarly well in all treatments (Shimizu-Inatsugi *et al.*, 2017). The three species thus appear to show niche divergence along hydrological gradients, characterized by two distinct stresses – water-logging and drought – at the two ends of the gradient at a scale smaller than 5 km^2. Microarray analyses from the laboratory experiment showed that the gene expression induction pattern of *C. flexuosa* was similar to that of *C. hirsuta* under drought and to that of *C. amara* under submergence (Shimizu-Inatsugi *et al.*, 2017). It is yet to be shown how the gene expression pattern of *C. flexuosa* compares to that of the diploid progenitor species in field. Additionally, a major limitation of the usage of the *Arabidopsis* microarray was that it allowed the quantification of only the sum of the expression levels of two homeologs of only *c.* 46% of the entire genes. Recently, HomeoRoq, a tool for analyzing the homeolog expression ratio change in allopolyploids (Akama *et al.*, 2014; Kuo *et al.*, 2020), and the genome sequence of *C. hirsuta* (Gan *et al.*, 2016) became available, enabling evaluation of the expression of homeologs across genomes of *C. flexuosa*. These technical advances and findings from the laboratory experiment make *C. flexuosa* an allopolyploid with distinct ecologies and genomic tools. Such a study system is still rate (Soltis *et al.*, 2016), and thus *C. flexuosa* offers a unique opportunity for studying ecological transcriptomics of an allopolyploid and its diploid progenitors. However, quantitative data from natural habitats are still lacking.

Here, we conducted a fine-scale empirical study over two seasons to quantify water availability, soil properties, and light availability in the habitats of *C. flexuosa*, *C. amara* and *C. hirsuta* within their native range in Switzerland, in three areas in and around Zurich, and to analyze homeolog expression patterns of *C. flexuosa* in comparison with diploid progenitor. Based on previous studies, we hypothesized that the three species occur in different habitat; that is, the species differ in realized niche (Hutchinson, 1957). We expect that *C. flexuosa* would inhabit a wide water-availability gradient, including sites with fluctuating water availability, and that it would differentially express homeologs similarly to either of the diploid progenitor species depending on environments. We addressed the following questions: (1) What is the relative contribution of different environmental factors to the occurrence of the allopolyploid *C. flexuosa* as well as the two diploid progenitor species? What are the key environmental factors? (2) Along the gradients of environmental factors contributing to the occurrence of the species, is *C. flexuosa* in a distinct and/or broader range than *C. amara* and *C. hirsuta*? (3) How does the gene expression of *C. flexuosa* relate to that of the coexisting diploid progenitor species? (4) Is the gene (homeolog) expression pattern of *C. flexuosa* associated with environmental

factor identified as the key for niche divergence based on ecological data?

Materials and Methods

Study species

Cardamine amara L. is a perennial widespread across most of central Europe (Marhold, 1998; Lihová *et al.*, 2000; Grime *et al.*, 2007). Populations of *C. amara* in lowland areas in Switzerland (< 1000 m asl) are reported to consist of diploids ($2n = 2x = 16$), and autotetraploid populations of subsp. *austriaca* have been observed at higher altitudes of the inner alpine valleys (Marhold, 1999; Lihová *et al.*, 2000; Marhold *et al.*, 2002). Our study sites were in the areas inhabited by only diploids. *Cardamine hirsuta* L. is a diploid ($2n = 2x = 16$) native to Europe (Marhold, 1995; Lihová *et al.*, 2006; Grime *et al.*, 2007). It is a winter annual and its seeds cannot tolerate submergence (Yatsu *et al.*, 2003). *Cardamine flexuosa* With. is an annual or perennial tetraploid ($2n = 4x = 32$) native to Europe (Lihová *et al.*, 2006), which originated from the cross of *C. hirsuta* and *C. amara* (Grime *et al.*, 2007; Mandáková *et al.*, 2014). Recently, plants that had been identified as *C. flexuosa* in eastern Asia turned out to belong to a distinct species, *Cardamine occulta* (Lihová *et al.*, 2006; Marhold *et al.*, 2016), making the ecology of *C. flexuosa* in Europe yet to be explored.

When not obvious, *C. flexuosa* and *C. hirsuta* were identified using morphological keys (Post *et al.*, 2011). The incidence of backcrossing is seemingly low, if at all possible, as we found none out of a total of 115 plants in the study sites screened for their ploidy level using flow cytometry (CyFlow Space; Sysmex Partec, Goerlitz, Germany; using CyStain UV Precise P reagents, Sysmex Partec). Specimens of the study species collected from the study sites have been donated to Zürich Herbaria, the herbarium of the University of Zurich and ETH Zurich, where they are registered with barcodes Z-000167685 to Z-000167691 for *C. hirsuta*, Z-000167692 to Z-000167697 for *C. flexuosa*, and Z-000167698 to Z-000167704 for *C. amara*.

Study sites

The study was conducted in the seminatural and anthropogenic areas of Irchel (IR), Wehrenbach (WBH), and Küsnacht-Tobel (KT) in and around Zurich, Switzerland (Fig. 1a). In 2013, we selected 18 sites (IR1–9, WBH1–3, KT1–5, 7) from the three areas (Fig. 1b–d). WBH1, WBH2 and WBH3 are separated by a hedge and a paved road. The composition of the species and the numbers of individuals of each species per site are summarized in Table S1. The study sites in 2014 were the same as in 2013, except for site KT6, which was newly added, and IR9, which was excluded because of its disappearance upon changed land use.

Measurement of habitat environment

The measurement was conducted on 17 sites where the species occurred in both 2013 and 2014 (excluding IR9 and KT6; see Study sites section). The data contained nine environmental factors: weight-based soil water content, soil carbon (C) to nitrogen (N) ratio (C/N ratio), soil pH, nitrate (NO_3^-) concentration in fresh soil, ammonium (NH_4^+) concentration in fresh soil, NO_3^- concentration in incubated soil, NH_4^+ concentration in incubated soil, sky openness at the beginning of the season, and sky openness at the end of the season. For the detailed method, see Soil properties and Sky openness in Method S1.

Principal component analysis on habitat

Out of the nine environmental factors measured, NO_{3-} concentration in fresh soil was excluded from analysis because most of the values were zeros and thus were not appropriate for principal component analysis (PCA). We conducted PCA using software R v.3.3.3 (R Core Team, 2017), with the VEGAN package (Oksanen *et al.*, 2017). To examine the extent of variance of habitat environmental conditions of each species, we calculated the root-mean-square error for PC1 values. For each species for each year, we subtracted the mean PC1 value from the PC1 value for each site, squared the outcome, summed the squared values to be divided by the number of sites, and then took the root of it.

Logistic regression based on data on species occurrence and environmental factors

To examine the contributions of environmental conditions to the occurrence of each species in each year, we performed logistic regression analyses using PROC LOGISTIC in SAS v.9.3 (SAS Institute Inc., Cary, NC, USA). With the presence or absence in a given site as response variable, we ran 255 logistic regressions consisting of all possible combinations of the eight of the nine environmental factors as explanatory variables. Sky openness at the end of the season was excluded from this analysis because of high correlation with sky openness at the start of the season (Table S2). Out of the 255 models examined, the model with the lowest Akaike information criterion (AIC) value was considered as the best one. We assessed the significance of the explanatory variables in the best models using the log-likelihood ratio test (Littell *et al.*, 1996).

Soil moisture monitoring

For regular nondestructive data collection, we monitored soil moisture weekly during growing seasons in 2013 and 2014 using an electrical sensor instead of sampling soil at every census. See Method S1 for details.

