Mendelian segregation of leaf phenotypes in experimental F_2 hybrids elucidates origin of morphological diversity of the apomictic *Ranunculus auricomus* complex

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Abstract Apomictic plant complexes exhibit a stunning morphological diversity. The *Ranunculus auricomus* complex with more than 800 morphologically recognized asexual species and just 4 known sexual species is a model group to study origins of high morphological diversity in asexually reproducing lineages. During the Pleistocene, the sexuals of the *R. auricomus* complex diverged in Central Europe into two morphological groups of contrasting basal leaf phenotypes, the undivided forms represented by diploid *R. carpaticola* and the deeply divided forms represented by diploid *R. notabilis*. An experimental interspecific crossing of *R. carpaticola* × *R. notabilis* revealed an extensive phenotypic diversity of the sexually formed F_2 hybrid generation. We hypothesize that apomictic species of the European *R. auricomus* complex might have established from hybrid derivatives of *carpaticola*- and *notabilis*-like sexual ancestors. Here we investigate segregation of F_2 basal leaf phenotypes from experimental *R. carpaticola* × *R. notabilis* crosses. We tested the hypothesis whether F_2 hybrid leaf phenotypes segregate in a Mendelian manner and whether any segregant phenotypes match *R. variabilis*, an apomictic species and natural hybrid derivative of *R. notabilis*. Using landmark-based geometric morphometrics of basal leaves, we recognized two major morphological clusters within the *R. carpaticola* × *R. notabilis* F_2 hybrid population. The *carpaticola*-like morphological cluster included undivided or less-divided leaf phenotypes without lateral sinuses and the *notabilis*-like morphological cluster contained deeply divided leaf phenotypes with 1–2 lateral sinuses. The overall ratio of the both morphological clusters within the F_2 hybrid population was approximately 1 : 1, suggesting Mendelian inheritance and partial dominance of the allele for the divided leaf phenotype. The morphological cluster of divided leaf phenotypes included 11% of F_2 individuals with a higher similarity to the apomictic species *R. variabilis* than to the parental sexuals. The *variabilis*-like F_2 segregant phenotypes often resulted from F_1 crosses of *carpaticola*-like mother plants with *notabilis*-like pollen donors; such crossing lines reached the highest morphological disparity. Equivalent hybridizations in nature followed by Mendelian segregation of phenotypes, which became later on fixed via the shift to apomictic reproduction, might have established the extant diversity of apomictic complexes. Microsatellite genotyping with six SSR loci revealed no correlation of genetic and phenotypic variation. We conclude that former "morpho-groups" or "main species" based on phenotypic similarity only are unsuitable units for taxonomic classification.

Keywords experimental hybridization; F_2 hybrids; geometric morphometrics; phenotype segregation; *Ranunculus auricomus* species complex

Suppelmentary Material The Electronic Supplement (Table S1; Figs. S1–S5) is available from https://doi.org/10.12705/676.6.S

■ INTRODUCTION

Apomictic species complexes are associated with a large morphological polymorphism (Grant, 1981; Stace, 1998; Hörandl & al., 2009). Morphological plasticity is crucial for static organisms such as plants and ensures adaptive possibilities in a chronically changing environment (Bradshaw, 1965; Rieseberg & al., 1999). Particularly leaf shapes show tremendous plasticity, which is possible due to less integrative plant morphogenesis enabling large morphological variation based on few genetic changes (Gottlieb, 1984). Plants generate morphological variation via interspecific hybridization and particularly closely related species are prone to crossing (Mallet, 2007). Hybrid swarms resulting from crossing events exhibit increased sensitivity to environmental variation and accelerated natural selection has frequently been studied in both herbs (Brochmann & al., 2000; Campbell, 2004) and trees (Benson & al., 1967; Potts & Reid, 1985). Ecological studies on hybrid swarms detected accelerated evolution of plant resistance against parasites (Fritz & al., 1999) and increase of animal diversity in hybrid-dominated *Eucalyptus* populations (Dungey & al., 2000). Interspecific hybridization in plants is often followed by polyploidization and shifts to apomixis and clonal reproduction (Arnold, 1992; Paun & al., 2006; Robertson & al., 2010).

Apomictic plant complexes usually originate from hybrids of divergent sexual progenitor species (Babcock & Stebbins, 1938; Grant, 1981). Their huge morphological diversity can

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originate from two different processes. First, sexual progenitor species cross to form hybrid offspring, which segregate in a Mendelian fashion and segregating phenotypes can be thereafter fixed by apomixis, resulting in stable and morphologically distinct apomictic lineages. This phenomenon was already detected by Gregor Mendel himself in his crossing experiments with *Pilosella* species (Mendel, 1869; Nogler, 2006). The second source of morphological diversity is linked to residual sexuality maintained after the origin of an apomictic lineage. Residual sexuality allows for intercrossing of lineages and their backcrossing to sexual species. In this way, novel genotypes and phenotypes can be produced continuously. While the latter process is in most apomictic complexes well known, little attention has been paid to morphological segregation patterns of early-generation hybrids directly after their origin.

López-Caamal & Tovar-Sánchez (2014) emphasized the importance of morphological characters for hybrid recognition even in the absence of genetic information. Recognition of hybrids via quantitative morphometrics can overcome the subjectivity of purely descriptive morphological approaches. Traditional morphometric tools (Marcus, 1990) played an important role in studies on natural hybrid populations in *Ranunculus* (Hörandl & al., 2009) and other model systems (Whiffin, 1973; Leach & Whiffin, 1978; Kirk & al., 2004; Lihová & al., 2007; Oberprieler & al., 2011). Rumpunen & Bartish (2002) noticed that leaf shape is a less biased estimator of genetic variation than metric leaf descriptors. We seek to contribute to the growing botanical literature in which geometric morphometrics is employed in order to take advantage of its power in quantifying of continuous morphological variation, which is characteristic for plant hybrids. Particularly landmark techniques were recognized helpful in ecological and taxonomic studies on interspecific hybrids in trees (Dickinson & al., 1987; Jensen & al., 2002; Viscosi & al., 2009a, b) and other plants (Hanušová & al., 2014; Hodač & al., 2014). Geometric morphometrics has also enabled evaluation of plant developmental plasticity (Young & al., 1995; Klingenberg & al., 2012), quantification of asymmetric leaf variation (Silva & al., 2012) and recognition of correlations between phenotypic variation and genome size (Hanušová & al., 2014). For the first time, in this study, we apply geometric morphometrics to uncover segregation patterns in experimentally produced interspecific F_2 hybrids. Excluding the influence of environment, under controlled garden conditions, we expect to quantify an unbiased Mendelian or non-Mendelian segregation of hybrid morphologies.

