Microsatellite Analyses of Artificial and Spontaneous Dogrose Hybrids Reveal the Hybridogenic Origin of Rosa micrantha by the Contribution of Unreduced Gametes

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Abstract

Dogroses are characterized by a unique meiosis system, the so-called canina meiosis, which facilitates sexual reproduction at odd-number ploidy. The mostly pentaploid somatic level of dogroses is restored by a merger of haploid sperm cells and tetraploid egg cells. We analyzed experimental hybrids between different dogrose species using microsatellites to determine pollen-transmitted alleles. This information was used to reconstruct the putative hybridogenic origin of Rosa micrantha and R. dumalis and to estimate the frequency of spontaneous hybridization in a natural population. We found no evidence for the hybrid origin of R. dumalis, but our data suggest that R. micrantha presumably arose by hybridization between R. rubiginosa and R. canina or R. corymbifera. We observed only hexaploid individuals of R. micrantha, thus the establishment of this hybridogenic species was favored when unreduced gametes contributed to their origin. We demonstrate that spontaneous hybrids originated infrequently from the parental species in a natural population, but hybridization was often associated with the formation of unreduced gametes. We postulate that unreduced gametes play a major role in the evolutionary success of dogrose hybrids because they provide highly homologous chromosomes crucial for bivalent formation during canina meiosis and thus ensuring this unique form of sexual reproduction.

Key words: anorthoploidy, hybridization, meiosis, polyploidy, sect. Caninae

Hybridization has become increasingly recognized as a major evolutionary driving force for the evolution of plant diversity (Rieseberg 1997; Rieseberg and Garney 1998; Soltis PS and Soltis DE 2009). It has an impact on many, if not most, speciation events in plants (Soltis PS and Soltis DE 2009); Recent genomic analyses have discovered several rounds of genome duplication during angiosperm evolution (Simillion et al. 2002; Blanc and Wolfe 2004), and genome doubling is involved in far most cases of experimentally proved hybrid speciation (allopolyploidy). Effects of hybridization and polyploidization vary remarkably between species (Hegarty and Hiscock 2005, 2009), and the mechanisms by which allopolyploid species evolve most frequently, for example, the impact of unreduced gametes, are not well understood (Soltis and Soltis 2009). Thus, studies on recently evolved natural hybrid systems will shed light on the factors and processes driving allopolyploidization.

The frequency of spontaneous hybridization has primarily been investigated in crops and their native relatives to assess the risk of gene flow from genetically modified crops to the wild (Darmency et al. 1998; Ellstrand et al. 2007; Kiær et al. 2007; Andersen et al. 2009). However, spontaneous hybridization varies between families and genera and is most common in outcrossing perennials with reproductive mechanisms that overcome hybrid sterility such as vegetative growth, agamospermy, or permanent odd-number ploidy (Ellstrand et al. 1996).

In the northern temperate region, Rosaceae genera (e.g., Malus L., Rosa L., Rubus L.) contain the highest number of hybrid taxa (Ellstrand et al. 1996). Dogroses (Rosa sect.
Caninae (DC. Ser.) comprising about 60 species distributed in Europe and west Asia are well characterized by a unique meiosis system (Täckholm 1920, 1922; Blackburn and Heslop-Harrison 1921; Blackburn 1925). The majority of dogrose species are pentaploid, but some are tetra- or hexaploid (2n = 4x, 5x, 6x = 28, 35, 42) with a chromosome base number of x = 7 (Figure 1). Irrespective of the somatic ploidy level, only 2 of the 5 homologous chromosomes form bivalents, whereas the other 3 homologous chromosomes remain unpaired during meiosis (Täckholm 1922). These univalents are discarded in the microsporogenesis but maintained in the megasporogenesis, resulting in haploid pollen grains and tetraploid egg cells in pentaploid plants, triploid egg cells in tetraploid, and pentaploid egg cells in hexaploid individuals (Figure 1). Molecular studies demonstrated that pentaploid dogrose species contained a maximum of 4 different nuclear ribosomal internal transcribed spacer types (Wissmann 1999; Ritz et al. 2005; Kovarik et al. 2008). Corresponding results were shown for microsatellite alleles in pentaploid and tetraploid species, with a maximum of 4 and 3 different alleles, respectively (Nyborg et al. 2006, 2004), suggesting that one of the microsatellite alleles of each locus has at least 2 identical copies. Furthermore, the chromosome sets that carry identical alleles are involved in bivalent formation. These results imply that chromosome pairing is not random between the homologous chromosomes. Instead, pairing proceeds between copies of the Proto-Caninae genome (Ritz et al. 2005; Kovarik et al. 2008), represented by black chromosome sets in Figure 1.

The peculiar meiosis system evolved probably in response to the multiple hybridogenic origin of dogroses (Ritz et al. 2005; Kovarik et al. 2008) because it restores sexual reproduction even at odd-number ploidy levels. However, it is unknown whether canina meiosis originated only once, and primary hybrids gave rise to many different species by incorporating various other rose genomes in subsequent hybridization events (Nyborg et al. 2006) or if it evolved several times independently (Wulff 1954), concordant with most other hybrid taxa (Solis PS and Solits DE 2009).

Taxonomy of dogroses is difficult because of this intensive reticulate history, their readiness to hybridize, and the plasticity of morphological characters (Wissemann and Ritz 2007; De Cock et al. 2008). Interspecific hybrids are difficult to detect because their phenotype is matroclinal with respect to most of the taxonomically important characters (Werlemark et al. 1999; Werlemark and Nyborg 2001; Ritz and Wissemann 2003). Peculiar exceptions among these characters are the persistence of sepals and the diameter of the orifice, which are paternally inherited (Keller 1931; Gustafsson 1944; Ritz and Wissemann 2003). The mixture of maternally and paternally inherited important taxonomic traits suggests that some dogrose species represent interspecific hybrids (Ritz and Wissemann 2003).

