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Hybrid zones between invasive *Rorippa austriaca* and native *R. sylvestris* (Brassicaceae) in Germany: ploidy levels and patterns of fitness in the field

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Hybrid zones may serve as natural laboratories for evolutionary studies. One common viewpoint is that hybrids may always be less fit than their parents due to genetic discontinuities. An alternative idea is that genotype–environment interactions influence the outcome of natural hybridization. Our comparative study of two different natural hybrid zones between the invasive diploid *Rorippa austriaca* and the native polyploid *R. sylvestris* in Germany identified the ploidy level as a major determinant of hybrid fitness. Different ploidy levels and patterns of fitness were detected in different hybrid zones. In one hybrid zone (Mülheim, Ruhr valley) hybrids were pentaploid and showed a relatively high seed set, whereas in the second hybrid zone (Randersacker, Main valley) hybrids were triploid and displayed extremely low fitness values. Analyses of fitness values in different natural hybrid zones between the same two species may lead to very different conclusions about the evolutionary significance of natural hybridization.
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Introduction

A central question in the study of hybrid zone evolution has been whether hybrids are always less fit than their parents due to genetic discontinuities or whether hybrid fitness varies (Arnold and Hodges, 1995). Most interspecific hybrids recognized in the field have been recorded as highly or completely sterile based on seed set (Stace, 1975). In contrast, many recent studies indicate that hybrids may show high variation in fitness (Arnold and Hodges, 1995; Arnold *et al*, 2001; Bleeker, 2004) and provide evidence for genotype–environment interactions (Campbell and Waser, 2001, Johnston *et al*, 2003).

To test the evolutionary importance of hybridization in a given species complex, it is critical that findings from greenhouse experiments be tested in the field (Arnold *et al*, 2001). Only very few measurements of hybrid fitness have been conducted under natural conditions (Rieseberg and Carney, 1998). Multiple hybrid zones between the same two species offer excellent opportunities for a comparative approach.

Rorippa austriaca is a diploid (2n = 16) perennial species native to western Asia and southeastern Europe (Jonsell, 1973). For several decades it has been invasive in Germany. *R. sylvestris* is a native perennial species which is common all over Germany and central Europe. Different ploidy levels, tetraploids (2n = 32), hexaploids (2n = 48), and rarely pentaploids (2n = 40), have been detected in *R. sylvestris* (Jonsell, 1968). Multiple introductions of *R. austriaca* into central Europe are leading to repeated possibilities for the formation of contact zones with native species. Independent local hybridization and bidirectional introgression between *R. austriaca* and the native *R. sylvestris* (L.) Besser (2n = 32, 48) have been detected in a recent study using molecular markers (Bleeker, 2003).

The goal of the present study was to test common predictions on hybrid fitness (hybrids are uniformly less fit, hybrid fitness varies) in different natural hybrid zones and to draw conclusions about the repeatability of hybridization processes along the invasive/native species border line. Therefore, we examined differences in pollen production, pollen viability, seed production, and seed germination in two hybrid zones located in Mülheim (Ruhr valley) and Randersacker (Main valley). Flow cytometry and molecular markers were used to investigate the ploidy level and the genetic constitution of hybrid genotypes. The *trn*L intron (chloroplast DNA) of *R. austriaca* shows a species specific deletion of 169 bp (Bleeker *et al*, 2002; Bleeker, 2003). This deletion was used to analyse the maternal parentage of hybrids. AFLP analysis was performed to prove hybrid origins based on the distribution of species-specific parental markers (Marhold *et al*, 2002; Bleeker, 2003; Lamont *et al*, 2003).

