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Hybridization between the locally endangered *Rosa spinosissima* and *Rosa mollis* results in the pentaploid *Rosa* × *sabinii* in western Norway

Heidi L. Andersen, Samson J. Næss and Per H. Salvesen

H. L. Andersen (heidi.andersen@uib.no), S. J. Næss and P. H. Salvesen, Arboretum and Botanical Gardens, Univ. Museum of Bergen, Univ. of Bergen, Bergen, Norway.

Rosa spinosissima is an endangered species in Norway, found only within a limited area on the southwestern coast. Presumed hybrid forms between *R. spinosissima* and rose species within *R.* sect. *Caninae* have been recorded from this area since the late 19th century. Here, analyses of such hybrid plants and selected populations of the tentative parent species were performed using AFLP markers, nuclear DNA content, pollen viability and seed germination rates, in addition to classic morphometric analysis. It is established that the hybrid rose represents a single hybrid taxon, viz *Rosa* × *sabinii*, formed by recurrent asymmetrical hybridization events between *R. spinosissima* and *R. mollis*, with the latter being the obligate ovule donor. The evidence presented does not indicate hybridization with other co-occurring rose species, or of introgression from *R. mollis* into *R. spinosissima* through backcrossing with *R.* ×*sabinii.*

Spontaneous hybridization between related species resulting in reproductive modes that are able to stabilize hybridity is frequently observed in outcrossing perennials. This is an important mechanism for speciation (Ellstrand et al. 1996). In areas where two compatible species coexist, diffuse hybrid zones with hybrid swarms are often observed (Harrison 1993). The morphological characters of the hybrids are frequently found to be intermediate to those of the two parental species (Anderson 1949), but as some morphological characters may be under simple genetic control, this is not always the case (Rieseberg et al. 1993).

Hybridization has played an important role in the evolutionary history of the genus *Rosa* (Ritz et al. 2005, Joly et al. 2006, Ritz and Wissemann 2011). Numerous hybrid forms are currently recognized, representing both inter-subsectional and inter-sectional crosses (Henker 2000, Graham and Primavesi 2005).

Introgression is frequently mediated by backcrossing of hybrids with either parent species, but the evolutionary implications of this process are still not clear (Rieseberg and Wendel 1993, Twyford and Ennos 2012). Introgression increases the genetic diversity in a population and can, by the transfer of beneficial genes, increase the fitness of an introgressed population (Choler et al. 2004, Martin et al. 2006). However, for rare species, introgression can also pose a threat, by causing a swamping of the genome, which may eventually lead to extinction (Levin et al. 1996, Rhymer and Simberloff 1996, Levin 2002, Kellner et al. 2012).

Wild rose species share the basic chromosome number x = 7 (Täckholm 1920, 1922, Blackburn and Harrison 1921), but ploidy levels range from diploid (2x) to octoploid (8x) (Hurst 1931). Species in *Rosa* sect. *Caninae* are characterized by the heterogamic or asymmertric *Canina*-meiosis (Gustafsson 1944), where only seven bivalents are formed, leaving the remaining chromosomes as univalents. Univalent chromosomes are included in egg cells, but not in pollen cells and the pollen donor will therefore only contribute seven chromosomes to the resulting embryo, while the ovule donor contributes 2n–7 (Täckholm 1920, 1922, Blackburn and Harrison 1921). It has been demonstrated that this mechanism can account for the pronounced matroclinal inheritance often observed for this section (Wissemann and Hellwig 1997, Ritz and Wissemann 2003). The seven bivalent-forming chromosomes are highly homologous and can be inter-exchanged among all *Caninae* taxa (Nybom et al. 2004, 2006), thereby facilitating the creation of new forms through hybridization.

Rosa spinosissima L. is widespread in Britain and western Europe, and distributed throughout Europe and western and central Asia (Mayland-Quellhorst et al. 2012). In Norway

it is considered native in the counties of Rogaland and Hordaland (Lid and Lid 2005).

Since the 19th century, plants occurring in western Norway perceived as hybrids between species of *Rosa* sect. *Caninae* and *R. spinosissima* have been referred to as *Rosa* × *involuta* Sm. (Smith 1804) according to Blytt (1874), or to *Rosa* × *sabinii* Woods (Woods 1818) following Blytt (1906). Currently, hybrids thought to involve *Rosa mollis* Sm. are referred to *R.* × *sabinii* while *R.* × *involuta* is reserved for plants where *Rosa sherardii* Davies. is the anticipated seed parent (Lid and Lid 2005, Stace 2010). *Rosa* × *sabinii* has been recorded from both Bømlo and Stord, while *R.* × *involuta* has only been recorded from a single location on Stord (Lid and Lid 2005).

Rosa mollis in northwest Europe is predominantly a tetraploid with 2n = 4x = 28 (see Table 1 for an overview of the chromosome numbers of the involved species), while *R. sherardii* mostly has 2n = 5x = 35 chromosomes (Table 1). Well documented chromosome counts are known from western and continental Europe, but no records exist from Norway. Both *R. mollis* and *R. sherardii* belong to subsection *Vestiate* Christ within section *Caninae,* and the taxonomic separation of the species within this group is known to be challenging (Henker 2000, Kellner et al. 2014). The hybrid taxa *R.* × *sabinii* and *R.* × *involuta* therefore represent intersectional hybridization events, involving a species with an unbalanced *Canina* meiosis as one parent.

Rosa ×sabinii occurs also in Britain, and the cytological behavior its hybrid origin was examined extensively by Blackburn and Harrison (1921, 1924, see also Täckholm 1922). In their material, *R.* × *sabinii* was found to be hexaploid (2n = 6x = 42), and their observations of the species in the field indicated total sterility, hence a hybrid origin of the taxon. Täckholm studied one plant he referred to as *R. mollis* × *pimpinellifolia* originating from Mosterhamn (Bømlo, Norway) with 2n = 5x = 35 chromosomes (Table 1).

Several recent attempts to reconstruct the relationship within *Rosa* using amplified fragment length polymorphism (AFLP) (Vos et al. 1995) have yielded encouraging results and underscores the usefulness of this technique for resolving relationships in groups with complex evolutionary history (De Cock 2008, Koopman et al. 2008, De Riek et al. 2013). AFLPs have also been successfully applied to detect hybrid ancestry in many plant taxa (Bateman et al. 2008, Schulte et al. 2010, van Hengstum et al. 2012).

Here we aim to establish the taxonomic status of the tentative hybrid taxon *R.* × *sabinii* from the island of Bømlo, Norway, using morphological, cytological, fitness and molecular data.

Material and methods

Plant material

Rosa spinosissima, *R. mollis* and *R.* × *sabinii* were collected in the islands Bømlo and Stord (County of Hordaland, Norway). The distribution of *R. spinosissima* in the study area is given in Fig. 1, and our specimens were collected in the three areas marked as '1' (Spyssøy), '2' (Lykling), and '3' (Steinsvåg). *Rosa mollis* has a continuous distribution in the entire area; our specimens were collected at several locations in the region, including the three sampling locations for *R. spinosissima*. The distribution of *R.* × *sabinii* is also given in Fig. 1. Every known location of this species was sampled, including sampling location 2 and 3. However, no specimen was found at location 1, even though it has previously been reported from this location. At Mosterhamn, the locality mentioned in Täckholm (1922), four plants were found and included in the present study. A complete list of specimens is given in Table A1 (Supplementary material, Appendix 1).

We have not been able to locate any extant occurrences of *R.* × *involuta*, and this previously reported taxon may thus be considered extinct in Norway.