Reference-guided assembly of *C. amara* genome and RNA sequencing

We used the published genome sequence of *C. hirsuta* (Gan *et al.*, 2016) and our *C. amara* genome sequence constructed by reference-guided *de novo* assembly based on *C. hirsuta* genome according to Lischer & Shimizu (2017). For the genome sequences, one specific line of *C. amara* from Switzerland (lab

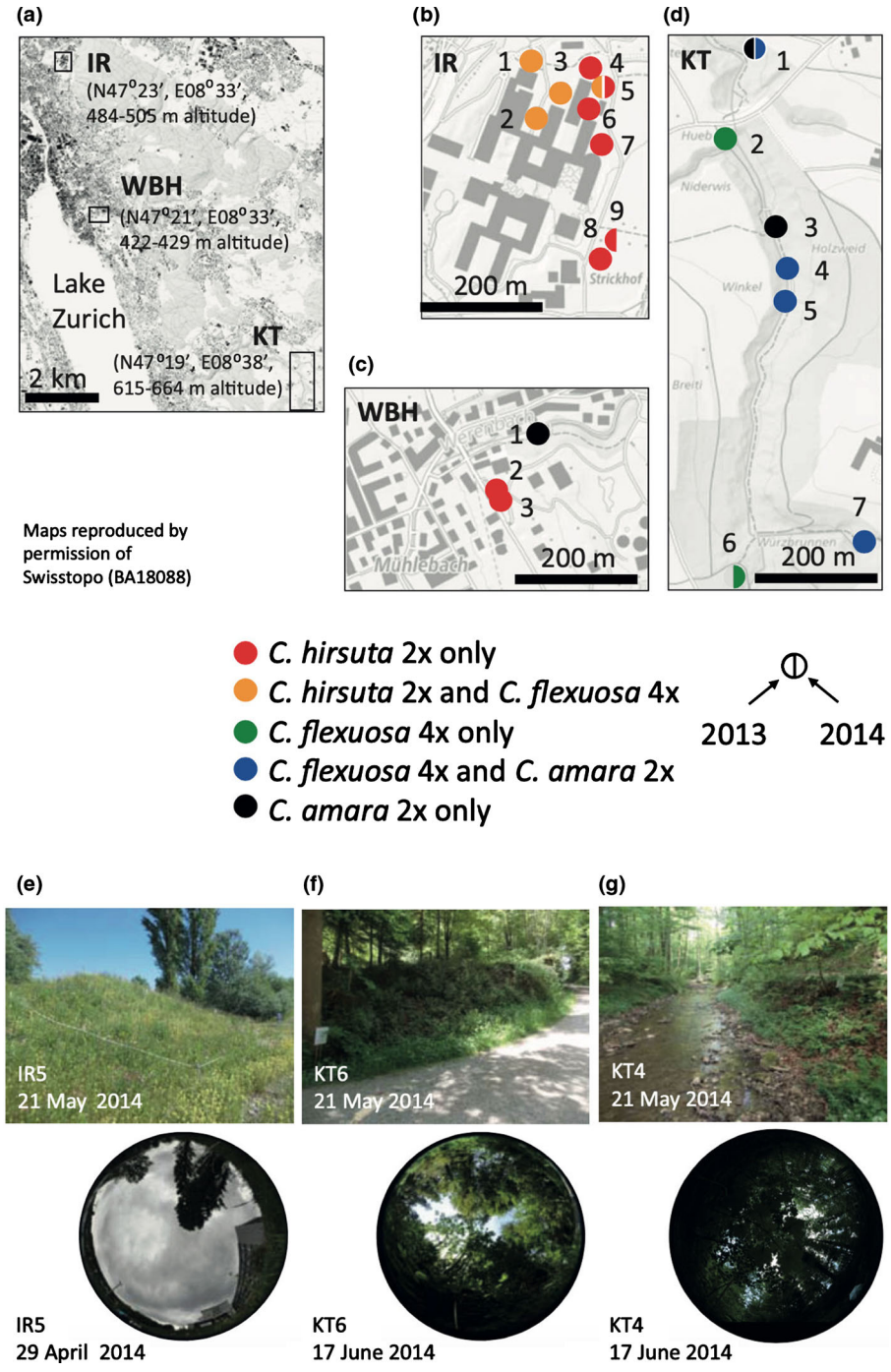


Fig. 1 Locations, species compositions, and photographs of representative sites of *Cardamine amara*, *Cardamine hirsuta*, and *Cardamine flexuosa*. (a) Locations of the study areas Irchel (IR), Wehrenbach (WBH), and Küsnacht-Tobel (KT) in and around Zurich, Switzerland. (b) Locations of sites at IR. (c) Locations of sites at WBH. (d) Locations of sites at KT. (e) The *C. hirsuta* site. (f) The *C. flexuosa* site. (g) The *C. amara* site. In (b–d), different colors of points indicate different species composition: red, *C. hirsuta* only; orange, *C. hirsuta* and *C. flexuosa*; green, *C. flexuosa* only; blue, *C. flexuosa* and *C. amara*; and black, *C. amara* only. The left and right halves of the circles represent the years 2013 and 2014, respectively, for the sites where the species composition differed between years. In (e–g), an overview (upper panels) and a hemispheric photograph used for light availability analysis (lower panels) are shown with date and site name.

ID: WEH ATS1) was clonally propagated and used for DNA extraction. We sequenced 368 million paired-end reads (three independent libraries with different insertion sizes of 150–-500 bp) and 548 million mate-pair reads (four independent libraries with different insertion sizes from 3–15 kb). To annotate genes on *C. amara* genome, RNA samples from eight organs (anther, filament, sepal, petal, pistil, flower bud, young flower bud, and leaf) of another individual from Switzerland (lab ID: 3-

19A) were sequenced. All sequence information used for assembly and annotation has been deposited in the DNA Data Bank of Japan (DDBJ; http://www.ddbj.nig.ac.jp) as DRA Accession ID: DRA006316, BioProject ID: PRJDB4989, BioSample ID: SAMD00098907. Details of the *de novo* genome assembly and gene annotation by AUGUSTUS (Stanke, 2014) are in Method S1.

To identify homologous genes of *Arabidopsis thaliana*, we applied a reciprocal best-hit method. We aligned gene regions of the assembled genome of *C. amara* onto *A. thaliana* gene regions (TAIR10: https://www.arabidopsis.org) using BLASTN+ v.2.2.30 (Altschul *et al.*, 1990) with 1×10^{-6} as a cutoff of *E*-value max_target_seqs was set at 5, yielding five hits that exceeded the cutoff *E*-value. Among those five, the *A. thaliana* gene with the smallest *E*-value and largest bit score was defined as the homologous gene of the *C. hirsuta* gene. We identified 29 458 homeolog pairs (Gene Set 1) annotated in both the *C. amara* genome and the *C. hirsuta* genome to be used for RNA sequencing (RNA-seq) analysis.