Materials and methods

Plant material

The hybrid zones that were analysed are located at riversides in Mülheim in the Ruhr valley between Essen

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and Duisburg (N 51°27′ E 06°50′) and Randersacker (N 49°45′ E 09°58′) at the river Main south of Würzburg. A map is given in Bleeker (2003). In spring 2002 individual plants were marked using waterproof plastic strips (Dymo label manager 100+; Esselte NV, Belgium). Owing to our experiences in 2000 and 2001, we were either able to identify *R. austriaca*, *R. sylvestris*, and their hybrids in the field or knew their exact locations from the results of molecular analyses. In the Mülheim hybrid zone, we marked 13 individuals of *R. austriaca*, 28 individuals of *R. sylvestris*, and five hybrids. In the Randersacker hybrid zone, eight individuals of *R. austriaca*, nine individuals of *R. sylvestris*, and eight hybrids were marked. Both hybrid zones are rather small explaining the unequal and low sample numbers. Additional populations of *R. austriaca* and *R. sylvestris* grew in the vicinity of both collection sides (appr. 100 m), but no additional hybrids were detected in a circle of 500 m around the sampled plants. Fresh leaf material of all marked individuals was collected in silica gel for molecular analyses. Both sites were revisited during the reproductive period to harvest pollen, seeds, and leaf material for flow cytometry analyses from the same individual plants.

Ploidy analysis

Flow cytometry was used for the determination of relative DNA amount. Fresh leaf material was harvested in the field and stored at 4°C for 1–2 days until further analyses. Approximately 0.5 cm^2 leaf material was chopped with a sharp razor blade in a DAPI solution and filtered into a sample tube. Subsequent flow cytometry analysis was performed on a Partec Ploidy Analyser-I (Partec, Münster, Germany). The diploid *R. islandica* (Oeder) Borbas (2n = 16) was used as an internal standard.

Chloroplast DNA analysis

Total DNA was isolated from silica-gel-dried leaf material using the CTAB method of Doyle and Doyle (1987). The *trn*L intron was amplified using the 'c' and 'd' primers of Taberlet *et al* (1991). Amplifications were performed following the protocol of Franzke *et al* (1998). Amplification products were resolved on 1.5% agarose gels and visualized by UV light after staining with ethidium bromide. The length of the amplification products was estimated using a standard 100 bp ladder.

AFLP analysis

AFLP analysis followed the protocol of Vos *et al* (1995) with minor modifications. DNA was isolated from silica-gel-dried material collected in the field (see above), digested with restriction enzymes *Eco*RI and *Mse*I for 2 h at 37°C and simultaneously ligated to double-stranded *Eco*RI and *Mse*I adapters. Preselective amplifications were performed using primers with a one base pair extension. In a second selective amplification, the number of fragments was further reduced by primers with a three base pair extension. For the second amplification, we used the primer combinations *Mse*I-CTA/*Eco*RI-AAC and *Mse*I-CTA/*Eco*RI-AAG, which revealed high resolution in a previous study (Bleeker, 2003). Selective amplification products were separated electrophoretically on an ABI 377 (Perkin Elmer) with an internal standard (GeneScan 500 Rox, ABI).

Fitness components

The mean number of pollen grains per flower was estimated by counting the pollen grains from five flower buds per individual plant. The flowers of *Rorippa* show, like most of the Brassicaceae, six anthers that are arranged in two rings (four anthers in the inner ring, two anthers in the outer ring). The pollen grains of two anthers of the inner ring and one anther of the outer ring from each bud were dissected on a microscope slide and counted using a counting grid. The number of pollen grains per bud was estimated using the following equation: Pollen grains per bud = 2 (number of grains in first inner anther + number of grains in second inner anther) + 2 (number of grains in outer anther).

Pollen viability was tested by lactophenol blue staining of pollen grains. Five freshly opened anthers of different flowers per individual plant were incubated in lactophenol blue solution for 2–3 days (Brochmann, 1992). The fraction of stained pollen grains was estimated under a microscope. The total number of seeds produced by each individual was investigated by destructive sampling. All ripe fruits were harvested before opening and the seeds were removed and counted.