![Figure 1. Genetic constitution of dogroses.](https://example.com/figure1.png)

Figure 1. Genetic constitution of dogroses. The hypothesized genetic constitution of dogroses at different somatic ploidy levels is presented according to cytological studies of Täckholm (1920, 1922). Species at all ploidy levels (A–C) are supposed to produce haploid sperm cells (1n = 1x = 7). Our data suggest that at least the hexaploid Rosa micrantha develops diploid pollen grains (C, dashed line). (A) Tetraploid species (2n = 4x = 28) produce triploid egg cells (1n = 3x = 21), (B) pentaploid species (2n = 5x = 35, most common) develop tetraploid egg cells (1n = 4x = 28), and (C) hexaploid species (2n = 6x = 42) produce pentaploid egg cells (1n = 5x = 35). Black chromosomes represent the 2 sets of bivalent-forming chromosomes that probably refer to the Proto-Caninae genome (Ritz et al. 2005; Kovarik et al. 2008). White, light gray, dark gray, and striated chromosomes refer to the univalent-forming genomes. A maximum of 2 different univalent-forming genomes is found in tetraploid species (A), a maximum of 3 in pentaploid species (B), and a maximum of 4 in hexaploid species (C).
Schanzer and Vagina 2007). For example, the hybrids between Rosa canina L. or R. corymbifera Borkh. as seed parent and R. rubiginosa L. as pollen parent are morphologically indistinguishable from R. dumalis Bechst., and the reciprocal hybrids R. rubiginosa × R. canina (or R. corymbifera) are morphologically identical to R. micrantha Borrer ex Sm. (Ritz and Wissemann 2003).

Given this example we want to answer the following questions:

1. Did R. micrantha and R. dumalis originated by recurrent hybridization between reciprocal crossings of R. rubiginosa and R. canina (or R. corymbifera)?
2. To what extent do spontaneous interspecific hybrids occur between R. rubiginosa and R. canina in a natural population?

To answer these questions, we identified pollen-transmitted microsatellite alleles in reciprocal artificial hybrids between R. canina (or R. corymbifera) and R. rubiginosa. We employed this information to detect species-specific alleles of the parental species in the putative hybridogenic species R. micrantha and R. dumalis. These species-specific alleles were used to screen seeds produced by open pollination of R. canina and R. rubiginosa in a natural population.

**Material and Methods**

**Plant Material**

In order to identify pollen-transmitted microsatellite alleles, 25 interspecific hybrids between different dogrose species and their parents (Wissemann and Hellwig 1997) cultivated in the Botanic Garden Gießen were investigated using flow cytometry and microsatellite analyses (Table 1). Parental plants were originally sampled from the sites “Einzelberg” and “Junkerberg” (both near Göttingen, Lower Saxony, Germany). Eight plants of R. canina (seed parent) × R. rubiginosa (pollen parent), 6 plants of R. corymbifera × R. rubiginosa, 1 plant of R. rubiginosa × R. canina, and 3 plants of R. rubiginosa × R. corymbifera originating from the parental plants of site “Junkerberg” were analyzed (Table 1, Supplementary Table 4). One plant of R. canina × R. rubiginosa, 3 plants each of R. micrantha × R. rubiginosa, and its reciprocal crossing of the parental plants from the site “Einzelberg” were investigated (Table 1, Supplementary Table 4).

To assess the frequency of spontaneous hybridization, we sampled offspring of R. canina and R. rubiginosa in a natural population “Himmelreich” (Jena, Thuringia, Germany). At this site, about 100 dogrose bushes grow arranged in hedges on a meadow of about 0.01 km² close to a pine forest. Most of these plants are R. canina and R. corymbifera, 9 are R. rubiginosa, and 1 is R. micrantha. Two bushes of R. elliptica also present at the site were not investigated here. Five bushes of R. canina and 4 bushes of R. rubiginosa of at least 1 m in height and diameter were sampled. Five seeds from 5 different hips per plant were analyzed (in total 125 seeds for R. canina and 100 seeds for R. rubiginosa). We analyzed the embryos directly from seeds to avoid effects of negative natural selection on hybrid establishment. For seeds with alleles deviating from the maternal ones, polymerase chain reactions (PCRs) were replicated to exclude PCR artifacts.

To estimate the intraspecific variation of the investigated species, we analyzed additional individuals of R. canina, R. corymbifera, R. micrantha, and R. rubiginosa at the sites “Junkerberg,” “Einzelberg,” “Gartetal,” and “Himmelreich” (Supplementary Table 1). Rosa dumalis was not present at the above sites, and 6 samples were collected at the site “Napoleonestein” (Jena, Thuringia, Germany). Specimens of parental plants of crossings, interspecific hybrids, parental plants of the offspring analyses, and representative plants of investigated species from all sample sites were deposited at the herbarium Gießen (GIE).

**Ploidy Estimation**

Leaf material of hybrids and of parental plants was subjected to flow cytometry analysis. Leaf material was chopped at room temperature in 0.4 ml extraction buffer (0.1 M citric acid, 0.5% Tween 20) with 10 g/l PVP 40 000) according to Yokoya et al. (2000). The suspension was filtered through a 30-µm nylon gauze filter (CellTrics, Partec GmbH, Münster, Germany), mixed with 1.6 ml staining buffer (Cystain UV precise P, Partec GmbH) and analyzed with the flowcytometer (Cell Counter Analyzer CCA II, Partec GmbH). Together with each sample, leaf material of R. arvensis Huds. (2n = 2x = 14) was analyzed as an internal standard. A minimum of 10 000 nuclei giving peaks with a coefficient of variation of approximately 10% were counted.