Tissue sample collection and RNA-seq

We selected three sampling sites in IR1, KT2 and KT5, where *C. flexuosa* coexisted with *C. hirsuta*, grew on its own, and coexisted with *C. amara*, respectively. Soil moisture was distinct for these sites (Fig. S2), allowing the comparison of the expression pattern of *C. flexuosa* among the dry site cohabited by one diploid, the intermediate site, and the wet site cohabited by the other diploid. Table S3 summarizes the growth stage of three individuals of each species at each sampling site and date of sampling, that are treated as biological replicates. Leaf tissue of average-sized *C. flexuosa* was collected from the same three individuals on 18 April, 2 May and 16 May in 2013. The samples of the diploid progenitor species were analyzed for only 2 May, as *C. hirsuta* dried up by 16 May and *C. amara* was too small for sampling on 18 April and was lost to flooding after 2 May (Tables S3, S4). At each site, one individual beside a spot for soil moisture monitoring was marked at the beginning of the season. The samplings were conducted between 10:00 h and 14:00 h on sunny days to reduce any variation introduced by nonquantified stimuli and circadian rhythm (Alvarez *et al.*, 2015). The leaf samples were stored in a tube with RNAlater (Qiagen), brought back to the laboratory and kept at 4°C overnight. Once RNAlater infiltrated through leaves, the sample tubes were stored at −80°C until RNA extraction by RNeasy Plant Mini Kit (Qiagen). Total RNA was used for the library synthesis using TruSeq Stranded mRNA Library Prep Kit (Illumina, San Diego, CA, USA) and sequenced using an Illumina HiSeq2500 at the Functional Genomics Center Zurich. On average, 7.8 million reads per sample were obtained from 32 samples (Table S4). RNA-seq reads with low quality were trimmed with TRIMMOMATIC (v.0.36) (Bolger *et al.*, 2014) and then mapped to the *C. amara* (described earlier herein) and *C. hirsuta* (Gan *et al.*, 2016) genomes with STAR (v.2.5.3a; Dobin *et al.*, 2013) to classify the origins with HOMEOROQ (Akama *et al.*, 2014; Kuo *et al.*, 2020). We counted the read number on each homeolog with FEATURECOUNTS (v.1.6.0; Liao *et al.*, 2014) and calculated the expression proportion of the homeolog pairs (Fig. S3; Table S4). Sequence information has been deposited in the DDBJ (http://www.ddbj.nig.ac.jp; DRA accession ID: DRA006314; BioProject ID: PRJDB4989; BioSample ID: SAMD00097398–-SAMD00097406).

PCA by subgenomes

We analyzed the expression level of each homeolog of *C. flexuosa* as well as the ortholog of cohabiting diploid progenitors, *C. hirsuta* at IR1 and *C. amara* at KT5 on 2 May. We calculated the fragments per kilobase per million (FPKM) of each homeolog and filtered out the homeolog pairs in which both homeologs had FPKM < 1. This process left 23 182 homeolog pairs out of Gene Set 1. We analyzed the homeologs in each subgenome and the orthologs in each progenitor genome by PCA, treating one ortholog/homeolog at each site (IR1 or KT5) on each date (18 April, 2 May, and 16 May for *C. flexuosa*, and 2 May for *C. hirsuta* and *C. amara*) as one sample (three samples; i.e., replicates per site per date), using software R v.3.5.0 (R Core Team, 2017), with prcomp in the STATS package.

Genes with homeolog expression ratio change and Gene Ontology analysis

We examined the changes in the ratio (or bias) of homeolog expression levels of *C. flexuosa* between sites or dates as follows. The homeolog expression ratio H is expressed by:

$$H(g_a, g_h) = \frac{g_a}{g_a + g_h}$$

(g_a and g_h, expressions (FPKM) of homeologous genes derived from *C. amara* and *C. hirsuta*, respectively). Whether the homeolog expression ratio differed among all pairs of the three sites (IR1 vs KT2, KT2 vs KT5, and KT5 vs IR1) on each sampling date (18 April, 2 May, or 16 May) or among all pairs of the three dates (18 April vs 2 May, 2 May vs 16 May, and 16 May vs 18 April) on each sampling site was tested using the HOMEOROQ pipeline (Akama *et al.*, 2014), with false discovery rate < 0.05 as the threshold for significance. Among Gene Set 1, we only analyzed genes whose SD among the sum of the biological replicates of the pair of concern was < 0.3 to remove false positives, according to the standard setting of HOMEOROQ. We also excluded extremely poorly expressed genes (FPKM < 0.2) from the analysis. This left 18 855 genes after filtering by SD and FPKM and subject to analysis to detect which genes showed significant homeolog expression ratio change between site-pairs or date-pairs and which genes did not. The analysis was performed using the SUSHI framework (Hatakeyama *et al.*, 2016). Dataset S1 summarizes the results.

At the next step, a Gene Ontology (GO) enrichment analysis of the biological process was conducted using TOPGO v.2.32.0 with the elim algorithm at a significance level of $P = 0.05$ (Alexa & Rahnenfuhrer, 2016) for each site-pair on each sampling date to examine which gene categories are overrepresented. Gene Set 1 was targeted as parent population. Dataset S2 shows the results of

all comparisons. GO terms with < 10 or > 500 annotated genes were excluded from interpretation.

Results

Habitat of the allopolyploid *C. flexuosa* and its diploid progenitor species

To characterize the habitat of the three species in this study, the relative contributions of soil properties and light availability to habitat were evaluated by PCA, which suggested three differences among the species. First, sites of two diploid progenitor species, *C. hirsuta* (red border encompassing red and orange points) and *C. amara* (blue border encompassing black and blue points), were separated (Fig. 2). The separation along the PC1 axis indicates that *C. amara* sites exhibited high soil water content and soil C/N ratios (Fig. 2; Fig. S4a,b) as well as a low degree of sky openness (Fig. 2; Fig. S5) compared with *C. hirsuta* sites. The high C/N ratios in *C. amara* sites likely reflect low N concentrations in fresh and incubated soils (Fig. 2; Fig. S4c–f). Second, in the PCA plot, sites of the allopolyploid *C. flexuosa* (green border encompassing orange, green, and blue points) overlapped with both diploid progenitor species, reflecting the coexisting sites. Importantly, the sites cohabited by *C. flexuosa* and *C. hirsuta* (orange dots) tend to be in the middle of the PC1 axis compared with sites with *C. hirsuta* alone (red dots). A similar tendency, although weaker, was found with *C. amara* (more pronounced in properties such as soil water content – see later and Fig. S4a). Third, compared with diploid progenitor species, the value range for PC1 was wider for the *C. flexuosa* sites. This was supported by larger root-mean-square error for PC1 values, which shows the average variance over samples: *C. flexuosa*: 0.68 and 0.54 in 2013 and 2014, respectively; *C. hirsuta*: 0.49; *C. amara*: 0.27; the same value for both years for diploid progenitor species due to consistency in the sites of occurrence.