No other possible parents of *R.* sect. *Caninae* subsect. *Vestitae* (Henker 2000) occur in this area (Lid and Lid 2005). Of *R.* sect. *Caninae* subsect. *Caninae*, *Rosa dumalis* Bechst., *R. subcanina* (Christ) Vuk. and *R. canina* L. are present. As judged from morphological traits, these are less likely involved as parental species.

The sampling resulted in a total of 114 specimens, of which 69 belongs to the species *R. spinosissima*, 20 to *R. mollis* and 25 to the tentative hybrid taxon, *R.* × *sabinii*, cf. Table A1 (Supplementary material, Appendix 1) for a complete list of specimens. An overview of all samples with numbers

Table 1. Overview of chromosome counts from literature for selected species of *Rosa*.

	Chromosome number	Geographical range		
	Ploidy level and meiotic behaviour	Scandinavia	Continental north Europe	British Isles
R. spinosissima	2n = 4x = 28; balanced meiosis		Małecka and Popek (1984)	Blackburn and Harrison (1921, 1924), Harrison (1930), Roberts (1977)
R. mollis	2n = 4x = 28; asymmetric meiosis	Täckholm (1922), Kellner et al. (2014)	Kellner et al. (2014)	Blackburn and Harrison (1921)
	2n = 5x = 35; asymmetric meiosis	Kellner et al. (2014)		
R. sherardii	2n = 4x = 28; asymmetric meiosis		Kellner et al. (2014)	
	2n = 5x = 35; asymmetric meiosis	Kellner et al. (2014)	Małecka and Popek (1982, 1984), Małecka et al. (1990), Kellner et al. (2014)	Rowley (1960)
R. × *sabinii*	2n = 5x = 35; distorted meiosis	Täckholm (1922)		
R. ×*involuta*	2n = 6x = 42; distorted meiosis			Blackburn and Harrison (1921, 1924), Täckholm (1922)

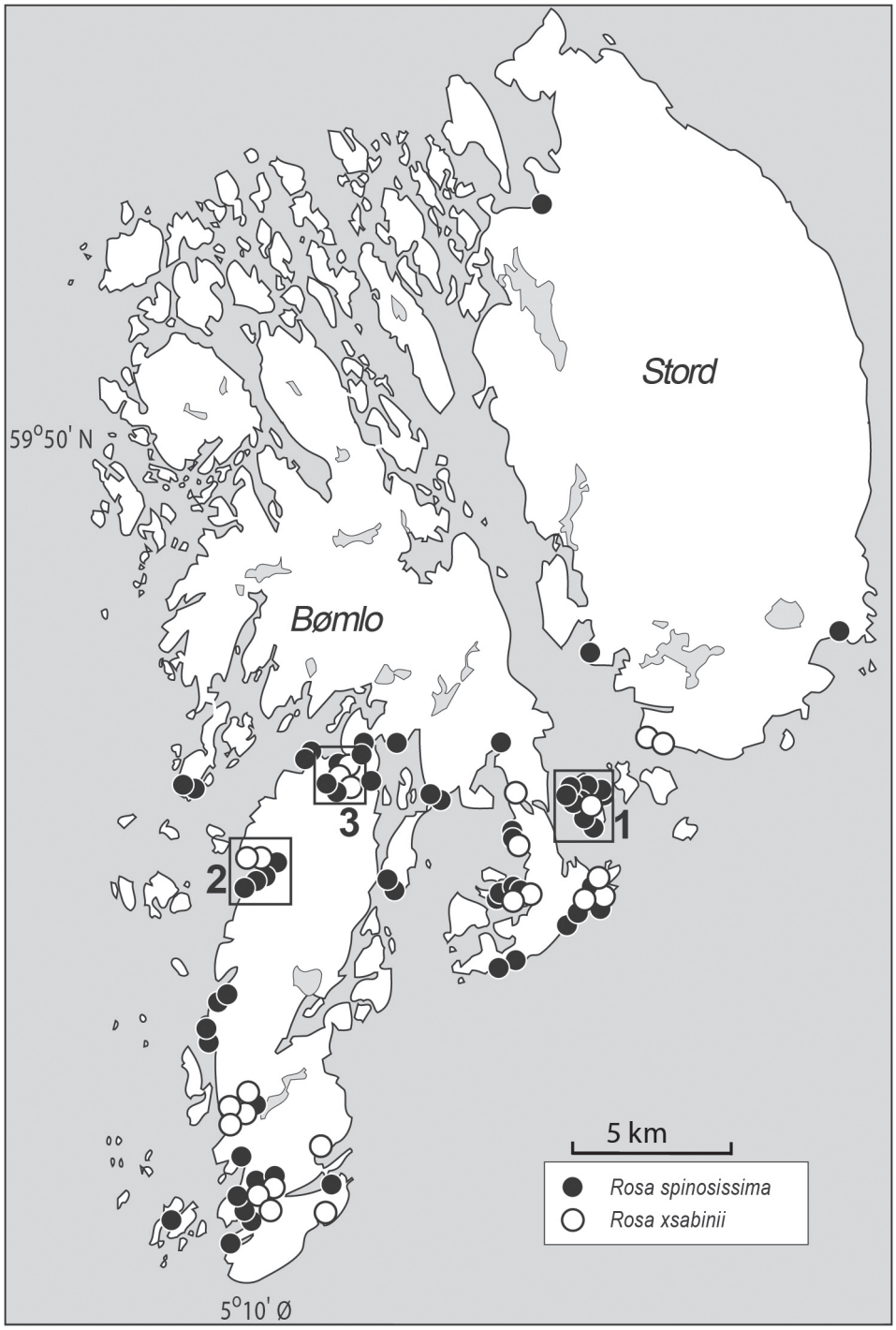

Figure 1. Distribution of *Rosa spinosissima* and *R.* × *sabinii* at Bømlo and Stord, western Norway. The localities are marked as 1 = Spyssøy, 2 = Lykling and 3 = Steinsvågen. The map is based on herbarium specimens in O and BG, as well as this study. Illustration: Beate Helle.

of individuals used for particular analyses for each species is given in Table 2. Plants were identified according to Lid and Lid (2005). In addition to the collected specimens, a total of 25 offspring plants, grown in the greenhouses of the Arboretum and Botanical Gardens (Univ. of Bergen, Norway), were included in the cytometric analysis.

A total of 21 voucher specimens were deposited in herbarium BG with the accession numbers BG-S-161835–161855.

Morphometric analysis

For morphometric analysis five leaves from each individual were studied. Five hips, if present, were collected from each specimen, and seeds were extracted for weighing and germination trials. A total of 29 morphological characters (Table 3) were recorded for 97 individuals belonging to *R. spinosissima* (62), *R.* × *sabinii* (18) and *R. mollis* (17).

Table 2. Numbers of individuals used for particular analyses for each species. For the germination trials, numbers of germinating seedlings are given, with total number of seeds tested in parentheses. For the ploidy measurements numbers are given for individuals, with number of samples in parentheses.