Germination experiments were performed in a controlled environment cabinet with a regime of 14 h daylight 20°C/10 h night 5°C. Seeds were germinated on sterile soil (FLORAGARD TKS 1/sand 3:1) using pots of 11 cm diameter. Light was supplied by PHILIPS lamps type MGR102-400. A maximum of 100 seeds were sown per pot. Pots were checked for newly emerging seedlings every 3 days. After 10 weeks, no new seedlings were observed and the experiment was stopped. The percentage of germinated seeds was calculated.

Data analysis

In the AFLP analysis, hybrids were identified by additive banding patterns of diagnostic parental markers. Markers were viewed as diagnostic when occurring in all analysed individuals of one parent species but not in the other parent species (Bleeker, 2003). The mean number of pollen grains per flower, pollen viability, seed number, and seed germination were analysed using a two-way analysis of variance (ANOVA) with fixed factors of class (*R. austriaca, R. sylvestris,* hybrids) and site (Mülheim, Randersacker). The mean number of pollen grains per flower was analysed on a log scale. The computations were performed using SPSS 11.0 (SPSS Inc.).

Results

Ploidy analysis

Five different groups could be distinguished based on the relative DNA amount of 69 individuals (Figure 1). The DNA amount of the 21 analysed individuals of *R. austriaca* did not differ from the internal standard (diploid *R. islandica* = 1s). *R. austriaca* was thus diploid in both invasive populations. *R. sylvestris* in Randersacker was tetraploid, and the relative DNA amount varied between 1.94s and 2.07s (n = 7). Two individuals of *R. sylvestris* from Randersacker were not analysed. *R. sylvestris* in Mülheim was hexaploid showing consider-

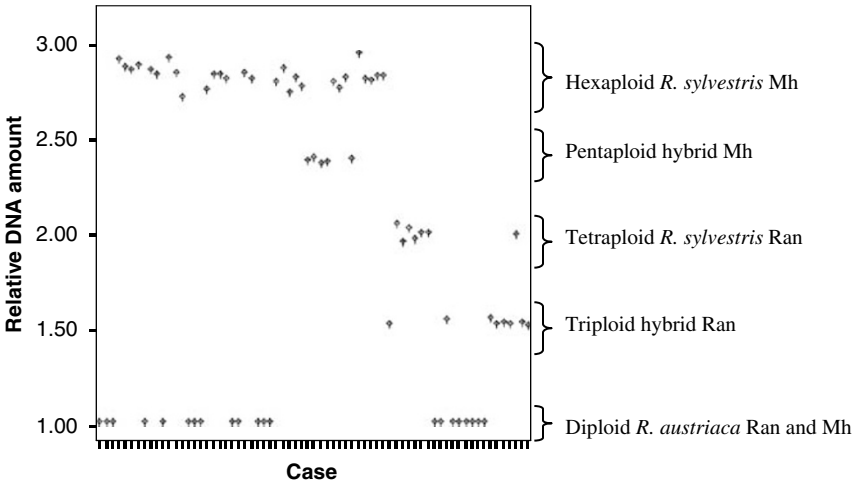

Figure 1 Relative DNA amount of 69 individuals collected in two natural hybrid zones between *R. austriaca* and *R. sylvestris* in Germany. The diploid *R. islandica* was used as an internal standard (=1s).

able variation in relative DNA amount (2.7s–2.92s; n=28). Tetraploid and hexaploid individuals of *R. sylvestris* with known chromosome numbers (2n=32, 2n=48) were used as additional reference material and gave peaks within the range of *R. sylvestris* plants collected in Randersacker and Mülheim, respectively, supporting our data interpretation. The hybrids in Randersacker and Mülheim showed different ploidy levels (Figure 1). In Randersacker, the hybrids were all triploid (1.5s–1.54s; n=8). The Mülheim hybrids showed much higher values than would be expected from a hybrid between a diploid and a hexaploid (2s). They were most likely pentaploid showing relative DNA amounts between 2.35s and 2.38s (n=5).