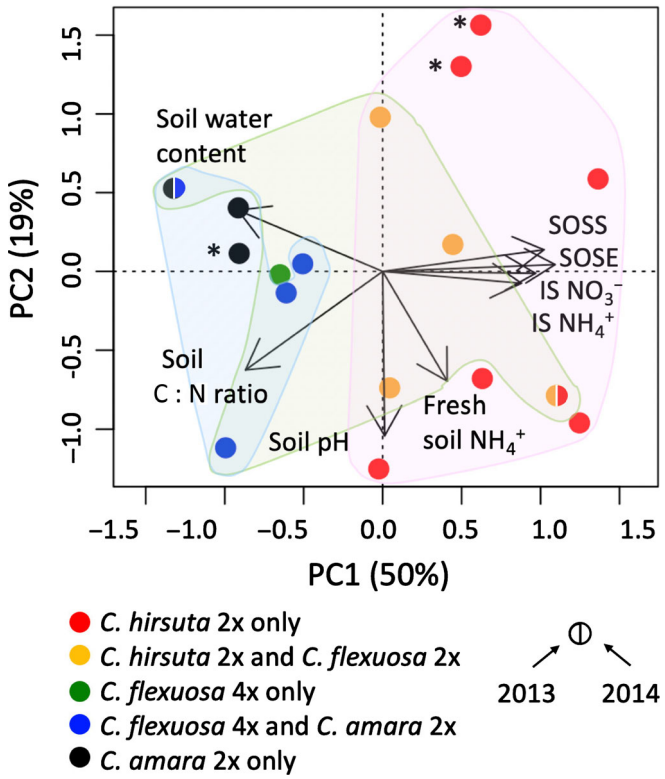


Fig. 2 The results of a principal component analysis of environmental factors from 17 sites of *Cardamine amara*, *Cardamine hirsuta*, and *Cardamine flexuosa* that were subject to the analysis. The relative impacts of different factors are illustrated as vectors. SOSS, sky openness at season start; SOSE, sky openness at season end; IS NO_3^-: incubated soil nitrate; IS NH_4^+: incubated soil ammonium; soil C/N ratio: soil carbon-to-nitrogen ratio. The first two principal component (PC) axes accounted for 50% and 19% of total variation, respectively. Different colors of the points indicate different species composition: red, *C. hirsuta* only; orange, *C. hirsuta* and *C. flexuosa*; green, *C. flexuosa* only; blue, *C. flexuosa* and *C. amara*; and black, *C. amara* only. Blue, green, and red background colors indicate the sites with *C. amara*, *C. flexuosa*, and *C. hirsuta*, respectively. The color of the point is halved for sites with species composition being different between 2013 and 2014. Three sites in the Wehrenbach area are indicated with asterisks.

Three sites in the area of WBH (indicated with asterisks in Fig. 2) exemplify the importance of the fine-scale environments. One was occupied by only *C. amara* (black point, WBH1) and the other two by only *C. hirsuta* (red points, WBH2 and 3). Given these sites were in close vicinity, this separation would not have been detected at a resolution of > 1 km^2 (Fig. 1a,c).

By examining each environmental factor separately, we found that the extent of overlap of the habitat of *C. flexuosa* and its diploid progenitor species varied among factors (Figs S4, S5). *Cardamine flexuosa* was in an intermediate value range, e.g. for soil water content (*C. amara* 30–60%, *C. flexuosa* 20–40%, *C. hirsuta* 20–35%, Fig. S4a) and sky openness (*C. amara* 5–35%, *C. flexuosa* 5–40%, *C. hirsuta* 10–70%, Fig. S5), all of which contributed to PC1. Therefore, soil water content, soil C/N ratio, and light availability seemed to correlate with habitat differentiation among species, and the habitat of *C. flexuosa* generally overlapped with that of the two diploid progenitor species in the intermediate value range.

Relative contributions of environmental factors to the occurrence of the study species

In all species, the best model with the lowest AIC to predict the occurrence of the species included water content, C/N ratio, and sky openness at season start. Importantly, the absolute value of the regression coefficient was the largest for water content, for the diploid progenitor species, and for the allopolyploid when sites in which *C. flexuosa* occurred in 2013 were concerned (Table 1). These results indicate that the three species share environmental factors that determine habitat differentiation and that soil water content is the most influential determinant.

The directions of the regression coefficients for the three factors differed among species (Table 1). The occurrence of *C. amara* was associated positively with water content and C/N ratio and negatively with sky openness at the season's start. By contrast, the occurrence of *C. hirsuta* was associated with the three factors in the opposite direction. In *C. flexuosa*, the

Table 1 Effects of the environmental factors in the best model on the occurrence of *Cardamine amara*, *Cardamine hirsuta* and *Cardamine flexuosa* in 2013 and 2014, analyzed with logistic multiple regression ($n = 17$).

			Water content		C/N		Sky openness at season start	
Species (ploidy level)	Year	AIC	$\beta \pm$ SE	χ^2	$\beta \pm$ SE	χ^2	$\beta \pm$ SE	χ^2
C. amara (2x)	2013, 2014	−2.34	0.11 ± 0.11	8.14**	0.06 ± 0.06	7.53**	−0.01 ± 0.06	5.68*
C. hirsuta (2x)	2013, 2014	−1.98	−0.10 ± 0.10	7.35**	−0.04 ± 0.05	6.86**	0.04 ± 0.06	7.01**
C. flexuosa (4x)	2013	6.38	−0.08 ± 0.08	5.25**	−0.005 ± 0.04	6.25*	−0.08 ± 0.05	10.22**
	2014	4.46	0.03 ± 0.06		−0.02 ± 0.04	6.32*	−0.08 ± 0.05	11.34***
	2013 and 2014	3.23	−0.06 ± 0.07	5.64*	−0.02 ± 0.04	6.02*	−0.10 ± 0.06	13.39***
	2013 or 2014	7.72	0.003 ± 0.06	5.56*	−0.003 ± 0.04	6.32*	−0.06 ± 0.04	8.76**

The site of occurrence was consistent in the two years for *C. amara* and for *C. hirsuta*. As *C. flexuosa* occurred in only one of the two years in some sites, we ran four models with different response variables: occurrence in 2013, occurrence in 2014, occurrence in both 2013 and 2014, and occurrence in either 2013 or 2014. Water content and carbon-to-nitrogen ratio (C/N) were measured for soil. For each model, Akaike information criterion (AIC), partial regression coefficient $\beta \pm$ SE for each variable, and chi-square value (χ^2) and significance (*) are given. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

directions of these regression coefficients consisted of a combination of those of its diploid progenitor species. For water content, negative and positive values were observed in 2013 and 2014, respectively, coinciding with the exclusion and inclusion of sites with extreme soil moisture (filled and open arrows in Fig. 3b,f; Table 1). Furthermore, the values of the regression coefficients for water content and C /N ratio of *C. flexuosa* were intermediate between those for *C. amara* and *C. hirsuta*. Hence, these quantitative analyses support the difference of the three species in the PCA plot (Fig. 2), in which the habitat of the two diploid progenitor species are distinct, and that of the allopolyploid *C. flexuosa* partly overlapped with those of its progenitors at the intermediate range.

Temporal fluctuation of the soil moisture

Because the key environmental factor for determining niche divergence turned out to be water content, which likely fluctuated naturally, we examined whether temporal patterns of habitat water availability varied among species. In 2013, the habitat's level of soil moisture for the diploid progenitor *C. amara* was high, in contrast to the other diploid progenitor *C. hirsuta* (Fig. 3a,c). At any given time, the level of soil moisture of sites inhabited by only *C. amara* (black points in the figure) and by only *C. hirsuta* (red points in the figure) did not overlap with each other. The habitat's level of soil moisture for the allopolyploid *C. flexuosa* overlapped partly with either of the diploid progenitor species in the intermediate range, but not at the high or low ends (Fig. 3b). The level of soil moisture in 2014 was generally similar to that in 2013, although at the beginning of the season the levels of soil moisture of the *C. amara* and *C. flexuosa* habitats were lower than they were in 2013 (Fig. 3a,b,e,f). Furthermore, the soil moisture range throughout the season was largest for *C. flexuosa* in both 2013 and 2014 (Fig. 3d,h). Thus, the habitats of *C. flexuosa* had a broad range of soil moisture overlapping with two diploid progenitor species at the intermediate range. The observed niche divergence along soil moisture provided a basis for studying homeolog expression patterns of *C. flexuosa* in comparison with its diploid progenitor species along a natural environmental gradient.

