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Research article

Hybrids between *Rubus caesius* and *Rubus* sect. *Corylifolii* (Rosaceae) and their relation to *R. cyclomorphus*, *R. tiliaster*, *R. glauciformis*, *R. slesvicensis* and *R. firmus*

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Using flow cytometry and microsatellite DNA analysis (MSDA), we studied hybrids between *Rubus caesius* and various other *Rubus* species, with an emphasis on Sweden. We show that hybrids between *Rubus caesius* and *Rubus* sect. *Corylifolii* arise easily. They show a large variation in morphology, but can normally be recognised by a number of characters. They are typically hexaploids, but ~13% of the hybrids are pentaploids and ~8% tetraploids. With MSDA, they are harder to identify than hybrids with *R. idaeus*, partly because all *Corylifolii* species have themselves arisen from *R. caesius* hybrids and therefore share many alleles with *R. caesius*, partly because hybridisation with *R. caesius* seems to give rise to some variation in microsatellite regions. Hybrids with species of *R.* sect. *Rubus* are much rarer in Sweden and we have only identified one such case. We show that *R. cyclomorphus* and *R. tiliaster* are not proper apomictic species, at least not in Sweden, but rather a collection of genetically unrelated hybrids between *R. caesius* and *R. raduloides* or *R. camptostachys*. MSDA analysis of *R. slesvicensis* and *R. firmus* shows that these species are problematic. We identify two distinct taxa, one from Skåne in Sweden, which we describe as a new species, *R. ruboculus* and another from the neighbourhood of Viborg and Schleswig, although the latter is probably not identical with *R. slesvicensis s.s.* All the other samples form a diverse group of putative *R. caesius* hybrids at three different ploidy levels. *Rubus glauciformis* is uniform in Småland, Öland and Blekinge, but becomes harder to distinguish from various hybrids in Skåne. We conclude that once *R. caesius* and *R. idaeus* hybrids are properly identified, along with a small number of new apomictic species (often with a local distribution), the genus *Rubus* does not pose any serious taxonomical problems in Sweden.

Keywords: apomixis, *Rubus*, *Rubus caesius* hybrids, *Rubus cyclomorphus*, *Rubus firmus*, *Rubus glauciformis*, *Rubus slesvicensis*, *Rubus tiliaster*

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Introduction

Rubus sect. *Corylifolii* Lindl. is a group of blackberries that have arisen from crosses between blackberries (*R.* sect. *Rubus* L.) and dewberry (*R. caesius* L.) (Gustafsson 1939). Like both parents, they are apomictic (aposporous and pseudogamous). However, the apomixis is not complete and therefore they often form hybrids, in particular with raspberry (*R. idaeus* L.) (Ryde et al. 2021) and *R. caesius*. Such crossings are important for the formation of new *Corylifolii* species (Lidforss 1914, Sochor et al. 2015, Ryde et al. 2021).

Hybrids between *R. caesius* and *Corylifolii* species are characterised by sepals that embrace the fruit, by rich, early maturing and well-developed fruits, many small prickles on the stem, pruinose stems, many glands on both the stem and in the inflorescence, broad stipules, irregular inflorescences and often broadly heart-shaped terminal leaflets (Lidforss 1914, Gustafsson 1939, Hylander 1958a, Ryde 2009). They are typically hexaploids (2n = 6× = 42) (Gustafsson 1939).

Lidforss performed extensive crossing experiments with various *Rubus* species (Lidforss 1899, 1901, 1907, 1914). He reported that the first generation (i.e. the primary hybrids) of crossings between species of *R.* sect. *Corylifolii* and *R. caesius* is rather uniform in morphology, showing intermediate characters between the two parents. The hybrids are highly fertile, both in terms of pollen viability and seed formation. This outcome is quite unexpected, because other hybrids are normally recognised for having reduced fertility. Notably, the *Corylifolii* parents themselves have rather poor pollen and fruits. In contrast to the first generation of hybrids, the second generation is extremely variable and also varies in fertility. In particular, characters are often encountered that are not present in any of the parents and sometimes characters are found that are characteristic for *R. idaeus* hybrids (e.g. seven-nate leaves, hairy fruits and conspicuously red prickles, Ryde et al. 2021). In later generations, the variability is reduced, but does not disappear fully. Gustafsson concluded from these hybridisation experiments that apomixis is due to genes with recessive expression (Gustafsson 1943).

It is notable that Lidforss and Dowrick did not obtain any hybrids with *R. caesius* as the mother plant, indicating that it is an obligate apomict (Lidforss 1914, Dowrick 1966). On the other hand, it has been shown that it actually forms embryo sacks by regular meiosis (Christen 1950, Dowrick 1961), but that it still produces embryos without fusion with a sperm cell, possibly as a result of automixis (Dowrick 1966). However, many *Corylifolii* representatives share a plastid haplotype with *R. caesius* (Sochor et al. 2015), which indicates that this species is frequently a maternal component. Thus, the exact mode of apomictic reproduction in *R. caesius* is still not fully resolved.

An important practical question is how to discern primary hybrids between *Corylifolii* species and *R. caesius* from apomictic species with similar origins. According to Lidforss (Lidforss 1899, 1901, 1907, 1914), apomictic species give rise to a constant, morphologically uniform progeny, whereas primary hybrids give rise to a strongly varying progeny. This difference allowed Lidforss to suggest that some taxa recognised at that time were hybrids. For example, *R. progenerans* Lidforss was suggested to be *R. camptostachys* G.Braun × *caesius*, *R. maximus* L. *v. rotundifolius* F.Aresch. was interpreted as *R. ruderalis* (F.Aresch.) Neuman × *caesius*, whereas *R. (nemoralis v.) acutus* Lindeb. was suggested to be *R. gothicus* Frid. & Gelert ex E.H.L. Krause × *caesius* and probably also partly *R. aureolus* Allander × *caesius* (Lidforss 1899, 1901).