*Trn*L intron analysis

The *trn*L intron of 71 individuals of *R. austriaca*, *R. sylvestris*, and the hybrid was amplified. Two different *trn*L intron amplification product length variants (approximately 420 and 590 bp, respectively) were detected by visual interpretation of the agarose gels (Table 1). *R. austriaca* was characterized by a *trn*L intron amplification product length variant of 420 bp; *R. sylvestris* always showed the 590 bp length variant. All five hybrid individuals in Mülheim showed the *R. austriaca* specific length variant (420 bp). Both length variants were detected in the Randersacker hybrids – three individuals showed the 420 bp length variant and five individuals were characterized by the 590 bp length variant.

AFLP analysis

A total of 71 individuals of *R. austriaca* (21 individuals), *R. sylvestris* (37 individuals), and their putative hybrid (13 individuals) were investigated. AFLP analysis yielded a total of 167 bands (AFLP loci). Of these 154 were polymorphic (92.2%) and 13 monomorphic (7.8%). In all, 15 different AFLP phenotypes (nine in Mülheim and six in Randersacker) were detected in *R. austriaca*; *R. sylvestris* showed 31 different AFLP phenotypes (22 in Mülheim and nine in Randersacker, Table 2). In both hybrid zones, the hybrids showed additive banding patterns of parental-specific AFLP markers. The distribution of the different diagnostic markers in Mülheim and Randersacker is shown in Figure 2. In the Mülheim hybrid zone the hybrids were characterized by three diagnostic *R. austriaca* markers and seven diagnostic *R. sylvestris* markers. In all, 10 diagnostic *R. austriaca* markers and 10 diagnostic *R. sylvestris* markers were detected in the Randersacker hybrids. In order to estimate the genomic constitution of the triploid and pentaploid hybrids, we analysed the total number of markers detected in the analysed taxa and the percentage

Table 1 Distribution of two *trn*L intron amplification product length variants in two hybrid zones between *R. austriaca* and *R. sylvestris*

	n	*420 bp*	*590 bp*
Hybrid zone Mülheim			
R. austriaca	13	13	—
R. sylvestris	28	—	28
Hybrids	5	5	—
Hybrid zone Randersacker			
R. austriaca	8	8	—
R. sylvestris	9	—	9
Hybrids	8	3	5

Table 2 Number of AFLP phenotypes and mean number of AFLP markers detected in two hybrid zones between *R. austriaca* and *R. sylvestris*

	n	*n phenotypes*	*Mean number of markers (±sd)*
Hybrid zone Mülheim			
R. austriaca	13	9	66.92±8.77
R. sylvestris	28	22	66.96±6.29
Hybrids	5	3	72.4±3.04
Hybrid zone Randersacker			
R. austriaca	8	6	65.5±4.93
R. sylvestris	9	9	73.5±3.71
Hybrids	8	5	90.25±3.99

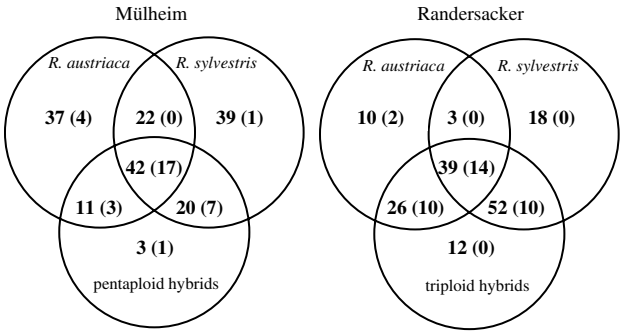

Figure 2 Distribution of the different AFLP markers detected in two natural hybrid zones between *R. austriaca* and *R. sylvestris* in Germany. The number of markers detected in all individuals of the respective classes or intersections is given in parentheses.