Our model system is the Euro-Siberian *Ranunculus auricomus* species complex with 4 sexual species in Europe and over 800 morphologically described apomictic species distributed throughout Europe from the Mediterranean mountains to the Arctic (Jalas & Suominen, 1989; Dunkel, 2015; Hörandl & Raab-Straube, 2015). The problems regarding species concepts in such asexual plant complexes were discussed in Hörandl (2018). In Central Europe, the *R. auricomus* species complex diversified during the Pleistocene with a split into two morphologically distinct lineages 0.9 million years ago (Hörandl, 2004). One lineage is characterized by deeply divided basal leaves (Electr. Suppl.: Fig. S1) and represented by only one extant described sexual species, *R. notabilis* Hörandl & Gutermann (Hörandl & Gutermann, 1998c). The species is diploid and distributed in a restricted and probably relict area on the Austrian-Hungarian border (Hörandl & Gutermann, 1998c; Hörandl & al., 2000; Hörandl, 2004). Another lineage of sexual species exhibits undivided basal leaves and two extant species, diploid *R. carpaticola* Soó distributed in the Carpathians and diploid and autotetraploid *R. cassubicifolius* W.Koch distributed in Prealps (Hörandl & Gutermann, 1998a). Hybridization among the sexuals was documented for diploid *R. carpaticola* and tetraploid *R. cassubicifolius*, whose hybrid is an apomictic hexaploid lineage, dated ca. 80,000 years ago (Paun & al., 2006; Pellino & al., 2013). A hybridogenous origin from *R. notabilis*-like progenitor was proposed for an allopolyploid apomictic species *R. variabilis* Hörandl & Gutermann (Hörandl & al., 2000; Hörandl, 2004; Hodač & al., 2014). *Ranunculus variabilis* has divided basal leaves similar to *R. notabilis* and partially sympatric but much larger distribution in the Prealps, Bohemian Massif (Hörandl & Gutermann, 1998c) and South Tyrol (Dunkel, 2005, 2010). Apart from the two documented hybridogenous origins, the vast majority of the extant polyploid apomictic species of the *R. auricomus* complex have an unknown evolutionary history. We hypothesized their hybridogenous origin as well and analyzed a garden cross between the diploid sexuals *R. carpaticola* × *R. notabilis*. The garden cross produced viable and sexual F_1 $2x$ hybrids which expressed apospory (i.e., a certain proportion of aposporous initial-like cells) but still had obligately sexual seed formation (Hojsgaard & al., 2014). The F_1 hybrids were genetically intermediate with mostly divided basal leaves (Hodač & al., 2014). Crossings of the F_1 hybrids revealed diploid F_2 hybrids with some facultative apomictic lineages (Barke & al., 2018), with a vast diversity of basal leaf phenotypes. The extensive morphological diversity of the F_2 hybrids and the establishment of facultative apomictic seed formation already in such an early generation let us speculate on the possible origin of the natural apomicts from similar hybridization events. In fact, the vast majority of apomictic *R. auricomus* species exhibits more or less divided leaf phenotypes, evidenced from Bavaria (Borchers-Kolb, 1985), western Germany (Schmelzer, 2015), Austria (Hörandl & Gutermann, 1998a, b, c, 1999), Scandinavia (Ericsson, 1992); JSTOR Global Plants (http://plants.jstor.org/) and European Russia (Moscow Digital Herbarium; https://plant.depo.msu.ru). Since *R. notabilis* is the only well-defined sexual species with divided leaf morphology in Central Europe (Hörandl & al., 2009) and because its crosses with diploid *R. carpaticola* produced viable hybrids (Hojsgaard & al., 2014), we selected both species as model progenitors of all putatively hybridogenous apomictic species. Applying a quantitative morphometric approach, i.e., geometric morphometrics, we aim to answer following questions: (1) Which are the main morphological trends within the F_2 hybrid morphospace? (2) Does the F_2 generation segregate according to Mendelian rules? (3) Is there a fraction of F_2 segregant phenotypes with a high similarity to a natural allopolyploid apomict *R. variabilis*?

■ MATERIALS AND METHODS

Experimental crossings. — We crossed two sexual diploid species of the *Ranunculus auricomus* complex. The female parents were *R. carpaticola* from the population 8483 (plants numbered 13 and 18 in our garden culture) originating from central Slovakia (Paun & al., 2006). The pollen parents were *R. notabilis* from the population 7224 (plants numbered 16 and 19) originating from eastern Austria (Hörandl & Gutermann, 1998c). We had produced two populations of the first-generation hybrids (F$_1$A and F$_1$B) from the following crossings: F$_1$A = *R. carpaticola* 8483/18 × *R. notabilis* 7224/19 (resulting in 4 F$_1$A plants) and F$_1$B = *R. carpaticola* 8483/13 × *R. notabilis* 7224/16 (resulting in 13 F$_1$B plants) as described by Hojsgaard & al. (2014). The first assessment of leaf shape variation of the F$_1$ was published in Hodač & al. (2014). In the next crossing step we randomly crossed F$_1$A hybrids with F$_1$B hybrids and also multiple F$_1$B (and a few F$_1$A) hybrids with each other. We generated three major lines of the second-generation hybrids (F$_2$A×B, F$_2$B×A, F$_2$B×B; 221 plants) according to the following crossing scheme (with female parent always first): F$_2$A×B = F$_1$A × F$_1$B (5 combinations, 65 plants), F$_2$B×A = F$_1$B × F$_1$A (3 combinations, 42 plants) and F$_2$B×B = F$_1$B × F$_1$B (13 combinations, 114 plants). The plants were cultivated in the botanical garden at the Göttingen University under identical conditions (in pots of the same size placed within a 16 m^2 garden bed without differences in solar irradiation and water supply). All F$_2$ plants were determined to be diploid (Barke & al., 2018).