In Sweden there are two hexaploid *Corylifolii* species: *R. cyclomorphus* H.E.Weber and *R. tiliaster* H.E.Weber (Gustafsson 1939). The former has broad stipules and rich fruits embraced by the sepals. Hylander and Allander have suggested that it has arisen from the hybrid *R. raduloides* (F. Aresch.) Neuman × *caesius* (Hylander 1958b). This hypothesis is supported by the reddish styles, stamens and petals, as well as the broad heart-shaped terminal leaflets. Two additional species with only few localities in Sweden, *R. slesvicensis* Lange and *R. firmus* Frid. & Gelert ex Utsch, also have some characters that indicate influence from *R. caesius* (Rydberg and Wanntorp 2001, Oredsson 2006, Tyler et al. 2007).

Another Swedish species suggested to have a hybrid origin is *R. glauciformis* Gustaf. ex H.Hyl. It was originally described as *R. caesius* × *wahlbergii* Arrh. *f. glauciformis* (Gustafsson 1938), but Hylander argued that it is a true-breeding species since it has an independent distribution in Blekinge, SE Sweden (Holmgren 1942, Hylander 1958b). This hypothesis is supported by the chromosome number, 2n = 28, notwithstanding that *R. caesius* hybrids may occasionally also have this ploidy level (Gustafsson 1943). Burén agreed with this interpretation and clarified its distribution and how it can be discerned from other species and *R. caesius* hybrids (Burén 2009).

In a recent article, we used microsatellite DNA analysis (MSDA) to clarify how primary hybrids between *R. idaeus* and *R.* sect. *Corylifolii* species can be discerned from apomictically reproducing species with similar origins (Ryde et al. 2021). We showed that apomictic species have an independent distribution distinct from the parental *Corylifolii* species, a uniform morphology and restricted genetic variability. By these criteria, we showed that there are four Swedish apomictic *Corylifolii* species that have arisen from *R. idaeus* hybrids, *R. pruinosus* Arrh. s.s., *R. lagerbergii* Lindeb., *R. onsalaënsis* Ryde and *R. cordatiformis* (Neuman) Ryde. In contrast, *R. rosanthus* Lindeb. was shown to be a collection of independently arisen *R. raduloides* × *idaeus* hybrids, and most of what has been called *R. pruinosus* in Sweden and other countries was shown to be a mixture of unrelated primary hybrids of *R. idaeus* and various *Corylifolii* species.

In the present study, we perform a similar investigation of *R. caesius* hybrids, mainly based on Swedish material. We show that the situation is similar to that of the *R. idaeus* hybrids. We also address a related question, viz. why some *R. caesius* hybrids seem to be particularly common in certain areas.

Methods

Five hundred seventy-six samples (young leaves from the tip of the annual shoot) of *R. caesius*, several *Corylifolii* species and putative *R. caesius* hybrids were collected, mainly from Sweden, but also from Denmark, Norway and Germany. The localities are specified in the Supporting information. Vouchers are deposited in LD. The samples were stored in a freezer at −80°C until they were used.

DNA was extracted with the CTAB method (Doyle and Doyle 1987). The concentration of the DNA was determined by a fluorometer and the samples were diluted with ddH_2O to ~14 ng μl^{-1}. Four pairs of microsatellite primers, described in the Supporting information, were used to analyse variable microsatellite loci. The three loci Ru105b, Ru117b and Ru275a were described in *R. idaeus* (Graham et al. 2004), whereas the locus mRaCIRRIV2A8 was described in *R. alceifolius* Poir. (Ansellem et al. 2001). They will here be referred to as 105b, 117b, 275a and 2A8, respectively. They have been successfully used also for other *Rubus* species (Stafne et al. 2005, Zhou 2014, Hedrén et al. 2020, Ryde et al. 2021). One primer in each pair was tagged with a fluorescing molecule (Cy5-) to enable the identification of the polymerase chain reaction (PCR) products in an automated sequencer (ALF Express II).

The PCR amplification consisted of 35 cycles of denaturation at 94°C for 30 s, annealing at varying temperatures (Supporting information) for 30 s and polymerisation at 72°C for 1 min (10 min in the last cycle). Each PCR was performed in a solution consisting of (in a total volume of ca 6.4 µl): 4.4 µl ddH_2O, 0.66 µl 10× reaction buffer (100 mM Tris–HCl, pH 8.3, 500 mM KCl and 15 mM $MgCl_2$), 0.56 µl dNTPs (2.5 mM for each nucleotide), 0.25 µl Cy5-tagged primer (10 µg ml^{-1}), 0.1 µl untagged complementary primer (25 µmol ml^{-1}), 0.03 µl Taq polymerase (5 µ μl^{-1}; Applied Biosystems) and 0.6 μl^{-1} template DNA (14 ng μl^{-1}).

The PCR products were mixed with formamide and appropriate size markers and were then heated to 94°C for 25 min before they were separated by electrophoresis in a polyacrylamide gel. The DNA fragments were detected and analysed with the help of an ALF Express II analysis system. Samples that resulted in poor PCR reactions were purified with a Qiagen purification kit, after which the PCR and electrophoresis were redone. Samples that still resulted in poor amplification (unspecific amplification, dominance of short fragments etc.) were excluded altogether.