	Armature	PCA leaf	Hairs	Hips	Seed	Pollen	Germination (total)	Ploidy dry leaves (DAPI)	Ploidy fresh leaves (PI)	Ploidy offspring (DAPI)	AFLP
R. spinosissima	55	62	62	57	56	5	16 (38)	60 (61)	6 (6)	17 (34)	39
R. mollis	8	15	14	8	8	2	8 (8)	13 (13)	7 (7)	7 (14)	9
R. ×sabinii	16	17	17	15	15	7	1 (13)	18 (18)	14 (14)	1 (1)	16
Total	79	94	93	80	79	14	34 (59)	91 (92)	27 (27)	25 (48)	64

Three classes of armature were defined (Fig. 2). Class one included stems carrying a mixture of straight slender prickles interspersed with numerous acicles. Acicles were also present near the shoot apex. The second class had straight, or slightly curved, paired prickles and stems were lacking acicles. The third class was armed with a mixture of straight slender prickles and acicles at the base, but with few or no acicles at the apex. Anonymized samples were classified by two independent co-workers.

Table 3. Measured morphological characters of *Rosa spinosissima*, *R. mollis* and *R. ×sabinii*. The characters marked with an asterisk are used in the ordination analysis, and transformations are given.

Morphological characters
Vegetative characters
Armature (Fig. 2)
Prickles on rachis (0 = absent, 1 = present)
Glandular hairs on leaflet margin (0 = absent, 1 = some, 2 = plentiful)
Glandular hairs on margin of stipule (0 = absent, 1 = some, 2 = plentiful)
Villose hairs on stipule (0 = absent, 1 = some, 2 = plentiful)
Glandular hairs on stipule (0 = absent, 1 = some, 2 = plentiful)
Villose hairs on rachis (0 = absent, 1 = present)
Glandular hairs on rachis (0 = absent, 1 = some, 2 = plentiful)
Villose hairs on dorsal side of terminal leaflet (0 = absent, 1 = some, 2 = plentiful)
Villose hairs on ventral side of terminal leaflet (0 = absent, 1 = some, 2 = plentiful)
Glandular hairs on dorsal side of terminal leaflet (0 = absent, 1 = some, 2 = plentiful)
Glandular hairs on ventral side of terminal leaflet (0 = absent, 1 = some, 2 = plentiful)
Leaf characters (Fig. 3)
Length of lamina (mm)* log-transformed
Width of lamina (mm)* log-transformed
Length of petiole (mm)* log-transformed
Number of leaflets per leaf*
Length of terminal leaflet (mm)
Width of terminal leaflet (mm)
Length/width ratio of terminal leaflet*
Length of distal leaflet (mm)
Width of distal leaflet (mm)
Length/width ratio of distal leaflet*
Length of proximal leaflet (mm)
Width of proximal leaflet (mm)
Length/width ratio of proximal leaflet*
Hip characters (Fig. 3)
Length of hip (mm)
Width of hip (mm)
Length/width ratio of hip*
Seed weight (g)* log-transformed

For each leaf and hip measurement (Fig. 3), the mean of up to five replicates from each specimen was calculated. Hairs, glands, and prickles were recorded as absent/present or absent/some/plentiful (Table 3). Mean seed weight was calculated from up to five fully developed seeds from each specimen. An unconstrained linear ordination, principal component analysis (PCA), based on nine of the morphometric characters (marked with an asterisk in Table 3) was carried out to explore the morphological variation. Prior to the analysis, normal distribution was ensured by log-transformation of some characters (Table 3). To avoid weighting the variables describing leaflet size, only the leaflet length/width ratio was included in the final data set. The analysis was performed with centering and standardization of characters, in CANOCO 5 (Ter Braak and Šmilauer 2012).

Pollen viability

To clarify whether the hybrid taxon had reduced pollen fertility, pollen viability was assessed as stainability in Lactophenol Blue solution (Stanley and Linskens 1974). Pollen was obtained from six specimens of *R. spinosissima*, six specimens of *R. ×sabinii* and two specimens from *R. mollis*, and the frequency of viable pollen was recorded.

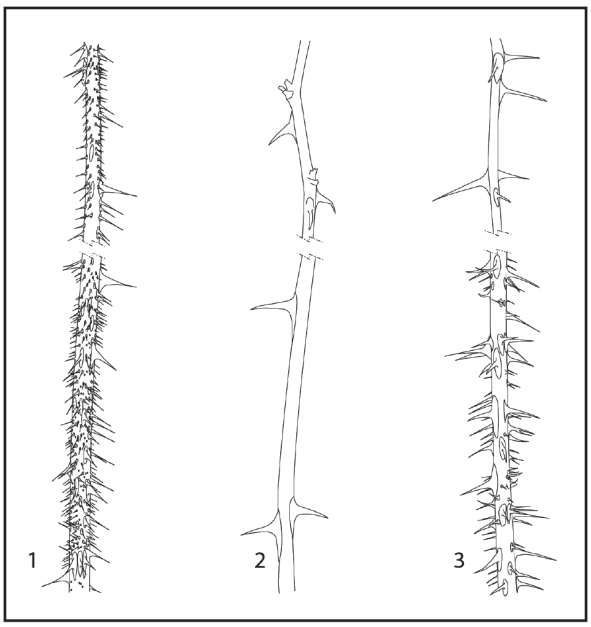

Figure 2. Armature on proximal and distal part of shoots, as displayed by the sampled specimens, where 1 = *Rosa spinosissima*, 2 = *R. mollis* and 3 = *R. ×sabinii*. Illustration: Beate Helle.