Basal stem leaves digitization. — Fresh basal leaves from F$_1$ hybrids (63 leaves) and F$_2$ hybrids (942 leaves) were harvested from flowering plants (Electr. Suppl.: Fig. S1A, B). The sampling of all leaves was conducted within one week in May 2016 and the leaves were scanned using a CanoScan 9950 immediately after the harvest to avoid possible bias due to preservation as suggested by Volkova & al. (2010) and Klingenberg (2015). Additional basal leaves of the species *R. carpaticola* (14 leaves), *R. notabilis* (89 leaves) and *R. variabilis* (305 leaves) were obtained from digitized herbarium specimens from the Herbarium of the University of Vienna (WU). For the reference species, we have used herbarium specimens because of the lack of fresh plant material in garden culture. Some of the populations, which we included in our study would be extremely difficult to resample, while others are known to have been extirpated. As reported by Volkova & al. (2010), drying of plant material affects mostly the size but less the shape.

Landmark digitization. — The species of the *R. auricomus* complex exhibit a strong basal leaf heterophylly (Electr. Suppl.: Fig. S1A), whereby only the basal leaves occurring during the flowering time are taxonomically informative (Borchers-Kolb, 1985; Hörandl & Gutermann, 1995). For geometric morphometric analyses, we sampled up to ten basal leaves per plant (four leaves per plant on average). Twenty-six two-dimensional homologous landmarks were digitized on leaf outlines in the program tpsDig v.1.40 (Rohlf, 2015). The landmarks were placed in order to capture all major morphological features of the basal leaf shape, i.e., the middle segment, the lateral segments, the blade base, the depth of the middle segment sinus (= incision) and the depth of the 1st plus 2nd lateral sinuses (Electr. Suppl.: Figs. S2A–D). Here we did not include stem leaves and receptaculum as potential taxonomic characters (Borchers-Kolb, 1985; Ericsson, 1992; Hörandl & Gutermann, 1995) as these are less differentiated between the parental species used here.

Geometric morphometrics. — Landmark configurations were superimposed using the Procrustes fit method (Zelditch & al., 2012) in the software package MorphoJ v.1.06d (Klingenberg, 2011). Shape variables were generated from the landmark configurations with the Thin-Plate Spline method (Bookstein, 1997). After having separated symmetric variation from asymmetry components our subsequent analyses concerned only the symmetric covariance matrix (Klingenberg & al., 2002). Variation among the leaf shapes (landmark configurations) was summarized using the relative warps analysis (Rohlf, 1993). We regressed the leaf scores on the first six relative warps on log-transformed leaf centroid sizes (Klingenberg, 2011) to test for effects of allometric variation (Gould, 1966; Bruner & al., 2003; Dujardin & al., 2003). Because the relative warps analysis did not reveal clearly separated clusters of F$_2$ hybrid leaf shapes but rather a continuum of forms, we sought a different strategy to explore the morphospace structure. Before the analysis, 25 F$_2$ hybrids have been excluded due to missing information about the phenotype of their F$_1$ parents and we have proceeded with 196 F$_2$ hybrids (F$_2$A×B, F$_2$B×A, F$_2$B×B; Electr. Suppl.: Table S1). We have averaged the leaf shapes within each of the 196 F$_2$ hybrid plants (2–10 leaves per plant, 4.4 on average) to obtain a single phenotype per plant. Subsequently, we have computed morphological dissimilarities (Procrustes distances) among the F$_2$ hybrids and three reference species, each represented by multiple populations, i.e., *R. carpaticola* (3 populations), *R. notabilis* (8 populations), *R. variabilis* (16 populations). In the case of the three reference species, we averaged leaf shapes within the populations instead of individuals. We then assigned F$_2$ hybrid plants to either of the three species if their Procrustes distance to any of species' population were smaller than the maximal Procrustes distance observed among the species' populations. In other words, plants were assigned to the species whose centroid in Procrustes shape space was closest. This way we delimitated two major morphological clusters and four morphological subclusters of the averaged F$_2$ hybrids. Cluster 1 included hybrids with undivided leaves similar to *R. carpaticola* (C-phenotypes) and hybrids with a shallow middle segment sinus similar to both *R. carpaticola* and *R. variabilis* (CV-phenotypes). Cluster 2 included hybrids with deeply divided leaves with 1–2 lateral sinuses similar to both *R. notabilis* and *R. variabilis* (NV-phenotypes) or hybrids similar only to *R. variabilis* (V-phenotypes). Overall morphological dissimilarities (Procrustes distances) among the averaged F$_2$ hybrids were summarized and visualized using principal coordinates analysis (PCoA) in the software PAST v.2.17c (Hammer & al., 2001). After assigning all F$_2$ hybrids into one of the four phenotypes we have performed a canonical variate analysis (CVA; 10,000 permutations) on the original dataset of all leaves to test for significant differences among the artificial subclusters. All analyses except for the PCoA were conducted with the software package MorphoJ v.1.06d (Klingenberg, 2011). In the same program using relative warps analysis, we also analyzed the major trend of asymmetric shape variation. The asymmetric variation was compared between *R. notabilis* (89 leaves), the experimental F_2 hybrids (942 leaves) and natural hybrid *R. variabilis* (305 leaves). The three groups were also compared based on their log-transformed centroid sizes. The group differences in mean centroid size and asymmetric variation were tested in the software PAST v.2.17c (Hammer & al., 2001). Morphological disparity sensu Perez & al. (2006) was compared among samples of individuals in the program DisparityBox8 from the IMP package (Sheets, 2001), considering unequal sample size correction.

Microsatellite genotyping. — Following Hojsgaard & al. (2014), we expected that the F_2 generation developed exclusively from sexual seeds and hence would exhibit non-maternal genotypes and Mendelian segregation. To test for these assumptions, we randomly selected 119 F_2 hybrids from ten crossing combinations (4× F_2A×B; 2× F_2B×A; 4× F_2B×B) and genotyped them using six microsatellite loci (simple sequence repeats, SSR) following methods previously described by Klatt & al. (2016) and Barke & al. (2018). Genetic dissimilarities among all multilocus genotypes were computed as Jaccard distances from a binary matrix (allele presence/absence dominant data) in the program FAMD v.1.31 (Schlüter & Harris, 2006). Based on the Jaccard distance matrix, we have tested whether the three crossing lines (F_2A×B, F_2B×A, F_2B×B) differ due to their genetic composition using a non-parametric MANOVA with 10,000 permutations and Bonferroni corrected *P*-values in the program PAST v.2.17c (Hammer & al., 2001). We quantified the genetic differentiation among the three crossing lines by computing F_{st} values from allelic (codominant) data in the program GenAlex v.6.501 (Peakall & Smouse, 2006). The 119 F_2 hybrids were tested for a correlation of their genetic and phenotypic variation using a Mantel test (based on Jaccard genetic distances and Procrustes phenotypic distances) with 10,000 permutations in the software PAST v.2.17c (Hammer & al., 2001). We have also tested a correlation between averaged genetic dissimilarities among the ten crossing combinations computed as chord distances based on the Bayesian method in FAMD v.1.31 (Schlüter & Harris, 2006) and their averaged Procrustes distances.