**Microsatellite Analysis**

Total DNA was extracted from silica gel–dried leaf material collected from living plants using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the user’s protocol. For the offspring analysis, the embryo was manually isolated from a single seed, and DNA was subsequently extracted following the same protocol as above.

We obtained primers of the microsatellite DNA loci developed for R. hybrida (Esselink et al. 2003) from the Department of Biodiversity and Identity, Plant Research International, Wageningen, The Netherlands. Hybrids and their parents were screened for unique paternally inherited alleles at 10 microsatellite loci, which have been the most variable ones in the studies of Nybom et al. (2004, 2006). We observed paternally inherited alleles at 3 loci, consisting of trinucleotide repeats (RhE506, RhP519, and RhP507), thus amplification of these loci was performed for all samples. The sequence tagged microsatellite site amplification was performed in 25 µl containing 2.5 µl 10-fold polymerase buffer (100 mM Tris–HCl pH 9.0, 500 mM KCl, 15 mM MgCl₂, 1.0% Triton X-100, 2.0 mg/ml bovine serum albumin), 2.5 µl 2 mM dNTP, 1 µl of each primer (10 pmol/µl), 1 unit of Taq polymerase (MBI Fermentas, St. Leon-Rot, Germany), and 50 ng DNA template from the
plants whose allele number matched their ploidy level are corrected with a factor obtained from allele peak areas of 700 bp (LICOR) size standard. Labels and lengths of alleles Fragment sizes were determined by comparison to the 50–automated DNA sequencer (LICOR 4000L, Lincoln, NE). National Diagnostics, Atlanta, Georgia, USA) on an lane on 41 cm polyacrylamide gels (6% Sequagel XR, chilled on ice, and then analyzed simultaneously in the same method (Esselink et al. 2004). Therefore, allele peak areas can be calculated to determine the allelic peak ratios were compared with the expected values of hypothetical configurations in the samples.

**Results**

**Ploidy Level**

The interspecific hybrids between *R. canina*, *R. corymbifera*, *R. rubiginosa* as well as the parental plants, and the 5 investigated plants of *R. dumalis* were pentaploid (Table 1, Supplementary Table 1). *Rosa micrantha* and hybrids originating from *R. micrantha* either as pollen donor or pollen receiver as well as the parental plant of the hybrids were hexaploid (Table 1, Supplementary Table 1).

**Microsatellite Analysis**

The pentaploid species *R. canina*, *R. corymbifera*, *R. dumalis*, and *R. rubiginosa* had 4 or less different alleles and the hexaploid *R. micrantha* had 5 or less different alleles at the investigated loci (Supplementary Table 1). The number of alleles lower than expected from the ploidy level imply that at least 1 allele of each locus has 2 or more identical copies in the majority of plants (Table 1). This was corroborated by calculating ratios between peak areas (MAC-PR approach, Supplementary Table 4). Peak areas could be unambiguously delimited because analyzed microsatellite loci consisted of trinucleotide repeats and rarely produced stutter bands.

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**Table 1** Tentative allelic configurations of parents, interspecific hybrids, and *Rosa micrantha*

<table>
<thead>
<tr>
<th>Parental plants and crossings</th>
<th>No. of plants</th>
<th>Ploidy level</th>
<th>RhE506</th>
<th>RhD201</th>
<th>RhP507</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>R. canina</em></td>
<td>1</td>
<td>5</td>
<td>ACEII</td>
<td>BBHEE</td>
<td>ADFGG</td>
</tr>
<tr>
<td><em>R. canina E</em></td>
<td>1</td>
<td>5</td>
<td>BCEII</td>
<td>BBFGG</td>
<td>DFGGG</td>
</tr>
<tr>
<td><em>R. canina J</em></td>
<td>1</td>
<td>5</td>
<td>BCEII</td>
<td>BBHEE</td>
<td>DFGGG</td>
</tr>
</tbody>
</table>

Tentative allelic configuration for 3 microsatellite loci *RhE506, RhD201,* and *RhP507* was determined based on the ploidy level of plants, the inheritance of alleles to offspring and allele peak ratios calculated by MAC-PR (see Supplementary Table 4). Parental plants were originally sampled from 2 populations: Einzelberg (E) and Junkerberg (J). The female parent is mentioned first in the designation of the interspecific hybrids. The putative bivalent-forming allele is marked in bold. The paternally inherited allele in artificial hybrids is underlined and in italics.

diluted extracts. The forward primers were modified at the 5’ end with infrared dyes DY-681 or DY-781 (Biomers.net GmbH, Ulm, Germany). The amplification was performed for 180 s at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 55 °C, and 60 s at 72 °C; and a final extension for 180 s at 72 °C. PCR products were diluted 1:20. Differentially labeled products were mixed prior to electrophoresis and diluted 1:1 in formamide loading dye (Amersham, Uppsala, Sweden). Samples were denatured for 2 min at 95 °C, chilled on ice, and then analyzed simultaneously in the same lane on 41 cm polyacrylamide gels (8% Sequagel XR, National Diagnostics, Atlanta, Georgia, USA) on an automated DNA sequencer (LICOR 4000L, Lincoln, NE). Fragment sizes were determined by comparison to the 50–700 bp (LICOR) size standard. Labels and lengths of alleles are given in Supplementary Table 2.