PCA of homeolog expression of *C. flexuosa* and its diploid progenitor species

To compare the whole genome gene expression pattern in the allopolyploid and diploid progenitor species, we analyzed RNA-seq data of *C. flexuosa* at three times at three sites with contrasting soil moisture levels, together with the cohabiting diploid progenitor species, *C. hirsuta* at IR1 and *C. amara* at KT5, on 2 May (Fig. S2; Tables S3, S4). The number of reads classified into each subgenome was almost equal in all *C. flexuosa* samples (Fig. S3), indicating no clear expression bias at subgenomic level.

When we treated the sum expression of two homeologs as gene expression and compared the pattern with that of the diploid progenitor species by PCA, the whole-genome pattern showed a loose cluster for each species, but the pattern was not clearly correlated with site (Fig. S6). We then examined the expression pattern of the subgenomes separately. Within *C. flexuosa* individuals, the two subgenomes were clearly divided into two large groups by PC1 with 30% contribution rate, and the diploid genomes were located close to each child subgenome of the allopolyploid (Fig. 4a). PC2 roughly grouped each subgenome by date with 13% contribution rate, from early (downside) to late (upside) date. In the PC2 and PC3 plots, each study site formed a loose group regardless of subgenomes (Fig. 4b). Similarly, the resemblance to the expression pattern of the progenitors was detected when the expression level of each homeolog of *C. flexuosa* was compared with that of the diploid progenitor species by clustering analysis (Fig. S7).

Genes with homeolog expression ratio changes between two homeologs and GO analysis

To examine the number of genes associated with habitat in the allopolyploid, we further analyzed the RNA-seq data of *C. flexuosa* to extract homeolog pairs with significant ratio change among habitats, assuming that they reflect the difference in key environmental conditions. We examined *c.* 20 000 homeolog pairs (Table S4; Dataset S1), which were nearly twice as many as the number of genes examined in the previous study (Shimizu-Inatsugi *et al.*, 2017). The number of genes with homeolog

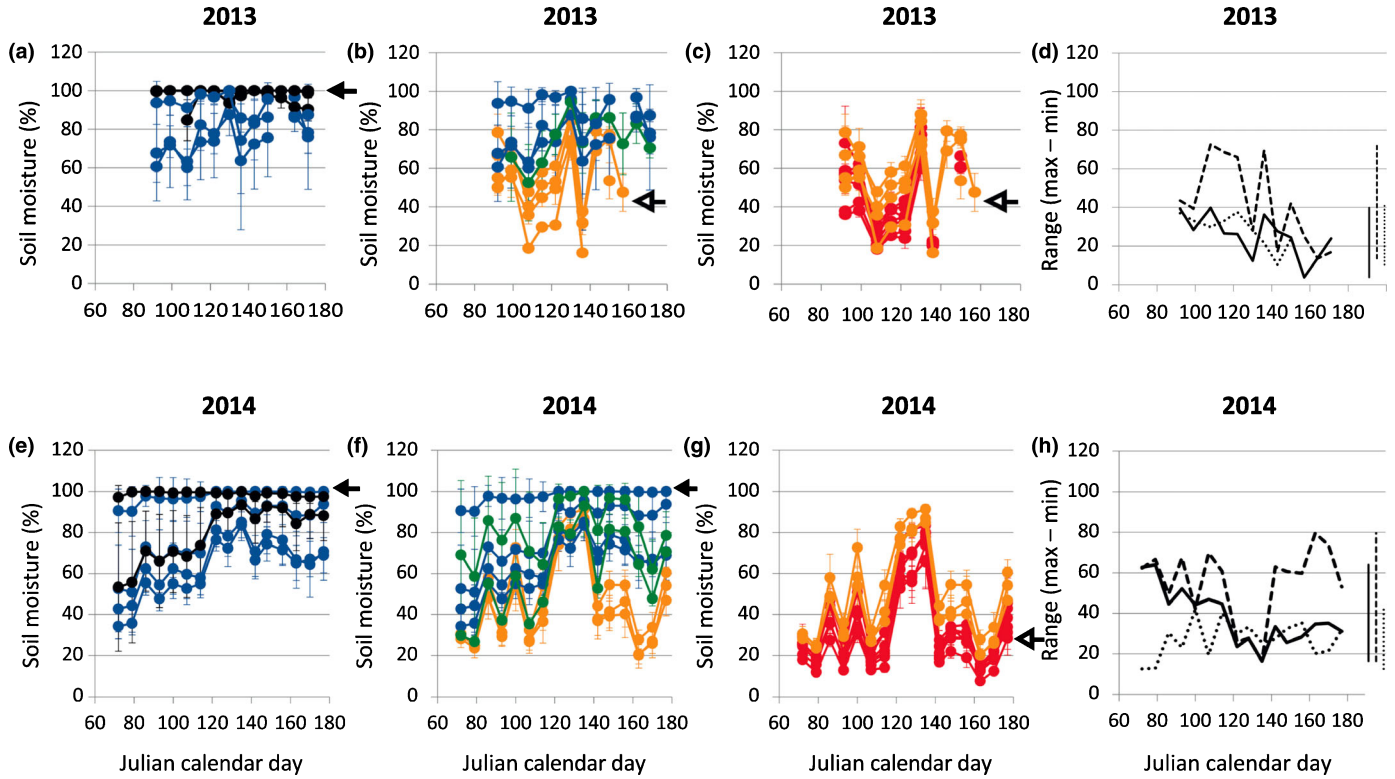


Fig. 3 The mean ± SD and the range (maximum–minimum) of soil moisture of *Cardamine amara*, *Cardamine hirsuta*, and *Cardamine flexuosa* sites during growing seasons in (a–d) 2013 and (e–h) 2014 measured with a soil moisture sensor at each site ($3 \leq N \leq 9$ per site). (a, e) Mean soil moisture of sites with *C. amara*. (b, f) Mean soil moisture of sites with *C. flexuosa*. (c, g) Mean soil moisture of sites with *C. hirsuta*. In (a–c) and (e–g), the color coding of points corresponds to that in Figs 1, 2, and open and filled arrows indicate the IR5 and KT1 sites, respectively. IR, Irchel; KT, Küsnacht-Tobel. (d, h) The range of soil moisture on census dates and for the entire growing season (vertical bars on the right) for each species; solid, dotted, and finely dotted lines indicate *C. amara*, *C. flexuosa*, and *C. hirsuta*, respectively. In 2013, *C. flexuosa* did not occur in the wettest site, KT1, inhabited by *C. amara* (a, filled arrowhead) but appeared in the driest site, IR5, inhabited by *C. hirsuta* (b, c, open arrowheads). The opposite was the case in 2014 (e–g).

expression ratio change differed among site-pair by date combinations and constituted 0.9–3.3% of all genes (Fig. 5a; Table S5). There was little overlap between site-pairs on a given date or between dates of a site-pair (Fig. 5c,d). Similarly, date-pair by site combinations constituted 0.2–3.0% of all genes, with little overlap between time points at a given site or between sites of a given date-pair (Fig. 5b,e,f; Table S6). The smallest number of genes was detected to be different between KT2 and KT5 on any date, and the biggest difference was found between IR1 and KT2 (Fig. 5a; Table S5). IR1 showed the largest difference among dates, coinciding with the largest temporal change of the soil moisture level at this site (Figs 5b, S2; Table S6).

The GO analyses of the genes with homeolog expression ratio change identified 15 to 72 GO terms enriched in each site-pair by date combination (Dataset S2). Among these GO terms, three terms, GO:0009414 (response to water deprivation), GO:0006979 (response to oxidative stress), and GO:0000302 (response to reactive oxidative species), were detected in the highest proportion; that is, in six site-pair by date combinations (Fig. 6; Table S7). GO:0006833 (water transport) was found in five combinations (Table S7).