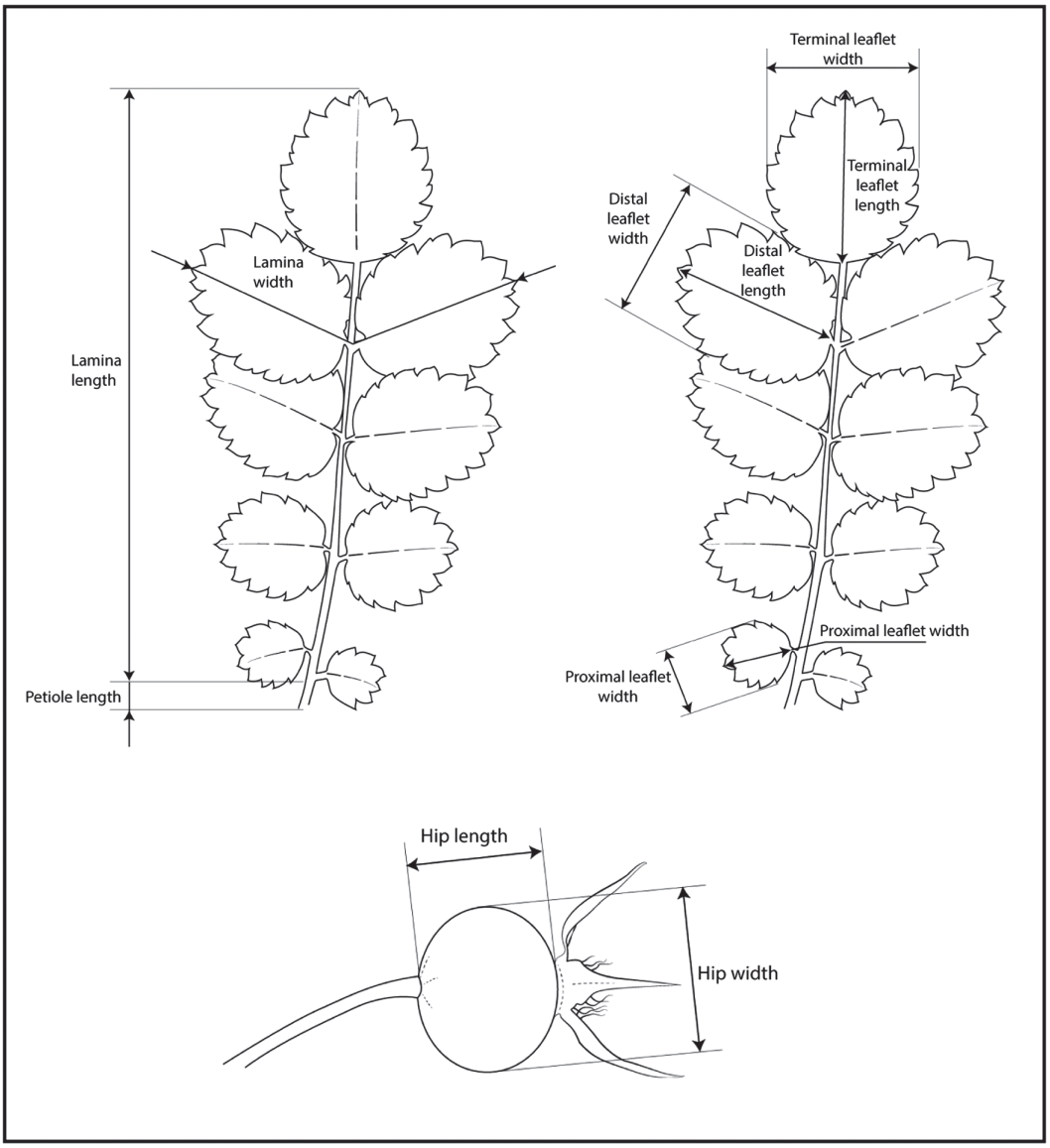

Figure 3. Leaf and hip characters of *Rosa spinosissima*, *R. mollis* and *R.* × *sabinii* measured in this study. Illustration: Beate Helle.

Seed germination

To examine whether the hybrid taxon was able to produce viable offspring, germination trials were conducted. These also included examination of variation in germination percentages between the hybrid and the tentative parental taxa. Up to 20 well-developed seeds from each specimen were sown in a moist soil-less potting mix. The seeds were then subjected to a cycle of 12 weeks of chilling at + 3–5°C to break dormancy, followed by 7 weeks in a heated greenhouse to stimulate germination. Seeds that failed to germinate after this treatment were given an additional treatment cycle of 16 weeks of chilling followed by 10 weeks in heated greenhouse conditions. The seeds that had not yet germinated were finally subjected to a third treatment cycle of 12 weeks chilling followed by 10 weeks of heating. The germinated seedlings were potted and grown separately in a heated greenhouse.

Nuclear DNA content

Absolute nuclear DNA content and DNA ploidy were measured using flow cytometry. A total of 168 samples were analyzed by Plant Cytometry Services (Schijndel, the Netherlands).

Nuclear DNA content was determined in leaves or buds from 119 specimens: 76 specimens of *R. spinosissima*, 23 of *R. mollis*, and 20 specimens of *R.* × *sabinii*. Analysis were initially performed on dried leaf samples, and subsequently fresh leaves from 27 plants were resampled and analyzed so that the data from the dried leaves could be calibrated. Nuclear DNA content was also measured in fresh leaves and winter buds from 22 seedlings raised in the greenhouses of the Arboretum and Botanical Gardens (Univ. of Bergen, Norway), counting 14 specimens of *R. spinosissima*, seven *R. mollis*, and one *R.* × *sabinii* (for details of analyzed material, see Table A1, Supplementary material, Appendix 1).

The plant material was prepared according to the standard protocol applicable to the flow cytometer CyFlow ML (<www.sysmex-partec.com/service/media-center.html>). An appropriate amount of plant tissue (a few cm^2 of leaf/20–50 mg) was chopped in a petri dish with a sharp razor blade in 500 µl ice-cold extraction buffer together with a leaf sample of the internal standard with known nuclear DNA content, *Vinca major* which has 4.2 pg DNA/2C (Bennett and Leitch 2012). After chopping, the buffer, containing cell constituents and large tissue remnants, was passed through a nylon filter of 40 µm mesh size, allowing only the nuclei to pass through.

The nuclei isolated from dried leaves and from the offspring plants were measured by the DAPI staining technique, using the kit cystain UV precise P from Partec GmbH with 0.1% DTT (dithiothreitol) and 1% PVP 10 (polyvinylpyrolidone). The absolute DNA content in the material of resampled fresh leaves was determined using the two-step method with cystain PI absolute buffer from Partec GmbH. After 30–60 sec incubation, 2.0 ml staining buffer, containing the fluorescent dye propidium iodide (PI) and RNA-se, was added. DTT (0.1%) and 1% PVP 10 were also added to the buffer.

After incubation for at least 30 min at room temperature, the intensity of fluorescence of the stained nuclei in the filtered solutions was recorded by a CyFlow ML with a high pressure mercury lamp for UV with the DAPI stained material, and with a green diode laser 30 mW 532 nm for the PI-stained material.

The intensity was analyzed by the software Flomax. The fluorescence recorded at the median of the histogram peak of the *Rosa* sample divided by the reading for the internal *Vinca* standard, give the DNA ratio, which with the PI stain binding to the double helix, is directly proportional to the absolute nuclear DNA content, while the DAPI stain binds preferentially to adenine and thymine bases of DNA, and the ratio will be biased if the amount of these bases differ significantly between the analyzed sample and the internal standard. The variation of the fluorescence readings for fresh leaf amounted to a CV (coefficient of variation) about 5%, while in the dried leaves peaks were produced with CV levels of 6–10%.

AFLP analysis

For the AFLP analysis, winter buds or young leaflets suitable for DNA-analysis were collected and stored in silica-gel until DNA-extraction. DNA was extracted from 20 mg of the dried plant material using the Qiagen DNeasy 96 plant kit.

The AFLP analysis was conducted according to Vos et al. (1995) with some modifications, allowing the restriction-ligation (RL) to be performed in a single step reaction. The reaction mixture consisted of 5 µl DNA-extract, 5 U *Eco*RI, 10 U *Mse*I, 1 U T4 ligase, 0.9 µM *Eco*RI-adaptor, 0.9 µM *Mse*I-adaptor, 1 × T4 ligase buffer, 0.05M NaCl and 5% bovine serum albumin, giving a total reaction volume of 10 µl. The reaction mixture was then incubated at 37°C for 3 h, and the resulting product was diluted 1:10.

Pre-amplification (PA) was performed with the *Eco*RI/*Mse*I primer pairs, each primer containing one selective nucleotide. The total reaction volume of 12.5 µl consisted of 1.5 µl diluted (1:10) RL- product, 0.2 µM *Eco*RI primer, 0.2 µM *Mse*I primer, 0.25 U AmpliTaq, 0.2 mM of each dNTP, 1 × Taq buffer and 1.5 mM $MgCl_2$. The samples were subjected to initial denaturation at 94°C for 2 min, then 30 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec and polymerization at 72°C for 2 min, followed by a final polymerization at 72°C for 10 min. The resulting PA-product was diluted 1:15. Finally 5 µl of undiluted PA-product was analyzed on a 1% agarose gel to verify the presence of amplified fragments.