Tif images of the polyacrylamide gels were analyzed with the software package Phoretix 1D v. 4.0 (Phoretix International, Newcastle upon Tyne, UK) to estimate the peak areas for the alleles in each sample which were afterward used for determination of the allelic genotype. Allele peak ratios can be calculated to determine the allelic configuration (genotype) of a plant exhibiting less alleles than the maximum number for its ploidy level using microsatellite DNA allele counting–peak ratio (MAC-PR) method (Esselink et al. 2004). Therefore, allele peak areas are corrected with a factor obtained from allele peak areas of plants whose allele number matched their ploidy level (e.g., pentaploids with 5 different alleles). These corrected allele peak ratios were compared with the expected values of hypothetical configurations in the samples.
To account for a bias of peak ratios caused by preferential amplification of a certain allele during PCR, we corrected peak ratios with a factor obtained from those samples that contained only alleles in single copy (pentaploid and hexaploid hybrids with 5 or 6 different alleles, respectively).

**Allelic Variation of the Crossings**

Allelic combinations and allele peak ratios of the interspecific hybrids and their parents are listed in Table 1 and Supplementary Table 4, respectively. In 3 of the 10 initially screened microsatellite loci, we found species-specific paternally inherited alleles in interspecific hybrids, but only in one direction of the crossing. In all plants of a crossing, the same alleles were transmitted by the pollen parent implying that the sperm cells of one plant always contained the same chromosome set. Crossing schemes based on the presumed allelic configuration of reciprocal hybrids and their parents are presented in Figure 2 (crossings between *R. canina*/*R. corymbifera* and *R. rubiginosa*) and Figure 3 (crossings between *R. rubiginosa* and *R. micrantha*). In the results presented below, crossings are presented as species of seed parent × species of pollen parent.

**R. canina × R. rubiginosa and R. corymbifera × R. rubiginosa**

At locus RhE506, hybrids had 5 different alleles (ACDEI and BCDEI). The D-allele was transmitted by the pollen grain because it was not present in the maternal plants *R. canina* or *R. corymbifera*. The allele peak ratios of both plants of *R. rubiginosa* used for the crossings suggest that the D-allele had 2 copies, which were located on the bivalent-forming chromosomes in *R. rubiginosa* because the ratio between the peak area of B-allele and the D-allele matched approximately the value 0.5 in the respective pollen parent (*R. rubiginosa* E: B/D = 0.59 and *R. rubiginosa* J: B/D = 0.53, Table 1).

At locus RhD201, hybrids had only 3 different alleles (BEH and BFG), which were the same as in the seed parents *R. canina* or *R. corymbifera*. Hybrids shared only the B-allele with the pollen parent, thus the B-allele was transmitted by the pollen grain and had presumably 2 identical copies in *R. rubiginosa* (*R. rubiginosa* E: A/B = 0.76; B/D = 1.60 and *R. rubiginosa* J: A/B = 0.79; B/D = 1.63).

At locus RhP507, hybrids from the site “Einzelpark” had 5 different alleles (ABDFG) and 4 different alleles at the site “Junkerberg” (BDFG). The B-allele was not present in the maternal plants *R. canina* or *R. corymbifera* but was transmitted by the pollen donor *R. rubiginosa*. We expect the configuration BBGGG in *R. rubiginosa* based on the allele peak ratios B/G = 0.52 (*R. rubiginosa* E) and B/G = 0.74 (*R. rubiginosa* J).

**R. rubiginosa × R. canina and R. rubiginosa × R. corymbifera**

At locus RhE506, hybrids had the same alleles as the maternal parent *R. rubiginosa* (BDGF), but we expect different allelic configurations. Hybrids shared the I- and the B-allele with *R. canina* and *R. corymbifera*. Allele peak ratios suggest that the I-allele had 2 copies in the hybrids (*R. rubiginosa* J × *R. canina* J: B/I = 0.79 and *R. rubiginosa* J × *R. corymbifera* J: B/I = 0.81) and the B-allele had 1 copy in the hybrids (*R. rubiginosa* J × *R. canina* J: B/D = 0.55; B/F = 1.31 and *R. rubiginosa* J × *R. corymbifera* J: B/D = 0.84; B/F = 1.20).

Thus, we expect that the I-allele had 2 copies in the paternal plants *R. canina* and *R. corymbifera*, (*R. canina* J: B/I = 0.32; C/I = 0.17 and *R. corymbifera* J: B/I = 1.16; C/I = 0.67). However, the ratios B/C = 1.90 (*R. canina* J) and 1.73 (*R. corymbifera* J) should be equal to 1 to match this expectation.

At locus RhD201, hybrids had 5 different alleles (ABCDG and ABCDE). The G- and the E-allele were transmitted by the pollen grain and had presumably 2 identical copies in *R. rubiginosa* but was transmitted by the pollen donor *R. rubiginosa* J: A/B = 0.81 and the B-allele had 1 copy in the hybrids (*R. rubiginosa* J × *R. canina* J: B/D = 0.59; B/G = 0.84; B/F = 1.16). Thus, we expect that the I-allele had 2 copies in the paternal plants *R. canina* and *R. corymbifera*, (*R. canina* J: B/I = 0.32; C/I = 0.17 and *R. corymbifera* J: B/I = 1.16; C/I = 0.67). However, the ratios B/C = 1.90 (*R. canina* J) and 1.73 (*R. corymbifera* J) should be equal to 1 to match this expectation.

At locus RhE506, hybrids had 5 different alleles (ABDFG) and 4 different alleles at the site “Junkerberg” (BDFG). The B-allele was not present in the maternal plants *R. canina* or *R. corymbifera* but was transmitted by the pollen donor *R. rubiginosa*. We expect the configuration BBGGG in *R. rubiginosa* based on the allele peak ratios B/G = 0.52 (*R. rubiginosa* E) and B/G = 0.74 (*R. rubiginosa* J).