■ RESULTS

Phenotypic variation. — The symmetric component of basal leaf shape variation accounted for 90% of the total variability within the *R. carpaticola* × *R. notabilis* F_2 hybrid population. The proportion of symmetric variation was slightly lower (79% and 77%) in the parental species *R. notabilis* and in the natural hybrid *R. variabilis*, respectively. The two major gradients (relative warps, RW1 & 2) of the symmetric basal leaf shape variation cumulatively described 77% of the total variability within the F_2 hybrid population (Fig. 1). The ordination of individual leaf shapes demonstrated a continuum of forms spanning from *R. carpaticola* to *R. notabilis* (Fig. 1). The most prominent phenotypic gradient (RW1; 61% of the total variation) described shape changes from undivided *R. carpaticola*-like leaves (Fig. 1A) to deeply divided *R. notabilis*-like leaves (Fig. 1B). The second most important shape change (RW2; 16%) described the transition from leaves without lateral sinuses and narrow blade base (Fig. 1C) to leaves with the 1st lateral sinus and broad blade base (Fig. 1D). The gradient from narrow to broad blade base affected both the *carpaticola*-like forms (Fig. 1E, F) as well as the deeply divided *notabilis*-like forms (Fig. 1G, H). Further relative warps (RW3–6) pointed out several less prominent features of leaf shape variation, including the

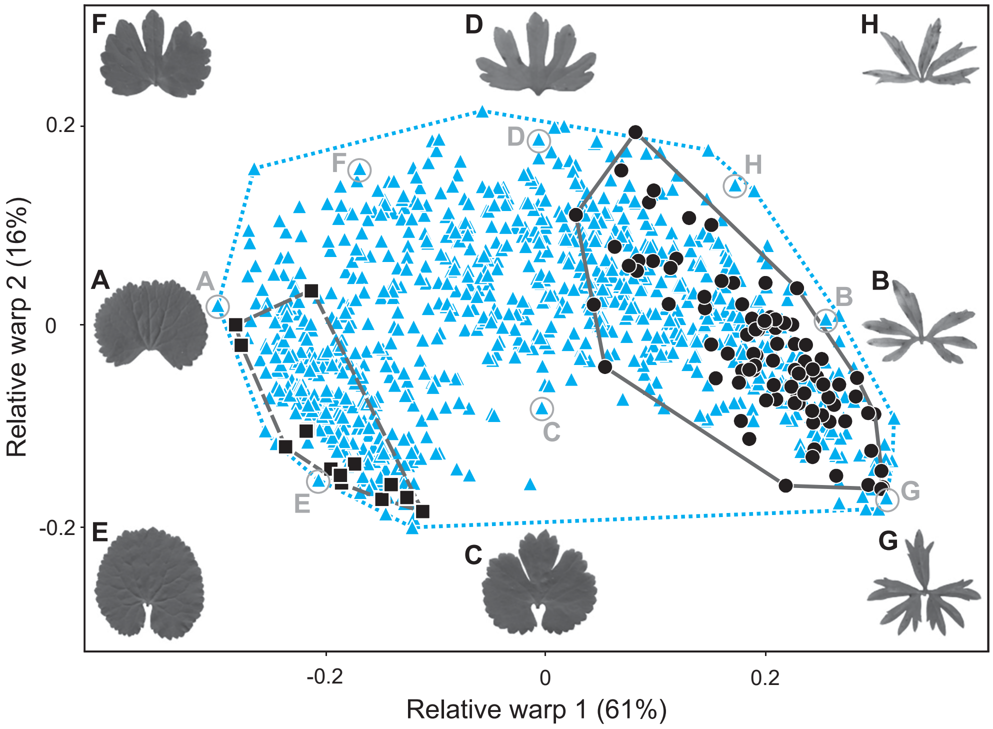

Fig. 1. Relative warps analysis of leaf shapes within the *R. carpaticola* × *R. notabilis* F_2 hybrid population and the parental species *R. carpaticola* and *R. notabilis*. The scatterplot shows ordination of leaf shapes along the first two major morphological gradients, i.e., relative warps (RW1 & 2). The relative warps were extracted from 942 F_2 hybrid leaves (triangles) and from leaves of the parental species (black squares = *R. carpaticola* with 14 leaves; black dots = *R. notabilis* with 89 leaves). Images of F_2 hybrid leaves A–H illustrate shape changes in the morphospace described by the two relative warps.

number of sinuses (RW3/5/6; Fig. 2) and changes affected by the gradient of blade base width (RW3–5). The sixth relative warp described a transition from horizontally versus vertically shrunk leaf blades. Allometric variation significantly affected multiple relative warps (RW1/2/4/5), but the only prominent effect (11%) was identified for RW4 (Fig. 2). The size factor, therefore, affected mostly leaves with one sinus and is linked to a narrowing of the blade base angle (Fig. 2).

Phenotypic segregation. — Applying Procrustes distances as a measure of morphological differentiation, we succeeded in assigning almost all F_2 hybrids into phenotypic clusters (Fig. 3). A multi-group discriminant analysis (i.e., canonical variate analysis, CVA) revealed significant differences ($P < 0.001$) for all pairwise comparisons. The F_2 hybrids segregated into the two major phenotypic clusters as follows: 51% corresponded to leaf phenotypes without lateral sinuses (C- or CV-phenotypes) and 49% exhibited divided leaves with at least one prominent lateral sinus. Among the individuals with divided leaves, 78% were phenotypically similar to both *R. notabilis* and *R. variabilis* (NV-phenotype) and 22% were more similar to *R. variabilis* (V-phenotype) than to *R. notabilis*. were more similar to *R. variabilis* (V-phenotype) than to *R. notabilis*. Crossings of mother plants exhibiting NV-phenotypes (expressed by F_1A genotypes) with pollen donors exhibiting non-dissected C-phenotypes (expressed by F_1B genotypes) resulted in a 4 : 3 ratio of leaves with and without lateral sinuses, respectively (F_2A×B phenotypes; Fig. 4). The reciprocal crossings generated a comparable ratio of 4 : 3 of more-divided (NV, V) and less-divided (CV, C) hybrids (F_2B×A; Fig. 4). When reciprocally crossing multiple F_1B hybrids exhibiting all four phenotypes, we reached the 4 : 5 ratio of more-divided and less-divided forms in the F_2 generation (F_2B×B; Fig. 4). Averaged over all three crossing lines, the total ratio of more-divided versus less-divided F_2 hybrids was 1 : 1 (F_2total; Fig. 4). The three crossing lines differed only slightly with respect to their morphological disparity, i.e., 0.0310 (F_2B×B) < 0.0323 (F_2A×B) < 0.0349 (F_2B×A), which was comparable with the parental species *R. notabilis* (0.0311) and overall higher than in the natural hybrid *R. variabilis* (0.0239).