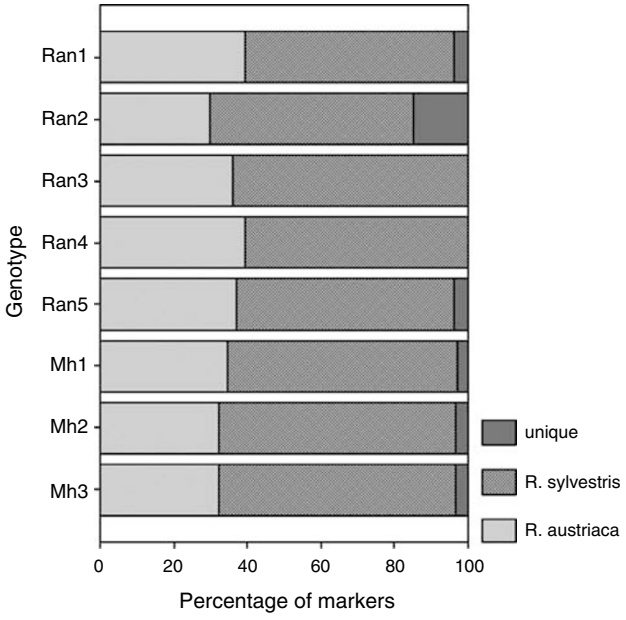

Figure 3 Percentage of *R. austriaca* markers, *R. sylvestris* markers, and unique markers in eight hybrid genotypes detected in two natural hybrid zones between *R. austriaca* and *R. sylvestris* in Germany.

of *R. austriaca* and *R. sylvestris* markers within the observed hybrid genotypes. The total number of markers varied between taxa and between hybrid zones (Table 2). The hybrids showed the highest number of markers in both hybrid zones. The triploid Randersacker hybrids displayed more bands than the pentaploid Mülheim hybrids. Tetraploid *R. sylvestris* from Randersacker showed more markers than hexaploid *R. sylvestris* from Mülheim. *R. austriaca* showed similar numbers of markers in both hybrid zones. Five different genotypes (AFLP phenotypes) were detected in eight triploid hybrid individuals in Randersacker, and three different genotypes were detected in five pentaploid hybrid individuals in Mülheim (Table 2). Figure 3 shows the percentage of *R. austriaca* markers, *R. sylvestris* markers, and unique markers within these eight hybrid genotypes. All hybrid genotypes showed higher percentages of *R. sylvestris* specific markers (55–64.5%) compared with *R. austriaca* specific markers (32.1–39.6%). With the exception of genotype Ran2, the triploid Randersacker hybrid genotypes (Ran1, Ran3–Ran5) showed a higher percentage of *R. austriaca* specific markers (36.2–39.6%) than the pentaploid Mülheim hybrid genotypes (Mh1–Mh3; 32.1–34.4%). Ran2 showed the highest number of unique markers (15%, Figure 3).

Fitness components

Unfortunately, not all marked individuals could be analysed. Some plants were lost due to the dramatic summer floods of 2002, others were grazed by cows or ducks and did not flower or no seeds could be harvested. Boxplots of the mean number of pollen per flower (a), the pollen viability as indicated by lactophenol blue staining (b), the number of seeds produced (c), and the germination rate of the seeds (d) including the number of individuals available for analyses are depicted in Figure 4. Pollen quantity, pollen viability, and germination rates differed significantly between classes (*R. austriaca*, *R. sylvestris*, hybrids) and sites (Mülheim, Randersacker; Table 3). Pollen quantity and pollen quality were lower in the Randersacker hybrid zone, possibly due to drought stress (Figure 4a, b). Significant between site differences in germination rates were due to the low germination rate of *R. austriaca* seeds from Mülheim (Figure 4d). The Randersacker hybrids showed the lowest pollen quantity (Figure 4a), low pollen viability (Figure 4b), and no seed set (Figure 4c). The Mülheim hybrids were also characterized by low pollen viability (Figure 4b), but showed surprisingly high seed set and germination rates of seeds (Figure 4c, d). Differences in performance, for example, between triploid and pentaploid hybrids and between tetraploid and hexaploid *R. sylvestris*, led to the detection of significant class × site interactions in all four fitness components (Table 3). Pollen viability and seed set of hexaploid *R. sylvestris* in Mülheim were much higher compared with tetraploid *R. sylvestris* in Randersacker (Figure 4b, c). Seed set and germination rate of *R. austriaca* in Mülheim were lower compared with *R. austriaca* in Randersacker (Figure 4c, d).