**R. rubiginosa × R. canina and R. rubiginosa × R. corymbifera**

At locus RhE506, hybrids had the same alleles as the maternal parent *R. rubiginosa* (BDGF), but we expect different allelic configurations. Hybrids shared the I- and the B-allele with *R. canina* and *R. corymbifera*. Allele peak ratios suggest that the I-allele had 2 copies in the hybrids (*R. rubiginosa* J × *R. canina* J: B/I = 0.79 and *R. rubiginosa* J × *R. corymbifera* J: B/I = 0.81) and the B-allele had 1 copy in the hybrids (*R. rubiginosa* J × *R. canina* J: B/D = 0.55; B/F = 1.31 and *R. rubiginosa* J × *R. corymbifera* J: B/D = 0.84; B/F = 1.20).

Thus, we expect that the I-allele had 2 copies in the paternal plants *R. canina* and *R. corymbifera*, (*R. canina* J: B/I = 0.32; C/I = 0.17 and *R. corymbifera* J: B/I = 1.16; C/I = 0.67). However, the ratios B/C = 1.90 (*R. canina* J) and 1.73 (*R. corymbifera* J) should be equal to 1 to match this expectation.

At locus RhD201, hybrids had 5 different alleles (ABCDG and ABCDE). The G- and the E-allele were transmitted by the pollen grains of *R. canina* and *R. corymbifera*, respectively. Allele peak ratios could only be calculated for the ratio B/E, which is 1.13 in *R. canina* E and 0.90 in

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**Figure 2.** Hypothesized diagram of artificial reciprocal crossings between *Rosa canina*/*R. corymbifera* and *R. rubiginosa*. White and black chromosomes represent the bivalent-forming chromosomes of *R. rubiginosa* and *R. canina*/*R. corymbifera*, respectively. Univalent-forming chromosomes are marked in gray tones (*R. rubiginosa*) and with stripes (*R. canina*/*R. corymbifera*). The different gray tones and types of stripes of univalent-forming chromosomes indicate that these chromosome sets can be genetically differentiated from each other.
For locus RhP507, alleles of hybrids were identical to those of *R. rubiginosa* (BG), but we assume that the allelic configurations differed between the hybrids and the maternal parent. For the hybrids, we hypothesize the allelic configuration BGGGG because we know from the reciprocal crossings that the B-allele was involved in the bivalent formation in *R. rubiginosa* and thus, there was only 1 copy of the B-allele in the egg cell of *R. rubiginosa*. The G-allele was transmitted by the pollen grain of *R. canina* and *R. corymbifera* because it was the only allele shared between hybrids and their paternal parent. However, the B/G values of the hybrids deviated from the expected value of the proposed configuration BGGGG (*R. rubiginosa* J/C2 *R. canina* J: B/G = 0.71 and *R. rubiginosa* J × *R. corymbifera* J: B/G = 0.36).

**R. rubiginosa** × **R. micrantha**

At locus RhE506, the hexaploid hybrids had 6 different alleles (BCDEFI). The C- and the D-allele were transmitted by a diploid pollen grain of *R. micrantha* because these alleles were not present in *R. rubiginosa*. The allele peak ratios of *R. micrantha* suggest that the C-allele had 2 identical copies (B/C = 0.60 and C/E = 1.31 and C/I = 1.54).

At locus RhD201, the hybrids had 5 different alleles (ABCDF). The F-allele was paternally inherited because it was not present in the maternal parent of the hybrids. The allele peak ratios imply that also a B-allele was transmitted by a diploid pollen grain of *R. micrantha* (*R. micrantha* E: A/B = 0.76 and B/D = 1.60). For locus RhP507, the hybrids in both directions and *R. micrantha* had the same alleles (BEG). We assume that the B-allele of both the pollen parent and the seed parent was involved in bivalent formation.

**R. micrantha** × **R. rubiginosa**

At locus RhE506, the allelic combinations of the hexaploid hybrids were identical to the reciprocal ones (BCDEFI), with the D-allele transmitted by *R. rubiginosa* (B/D = 0.59) and only 1 C-allele, which instead occurred in 2 copies in *R. micrantha* (B/C = 0.60 and C/E = 1.31 and C/I = 1.54).

At locus RhD201, the hybrids in both directions and *R. micrantha* had identical allelic configurations (ABBCDF). The B-allele was transmitted via the pollen grain because it had 2 identical copies in *R. rubiginosa* (A/B = 0.76 and B/D = 1.60). For locus RhP507, the hybrids in both directions and *R. micrantha* had the same alleles (BEG). We assume that the B-allele of both the pollen parent and the seed parent was involved in bivalent formation.

**Genetic Variability within and between Species**

Samples of *R. rubiginosa* were genetically less variable than those of *R. canina* and *R. corymbifera* with respect to the number of different alleles and allelic combinations (Supplementary Table 3). The number of different genotypes was also very low in *R. micrantha* and *R. dumalis*, but only 8 and 6 plants were sampled, respectively (Supplementary Table 3).
*Rosa canina* and *R. corymbifera* could not be differentiated by the analyzed microsatellite loci because many samples shared allelic combinations (Supplementary Table 1).

Allelic combinations of the putative hybridogenic *R. micrantha* were similar to those of *R. rubiginosa*; however, all individuals contained the E-allele at the locus RhE506 and the F-allele at locus RhD201 specific for *R. canina* and *R. corymbifera* (Table 3). The 6 investigated individuals of putative hybridogenic *R. dumalis* had the same or similar allelic combinations as *R. canina* and *R. corymbifera* but did not contain alleles species specific for *R. rubiginosa* (Supplementary Table 1).

### Allelic Variation in Offspring of *R. canina* and *R. rubiginosa*

In most offspring, alleles were identical to those of the maternal parent (Table 2). Of 225 seeds of *R. canina* and *R. rubiginosa*, 10 seeds differed from the parental genotype at locus RhE506, 8 seeds at locus RhD201, and 5 seeds at locus RhP507.