Phenotypes vs. genotypes. — As already seen in the analysis of 196 F_2 hybrids (Fig. 3), the more-divided phenotypes (NV, V) were morphologically differentiated from the less-divided phenotypes (C, CV). The same result has been obtained for a subset of 119 F_2 hybrids (Fig. 5A), which we have genotyped with six SSR markers. The morphological structure of the F_2

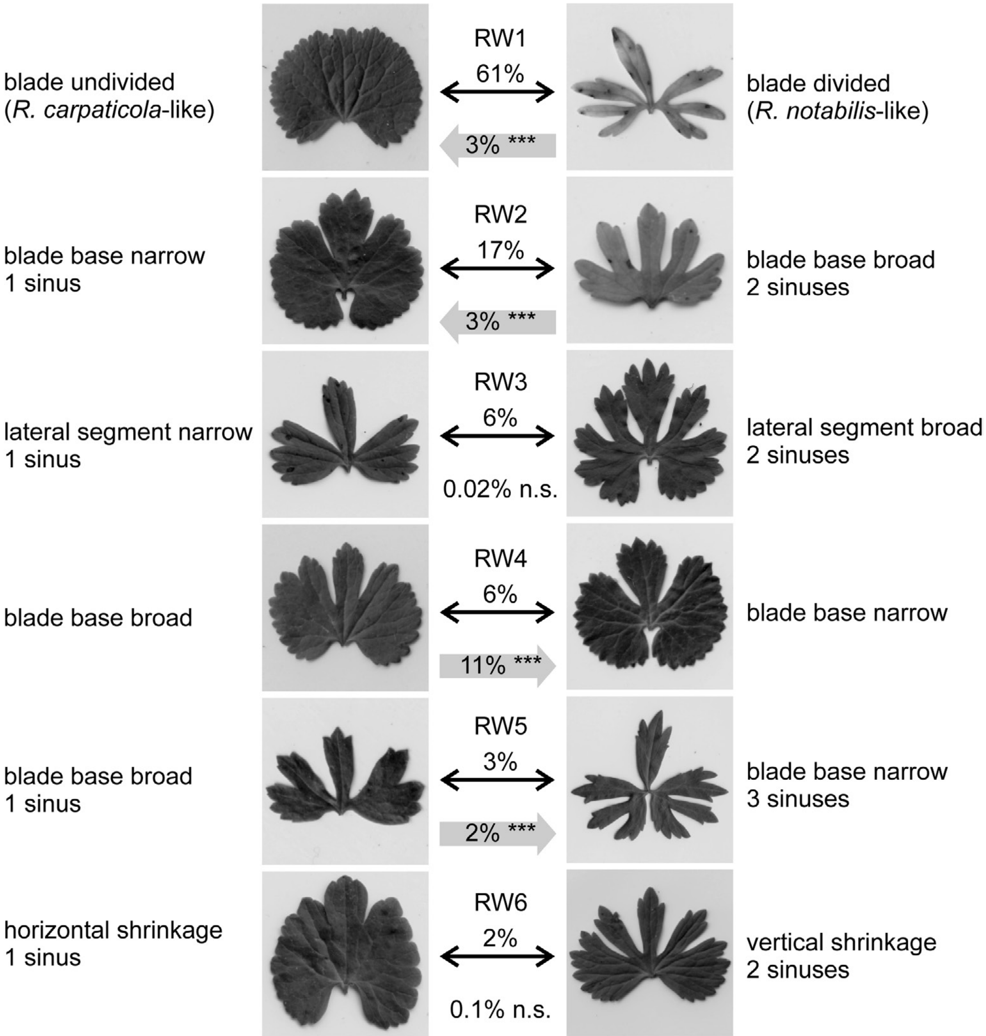

Fig. 2. Relative warps analysis of leaf shapes within the *R. carpaticola* × *R. notabilis* F_2 hybrid population. Six relative warps (RW1–6) show major leaf shape changes within the F_2 population (221 plants; 942 leaves) and the leaf images illustrate F_2 hybrid shapes at the extremes of each relative warp (represented by thin black arrows). Percentages above the arrows give a proportion of variation described by relative warps. Percentages within grey arrows give variation due to allometry. Significant allometry effects are indicated by asterisks: $P < 0.001$ (***), n.s. = not significant. The grey arrows are oriented with respect to the size gradient.

hybrids was not reflected by their genetic differentiation because neither the deeply divided phenotypes (NV, V) nor the less-divided phenotypes (C, CV) formed any separated genetic clusters (Fig. 5B). The distribution of phenotypes across the three crossing lines ($F_2A×B$, $F_2B×A$, $F_2B×B$) was also unstructured (Fig. 5C), although the crossing lines were genetically differentiated (Bonferroni corrected $P < 0.001$ for all pairwise comparisons), yet largely overlapping (Fig. 5D). The degree of genetic differentiation (F_{st}-value) among the three crossing lines was low, ranging from 0.074 ($F_2A×B × F_2B×A$; $P < 0.001$) to 0.137 ($F_2B×B × F_2B×A$; $P < 0.001$). Mantel tests revealed no significant correlation between the genetic and phenotypic variation neither within the entire subset of 119 F_2 hybrids ($P = 0.1604$) nor among the 10 particular crossings ($P = 0.6962$).