Discussion

Our comparative study revealed different ploidy levels and patterns of fitness in different hybrid zones. Triploid hybrids detected in a hybrid zone in Randersacker had no seed set whereas pentaploid hybrids detected in a hybrid zone in Mülheim had high seed set.

Genomic constitution of hybrids

The outcome of hybridization between the invasive *R. austriaca* and the native *R. sylvestris* in central Europe may largely be determined by the ploidy level of the hybridizing native species (*R. sylvestris*).

The formation of the triploid Randersacker hybrids can easily be explained by the fusion of reduced gametes of both parental species. The fusion of a haploid *R. austriaca* (A) gamete with a diploid *R. sylvestris* (RR) gamete would lead to a triploid F1 hybrid (RRA). The results of the chloroplast DNA analysis showed that both parental species may serve as the maternal parent, since

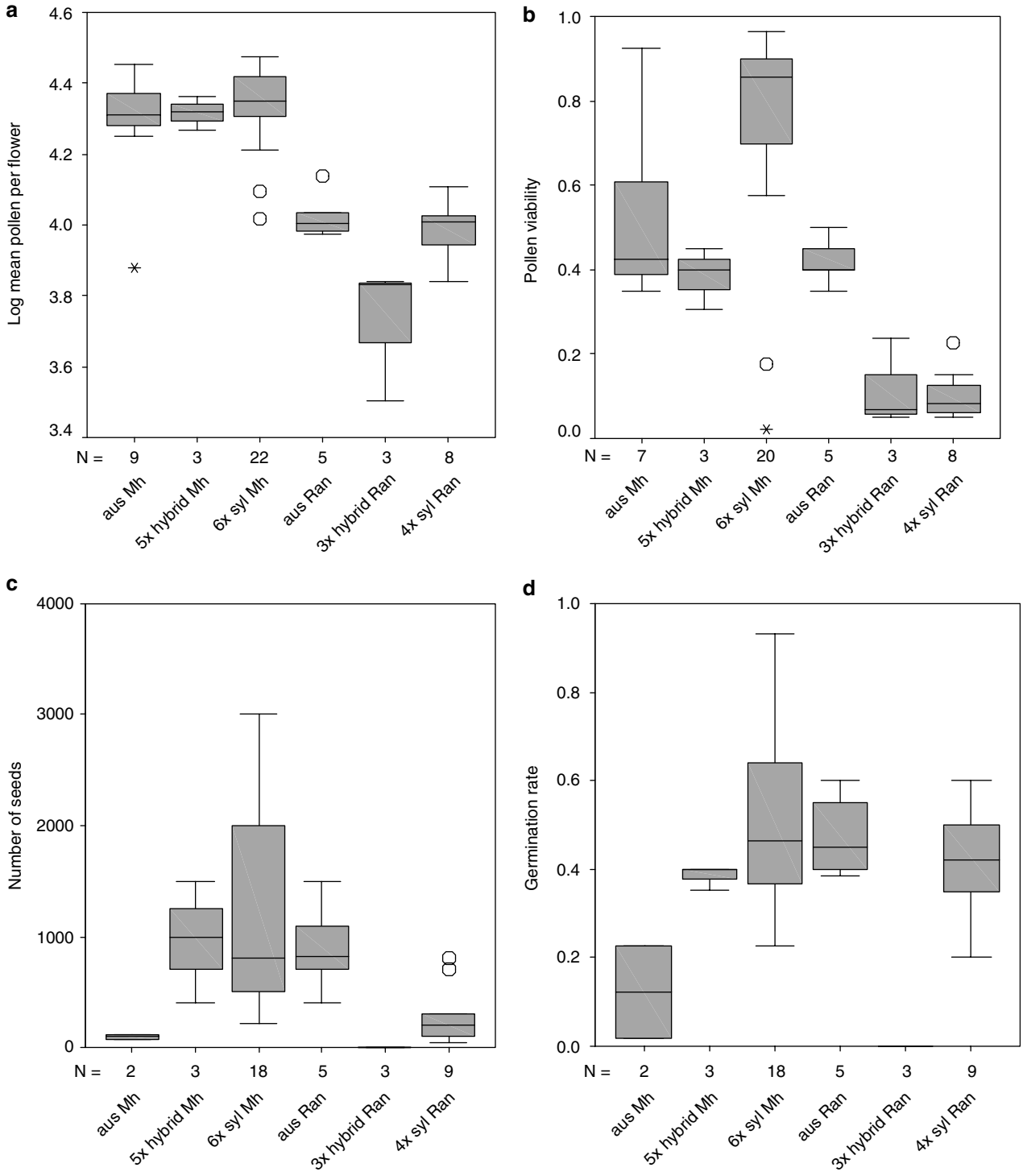

Figure 4 Boxplots of the mean number of pollen per flower (**a**), the pollen viability (**b**), the number of seeds produced (**c**), and germination rates (**d**) of *R. austriaca*, *R. sylvestris*, and their hybrid in two natural hybrid zones in Germany. Rectangles define 25 and 75 percentiles; horizontal lines show median; whiskers are from 10 to 90 percentiles.

chloroplast DNA is usually maternally inherited in the Brassicaceae (Harris and Ingram, 1991). The AFLP data are in accordance with the RRA hybrid genome, with triploid hybrids showing higher percentage of *R. sylvestris* markers than *R. austriaca* markers (Figure 3).

The formation of the pentaploid Mülheim hybrids is more difficult to explain, since octoploid *R. sylvestris* has never been observed in this well-studied species. At least one parental species must have contributed unreduced gametes. Unreduced gamete formation is a major

Table 3 Two way ANOVA on mean number of pollen per flower, pollen viability, seed production, and seed germination with fixed factors class and site