Selective amplification (SA) was performed with three selective nucleotides on each primer. All 64 possible primer combinations were tested on four different individuals. The resulting chromatograms were evaluated and the four primer combinations yielding the clearest and most variable bands were used in the final analysis (*Eco*RI-ACA-(FAM)/*Mse*I-CTC, *Eco*RI-ACA-(FAM)/*Mse*I-CAA, *Eco*RI-ACC-(VIC)/*Mse*I-CTT, and *Eco*RI-AGC-(VIC)/*Mse*I-CAA). The selective amplification reaction mixture consisted of 2.5 µl diluted PA-product, 0.008 µM fluorescent *Eco*RI primer (E-ANN), 0.01 µM *Mse*I primer (M-CNN), 0.5 U AmpliTaq, 0.2 mM of each dNTP, 1 × Taq buffer, 2.5 mM MgCl2 and 8‰ bovine serum albumin. PCR reactions were performed with initial denaturation at 95°C for 2 min, 35 cycles of: denaturation at 94°C for 30 sec, annealing at 65°C decreasing by 0.7°C for 12 cycles, ending at 56°C for 23 cycles each of 30 sec, polymerization at 72°C for 1 min, followed by a final polymerization at 72°C for 10 min. The resulting SA-product was diluted 1:50 before analysis on an ABI sequencing machine using LIZ500 as size standard. The analyses of the SA-products were carried out by the Sequencing Facility at the Univ. of Bergen (Norway).

The resulting chromatograms where visualized using the software GeneMapper 4.0. Bands in the range of 50–300 base pairs were manually binned, and registered peaks were controlled visually before further analysis. Fragments were scored, and the mismatch-error rate estimated using the statistical software R (R Core Team 2016) and the software package AFLPScore (Whitlock et al. 2008). The resulting binary matrix was analyzed by principal coordinates analysis (PCoA) with SQRT distance measure, using Canoco 5 (Ter Braak and Šmilauer 2012), and the phylogenetic relationships were explored further in a NeighborNet (NN) analysis using Dice distance in SPLITSTREE4 (Huson and Bryant 2006) with 1000 bootstrap replicates.

Results

Morphometric analysis

Armature was classified into three classes (Fig. 2). All samples of *R. spinosissima* were assigned to class 1, while those of *R. mollis* to class 2, and those of *R.* × *sabinii* to class 3.

The presence of hairs, glands and prickles were most prominent in *R. mollis* and clearly less prominent in *R. spinosissima* as seen in Table 4. *Rosa* × *sabinii* displays intermediate character states for many traits, but is clearly

Table 4. Type of hairs measured as frequencies (%) in *Rosa spinosissima* (n = 62), *R.* ×*sabinii* (n = 17) and *R. mollis* (n = 14). Scores: 2/1/0 = frequent/sparse/absent or 1/0 = present/absent.

	Score	*R. spinosissima*	*R.* × *sabinii*	*R. mollis*
Rachis				
Glandular hairs	2	1	96	100
	1	49	2	0
	0	50	1	0
Villose hairs	1	0	65	100
	0	100	35	0
Bristles	1	4	83	95
	0	96	17	5
Stipule margin				
Glandular hairs	2	1	95	100
	1	98	5	0
	0	1	0	0
Stipule surface				
Villose hairs	1	0	36	99
	0	100	64	1
Glandules	2	2	14	79
	1	0	39	17
	0	98	47	4

approaching *R. mollis* with regard to the distribution of glandular hairs on the rachis and margins of leaflets and stipules. The difference between the two species *R. spinosissima* and *R. mollis* in the presence and frequency of villose and glandular hairs is conspicuous, no matter if the dorsal or ventral sides of distal or proximal leaflets are studied.

The results of the PCA analysis are displayed as a scatterplot of the morphological variation in Fig. 4a, which shows that individuals of *R. spinosissima* and *R. mollis* are grouped in two separated clusters, with *R.* × *sabinii* as a group in between the two, partially overlapping both. The first component axis explains 49.1% of the variation in the dataset and the second 15.5%, combined they explain 64.6%.

Rosa spinosissima is characterized by a larger number of leaflets than the others, while *R. mollis* has larger and wider leaves, with longer petioles. Also, *R. mollis* has slightly higher seed weight than *R. spinosissima*.

When the same PCA analysis was sorted by the three populations of *R. spinosissima* (Fig. 4b), it became clear that the subpopulations are indistinguishable with respect to these characters.

Pollen viability

Pollen viability varied greatly among taxa. *Rosa spinosissima* had the highest frequency of well-developed pollen (67–94%). *Rosa mollis* had 25–33% viable pollen, while *R.* × *sabinii* had a very low frequency of viable pollen, with only 0–33% of pollen grains stained in Lactophenol Blue Solution (Fig. 5).

Seed germination

After a total of three chilling cycles, 91 out of 943 seeds had successfully germinated. *Rosa mollis* had the highest germination rate with 31.9% of the seeds germinating, *R. spinosissima* had a germination rate of 5.6%, while only one seed from *R.* × *sabinii* germinated, resulting in a germination rate of only 1.0%. Based on general morphology, all progeny appeared closely similar to their seed parent. The single seedling resulting from the *R.* × *sabinii* seeds was consistent with the seed parent in terms of leaf color and serration.

Nuclear DNA content

The mean total DNA-content measured as DNA ratio in relation to the internal standard *Vinca major* was determined with PI fluorescent dye amounted to 0.357 ± 0.023 in *R. spinosissima*, 0.420 ± 0.006 in *R. mollis* and 0.500 ± 0.006 in *R.* ×*sabinii*. These ratios correspond to absolute DNA contents of 1.915 ± 0.034 pg/2C in *R. spinosissima*, 2.180 ± 0.031 pg/2C in *R. mollis* and 2.606 ± 0.006 pg/2C in *R.* ×*sabinii*. DNA-ratios measured with the DAPI dye in fresh plant tissues were 0.368 ± 0.008 and 0.422 ± 0.006 for *R. spinosissima* and *R. mollis*, respectively, whereas dried leaves in *R. spinosissima* had a ratio of 0.360 ± 0.020, *R. mollis* of 0.415 ± 0.032, and *R.* ×*sabinii* of 0.498 ± 0.025. The differences in mean DNA ratios observed between dried leaf samples and fresh tissues, as well as between the two dyes utilized, were insignificant (pairwise t-tests, $p > 0.01$, given in Table A2, Supplementary material, Appendix 1), except for the comparison between fresh leaves and fresh buds in *R. spinosissima* yielding a significant t-test ($t = 4.87$; $p < 0.001$). The differences observed between the three species are, however, consistent and highly significant (pairwise t-tests, $p << 0.001$).

Analysis of the only offspring plant of *R.* × *sabinii* resulting from the germination trials yielded strongly deviating results from the other samples, with a DNA-ratio of 0.58 that corresponds to a total DNA content of 3.06pg/2C. This sample was therefore excluded from the aforementioned calculations but will be treated more thoroughly in the discussion.

AFLP analysis

A total of 93 markers were obtained from four primer combinations of the 64 specimens (Table A1, Supplementary material, Appendix 1). The number of scored bands per primer combination varied from 9 to 36. Error mismatch-rates were estimated from a minimum of six replicates (7%), and varied between 3.6 and 4.2%. No species specific markers were found. Eight markers were only found in *R. spinosissima* and *R.* × *sabinii*, but not in *R. mollis*. On the other hand, 3 markers were found only in *R. mollis* and *R.* × *sabinii*, and not in *R. spinosissima*. No genetically identical samples existed.

The principal coordinates analysis (PCoA) of the 0/1 matrix for the 93 AFLP markers on the 63 complete samples are displayed as a scatter diagram of the two first axes in Fig. 6. The variation of the markers clustered the samples into three groups according to the predicted taxon.