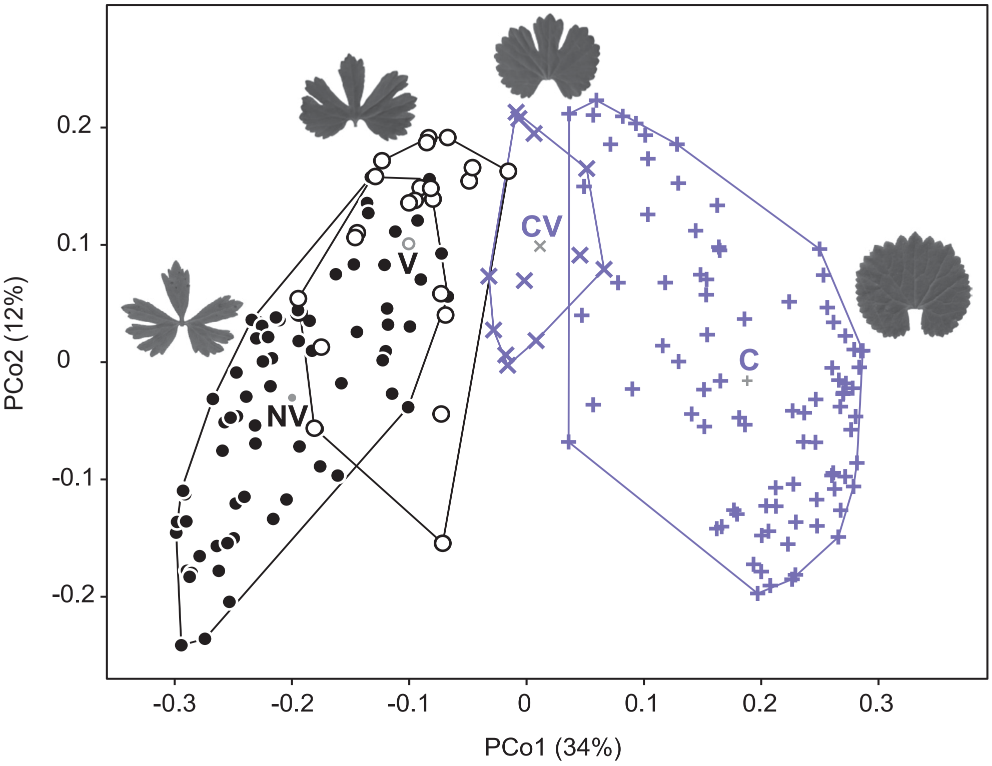

Fig. 3. Morphological segregation of *R. carpaticola* × *R. notabilis* F_2 hybrid plants. Principal coordinates analysis (PCoA) scatterplot summarizes morphological dissimilarities (Procrustes distances) among 196 F_2 hybrid plants. Black dots = F_2 hybrid plants similar to *R. notabilis* and *R. variabilis* (NV phenotypes); white dots = F_2 hybrids similar to *R. variabilis* (V phenotypes); cross marks = F_2 hybrids similar to *R. carpaticola* and *R. variabilis* (CV phenotypes); plus signs = F_2 hybrids similar to *R. carpaticola* (C phenotypes). Smaller grey symbols are group centroids and four leaf images illustrate F_2 hybrid phenotypes close to the four centroids (group means shapes).

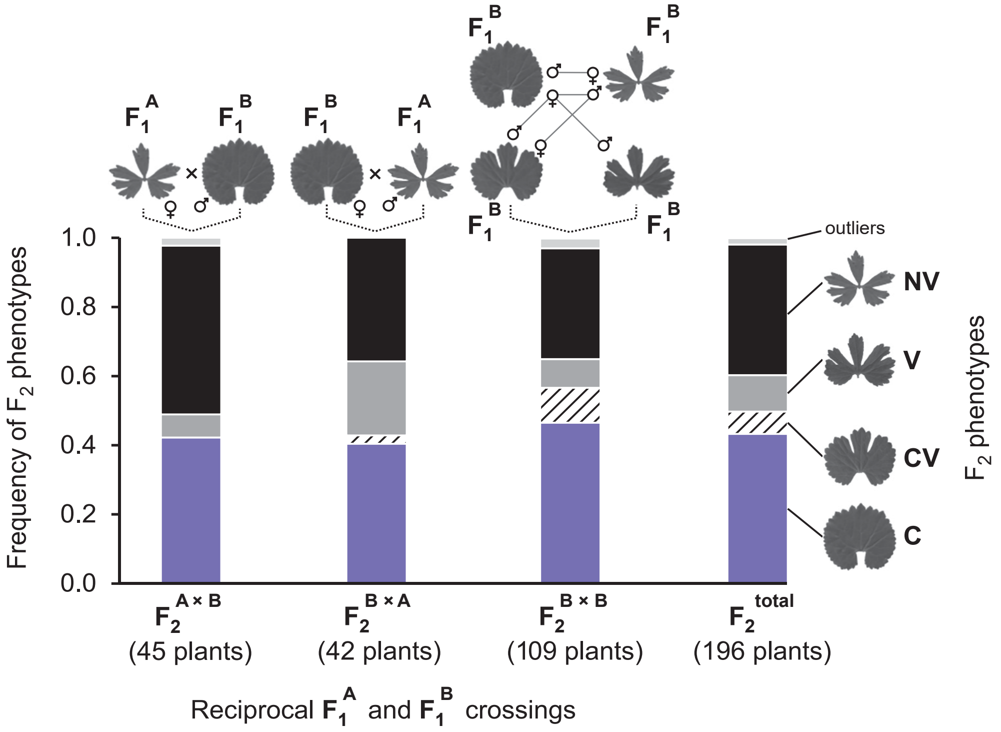

Fig. 4. Morphological segregation of *R. carpaticola* × *R. notabilis* F_2 hybrid plants. Bars show proportions of F_2 hybrid phenotypes segregating from different F_1A and F_1B crossing lines. NV = F_2 hybrids similar to *R. notabilis* and *R. variabilis*; V = F_2 hybrids similar to *R. variabilis*; CV = F_2 hybrids similar to *R. carpaticola* and *R. variabilis*; C = F_2 hybrids similar to *R. carpaticola*. Leaf images above the bars illustrate F_1 phenotypes used for the crossing experiments. All F_1 crossings and the resulting F_2 phenotypes are listed in Table S1 in the Electronic Supplement.