Source of variation	*df*	*Type III MS*	F	P
Log mean pollen per flower				
Class	2	0.13	3.69	0.031
Site	1	1.52	86.56	0.000
Class × site	2	0.19	5.52	0.006
Pollen viability				
Class	2	0.35	4.70	0.013
Site	1	1.16	31.10	0.000
Class × site	2	0.69	9.33	0.000
Seed production				
Class	2	700560.10	0.89	0.415
Site	1	322572.61	0.83	0.368
Class × site	2	5265567.50	6.75	0.003
Germination rate				
Class	2	0.231	4.49	0.018
Site	1	0.136	5.27	0.027
Class × site	2	0.388	15.1	0.000

mechanism in the evolution of polyploid plants (Bretagnolle and Thompson, 1995; Ramsey and Schemske, 1998). The high percentage of *R. sylvestris* markers in the pentaploid hybrids (Figure 3) suggests an RRRAA or RRRRA genome. *R. austriaca* was the maternal parent of all Mülheim hybrids since they contain the 420 bp variant of the *trn*L intron. A first generation RRRAA hybrid genome could be explained by unreduced gamete (egg) formation in *R. austriaca*. However, the low total number of AFLP markers detected in the pentaploid Mülheim hybrids compared with the triploid Randersacker hybrids (Table 2) does not support such a scenario. First generation hybrids will show high numbers of markers due to additivity while in future generations there will be recombination and a potential loss of markers. It is possible that the pentaploid hybrids evolved via a first generation RRRA hybrid (*R. austriaca* served as the maternal parent) and subsequent backcrossing with *R. sylvestris*. The fusion of a reduced *R. sylvestris* RRR pollen with a reduced RA egg of the F1 hybrid would lead to an RRRRA pentaploid hybrid showing the *R. austriaca* chloroplast. Hybrids between *R. austriaca* and *R. sylvestris* are common in the middle Elbe region in eastern central Germany extending their range into northern Germany. Most of the existing hybrid populations are tetraploid (Jonsell, 1968; Bleeker, unpublished data); thus the formation of tetraploid hybrids between *R. austriaca* and *R. sylvestris* is possible.