The relative height of the amplified fragments on the resulting electropherogram was recorded on a five-graded scale for each sample to obtain an estimate of band intensity. Differences in relative band intensity between plants with overlapping banding patterns were matched with information on ploidy levels and interpreted as possibly due to variation in allele copy numbers. Similarly, differences in relative band intensity between plants of the same ploidy expressing different numbers of bands were also interpreted as due to variation in allele copy numbers. Several samples were re-amplified by PCR and rerun to verify the patterns of relative band intensity. Such analyses gave rise to closely similar differences in band intensity as the original runs, with only minor variation in peak height (typically, at most by a single unit along the five-graded scale used by us).

DNA-ploidy levels were estimated by flow cytometry for 173 samples. This analysis was performed by Plant Cytometry Services (<www.plantcytometry.nl>). Fresh leaf material was homogenised and mixed with an ice-cold buffer with DAPI (Arumuganathan and Earle 1991). The amount of DNA per cell nucleus was determined in a CyFlow ML with iceberg lettuce *Lactuca sativa* as an internal standard. The estimates were calibrated by using *Rubus* samples with known chromosome numbers. The DNA-ploidy levels are presented as fractional chromosome numbers from this calibration line between the ratio in fluorescence between the samples of known chromosome numbers and the internal standard.

The search for the origin of the various putative hybrids based on the MSDA results was facilitated by the use of computer programs taking into consideration both the length of the various microsatellite alleles and the relative intensity of the PCR products. Principal-component analyses were performed with the NTSYSpc ver. 2.1q software (Rohlf 1994), using a pair-wise comparison of Jaccard coefficients (Jaccard 1908) based on the occurrence of alleles for all four loci.

Result and discussion

DNA-ploidy levels

DNA ploidy levels of 125 samples of putative *Rubus caesius* hybrids were determined by flow cytometry. Of these, 99 (79%) were hexaploids with $2n = 6x \approx 42$. This is the expected chromosome number for *R. caesius* hybrids, reflecting that they have arisen from the fusion of reduced pollen from *R. caesius*, with $n = 14$, and unreduced egg cells from the *Corylifolii* parent with $2n = 28$ (Gustafsson 1939). From the calibration line, they gave an average estimated chromosome number of 42.3, i.e. close to the expected value of 42. Only a few apomictic Swedish *Corylifolii* species have been reported to be hexaploids and they will be discussed below.

Sixteen samples (13%) were pentaploids. Gustafsson also reported this chromosome number for *R. caesius* hybrids (Gustafsson 1943) and our results quantify how common this is. It is not clear how this chromosome number arises, especially as the same parents may give hybrids with either $2n = 42$ or 35 (or even 28). Dowrick (1961, 1966) described that apomixis in tetraploid *Rubus* species passes through stages of meiotic division and fusion of reduced nuclei prior to embryo formation, so-called automixis. In artificial crosses between tetraploid apomicts and diploid sexual species, he obtained offspring with lower ploidy levels than expected, which must have resulted from the fertilisation of reduced egg cells in the normally apomictic *Rubus* parent. Although the pentaploid plants identified here must also involve the fusion of partially reduced gametes, it is still unclear how the results reported by Dowrick can explain our findings.

The average estimated chromosome number from the calibration line of the pentaploid samples is 35.1, i.e. close to the expected value (35). This number differs from that of the *R. idaeus* hybrids, which consistently give results below the calibration line, typically around 34 (Ryde 2011, Ryde et al. 2021), indicating that the amount of DNA is slightly less in *R. idaeus* than in the *Corylifolii* species and in *R. caesius*. Consequently, the estimated chromosome number also gives an indication whether a sample is a *R. idaeus* or *R. caesius* hybrid.

The lowest estimated chromosome number among the 16 samples of pentaploid *R. caesius* hybrids was 33.3 for a sample from Kalmar in Småland that initially was interpreted as a *R. caesius* × *idaeus* hybrid. The low chromosome number may indicate that this interpretation is correct and that the hybrid might have been formed from an unreduced egg cell of *R. caesius*, rather than a reduced one (which gives rise to the normal chromosome number of 2n = 21 for the *R. caesius* × *idaeus* hybrid) or that a tetraploid cultivar of *R. idaeus* was involved. Four additional samples gave estimated chromosome numbers of 34.3–34.5, but two of them gave higher estimates (35.4) when the flow cytometry was repeated on new material.

Pentaploid hybrids include samples of *R. eluxatus* Neuman × *caesius*, *R. fasciculatus* P.J.Müll. × *caesius*, *R. gothicus* × *caesius* and *R. raduloides* × *caesius*. The latter hybrid is conspicuously common: eight samples are of this hybrid, which is slightly more than half of the number (14) of the same hybrid with 2n = 42. The background to the observed ratio is currently unclear, but might indicate that the process that leads to pentaploids is especially important for *R. raduloides*. Another interpretation is that the hybrids represent *R. raduloides* × *idaeus* instead, but neither the rather high estimated chromosome numbers (35.3 on average), nor the MSDA are in favour of this interpretation.

A number of the putative *R. caesius* hybrids were tetraploids. This finding might indicate that they are not true hybrids, but pure *Corylifolii* species. However, in 10 cases (8%), we are quite confident (from both the morphology and from the MSDA) that the samples represent *R. caesius* hybrids. A tetraploid chromosome number has also been reported before for *R. caesius* hybrids (Gustafsson 1943). They might arise from the combination of reduced pollen and egg cells from the parents, but it seems more likely that they have arisen from some sort of chromosome loss in hexaploid hybrids. The tetraploid hybrids have *R. camptostachys*, *fasciculatus*, *gothicus* and *raduloides* as the *Corylifolii* parent, and in one case *R. insularis* J.Presl & C. Presl, which belongs to *R.* sect. *Rubus*. Chromosome numbers will be further discussed below for specific hybrids.