The NeighbourNet analysis of the AFLP data revealed a clear internal structure and all the samples were clustered into three groups according to taxon (Fig. 7). These main clusters showed strong bootstrap support. No internal structure was apparent within the three different subpopulations

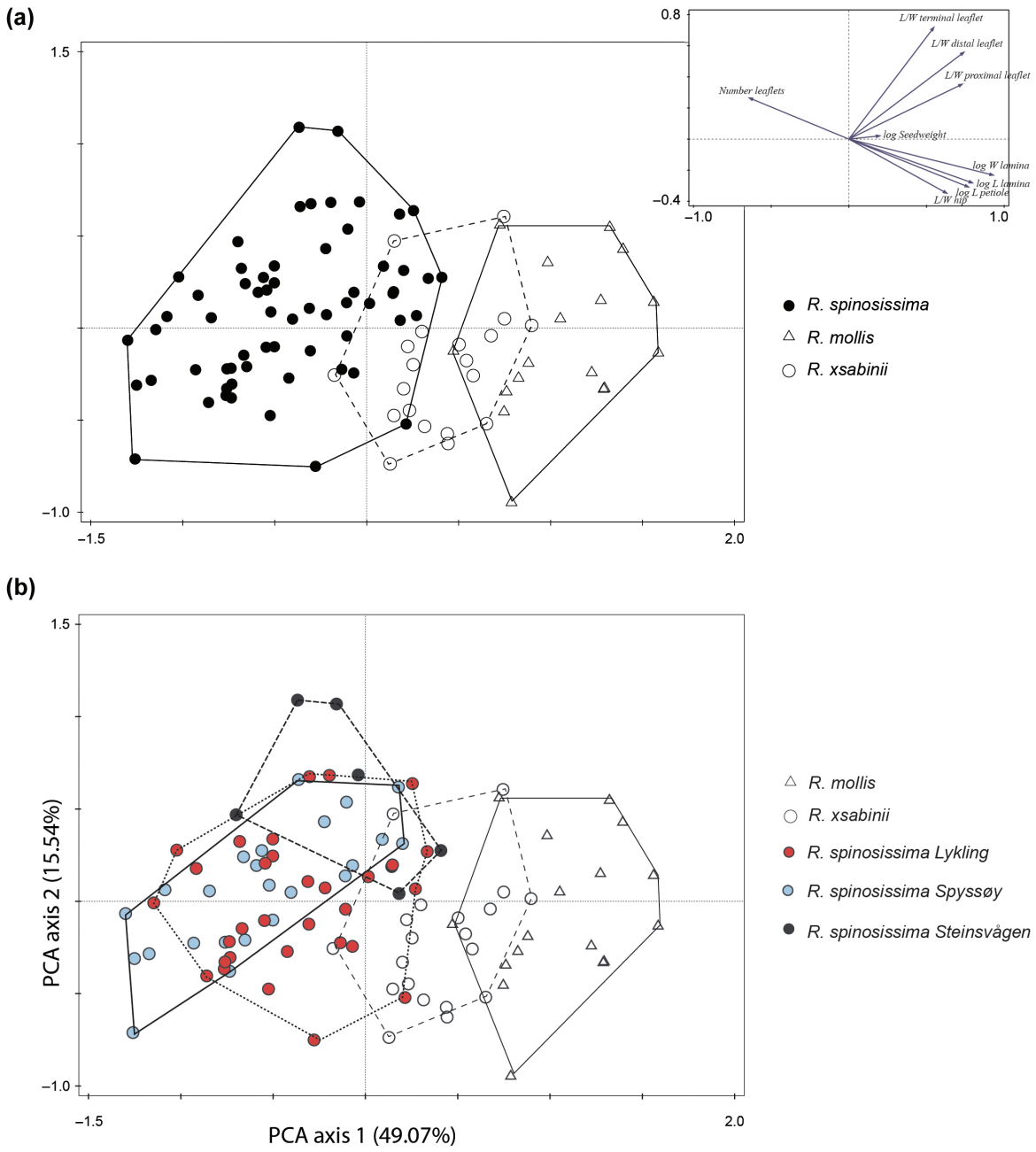

Figure 4. Scatter plot of the two first axes from a principal components analysis (PCA) of morphological characters of *Rosa spinosissima*, *R. mollis* and *R.* × *sabinii*. Lines connect the most divergent individuals of a cluster. (a) the three species, with the inset of the explaining morphological characters, marked as arrows. (b) the different populations of *R. spinosissima* from Bømlo.

of *R. spinosissima* (Fig. 7). All samples of *R. spinosissima* were clearly separated from *R.* × *sabinii*.

Discussion

In this study we investigated the parentage of the tentative hybrid rose *Rosa* × *sabinii* (sensu Lid and Lid 2005) from Bømlo. Plants assigned to this taxon in its Norwegian range co-occurs with the two anticipated parental species *R. spinosissima* and *R. mollis*, and it was therefore hypothesized that *R.* × *sabinii* is the result of crossings between these two species. We also examined whether the locally endangered *R. spinosissima* could be subject to introgression from *R. mollis*.

The parentage and hybrid status of *Rosa* × *sabinii*

It has long been recognized that hybrid forms often display morphological characteristics intermediate between their parents (Grant 1971). The findings in this study support the hybrid hypothesis as many morphological characters of *R.* × *sabinii* are intermediate between *R. spinosissima* and *R. mollis*, such as the presence of villose and glandular hairs on leaflets and stipules (Table 4). Ritz and Wissemann (2003) demonstrated that the presence of hairs and glands

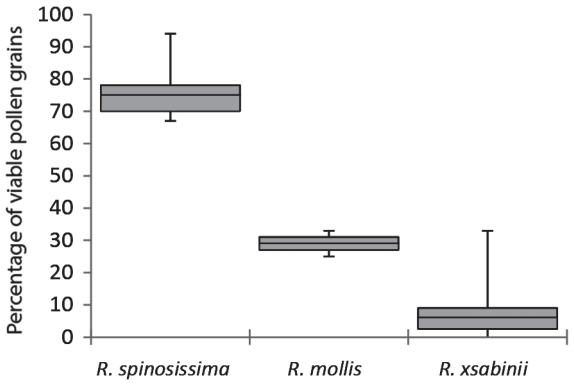

Figure 5. Box plots of the total range of the data with whiskers, showing the lower and upper quartiles. The line on the box gives the median value of the frequency of viable pollen from *Rosa spinosissima*, *R. mollis* and *R.* × *sabinii*.

on leaves were subjected to matroclinal inheritance. Our findings would then point to *R. mollis* as the ovule donor in the hybrid forming cross. The morphologically intermediate nature of *R.* × *sabinii* in relation to the hypothesized local parent taxa becomes particularly evident when analyzing armature. The samples studied were clearly divided into three separate classes reflecting the three taxa considered (Fig. 2). The armature of *R.* × *sabinii* resembles a combination of traits from *R. spinosissima* and *R. mollis*, with the lower part of the shoot covered by straight slender prickles and numerous acicles like in *R. spinosissima*, while the apical part only displays the paired, slightly curved, slender prickles characteristic of *R. mollis*.

In the PCA analysis of measured morphological characters, *R. mollis* and *R. spinosissima* form two separated groups, while *R.* × *sabinii* forms an intermediate group overlapping with the two other species, as would be expected for a hybrid taxon (Fig. 4a).