DISCUSSION

The experimental F_2 hybrids of closely related sexuals *Ranunculus carpaticola* and *R. notabilis* represented a phenotypically highly diverse array of transgressive and intermediate recombinant phenotypes of basal leaves. The most phenotypic variability concerned the symmetric component and although asymmetries certainly affected all segments of the basal leaf variation, their distribution over the whole F_2 population suggests fluctuating asymmetry (Klingenberg, 2015). Therefore, in the following, we focus exclusively on the symmetric variation. The *R. carpaticola* × *R. notabilis* F_2 hybrids are not predominantly intermediate, as has been observed in F_2 hybrid swarms of some Asteraceae (Levin, 1968; Brochmann & al., 2000). Instead, the *Ranunculus* hybrids rather encompass the whole range of both parental species and a large proportion of them exhibit phenotypes not found in the parents, a phenomenon already discussed by Rieseberg & al. (1993). Considering the entire dataset of all F_2 hybrid leaf phenotypes, the continuum of forms made it difficult to define phenotype clusters in order

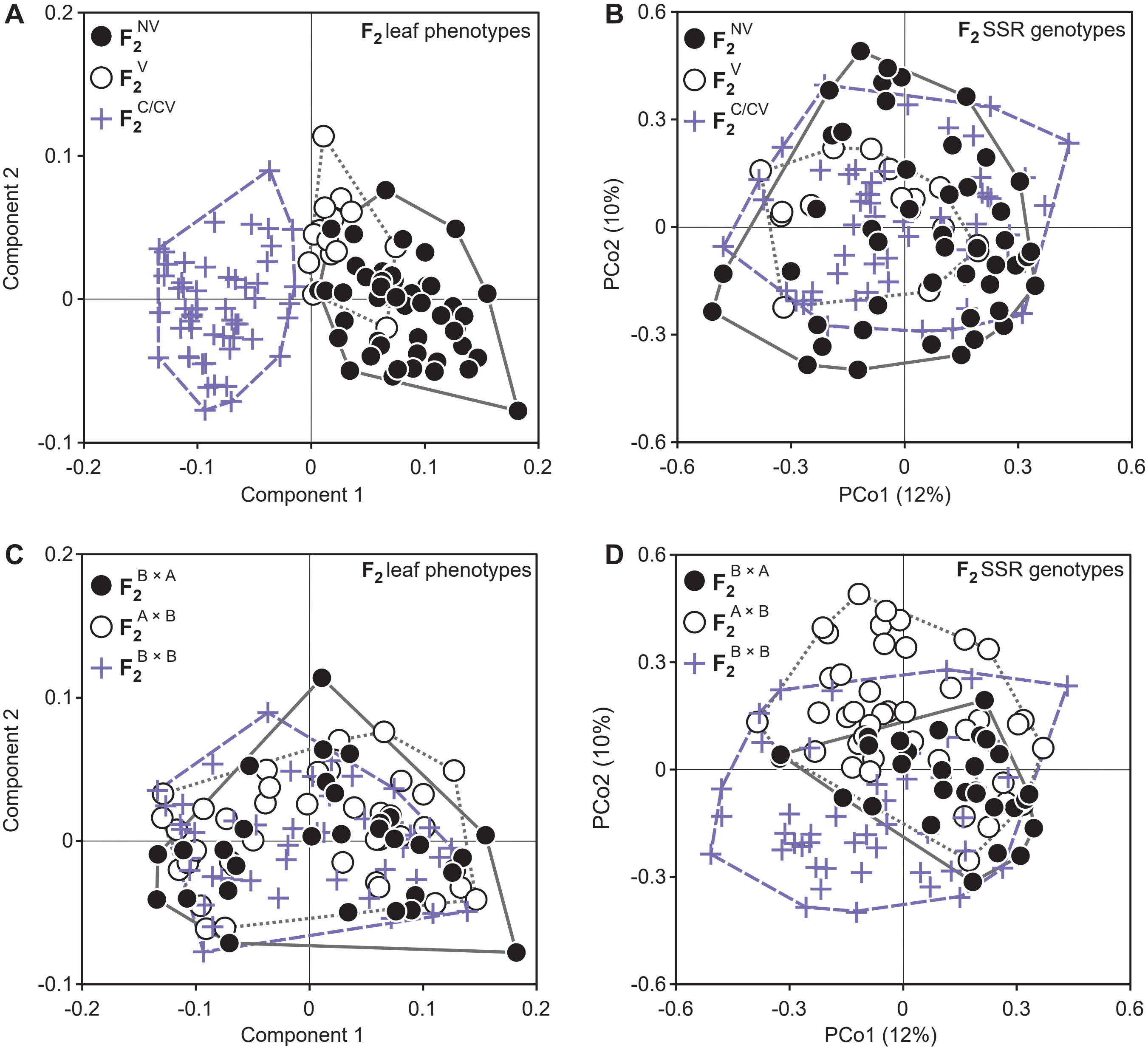

Fig. 5. Segregation of phenotypes and genotypes in *R. carpaticola* × *R. notabilis* F_2 hybrid plants. **A,** Non-metric multidimensional scaling (NMDS; 2D) based on morphological dissimilarities (Procrustes distances) within a subset of 119 F_2 hybrid plants (NMDS stress = 0.1334). Plus signs = F_2 hybrids similar to *R. carpaticola* and *R. variabilis* (C and CV phenotypes); black dots = F_2 hybrids similar to *R. notabilis* and *R. variabilis* (NV phenotypes); white dots = F_2 hybrids similar to *R. variabilis* (V phenotypes). **B,** Principal coordinates analysis (PCoA) based on genetic dissimilarities (Jaccard distances among multilocus SSR genotypes) within the same subset of 119 F_2 hybrids as shown in (A). **C,** Identical NMDS as in (A) with F_2 hybrids affiliated to the F_1 crossing lines. **D,** Identical PCoA as in (B) with F_2 hybrid affiliated to the F_1 crossing lines.