Microsatellite DNA characterisation of *R. caesius*

One hundred twenty-one samples of *R. caesius* were analysed with microsatellite DNA analysis (MSDA). They gave somewhat varying results as is illustrated in Fig. 1. For the 105b locus, all samples had two identical alleles with lengths of 146 and 152 bp (Fig. 1a). These two alleles are also found in all Swedish *Corylifolii*, whereas the two alleles are not found in any indigenous Swedish species of *R.* sect. *Rubus* and only

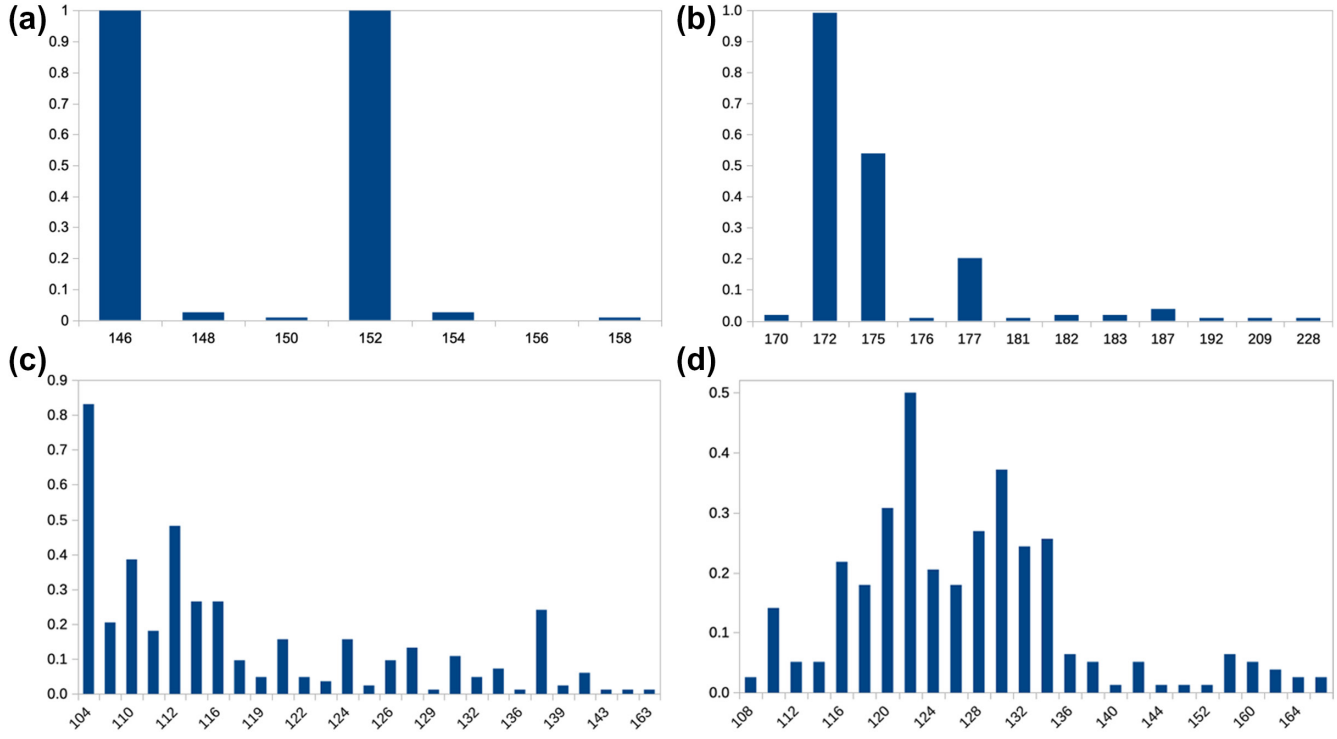


Figure 1. Frequency of the various alleles for *R. caesius* for the four studied loci, (a) 105b, (b) 2A8, (c) 117b and (d) 275a.

in a few introduced species, e.g. *R. allegheniensis* Porter, *R. gratus* Focke and the cultivar *R.* 'Bedford Giant'. This pattern strongly supports the suggestion that all *Corylifolii* species have arisen from hybrids involving *R. caesius*. Eight of the samples (7%) exhibit one additional allele.

At locus 2A8, all *R. caesius* samples except one have an allele with length 172 bp (Fig. 1b). In 68% of the samples, one additional allele is found, typically with a length of 175 or 177 bp. However, 23% of the samples have only a single allele, whereas 8% have three alleles. About 70% of the Swedish *Corylifolii* have the 172 bp allele, but it is only found in one Swedish *R.* sect. *Rubus* species (*R. pseudopallidus* (Gust.) Å.Gust.). On the other hand, nearly all *R.* sect. *Rubus* have an allele of length 182 bp (except *R. scissus* W.C.R.Watson and *R. nessensis* Hall.). This allele is also present in 85% of the Swedish *Corylifolii* species, but only in two of the *R. caesius* samples.

At locus 117b, the results are more varying. Most samples have 3, 4 or 5 alleles (27, 39 and 27%, respectively). Twenty-seven alleles were observed with sizes between 104 and 163 bp. The most common ones have lengths of 104, 110 and 112 bp (observed in 83, 32 and 40% of the samples).

The results at locus 275a are similar. Most samples have 3 or 4 alleles (38 and 37%), and 26 alleles of sizes between 108 and 166 bp were observed. The most common alleles have lengths of 120, 122, 128, 130 and 134 bp (observed in 26–50% of the samples).