In *R. rubiginosa*, only 1 seed deviated at 2 loci from the maternal genotype (ABCE1 and ABG for RhD201 and RhP507, respectively). This seed contained alleles that were observed in *R. canina* (I-allele at locus RhD201; A-allele at locus RhP507, respectively). The seed contained alleles that were observed in *R. canina* (I-allele at locus RhD201; A-allele at locus RhP507), thus it probably originated from an interspecific hybrid between the 2 species. Moreover, its allelic combination at locus RhP507 was equal to that of *R. micrantha* from site “Gartetal” (ABG, see Table 3).

Allelic configurations of 15 embryos deviated from the maternal genotype in *R. canina*. Ten embryos with deviating allelic configurations were produced by 2 hips of 1 plant (data not shown). Six embryos of these 2 hips contained 6 different alleles (BCDFHI) at locus RhD201 and were most likely hexaploids. These seeds had an H- and an I-allele not present in the maternal plant hca1 but were observed in other samples of *R. canina* in the same population (Supplementary Table 1).

We sampled 2 deviating embryos in the mother plant hca2 of which 1 was most likely hexaploid (ABDFG and ABDEFG at locus RhP507). Both seeds had B-alleles, which occurred mostly in *R. rubiginosa* and were shown to be involved in bivalent formation in this species (Table 1). The seed with the allelic combination ABDFG corresponded to one of the interspecific hybrids between *R. canina* and *R. rubiginosa* (Table 1). Both seeds contained an A-allele and one also contained an E-allele not present in the mother plant. Possibly they were pollinated by diploid or even triploid pollen grains of *R. rubiginosa*.

We found 2 seeds with a deviating genotype from the maternal plant hca10. The seed with allelic combination ABDFG at locus RhP507 arose presumably by hybridization because its genotype was identical to one of the interspecific hybrids between *R. canina* and *R. rubiginosa* (Table 1).

### Discussion

The aim of the study was to unravel the hybridogenic origins of *R. micrantha* and *R. dumalis* and to retrace their recurrent origins in a natural population. Therefore, we employed species-specific pollen-transmitted microsatellite alleles, which were identified from experimental crossings between putative parental species, as genetic markers.

<table>
<thead>
<tr>
<th>Locus</th>
<th>RhE506</th>
<th>RhD201</th>
<th>RhP507</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of deviating offspring and their allele combination</td>
<td>Parental allele combination</td>
<td>No. of deviating offspring and their allele combination</td>
<td>Parental allele combination</td>
</tr>
<tr>
<td><strong>Rosa canina</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hca1</td>
<td>10 ACJEM</td>
<td>BCDF</td>
<td>ABG</td>
</tr>
<tr>
<td>hca2</td>
<td>ACEI</td>
<td>BHI</td>
<td>DFG</td>
</tr>
<tr>
<td>hca9</td>
<td>ACEI</td>
<td>BCI</td>
<td>EFG</td>
</tr>
<tr>
<td>hca10</td>
<td>BCEIJ</td>
<td>BEH</td>
<td>ACDG</td>
</tr>
<tr>
<td><strong>Rosa rubiginosa</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hru3</td>
<td>BDFI</td>
<td>ABCE</td>
<td>BG</td>
</tr>
<tr>
<td>hru4</td>
<td>BCEI</td>
<td>ABCE</td>
<td>BG</td>
</tr>
<tr>
<td>hru6</td>
<td>BCEI</td>
<td>ABCE</td>
<td>EG</td>
</tr>
<tr>
<td>hru7</td>
<td>BDFI</td>
<td>ABCE</td>
<td>—</td>
</tr>
</tbody>
</table>

Allelic combinations of 5 parental plants each of *Rosa canina* and *R. rubiginosa* and their offspring are presented for the loci RhE506, RhD201, and RhP507. For each parental plant, 25 seeds from 5 different hips were analyzed. Seeds with allelic combinations that arose presumably by bidirectional hybridization between *R. canina* and *R. rubiginosa* are marked in bold. Alleles not present in the maternal parent but transmitted by a pollen grain of a plant with different alleles or originated by mutation are underlined and in italics. One seed produced deviating allele combinations in 2 loci (marked by an asterisk). All other deviating allele combinations of one maternal originated from different seeds.
Microsatellite alleles of *R. micrantha* which originated presumably by hybridization between *R. rubiginosa* (seed parent) and *R. canina* or *R. corymbifera* (pollen parent) are presented for the loci RhES06, RhD201, and RhPS07. Alleles detected in *R. canina* or *R. corymbifera* but not in *R. rubiginosa* are underlined and in italics. The E-allele marked in bold at locus RhD201 were located on the pollen-transmitted chromosome than in *R. canina* or *R. corymbifera* plants at the sampling sites. The low genetic differentiation between *R. canina* and *R. corymbifera* does not allow for differentiation between both species as paternal parent of *R. micrantha*. Indeed, discrimination between *R. canina* and *R. corymbifera* refers only to hairs of the lower leaf surface and the rhachis (Henker 2000). The hybridogenic origin of *R. micrantha* is also corroborated by a phylogenetic analysis based on AFLP data (Koopman et al. 2008). In this phylogeny, *R. micrantha* is not a sister species of *R. rubiginosa* and part of subset Rubigineae, but sister to *R. balsamica* Will., which itself might have arisen by hybridization between species of subset. Caninae and Rubigineae (Wissmann 2000; De Cock et al. 2008).