to identify a clear segregation pattern. As already mentioned by Hardig & al. (2000), morphological characters are often not expressed in a simple additive fashion. Combined effects of two shape gradients can be seen in the ordination of F_2 hybrid leaves in the plane described by the first two relative warps (RW1 & 2; Fig. 1). The relative warps analysis of the entire dataset of all possible hybrid leaf shapes might not be appropriate to detect an overall segregation pattern of the F_2 plant individuals. Averaging of leaf shapes within each plant individual and analyzing Procrustes distances among the plants enabled the assignment of half of the F_2 hybrids to phenotypes with lateral sinuses (i.e., *R. notabilis*- and *R. variabilis*-like) and the others to *R. carpaticola*-like phenotypes. In spite of the great phenotypic diversity of the F_2 hybrids, their leaf shape might be controlled by a single pair of alleles according to the single gene model for determination of leaf shape (Elmore, 1986; Campitelli & Stinchcombe, 2013; Campitelli & al., 2013; López Anido & al., 2016; Huang & Chu, 2017). All above-mentioned studies concordantly identified the lobed or divided leaves as dominant trait over the recessive undivided state. While other plants, e.g., *Cynara* L., showed full dominance of the divided shape allele with 3 : 1 segregation pattern (López Anido & al., 2016), our *R. carpaticola* × *R. notabilis* F_2 hybrids exhibited a segregation ratio of 1 : 1, suggesting partial dominance of a putative divided-leaf allele, recorded also for *Ipomoea* L. (Elmore, 1986; Bright, 1998). Similar to the case of the *R. auricomus* heterophylly (basal leaves exhibit different phenotypes during one vegetation period; Electr. Suppl.: Fig. S1), the lobed forms of *Ipomoea* appear only from the 5th leaf of a heterophyllous sequence (Campitelli & al., 2008, 2013). The *Ipomoea* model system also showed an uneven distribution of lobed/unlobed leaf phenotypes in nature and a relationship between leaf shape variation, thermoregulation and frost tolerance was discussed (Campitelli & al., 2013). Regarding the distribution of the European *Ranunculus auricomus* complex, some authors reported on the north–south and west–east cline of species with divided leaf phenotypes (Borchers-Kolb, 1985; Jalas & Suominen, 1989). However, the evolutionary significance of this geographical gradient needs to be tested. According to Bright (1998), the role of variation in plant leaf shape for adaptive evolutionary processes is still questionable.

The *R. carpaticola* × *R. notabilis* F_2 hybrids originated from three crossing lines (F_2A×B, F_2B×A, F_2B×B), which differed in their genetic background as inferred from microsatellite genotyping. In contrast to the very low yet detectable genetic differentiation, the three crossing lines did not exhibit a significant phenotypic differentiation. The genetic segregation inferred from microsatellites does not necessarily reflect the segregation of shape-altering alleles, but, on the other hand, discordance in genetic and phenotypic patterns has been observed in other plants as well (Lihová & al., 2007). Besides a potential bias due to genotyping of only a few SSR loci, also epigenetic control factors of leaf shape may explain the discordance. Furthermore, the effects of different parental F_1A and F_1B genotypes (Electr. Suppl.: Fig. S3A) may have played a role because multiple F_2 hybrids originating from different crossing lines differed in shape. This might be an indication that parental genotypes do affect hybrid phenotypes, as previously reported for other plants (Lord & Richards, 1977; Iwata & al., 2002). The F_1A and F_1B plants also slightly differed in shape (Electr. Suppl.: Fig. S3B), whereby the F_1A plants were phenotypically closer to *R. notabilis* and the F_1B were more similar to *R. carpaticola*. The F_2B×A crossing line with *R. notabilis*-like pollen donors and *R. carpaticola*-like mother plants exhibited multiple conspicuous features, e.g., the highest morphological disparity among the three crossing lines, the smallest mean centroid size and the highest proportion of *R. variabilis*-like segregant phenotypes. These results can be interpreted as an additional indication that *R. variabilis*-like species might have originated preferentially from crosses with *R. notabilis*-like pollen donors, which contributed the partially dominant divided-leaf allele. A close morphological relationship of the sexual species *R. notabilis* and its natural hybrid *R. variabilis* has been supported by analyzing their morphospace, showing identical morphological trends along the major relative warps (Electr. Suppl.: Figs. S4A, B). Although the relative warps described practically the same shape changes in both species, the interspecific differences remained preserved, i.e., *R. notabilis* exhibited finer segment sinuses and smaller leaves ($P = 0.0148$; Electr. Suppl.: Fig. S5A). The contribution of the phenotypically opposite *R. carpaticola* to the F_2 hybrid morphospace (Fig. 2) was expressed by the segregation of leaf phenotypes without lateral sinuses. In comparison, the *R. notabilis* morphospace almost entirely lacked the less-divided leaf forms (Electr. Suppl.: Fig. S4A). In contrast to *R. notabilis* but similar to the F_2 hybrids, the natural allopolyploid hybrid *R. variabilis* exhibited also leaf phenotypes without lateral sinuses (Electr. Suppl.: Fig. S4B; RW4). *Ranunculus carpaticola*-like parental species affected the F_2 hybrid morphospace by increased leaf size (Electr. Suppl.: Fig. S5A), increased phenotypic disparity and decreased asymmetric variation (Electr. Suppl.: Figs. S5B–E) as compared to *R. notabilis* and *R. variabilis*. The relative warps of the F_2 hybrid morphospace also uncovered shapes, which are dissimilar from *R. carpaticola*, *R. notabilis*, and *R. variabilis*, but highly resemble other polyploid apomictic species of the *R. auricomus* complex. A prominent example of a novel hybridogenous leaf form, not present in either of the parental species morphospace, showed the 3rd relative warp of the F_2 hybrid morphospace, depicting leaf phenotypes highly resembling *R. alsaticus* W.Koch (Koch, 1939; Borchers-Kolb, 1985).

In conclusion, our study confirms the importance of Mendelian segregation in early hybrid offspring for creating phenotypic diversity in apomictic plant complexes. Since the establishment of functional apomictic seed formation takes several generations (Hojsgaard & al., 2014), sexual processes after the first hybridization events do have a major influence on the diversity of a complex as a whole. We demonstrate with a quantitative method that a single cross of two divergent sexual progenitor species can result in a morphospace with a continuum of phenotypes between the parents and can generate new forms not present in the progenitors. From this morphospace, apomictic lineages can evolve and eventually certain phenotypes can be stabilized. Groups of similar phenotypes (C, CV, NV, V), however, are neither monophyletic groups nor can they be clearly circumscribed. Hence our results confirm experimentally the conclusions made by Ericsson (1992), Hörandl & al. (2009) and Hörandl (2018), that former "morpho-groups" or "main species" (Marklund, 1961, 1965) based on phenotypic similarity are unsuitable units for taxonomic classification.

■ AUTHOR CONTRIBUTIONS

LH and EH conceived and designed the study. EH did the crossings, BHB did the cytotyping, LH did the geometric morphometric analyses and genotyping. LH, BHB and EH wrote the manuscript. — ORCID: LH, https://orcid.org/0000-0002-6885-1317; BHB, https://orcid.org/0000-0001-5379-6055; EH, https://orcid.org/0000-0002-7600-1128

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