The amount of variation in *R. caesius* is intermediate between what is observed for the sexual species *R. idaeus* and that for the apomictic *Corylifolii* species. If we quantify the variation as the Shannon entropy ($S = \sum_{i=1}^{n} -p_i \ln(p_i)$, where the sum is over all observed alleles and p_i is the frequency of the allele of size i), *R. caesius* has an intermediate variation at loci 105b and 2A8 ($S = 0.2$ and 1.2), compared to 2.5 and 6.0 for *R. idaeus*, and giving it a rank of 4 and 10 among the 12 species with 20 or more samples). However, for the other two loci investigated, *R. caesius* has the largest variation among all species ($S = 5.0$ and 5.3, compared to 4.2 and 4.5 for *R. idaeus* and 4.4 and 3.7 for the *Corylifolii* species with the largest variation). It should be noted that the reason why *R. caesius* has a larger variation than *R. idaeus* at these two loci is that it is probably an allotetraploid combining two divergent parental genomes and that it has twice as many alleles as *R. idaeus*. The variation found is consistent with automixis, as described by Dowrick (1961, 1966). However, it is still unclear why the variation is higher in *R. caesius* than in the apomictic *R.* sect. *Corylifolii* species.

Rubus fasciculatus × *caesius*

We have identified *R. caesius* hybrids with 16 different *Rubus* species. We will start by discussing *R. fasciculatus* × *caesius* because we have much material of this hybrid and many samples with chromosome-number determinations, making the assignment quite certain. Therefore, we use this hybrid to describe what MSDA pattern can be expected from *R. caesius* hybrids.

As mentioned above, most of the hybrids between *Corylifolii* species and *R. caesius* are hexaploids. They are supposed to arise from reduced (2n = 14) *R. caesius* pollen combined with unreduced (normally 2n = 28) egg cells from the *Corylifolii* parent. Consequently, we expect to find all alleles from the *Corylifolii* parent, together with two alleles from *R. caesius* in the hybrid. However, for the 105b locus, the two invariant *R. caesius* alleles are also present in all Swedish *Corylifolii* species, so no new alleles are expected. Still, the hybrids may be identified by a change in the band intensity patterns of the alleles (the two alleles from *R. caesius* should gain increased intensity, relative to the other alleles). Likewise, for the 2A8 locus, most of the *Corylifolii* species already have alleles of 172 and 174 or 176 bp, so new alleles are not always expected. The situation is further complicated by the fact that ~20% of the *R. caesius* hybrids are tetra- or pentaploids; therefore, they probably do not have inherited all alleles from the *Corylifolii* parent.

Fourteen samples of *R. fasciculatus* and 23 samples of putative *R. fasciculatus* × *caesius* hybrids have been examined by MSDA. Flow cytometry showed that 11 putative hybrids are hexaploids, whereas one is a pentaploid and one is tetraploid (the others were not tested). It should first be mentioned that the initial analysis showed that the Swedish material of *R. fasciculatus s.l.* can be divided into two species, as has previously been suggested by Hylander (1958b). One occurs in the western part of Sweden and the other in the eastern part; they meet in eastern Skåne and especially in Blekinge. This situation will be clarified in a separate publication. Here, we will consider only the taxon with the western distribution, which probably is identical with *R. fasciculatus s.s.*, described from the border between France and Germany (Weber 1981).

At locus 105b, 17 of the putative *R. fasciculatus* × *caesius* hybrids had the same four alleles as *R. fasciculatus*. This is the expected pattern for the hybrid, because all *R. caesius* samples have the same two alleles as all the *Corylifolii* species, with lengths of 146 and 152 bp (Fig. 1a). However, the band intensity pattern is different. The hexaploid hybrids should have double copies of the 146 and 152 bp alleles. In *R. fasciculatus*, the 152 bp allele is most intense but only slightly more than the other three alleles (the pattern for the relative intensities of the four alleles is typically 4–4–4–5). For *R. caesius*, the allele band at 152 bp is normally slightly more intense than that at 146 bp (the pattern is typically 4–5). Therefore, for the hybrids, the allele of 152 bp is most strongly amplified, followed by the 146 bp allele and then the other two (the intensity pattern is typically 2–3–2–5). Such a pattern is found for 16 of the putative hybrids.

Likewise, at locus 2A8, 20 samples of the putative hybrids have the same 3–4 alleles as *R. fasciculatus*. Again, this pattern is expected, because *R. caesius* normally has only two alleles, one at 172 bp and the other at 175 or 177 bp, but the 177 bp allele is not found in any of the putative hybrids. The hybrids can be recognised by a different intensity pattern, 5–2–2–1, compared to 5–3–4–2 for *R. fasciculatus* (reflecting that the 172 bp allele normally is appreciably more strongly amplified than the other allele in *R. caesius*, typically having an

intensity pattern 5–2). Eighteen of the putative hybrids have this pattern.

At locus 117b, most *R. fasciculatus* samples have three alleles (one allele is missing in two of the samples). Seventeen putative hybrids share these alleles, although one of them is missing in five cases. However, in all except four cases, there are one, two (three samples) or three (one sample) additional alleles, with lengths observed for *R. caesius*. In the remaining samples, the alleles from *R. caesius* may overlap with those from *R. fasciculatus*, as is often indicated by a change in the intensity pattern.

At locus 275a, most *R. fasciculatus* samples have four alleles. These were also found in most of the putative hybrids. In addition, they have one (seven samples), two (6), three (2) or even four (1) extra alleles, which mostly overlap with alleles observed for *R. caesius*. However, for three samples, no additional alleles were observed. Several putative hybrids have lowered intensities for the longest alleles, but the difference is rather small. This pattern may reflect that the 122 bp allele is the most common allele in *R. caesius*.

Consequently, we conclude that the *R. caesius* hybrids can be identified with MSDA. However, their identification is somewhat more complicated than for the *R. idaeus* hybrids (Ryde et al. 2021), mainly because the 105b and 2A8 loci, which normally give the most uniform and easily interpreted pattern, do not give any new alleles from *R. caesius*. Therefore, the identification of hybrids needs to be based on subtle differences in the intensity pattern or by extra alleles for loci 117b and 275a, which typically give quite some variation also for the pure *Corylifolii* species.