*Rosa micrantha* samples collected by us were hexaploid (Table 3), but the artificial hybrids *R. rubiginosa* × *R. canina* and *R. rubiginosa* × *R. corymbifera* were pentaploid (Table 1, Figure 2). We therefore hypothesize that hexaploid *R. micrantha* originated either by a merger of a tetraploid egg cell of *R. rubiginosa* and a diploid pollen grain of *R. canina/R. corymbifera* or by fertilization of an unreduced egg cell of *R. rubiginosa* by a haploid sperm cell of *R. canina/R. corymbifera* (Figure 4). Our results demonstrate the occasional production of unreduced pollen grains in dogroses (Table 2), which was also reported for *R. rubiginosa* × *R. sherardii* (Nybomb et al. 2006), but there is only one documented case on the occurrence of unreduced eggs (Nybomb et al. 2006).

Allele peak ratios imply that hexaploid samples of *R. micrantha* contained 2 highly homologous chromosome sets, which should facilitate correct bivalent formation (Table 1, Supplementary Table 4, Figure 4) and thus will be favored by natural selection against pentaploid hybrids (Figure 2). Correct bivalent formation of 2 highly homologous chromosomes is essential for sexual reproduction in dogroses because it has an impact on the production of viable pollen grains (Blackhurst 1948; Roberts 1975; Werlemark 2000). The maternal lineage seems to be less affected because experimental pentaploid hybrids did not produce fewer and less viable seeds than their parental species (Werlemark 2000; Ritz and Wissmann 2003).

*Rosa micrantha* transmitted diploid pollen grains because the backcrossing *R. rubiginosa* × *R. micrantha* harbors 2 alleles of the pollen parent (Table 1, Figure 3). Allele peak ratios demonstrated that one of the paternally inherited alleles was located on one of the bivalent-forming chromosomes and the other allele was located on one of the univalents (Table 1, Supplementary Table 1).

**Table 3  Microsatellite alleles of Rosa micrantha**

<table>
<thead>
<tr>
<th>Sample origin</th>
<th>No. of plants</th>
<th>Ploidy level</th>
<th>Alleles at locus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Einzelberg (LS)</td>
<td>6</td>
<td>6</td>
<td>BC/FFI ABCDF/ E</td>
</tr>
<tr>
<td>Gartetal (LS)</td>
<td>1</td>
<td>nd</td>
<td>BDEFI ABCDF/ ABG</td>
</tr>
<tr>
<td>Himmelreich (T)</td>
<td>1</td>
<td>nd</td>
<td>BDEFI ABCDF/ E</td>
</tr>
</tbody>
</table>

Detection of Species-Specific Pollen Transmitted Alleles in Experimental Crossings

We detected species-specific pollen-transmitted alleles for *R. canina*, *R. corymbifera*, and *R. rubiginosa* in 3 microsatellite loci (Table 1). This information was used to unravel the presumed hybridogenic origin of *R. micrantha* and *R. dumalis* and to detect spontaneously occurring putative hybrids between these species.

In accordance with the studies of Nybomb et al. (2006, 2004), we observed a maximum of 4 different microsatellite alleles in pentaploid species and 5 or less different alleles in hexaploid species (Tables 1 and 3, Supplementary Table 1) implying that at least 1 allele has 2 or more identical copies as was also proposed for ribosomal DNA loci (Wissmann 1999; Lim et al. 2005; Kovarik et al. 2008). In all plants of a crossing, the same alleles were inherited by the pollen parent and the allele peak ratios revealed that the paternally inherited allele has at least 2 identical copies in the pollen parent (Table 1, Supplementary Table 4). These results support the hypothesis of Nybomb et al. (2006, 2004) that the sperm cells of one plant always contain the same chromosome set, which is involved in bivalent formation during canina meiosis.

Calculated allele peak ratios do not always match the expected values of the proposed allelic configurations. However, this does not contradict the general interpretation of the data because the pollen-transmitted allele in the hybrid was in most cases unambiguously identified because it was absent in the maternal parent. The correct estimation of allele peak ratios using the MAC-PR method depends on the scoring quality of the loci (Esselink et al. 2004) and relatively large ranges of allele peak ratios for some alleles were also reported in other studies (Nybomb et al. 2006, 2004) or could not be obtained for some loci (Esselink et al. 2004; Garcia-Verdugo et al. 2009). Peak areas may vary because PCRs can be biased toward certain alleles for several reasons. During this study, we analyzed allele peak ratios via measuring the densities of image pixels because the use of an LICOR sequencer does not allow for determination of peak areas directly.
Figure 4. Hybridogenic origin of *Rosa micrantha*. Diagram of the presumed hybridogenic origin of *R. micrantha* by a merger of an unreduced egg cell of *R. rubiginosa* and a haploid pollen grain of *R. canina/R. corymbifera* (dot-dash arrows) or by fertilization of a tetraploid egg cell of *R. rubiginosa* by a diploid sperm cell of *R. canina/R. corymbifera* (dotted arrows). White and black chromosomes represent the bivalent-forming chromosomes of *R. rubiginosa* and *R. canina/R. corymbifera*, respectively. Univalent-forming chromosomes of *R. rubiginosa* are marked in gray tones those of *R. canina/R. corymbifera* are marked with stripes. The different gray tones and types of stripes of univalent-forming chromosomes indicate that these chromosome sets can be genetically differentiated from each other.

Table 4). These results imply that meiosis in the hexaploid individual of *R. micrantha* might be less effective than canina meiosis of pentaploid and tetraploid dogrose species resulting in haploid sperm cells (Figure 1, dashed line). However, success of crossings involving *R. micrantha* demonstrates that the species is fully sexual and apomixis is not its major form of reproduction. Interestingly, the establishment of an intersectional hybrid at hexaploid level has also been documented for *R. marginata Wallr.* (Täckholm 1922), which originated by hybridization between pentaploid *R. canina* (seed parent) and the tetraploid *R. gallica* L. (pollen parent producing diploid pollen grains, Wissemann 1999).