Various levels of pollen sterility are common in hybrid taxa, and this is commonly used to identify hybrid forms from their parental species (Stace 1980). The frequency of viable pollen within the genus *Rosa* is highly variable (Erlanson 1931), and within *R.* sect. *Caninae* it is assumed that this variation is caused by the many hybridization events in the evolutionary history of this genus (Wissemann and Hellwig 1997). This variation, and the fact that hybrids are

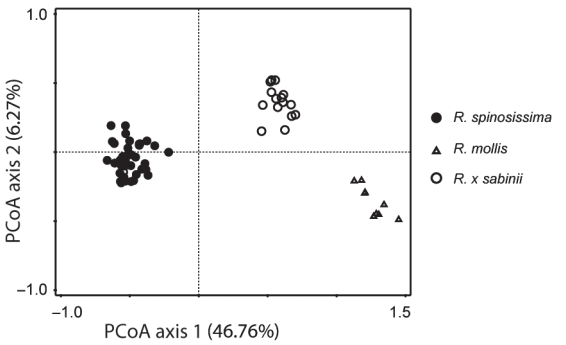

Figure 6. Scatter plot of the two first axes from a principal coordinates analysis (PCoA) of AFLP markers of *Rosa spinosissima*, *R. mollis* and *R.* × *sabinii*.

not necessarily pollen sterile (Gustafsson 1944), makes it difficult to recognize hybrid roses based solely on low pollen viability (Wissemann and Hellwig 1997). Nonetheless, by comparing the frequency of viable pollen in possible hybrids with that of the tentative parental taxa, indications regarding the hybrid nature of these individuals may be obtained. The frequency of stained pollen was found to be variable but high in *R. spinosissima* (67–94%), but lower in *R. mollis* (25–33%). That pollen viability was found to be lower in the species belonging to *R.* sect. *Caninae* is in accordance with Jičínská (1976), who concluded that the low frequency of viable pollen was caused by the unusual nature of the *Canina*-meiosis. Still, the frequencies of viable pollen recorded by us for *R.* × *sabinii* are all even much lower, ranging between 0 and 33%, a clear indication of a disturbed meiosis, a trait frequently associated with hybrid taxa (Grant 1971).

Blackburn and Harrison (1921) state that in Britain, *R.* × *sabinii* is completely barren. Based on our observations this is not always the case for the plants found in Norway. Hybrid plants were observed to produce a large number of flowers, but when revisited later in the season, it appeared that most hips were aborted at an early stage of development. Nevertheless, some individuals were able to produce a few well-developed and ripe fruits and also some mature seeds. However, results from germination trials show a clear difference in seed viability between the three taxa, with germination rates varying from 31.9% (*R. mollis*) to 5.6% (*R. spinosissima*) and 1.0% (*R.* × *sabinii*). Also *R. mollis* has a slightly higher seed weight than *R. spinosissima* (Fig. 4a). For *R.* × *sabinii*, the germination rate is notably low. Combined with the low pollen viability recorded in *R.* × *sabinii*, evidence point in the direction of an imperfect meiosis causing partial gametic sterility, as often observed in hybrid taxa (Grant 1971). The unexpectedly low germination rate of *R. spinosissima* can possibly be explained by the unusually thick pericarp produced by this species. The overall low germination rates might be due to the delayed achene extraction, as this is proven to increase the concentration of germination inhibitors in the pericarp and testa, and thereby cause prolonged seed dormancy (Anderson 2007).

The genetic relationship between the three rose taxa was inferred by an analysis of 93 AFLP markers. In the resulting NeighbourNet (Fig. 7), all three groups have strong bootstrap support. Based on genetic distances, *R.* × *sabinii* appears to be somewhat closer to *R. mollis* than to *R. spinosissima.* These results strongly support the hypothesis that *R.* × *sabinii* is of hybrid origin, with *R. spinosissima* as one of the parent species. As *R. mollis* is the only species representing *R.* sect. *Caninae* in this analysis, it is difficult to determine the taxonomic resolution provided by the AFLP dataset alone. De Cock (2008) analysed the taxonomic relationships within the genus *Rosa* using AFLP markers. She found that the genetic distances within *R.* sect. *Caninae* were small compared to the distance between the sections *Caninae* and *Pimpinellifoliae*. It is therefore not clear whether the results of the AFLP-analysis should be interpreted as support for *R. mollis* as the second parent species, or if they only indicate a parent from *R.* sect. *Caninae*. Under the latter assumption, *R. mollis* and *R. sherardii* are the only plausible candidates to account for many of the morphological features

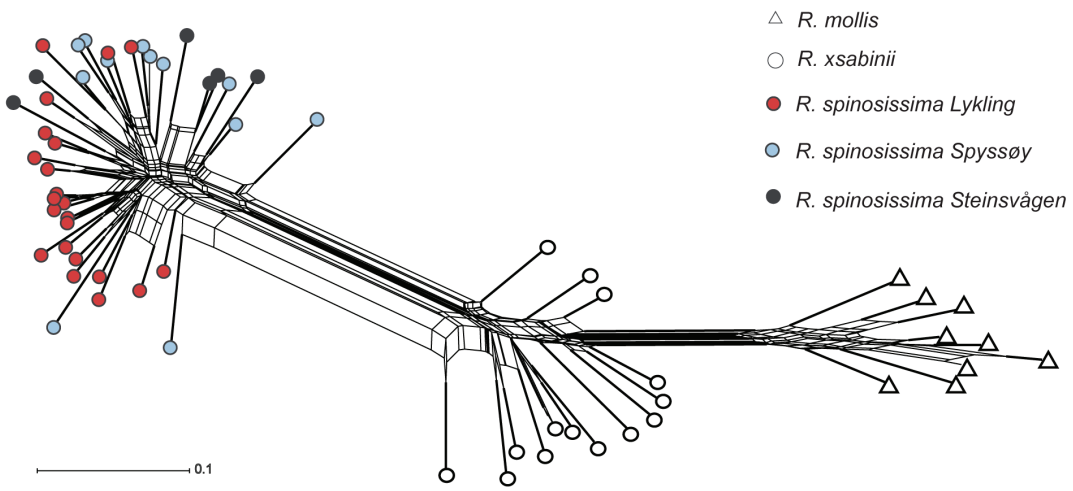

Figure 7. NeighborNet analysis of AFLP markers calculated using Dice coefficient. Bold lines mark bootstrap support > 95, with 1000 replicates. The five groups that are highlighted are the three different populations of *R. spinosissima* and the species *R.* ×*sabinii* and *R. mollis.*

characterizing the hybrid taxon, namely villose hairs, resinous glands and bristly hips. During the survey leading to the present study, no specimens fitting to the description of *R. sherardii* were encountered within the study area or even in the region, while *R. mollis* is relatively common in most of the surveyed areas, making it the most likely partner for the hybrid forming crossings.

From the AFLP analysis it is also evident that the sampled individuals of *R.* ×*sabinii* represent several distinct genotypes, and not a single clone. From this, it can be concluded that the hybrid plants are the result of separate and recurrent in situ hybridization events. Although the hybrid plants are capable of strong vegetative spread by means of suckering shoots, the number and distribution of unique hybrid genotypes indicate that vegetative dispersal is only effective on a very local scale, and thus, that hybridization events are occurring quite frequently. Both the morphometric analysis and the analysis of AFLP markers group the hybrid samples into one cluster. This indicates that all samples represent a single hybrid taxon, created by recurrent hybridization between different individuals of the same two parent species.