One sample from Släp in northern Halland is undoubtedly the *R. fasciculatus* × *caesius* hybrid (based on morphological characters and the fact that the two parents appear in the neighbourhood). However, the cytometry shows that it is a pentaploid, in contrast to the other hexaploids. In the MSDA, this specimen gives results similar to those of the other hybrids, with an extra allele for 117b and one or two additional alleles for 275a (compared to *R. fasciculatus*), and the characteristic change in intensity pattern for both 105b and 2A8. The only clear difference is the lack of one of the four alleles at locus 105b. Thus, the reduced ploidy seems to be caused by a loss of one set of chromosomes from the *R. fasciculatus* parent (the longest allele at locus 275a is also missing). Another sample with an unknown chromosome number is also missing the same allele for 105b.

Based on this discussion and considering the results of all four loci together, we suggest that 21 samples are hybrids. However, two samples are more questionable because they are missing some alleles from *R. fasciculatus* and have new alleles that are not found in *R. caesius*. Yet, both grow together with *R. fasciculatus*, so they most likely represent hybrids, possibly with a ploidy level lower than hexaploid (explaining the missing alleles). No cytometry results are available for these samples.

Interestingly, ten of the unambiguous samples of the *R. fasciculatus* × *caesius* hybrids (samples fac1–fac12 in Fig. 2) come from a small area in the north-western part of Halland (parishes of Släp and Onsala), where it is as common as pure *R. fasciculatus* (15 localities of the hybrid and 13 of *R. fasciculatus*; a distribution map was presented in Ryde (2009)). The question then arises whether they represent primary independent hybrids or a new apomictic species that originates from a *R. fasciculatus* × *caesius* hybrid and currently spreads in that area. The MSDA shows that the hybrids are uniform for loci 105b, but they show quite some variation for the other three loci. There are no pair of samples with identical results for the three loci. Accordingly, we conclude that none of the hybrid samples from Halland are genetically identical and therefore most likely have arisen independently. This conclusion is also reinforced by the fact that one of the samples shows a differing chromosome number. Thus, the most natural interpretation is that this hybrid arises easily when the two parent species meet. This conclusion is also supported by the fact that the hybrids are scattered throughout the area and closely follow the distribution of *R. fasciculatus*. However, it is somewhat puzzling that the hybrids are not as frequent in the province of Skåne, where both *R. caesius* and *R. fasciculatus* are more common.

Most samples of the *R. fasciculatus* × *caesius* hybrid are morphologically quite similar to *R. fasciculatus*, but the plants are rather small and weak. However, there are three samples from the same area in Halland that are different in appearance, forming large and strong thickets but still combining morphological characters of *R. fasciculatus* and *R. caesius*. This interpretation is confirmed by the MSDA, which shows that also these samples (csx28, csx29 and csx131) fall well into the variation for the *R. fasciculatus* × *caesius* hybrids. Interestingly, two samples (csx29 and csx131) are the only two that give the same patterns for all four loci. They were collected from two distinct localities 800 m apart, but along the same country road. It is possible that they have spread vegetatively by human assistance during road construction. At both localities, they expand vegetatively, but there is no sign that they can spread via seeds.

One deviating sample from Smedstorp in Skåne does not show many morphological characteristics of a *R. caesius* hybrid and the cytometry shows that it is tetraploid. However, in ordination plots derived from MSDA, it clusters together with the *R. fasciculatus* × *caesius* hybrids. It also has a similar MSDA pattern (Fig. 2, sample ma1n, fourth from the bottom). For each of the four loci, it shares two or three alleles with *R. fasciculatus*, but one or two expected alleles are missing. This pattern is expected for a tetraploid sample, for which two sets of alleles have been lost. For all loci, except 2A8, one or three extra alleles are found, probably originating from *R. caesius*. The only detail that contradicts an interpretation as a *R. fasciculatus* × *caesius* hybrid is that the extra allele at locus 105b has a length of 164 bp, a length that is not observed for *R. caesius* (cf. Fig. 1a).

At the same locality as one of the weak *R. fasciculatus* × *caesius* hybrids (Kläppa in Släp), a *Corylifolii* plant is found that is not similar to any other taxon in Sweden (Ryde 2009, 2011). It was previously interpreted as a derivative from a *R. fasciculatus* × *caesius* hybrid and it is tetraploid. However, the MSDA results do not support such a suggestion (last sample