Given that the genes included in GO:0009414 could be most relevant to the fluctuating soil moisture, we examined the expression pattern of the 32 genes in GO:0009414 (Table S8). Different homeologous pairs changed the ratio depending on sites and times (Fig. S8). To illustrate different patterns of expression changes, we visualized the expression levels of the 32 genes together with those of cohabiting diploids in IR1 and KT5 (Fig. S9). In the gene CARHR155190, a homologue of *Arabidopsis RDUF1* responsible for the response to a plant hormone ABA (Kim *et al.*, 2012), F^a (*C. amara* homeolog of *C. flexuosa*) had low expression at KT5. In the genes CARHR073790 and CARHR261630, the expression levels of F^h (*C. hirsuta* homeolog of *C. flexuosa*) and F^a were similar to those of the cohabiting diploid. Which of F^a and F^h was more expressed than the other differed between IR1 and KT5 in the genes CARHR261630 and CARHR000120, whereas this was not the case in most other genes. These results suggest no single dominant pattern of homeolog regulation in the natural environments.

Discussion

Fine-scale niche acquisition by the allopolyploid *C. flexuosa* by combining the legacy of the progenitors

We documented the various environmental factors of the habitats of the allopolyploid and its diploid progenitor species (Figs 2, 3,

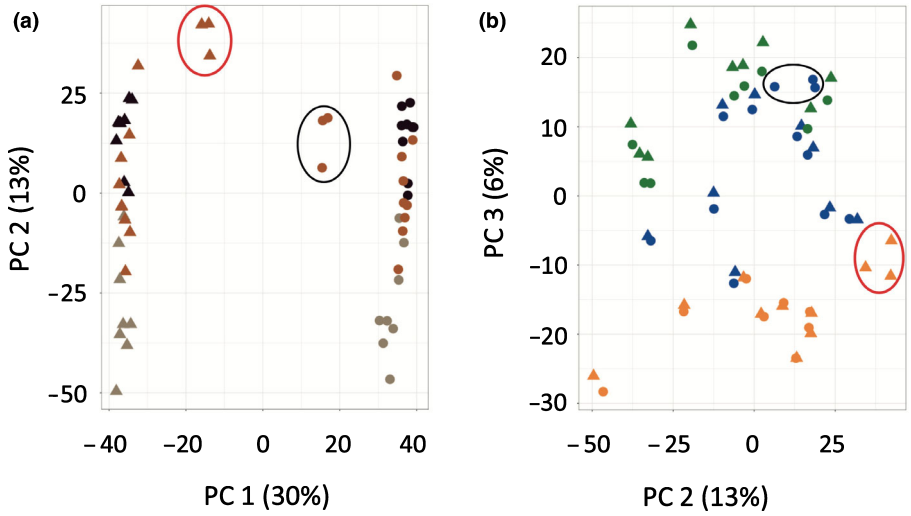


Fig. 4 The plot of (a) principal component (PC)1 and PC2 and (b) PC2 and PC3 of the result of a principal component analysis of 23 182 homeolog or ortholog expression in *Cardamine flexuosa* and its diploid progenitor *Cardamine amara* and *Cardamine hirsuta*. Points indicate *C. flexuosa* A (*C. amara*-type) subgenome and *C. amara* (circled in black), whereas triangles indicate *C. flexuosa* H (*C. hirsuta*-type) subgenome and *C. hirsuta* (circled in red). Different colors in (a) indicate different dates: light brown, 18 April; brown, 2 May; dark brown, 16 May. The colors in (b) correspond to the sites in Fig. 1.

S4, S5; Table 1). Together with previous growth experiments with water stress treatments (Shimizu-Inatsugi *et al.*, 2017), these data suggest the following information on niche divergence of the two diploid progenitor species in *Cardamine* and the allopolyploid derived from them. First, the habitats of the two diploid species are distinct. The environmental conditions of the two species were separated in the PCA (Fig. 2), among which soil water content contributed the most to habitat differentiation (Table 1). The temporal data of water availability (Fig. 3) indicated that the habitats of *C. amara* and *C. hirsuta* were wet and dry, respectively. Second, the habitat of the allopolyploid was broad, as indicated by the larger root-mean-square error along PC1 of Fig. 2, but the overlap with the progenitors was limited to the intermediate range, and thus excluded extremely wet or dry conditions (Fig. 2; Table 1). The broad range itself does not guarantee that the allopolyploid establishes its own niche. In the case of *C. flexuosa*, the temporal monitoring of habitat water availability revealed high fluctuation (Fig. 3), suggesting that the adaptation to a broad range of conditions can be advantageous in acquiring robustness in fluctuating environments. Consistent with this observation, *C. flexuosa* grew similarly well under the submergence, drought, and the fluctuation of the two treatments in the laboratory experiment (Shimizu-Inatsugi *et al.*, 2017). Given these data, the allopolyploid *C. flexuosa* seems to have obtained a new distinct niche with fluctuating water availability (Fig. 3), which is in accordance with a previous record on occurrence along water gradient in Britain (Grime *et al.*, 2007).

Empirical information lies central in the current study. As such, the scale of our data set corresponds to what manual data collection in the field can cover and does not suffice for niche modeling. Despite the difference in approaches, there is an intriguing similarity in the findings from current and previous studies. In this study, PCA and multiple regression on empirical data of habitat at fine scale detected the allopolyploid occupying intermediate and broader environmental conditions. Previous studies using ecological niche modeling at continental scale reported intermediacy (Theodoridis *et al.*, 2013; Harbert *et al.*, 2014; Lopez-Alvarez *et al.*, 2015; Marchant *et al.*, 2016) or broader niche (McIntyre, 2012; Lopez-Alvarez *et al.*, 2015; Marchant *et al.*, 2016; Huynh *et al.*, 2020). In contrast to previous studies, we observed species occurrence and habitat at small geographical scale. In some cases, the species segregated < 1 km^2 in a contrasting habitat; for example, *C. amara* in WBH1 vs *C. hirsuta* in WBH2 and 3 (Fig. 1). We did not capture this habitat differentiation at a larger geographic scale (IR, WBH, and KT; Fig. 1a). It is noteworthy that the significance of fine spatial scale has been recently highlighted in ecological niche modeling (Mertes & Jetz, 2018; Graham *et al.*, 2019). Ecological niche modeling may also benefit from incorporating more diverse environmental factors. In this study, soil properties, light availability, and fluctuation of water availability during growing seasons were associated with the occurrence of the study species (Figs 2, 3, S4, S5 Table 1), confirming the importance of these factors in niche divergence (Silvertown, 2004). Overall, our results underscore that empirical data on environmental factors contributing to species occurrence at a fine spatial scale are informative.