*Rosa micrantha* is a rare species (Henker 2000). This might be explained by either a low number of hybridization events or selection against it (Arnold 1997). The occasional production of *micrantha*-like seeds in *R. rubiginosa* (Table 2) suggests its recurrent independent origin as reported for many allopolyploids (Solis PS and Solis DE 2009). Even a few hybridization events would suffice to establish a hybrid population if favored by selection. However, the formation of hybrids from *R. rubiginosa* (seed parent) and *R. canina* (pollen parents) is limited in contrast to the reciprocal hybrid, presumably due to cytonuclear incompatibility (Table 2, Wissemann and Hellwig 1997). This reciprocal hybrid between *R. canina/R. corymbifera* (seed parent) and *R. rubiginosa* (pollen parent) is morphologically identical to *R. dumalis* (Ritz and Wissemann 2003). Despite the fact that we observed *R. rubiginosa*-specific alleles in the seeds of *R. canina* (Table 2), we did not find any genetic evidence for the hybridogenic origin of *R. dumalis* so far, as no such alleles were found in the species (Supplementary Table 1).

Spontaneous Hybrids in the Seed Set of *R. canina* and *R. rubiginosa*

Eight percent of the investigated offspring of *R. canina* and *R. rubiginosa* deviated from the maternal genotypes. For 4 seeds (1.7% of the offspring), we hypothesize a hybridogenic origin: One seed of *R. rubiginosa* contained alleles found in *R. canina* and 3 seeds of *R. canina* contained alleles present in *R. rubiginosa* (Table 2). The observed frequency of spontaneous hybridization in this dogrose population is rather low. This result has to be taken with caution because we investigated only one population and hybridization rates vary considerably between species and populations (Rieseberg and Carney 1998). Hybridization rate may be underestimated because the remaining seeds deviating from the maternal genotype (Table 2) could also have arisen by 1) hybridization with dogrose species not investigated in the crossing experiment but occurring in the sample area, 2) pollination with the same species used for the experimental crossings but with different alleles involved in bivalent formation, 3) pollination with the same species as the maternal parent with a genotype not sampled in this analysis, or 4) mutation in the microsatellite locus.

The low number of deviating seeds suggests that pre- or postzygotic isolation mechanisms exist between dogrose species or the ratio of seeds produced by apomixis or selfing is higher than previously assumed (Wissemann and Hellwig 1997). However, other studies also demonstrated that apomictic reproduction occurs only occasionally (Werlemark and Nybom 2001; Nybom et al. 2006, 2004). Experimental crossings (Wissemann and Hellwig 1997) and a population genetic analysis of *R. canina* (Jürgens et al. 2007) revealed a high rate of cross-pollination in dogroses. This implies that in nature, species boundaries are maintained to some extent in dogroses.

The percentage of deviating seed genotypes was considerably lower in *R. rubiginosa* than in *R. canina* (Table 2), which is also in accordance with the low genetic variability detected generally in *R. rubiginosa* (Supplementary Table 1). The late flowering time of *R. rubiginosa* may at least partially prevent gene flow with other dogrose species at the sample...
site. The success of experimental reciprocal crossings between R. canina and R. rubiginosa was strongly reduced when using R. rubiginosa as seed parent (Wissemann and Hellwig 1997). Asymmetrical reproductive isolation toward one parental species has frequently been observed (Rieseberg and Carney 1998) and is believed to be caused by cytonuclear incompatibilities (Cruzan and Arnold 1999; Bartish et al. 2000; Fishman and Willis 2006).

Interestingly, at least 6 of 15 deviating embryos of R. canina were hexaploid (Table 2). Hybrids may thus often originate by pollination involving diploid pollen grains. Unreduced gametes are a major route of polyploidy formation in plants and are frequently produced in hybrid systems due to meiosis irregularities (Ramsey and Schemske 1998). Pollen grains produced by dogroses are to a large extent unviable due to unbalanced chromosome numbers (Täckholm 1922), but also the viable fraction of pollen grains may be not uniform genetically. Unfortunately, our experimental set up could not control for the occurrence of unreduced egg cells, but the few existing studies predict an equal frequency of unreduced eggs and pollen grains (Ramsey and Schemske 1998). We hypothesize that the production of unreduced gametes is probably a crucial point in hybrid establishment between dogrose species because an increase of the ploidy level ensures that 2 highly homologous chromosomes form correct bivalents during canina meiosis and thus ensure sexual reproduction. Diploid pollen grains detected in this study contained 2 different chromosome sets (Tables 1 and 2). However, this might also occur with 2 highly homologous sets; or unreduced egg cells may play a major role in hybrid establishment. Hybrids with a balanced genetic constitution originate probably infrequently in dogroses, but these will be favored by natural selection, leading to stable new hybrid lineages as shown also for other plant hybrid systems (Arnold 1997 and literature therein).

Conclusions

We identified species-specific pollen-transmitted microsatellite alleles in experimental dogrose hybrids. These alleles were present in at least 2 copies and were located on highly homologous chromosomes involved in bivalent formation. Using this information, our data suggest that R. micrantha arose by hybridization between R. rubiginosa and R. canina or R. corymbifera, but the establishment of this hybridogenic species requires also the participation of unreduced egg cells or pollen grains in its formation. Screening for pollen-transmitted microsatellites in offspring of the parental species in a natural population, we demonstrated that spontaneous hybridization was rare but frequently associated with the formation of unreduced gametes. We postulate that unreduced gametes play a major role in the evolutionary success of dogrose hybrids. They provide genotypes on which selection favors those containing 2 highly homologous chromosomes crucial for bivalent formation during canina meiosis, thus ensuring this unique mode of sexual reproduction. Population genetic studies on R. micrantha are needed to investigate its presumed recurrent origin.

Supplementary Material

Supplementary material can be found at http://www.jhered. oxfordjournals.org/.

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