Hybrid fitness

Hybrids between *R. austriaca* and *R. sylvestris* were not generally less fit than their parents. The ploidy level has been identified as a major determinant of hybrid fitness.

Many recent studies indicate that hybrids are not always characterized by low fitness and that hybrid fitness may be environment dependent (Burke *et al*, 1998; Johnston *et al*, 2001, 2003; Campbell and Waser, 2001; Emms and Arnold, 1997; Hauser *et al*, 2003). We were not able to test whether hybrid fitness is affected by the genomic constitution alone or also by environmental factors since we analysed different genotypes in different natural environments. However, because of their different ploidy levels, it is likely that the genomic constitution affects the fitness differences between the Mülheim and the Randersacker hybrids.

The low fertility of the triploid Randersacker hybrids is in accordance with expected problems in performing normal meiosis. Based on the extremely low fitness of the triploid (F1) hybrids, these genotypes could be interpreted as an evolutionary dead end. However, even though F1 progeny may be produced rarely and be of limited fertility, further hybrid generations may frequently be produced if there are repeated opportunities for hybridization (Arnold and Hodges, 1995). Very few or even single hybrids can be of high evolutionary importance. A well-studied example for such processes is from *Senecio* in the British Isles. The triploid hybrid between the native tetraploid *S. vulgaris* L. and the invasive diploid *S. squalidus* L. is highly sterile, but has acted as a bridge for introgression and also in the formation of the allohexaploid *S. cambrensis* Rosser (Abbott *et al*, 1992, Abbott, 1992, Abbott and Lowe, 1996).

Our finding of high seed set in pentaploid hybrids is surprising. High seed set in a pentaploid hybrid would be consistent with agamospermy. However, apomixis is not known in *Rorippa* or closely related genera *(Cardamine* L., *Nasturtium* R. Br., *Barbarea* WT Aiton). Flow cytometry data indicate that the high seed production in the pentaploid Mülheim hybrids is most likely due to backcrossing with hexaploid *R. sylvestris* (Bleeker, unpublished data). Several examples of fertile hybrids with irregular chromosome numbers are known from the Brassicaceae. The triploid hybrid *Cardamine xinsueta* is capable of producing fertile gametes via polarized segregation of chromosomes during meiosis (Urbanska *et al*, 1997). Aneuploid individuals of the watercress hybrid *(Nasturtium xsterile)* sometimes show a high seed set and considerable pollen viability (Bleeker *et al*, 1999).

As hybrid fitness parental performance is expected to vary between genotypes and between environments. Hybrids may show higher relative fitness where parents suffer from suboptimal conditions, for example, at the edge of the natural distribution areas or in invasive populations. *R. austriaca* is an invasive self-incompatible species and showed an extremely low seed set in the Mülheim population. Natural populations of self-incompatible *Rorippa* species often show a low seed set. This is likely due to a low number of self- incompatibility alleles within populations (Mulligan and Munro, 1984; Bleeker, 2004). In Randersacker, the geographical distance to other *R. austriaca* populations was much lower than in Mülheim, presumably resulting in a higher number of cross-compatible partners and a higher seed set.

Our study shows that it is difficult to generalize or to predict the outcome of natural hybridization even between the same two species and highlights the importance of measuring multiple fitness components. Results from different natural hybrid zones or of different fitness components may lead to very different conclusions concerning the evolutionary significance of hybridization. The formation of hybrid zones between *R. austriaca* and *R. sylvestris* in Germany is a recent phenomenon and it will be interesting to see what it might lead to in the future.

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