Expression pattern of homeologs in the allopolyploid *C. flexuosa* associated with the key factor in niche divergence

The comparison between transcriptomes of the diploids and the allopolyploid has shown that the most dominant factors shaping expression patterns were genome type and date, and the effect by site was less dominant (Figs 4, S6). This suggests that most homeologs in the allotetraploid are strongly regulated by the legacy of progenitor genomes and subsequently by growing stage

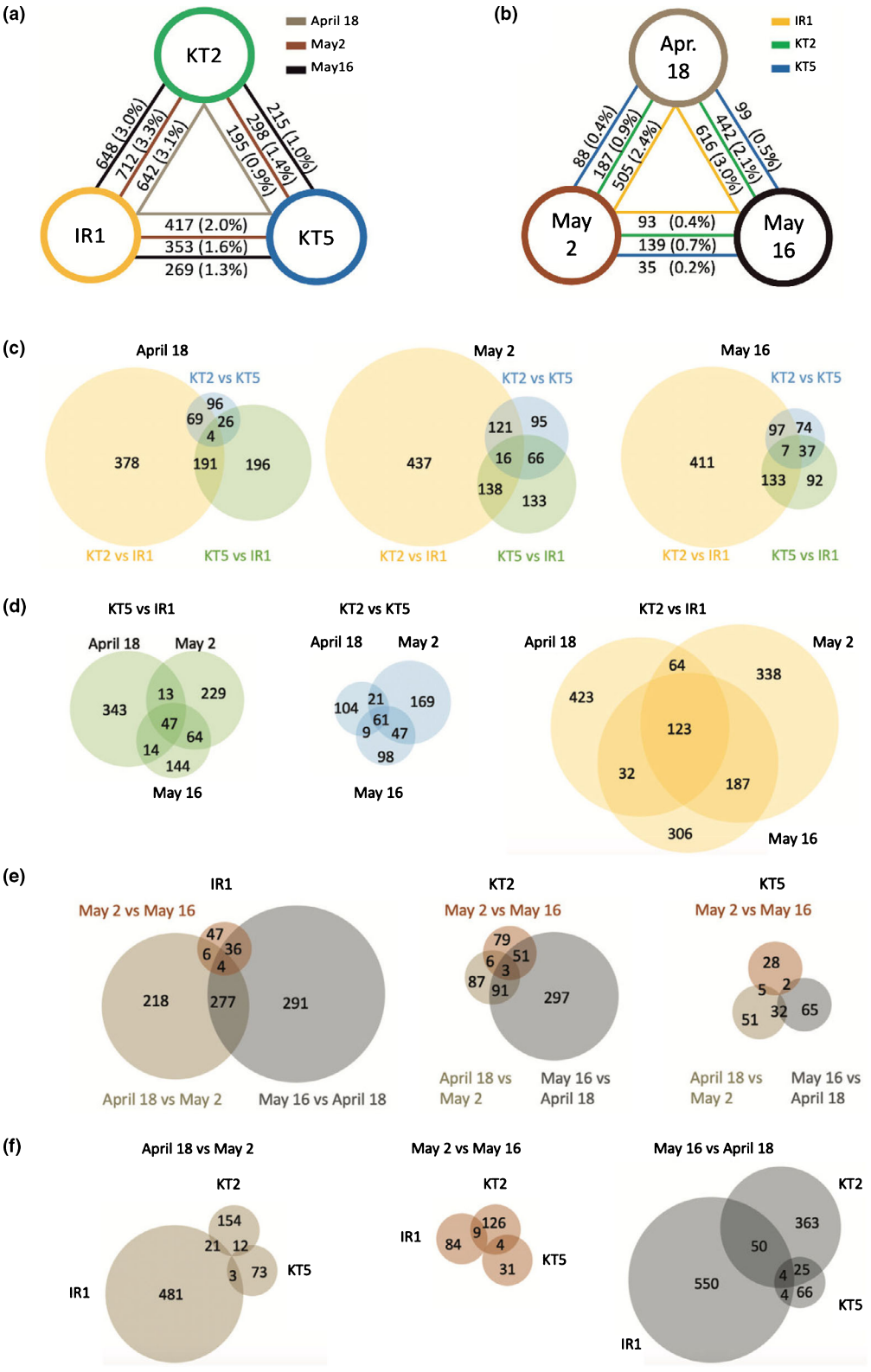
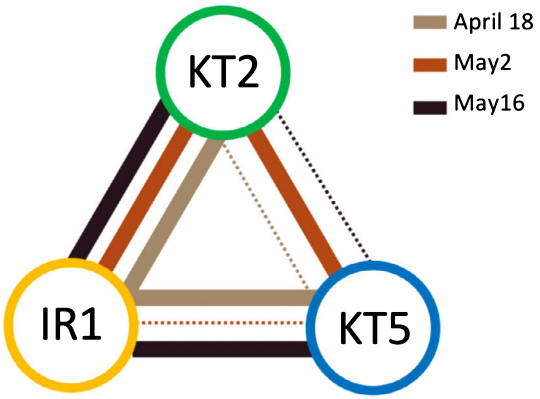
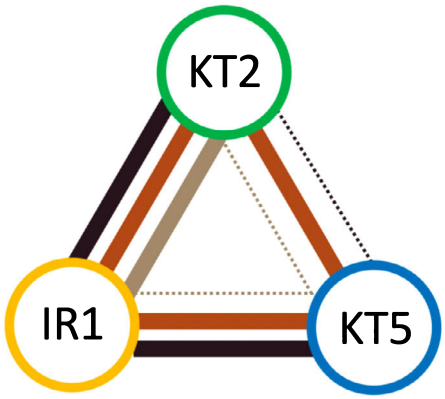


Fig. 5 The numbers and proportional Venn diagrams for the number of genes with homeolog expression ratio change in *Cardamine flexuosa*. (a, b) A summary of the number of genes with homeolog expression ratio change (a) between sites (IR, Irchel; KT, Küsnacht-Tobel) and (b) between dates among 23 182 homeologous pairs analyzed. Different sites and dates are indicated with different colors: IR1 in orange, KT2 in green, and KT5 in dark blue; light brown, 18 April; brown, 2 May; dark brown, 16 May. (c) The number of genes overlapping between site-pairs on each date. (d) The number of genes overlapping between dates for each site-pair. (e) The number of genes overlapping between date-pairs on each site. (f) The number of genes overlapping between sites for each date-pair. In (c–f), the size of circles with less than 190 genes is disproportionally enlarged to make numbers visible.



GO: 0009414 response to water deprivation



GO: 0006979 response to oxidative stress
GO: 0000302 response to reactive oxygen species

Fig. 6 Schematic diagram of the Gene Ontology (GO) categories detected at highest proportion for site-pair by date combination by analysis on genes of *Cardamine flexuosa* with homeolog expression ratio change using HomeoRoq. Bold lines between sites indicate that the term was significantly enriched between them at each date, and dotted lines indicate no enrichment.

or season, and the contribution of other nonseasonal environmental factors is weaker compared to them. Considering the separation between diploids along environmental gradients, the key genes related to nonseasonal environmental fluctuation may show a distinct expression pattern between homeologs among sites. In the current study, the number of homeolog pairs with significant expression ratio change among sites constituted of only a few (0.9–3.3%) of total pairs (Fig. 5; Table S5), and many of them were related to water stress and oxidative stress, as shown by the GO analyses (Table S7). Therefore, the response to environmental fluctuation may be affected by a relatively small group of genes that alters the homeolog expression ratio and possibly also by genes whose total expression level of both homeologs changes among sites, without a difference between homeologs.

It is beyond the scope of this study to confirm the role of the genes with homeolog expression ratio change in niche divergence. Yet, some GO terms of the genes detected by HomeoRoq (Table S7) were associated with water availability, the key environmental factor for niche divergence (Table 1). Possibly, homeolog expression-ratio-change genes in GO:009414 (response to water deprivation) are involved in responding to key environmental factors, even though there is no dominant pattern of homeolog regulation among them (Fig. S9), because they may be attributed to the genes of diverse functions (e.g. drought response and submergence tolerance). GO:0009414 was the same in our HomeoRoq analyses as in laboratory experiments manipulating water availability in *A. thaliana* (e.g. Li *et al.*, 2008; Lowry *et al.*, 2013; Yang *et al.*, 2014). Therefore, seemingly, genes covered by GO:0009414 are commonly used in water responses in a range of environments in multiple species. These genes might have also been involved in the niche diversification between *C. amara* and *C. hirsuta*, and thus in the establishment of *C. flexuosa* in habitats of a wider range of water availability between progenitors. Diversification of the total expression levels as the result of differentially expressed homeologs might have conferred the plasticity for *C. flexuosa* to persist in a broad range of water availability.