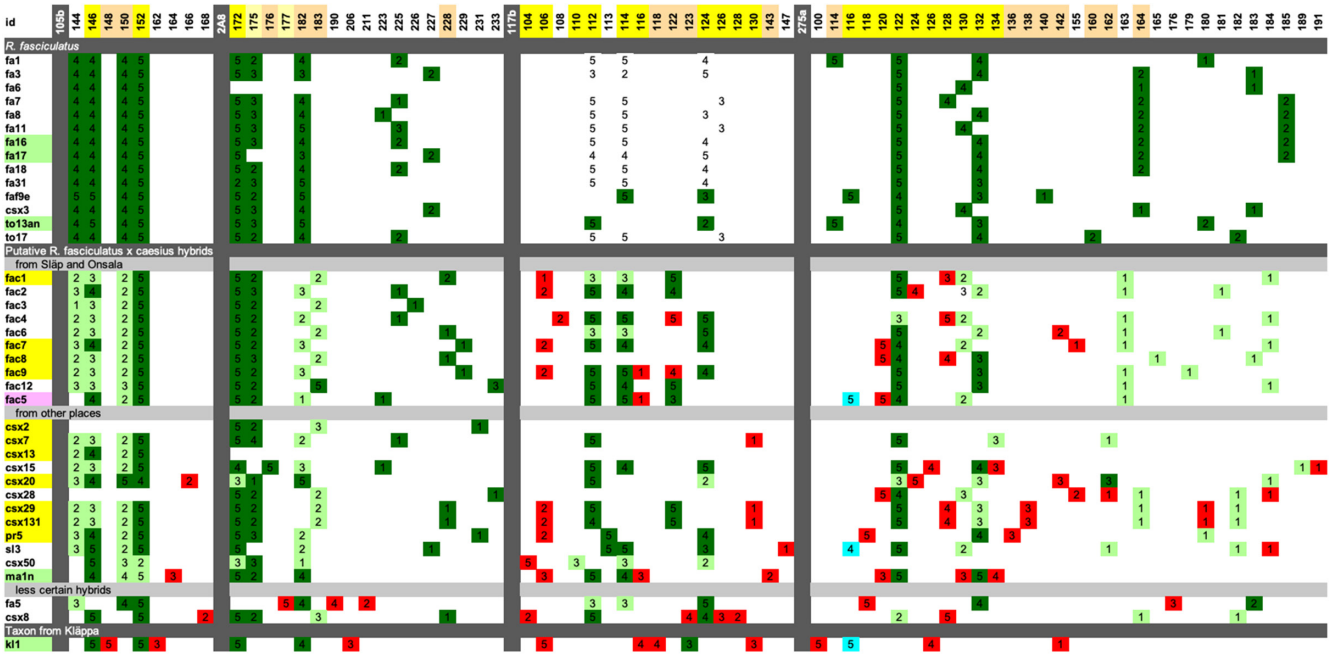


Figure 2. MSDA results for *R. fasciculatus* and the putative *R. fasciculatus* × *caesius* hybrids. The upper 14 samples are *R. fasciculatus* and other samples are putative *R. fasciculatus* × *caesius* hybrids; the ten samples fac1–fac12 above the light grey area are all from Släp in Halland. The figure shows the various alleles observed in MSDA, together with the relative intensities (1–5) of the PCR products. The lengths in bp are shown in the first row. Those shown in yellow are frequently observed for *R. caesius*, whereas those in orange are only found for a few samples. The first column shows a sample code, which is coloured according to the ploidy level: light green – tetraploid, magenta – pentaploid, yellow – hexaploid and white – chromosome number not determined. The alleles are also colour coded: dark green – allele probably from *R. fasciculatus*, light green – allele probably from *R. fasciculatus*, but with a lowered intensity, red – allele probably from *R. caesius* and cyan – an allele that occasionally occurs for *R. fasciculatus* but may also come from *R. caesius*.

in Fig. 2). At loci 105b and 2A8, it shares two alleles with *R. fasciculatus* (as may be expected in a tetraploid plant) and it has two or one extra allele, although only one of them overlaps with alleles from *R. caesius*. However, at the other two loci, only one allele each is close to alleles from *R. fasciculatus*. Thus, the Kläppa taxon remains unidentified. As it is found only in a single locality, we see no reason to give it a taxonomic name.

Rubus cyclomorphus has been reported a few times from Skåne (Lidforss 1901, Gustafsson 1923, Weber 1981). In particular, a locality in Kabbelljung, near Holmeja, was mentioned by all three authors and described in detail by Gustafsson (1923). The locality can still be identified, but the population there undoubtedly represents a *R. fasciculatus* × *caesius* hybrid, with both parents growing in the neighbourhood and flow cytometry analysis confirm that it is a hexaploid. However, Gustafsson reported a *wahlbergii*-like *Corylifolii* morphotype from the locality, not *R. fasciculatus*. We have not been able to confirm any occurrence of *R. cyclomorphus* in Skåne (Tyler et al. 2007).

Rubus gothicus × *caesius*

The hybrid *R. gothicus* × *caesius* is known from many localities in southern Sweden, but in particular from the parish of Brunnby in north-western Skåne, where we have observed it at eight rather nearby localities. We have analysed 29 samples of *R. gothicus* and 18 samples of the putative *R. gothicus* × *caesius* hybrid. The results are presented in Fig. 3.

Based on the results (a detailed discussion is given in the Supporting Information), we judge that all 18 samples are *R. gothicus* × *caesius* hybrids. In some cases (especially when a fifth 164 bp allele is observed at locus 105b), it becomes somewhat difficult to discern the hybrids from some variants within the *R. wahlbergii* complex. This may indicate that the latter species actually has arisen from this hybrid, but this will be further scrutinised in a separate study devoted to this challenging complex. Three samples of the hybrid are pentaploid according to the cytometry. They typically have more missing alleles than the other hybrids, but also show a considerable variation.

Four samples come from the same area in Brunnby (the first four samples for the hybrid in Fig. 3). They show a large variation in the alleles and no pair of samples show an identical pattern. One of them even has a deviating chromosome number. Therefore, we conclude that the four samples are not genetically related, but seem to have arisen independently. Thus, there is no indication of any apomictic local species in this area that has arisen from the *R. gothicus* × *caesius* hybrid. The same applies to the other samples of the hybrid: no pair of samples share the same alleles for the four loci, showing that each is an independent primary hybrid.