The hybridization mechanism

According to the data currently available (Table 1), both *R. spinosissima* and *R. mollis* are tetraploid in Norway. In southeastern Scandinavia, Great Britain and on the European continent, other chromosome numbers exists in *R. mollis* and other taxa of the *R.* subsect. *Vestitae* (Table 1; Henker 2000). For Norwegian *R.* ×*sabinii*, the chromosome number 2n = 5x = 35 was found by Täckholm (1922, p. 222) in the hybrid *R. mollis* × *pimpinellifolia* originating from Mosterhamn (Bømlo, Norway). Examination of herbarium specimens of the plant studied by Täckholm ('Innplanterad från Norge', Hortus Bergianus 24.7.1917 leg. G. A. Ringselle (SBT)), confirms that it belongs to the taxon currently referred to as *R.* ×*sabinii* in Norway.

In the investigated samples, three distinctly different levels of total DNA content were found, consistent with the taxonomic grouping of the samples. The average observed values were 1.91, 2.18 and 2.60 pg for *R. spinosissima*, *R. mollis* and *R.* ×*sabinii*, respectively. For *R. spinosissima* the value is in accordance with that found by Yokoya et al. (2000). No references are available for the other taxa, but both this value and the value observed for *R. mollis* is well within the range 1.85–2.30 pg/2C found by other workers in tetraploid rose species (Bennett and Leitch 2012). The observed value for *R.* ×*sabinii* is discussed below.

These results indicate that the different hybridization mechanisms or partners in Norway differ from those documented in Britain (Blackburn and Harrison 1921, Stace 2010). This may potentially challenge the scientific name currently used for the hybrid taxon in Norway, a problem that should be examined more thoroughly in future research. Taking *R.* ×*sabinii* as a pentaploid, the following hybridization mechanism can be suggested. Due to the special *Canina*-meiosis, tetraploid *R. mollis* produces ovules that are triploid (1n = 3x = 21) rather than diploid (1n = 2x = 14) (Täckholm 1922, Hurst 1931). When fertilized by a diploid (n = 14) sperm from *R. spinosissima* this would yield a pentaploid hybrid embryo (2n = 5x = 35). One may substantiate whether this is indeed the process taking place in the present case on Bømlo by utilizing the relationship between chromosome size and number, and measured total DNA amount. If a hybrid was formed as suggested here, we can predict that it's total DNA amount would be a combination of fourteen chromosomes from *R. spinosissima*, with an average DNA amount per chromosome of 0.068 pg/nucleus and 21 chromosomes from *R. mollis* with an average of 0.078 pg/nucleus. This would give a total DNA amount of 2.59 pg for a hypothetical pentaploid hybrid, which is almost identical to the average value actually observed in *R.* ×*sabinii* (2.60 pg). As a corollary the model accounts for the value observed in the hybrid being distinctly lower than found in pentaploid *Rosa canina* (2.85–2.90 according to Bennett and Leitch 2012). The model would also explain why the hybrid taxon appears to share more AFLP markers with *R. mollis*, than with *R. spinosissima* (Fig. 7). As all hybrids were clustered together

in the NeighbourNet analysis, and because they all have the same level of total DNA content, it appears as *R.* × *sabinii* is only produced by the mentioned mechanism and not by reciprocal hybridisation. This is probably due to the strong asymmetrical crossing barrier created by the *Canina*-meiosis (Tiffin et al. 2001).

A hexaploid offspring plant of *Rosa* × *sabinii*

The germination trials produced only a single offspring of *R.* × *sabinii*. This plant appeared morphologically similar to the seed parent in terms of leaf color and serration. Its total DNA content was determined to 3.06 pg, using flow cytometry. This is significantly higher than for the other taxa, and suggests that the plant may be a hexaploid (2n = 6x = 42), which may have been formed by fertilization of an unreduced embryo sac in *R.* × *sabinii* (2n = 5x = 35) by a haploid sperm (1n = 1x = 7) from *R. mollis*, or possibly from another member of the section *Canina* present. A back-cross like this was suggested as a possible mechanism for the evolution of *R. micrantha* Sm. by hybridization of *R. rubiginosa* L. and *R. canina* (Ritz and Wissemann 2011). Although we were able to detect a F2 hybrid plant in an artificial environment, hexaploid hybrid plants have so far not been encountered in the study area. This indicates that the F2 hybrids, even if they can form in seeds, will have difficulties surviving under natural environmental conditions.

No introgression into *Rosa spinosissima* and conservational implications

Due to its limited distribution and small populations, *R. spinosissima* has been included on the Norwegian red list of endangered species (Kålås et al. 2010). Considering this status, it is of interest to clarify whether hybridization and potential introgression with *R. mollis* pose a threat to the Norwegian populations, as frequent hybridization events can reduce reproductive success of both species involved (Levin et al. 1996) and introgression may lead to genetic extinction, i.e. loss of genetic diversity within a species (Rhymer and Simberloff 1996). Further, in certain cases the hybrids can have a higher fitness than one or both parent species, and drive them to extinction through interspecific competition (Ayres et al. 2004). However, based on our results, none of these mechanisms seems to pose a threat to the populations of *R. spinosissima* on Bømlo. It has been demonstrated that the hybridization between *R. spinosissima* and *R. mollis*, which produces the hybrid taxon *R.* × *sabinii*, is most likely asymmetrical, with *R. spinosissima* as the pollen parent. This process is therefore not considered to lower the reproductive output of *R. spinosissima*. Although not investigated in the present study, there is a possibility that reciprocal hybridization does occur, but that the resulting embryos are later aborted. This could affect the reproductive success of *R. spinosissima*, but further research is needed to detect and potentially quantify this.

The morphometric analysis of the three populations of *R. spinosissima* revealed no clear structure within this species (Fig. 4b). No distinct groups could be identified and all populations appeared to be equally distinct from *R. mollis*. Also, the NeighbourNet analyses of AFLP markers did not reveal any indication of introgression taking place (Fig. 7): the clustering of individuals belonging to *R. spinosissima* has strong bootstrap support and there are no signs of gene flow from *R. mollis* to *R. spinosissima*. It is, however, important to note that introgression can be difficult to detect with molecular markers and that cryptic introgression might be overlooked if insufficient molecular markers are studied (Currat et al. 2008, Twyford and Ennos 2012). Some weakly supported subclusters containing individuals from the same populations, reflects the higher level of relatedness expected within local populations, but they do not appear to be genetically isolated from each other.

Thus, no signs of introgression were found, indicating that this poses a neglible risk to the genetic variation within *R. spinosissima* in Norway. A scenario where the hybrid *R.* × *sabinii* outcompetes its parental species is therefore unlikely. The major threats to *R. spinosissima* are thus not currently posed by hybridization with other rose species, but by changing land use and development.

Conclusions

It has been established that the intermediary rose form from the island Bømlo in western Norway represents a single hybrid taxon, formed by recurrent asymmetrical hybridization events. *Rosa spinosissima* was found to be the pollen parent of this hybrid taxon, while a member of section *Caninae*, most likely *R. mollis*, was found to be the ovule donor. These findings are supported by morphometric, cytological and genetic data. The Bømlo hybrid was found to be most likely a pentaploid (2n = 5x = 35), and not a hexaploid as is recorded in *R.* × *sabinii* on the British Isles.

No signs of introgression from *R. mollis* into *R. spinosissima* were detected in native populations, and it is therefore assumed that the process does not pose a threat to the genetic composition of *R. spinosissima*. The successful germination of a hexaploid offspring plant in the greenhouse, however, suggests that back-crossings might be occurring, but that this may be a rare event and that the resulting progeny may have a strongly reduced fitness, which prevents introgression from taking place under field conditions.

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Supplementary material (Appendix njb-01070 at <http://www.nordicjbotany.org/appendix/njb-01070>. Appendix 1.