The allopolyploid species obtained a generalist niche by transcriptomic plasticity

A fundamental question on niche divergence is how the species coexist (Silvertown, 2004). We propose two mutually nonexclusive reasons for the coexistence of the trio of *Cardamine* species. First, unlike diploid progenitors, *C. flexuosa* can thrive in fluctuating environments, probably thanks to the transcriptomic plasticity achieved by the combination of two progenitor genomes specialized in contrasting environments (Madlung, 2013; Paape *et al.*, 2016). The majority of the homeolog pairs did not change the ratio between site-pairs or time points in the current study; however, those with the ratio change showed little overlap across sites or over time (Fig. 5c–f; Tables S5, S6). This suggests that the allopolyploid flexibly expresses diverse homeologs at a given place or time in responding to fluctuating environment. Second, although *C. flexuosa* can occur in both wet and dry habitats, it may not be able to withstand extreme environments as much as the diploid progenitor species can. This can be attributed to the diploid progenitor species being better adapted to their respective niches compared with the allopolyploid, and thus the allopolyploid could not compete/displace its progenitor species from those niches (e.g. Lopez-Alvarez *et al.*, 2015). It can also be due to attenuated response of the allopolyploid to environments at the transcriptomic level. Indeed, a previous transcriptomic study showed that the degree of upregulation of stress response genes was, in general, lower in *C. flexuosa* than in the advantageous diploid (Shimizu-Inatsugi *et al.*, 2017). Thus, it seems that the allopolyploid *C. flexuosa* is a generalist, both ecologically and transcriptomically, filling the open niche between diploid progenitor species (Parisod & Broennimann, 2016). At the same time, becoming a generalist has a trade-off, in that its stress tolerance might also be attenuated, which may lead to *C. flexuosa* not being able to compete against progenitors in the extremes of the environmental gradient.

The generalist niche and transcriptomic plasticity of an allopolyploid we proposed for *C. flexuosa* can be common to other allopolyploid species. For example, the genus *Cardamine* has been proposed to have experienced adaptive radiation along water availability gradients by recurrent allopolyploidization, and the anecdotally described habitats of allopolyploid species including *Cardamine scutata* and *Cardamine insueta* are intermediate and/or fluctuating (Shimizu-Inatsugi *et al.*, 2017), consistent with a generalist niche. At the transcriptomic level, genes involved in abiotic stress responses might be under *cis*-regulation in *C. flexuosa* and in other allopolyploid species, such as cotton, coffee, and *Arabidopsis* (Chaudhary *et al.*, 2009; Dong & Adams, 2011; Combes *et al.*, 2013; Yoo *et al.*, 2013; Carlson *et al.*, 2017). Both in the present study (Table S5) and previous studies on Brassicaceae in the laboratory (Akama *et al.*, 2014; Paape *et al.*, 2016), only a small portion (< 4%) of all genes exhibited the homeolog expression ratio change. Future studies may address the role of a limited number of distinctively regulated environment-response homeologs in the establishment and persistence of an allopolyploid in a generalist niche.

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Author contributions

RA, KKS and RS-I conceived the study, RA, HK and RS-I designed the study and collected the data, HELL, RVB, AH, XG, and MT provided the sequence data, RA, JSun, MH and JSese analyzed the data, RA, JSun, MH, MMK, KKS and RS-I wrote the manuscript with input from the other authors.

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Data availability

The sequence information used in this study will be openly available as of 31 December 2020 in the DDBJ (http://www.ddbj.nig.ac.jp) as DRA Accession ID: DRA006316, BioProject ID: PRJDB4989, BioSample ID: SAMD00098907 as well as DRA006314, BioProject ID: PRJDB4989, BioSample ID: SAMD00097398-SAMD00097406. The data that support the findings of this study are available in Dryad at https://doi.org/10.5061/dryad.4qrfj6q8h

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Dataset S1 List of genes whose homeolog expression ratio of *Cardamine flexuosa* differed between site-pairs on each sampling date and date-pairs at each sampling site.

Dataset S2 List of gene ontology (GO) terms for the biological process that were significantly enriched for each site-pair on each sampling date in *Cardamine flexuosa*.

Fig. S1 The distribution of the study species in *Cardamine* in Switzerland.

Fig. S2 Soil moisture of the *Cardamine flexuosa* sites where RNA samplings were conducted.

Fig. S3 The proportion of *Cardamine hirsuta* origin and *C. amara* origin reads in RNA-seq samples.

Fig. S4 Soil properties from 18 *Cardamine* sites in 2013.

Fig. S5 Sky openness from 18 sites measured at the season's start (around Julian calendar day 80) and season's end (around Julian calendar day 120 for *Cardamine hirsuta* sites and 170 for *C. flexuosa* and *C. amara* sites, respectively) in 2014.

Fig. S6 The result of principal component (PC) analysis on the expression level of 23 065 genes.

Fig. S7 The result of a clustering analysis based on $\log_{10}$FPKM calculated for 23 182 genes in *Cardamine amara* (A), *C. hirsuta* (H), *C. amara* homeolog of *C. flexuosa* (F^a), and *C. hirsuta* homeolog of *C. flexuosa* (F^h) on 2 May.

Fig. S8 Proportional Venn diagrams for the number of genes in the gene ontology (GO) category GO:0009414 (response to water deprivation) based on the GO enrichment analysis on the genes with homeolog expression ratio change in *Cardamine flexuosa*.

Fig. S9 The expression level of 32 genes in the gene ontology category GO:0009414 (response to water deprivation) for which the homeolog expression ratio of *Cardamine flexuosa* significantly differed between IR1 and KT5.

Methods S1 Detailed methods of data collection and analysis.

Table S1 The name of the study areas, the name and species composition (Type) of the study sites, and the number of individuals of the study species in *Cardamine* in 2013 and 2014.

Table S2 Pearson coefficients from pairwise correlations between environmental variables of *Cardamine* habitats.

Table S3 The developmental stage at sampling of the three individuals of *Cardamine hirsuta*, *C. flexuosa*, and *C. amara* in IR1, KT2, and KT5 in 2013.

Table S4 The numbers of reads mapped by HomeoRoq (H-origin: mapped to *Cardamine hirsuta* (2x); A-origin: mapped to *C. amara* (2x) and the number of expressed homeologs (FPKM > 1) in parenthesis shown for each individual of *C. hirsuta*, *C. flexuosa* (4x), and *C. amara*.

Table S5 The summary of the number of genes of *Cardamine flexuosa* examined and detected in analyses on site pairs.

Table S6 The summary of the number of genes of *Cardamine flexuosa* examined and detected in analyses on date pairs.

Table S7 Summary of gene ontology (GO) IDs and terms that were detected for the majority of site-pair by time combinations for genes whose homeolog expression ratio significantly changed in the allopolyploid *Cardamine flexuosa* (4x).

Table S8 List of *Cardamine hirsuta* genes and description of their *Arabidopsis thaliana* homologs belonging to the gene ontology (GO) ID GO:0009414 identified in the GO analysis on the homeolog expression ratio between at least one pair of sites on at least one date.

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