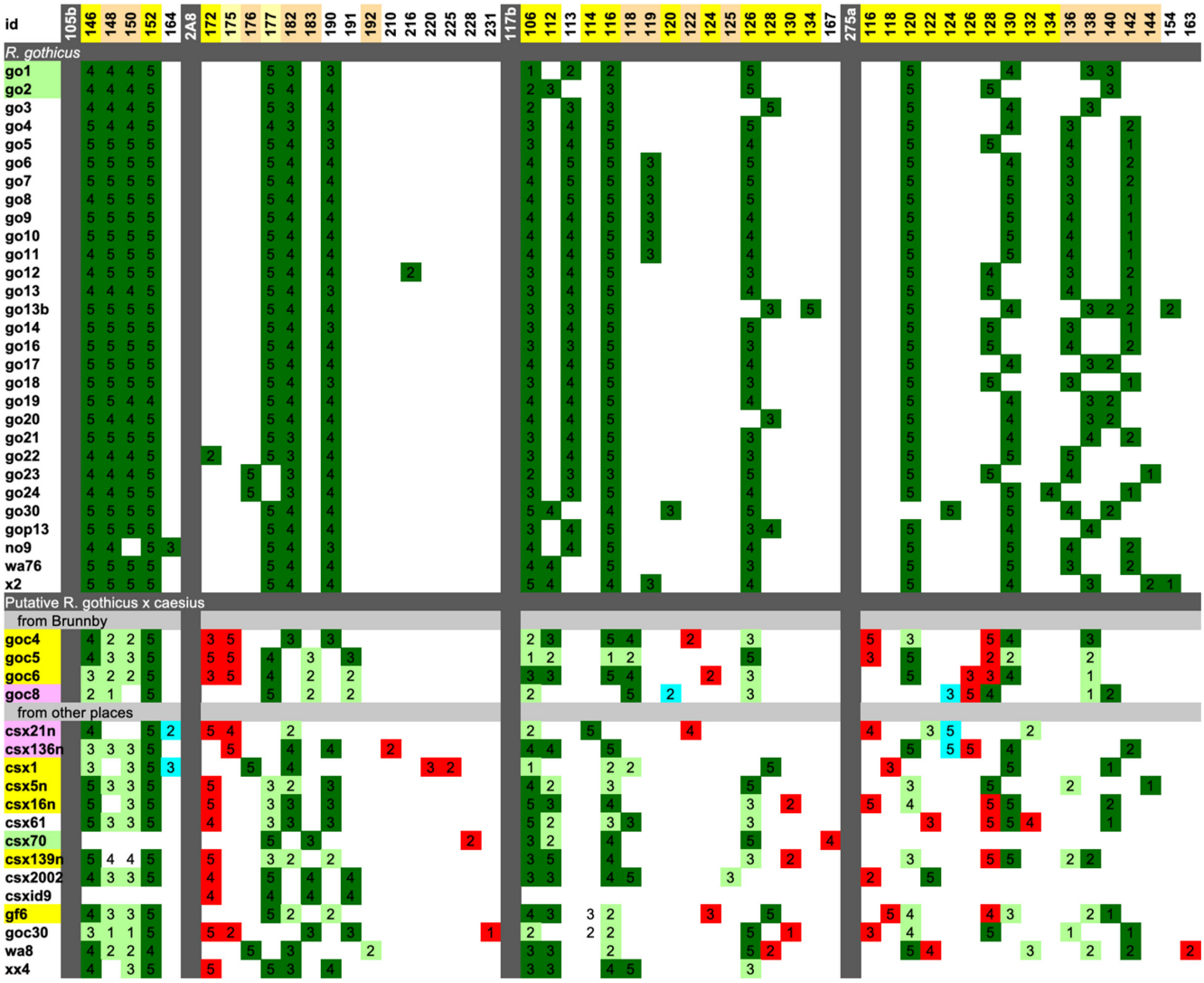


Figure 3. MSDA results for *R. gothicus* and the putative *R. gothicus* × *caesius* hybrids. The upper 29 samples are *R. gothicus* and other samples are putative *R. gothicus* × *caesius* hybrids; the four first samples goc4–goc8 above the light grey area are all from Brunnby in Skåne. The figure shows the various alleles observed in MSDA, together with the relative intensities (1–5) of the PCR products. The lengths in bp are shown in the first row. Those shown in yellow are frequently observed for *R. caesius*, whereas those in orange are only found for a few samples. The first column shows a sample code, which is coloured according to the ploidy level: light green – tetraploid, magenta – pentaploid, yellow – hexaploid and white – chromosome number not determined. The alleles are also colour coded: dark green – allele probably from *R. gothicus*, light green – allele probably from *R. gothicus*, but with a lowered intensity, red – allele probably from *R. caesius* and cyan – allele that occasionally occurs in *R. gothicus* but may also come from *R. caesius*.

Rubus cyclomorphus and *R. raduloides* × *caesius*

Rubus cyclomorphus H.E.Weber was initially described as *R. cyclophyllus* by Lindeberg (Lindeberg 1885). It occurs along the coast of Skagerack from southeast Norway to Bohuslän in Sweden (Weber 1981, Blomgren et al. 2011), but it has also been reported in the provinces of Skåne and Halland in Sweden (Lidforss 1901, Gustafsson 1923, Weber 1981), although this has later been revised (Tyler et al. 2007, Ryde 2009). For example, as was discussed above, reports of *R. cyclomorphus* from Skåne correspond to the *R. fasciculatus* × *caesius* hybrid. For the samples from Bohuslän, the morphological characters, especially the heart-like terminal leaflets and the pink flowers, styles and filaments, indicate that *R. raduloides* is involved. Therefore, we examined 15 samples of *R. raduloides* and 14 samples of putative *R. cyclomorphus*. Eight of the latter are hexaploids, two are pentaploids, whereas the remaining four were not examined by cytometry.

The results are presented in Fig. 4. They show that all the putative *R. cyclomorphus* samples originate from *R. raduloides* × *caesius* hybrids (discussed in detail in the Supporting Information). However, no pair of the putative *R. cyclomorphus* samples give identical patterns in MSDA. The same is observed by standard clustering analysis, in which the putative

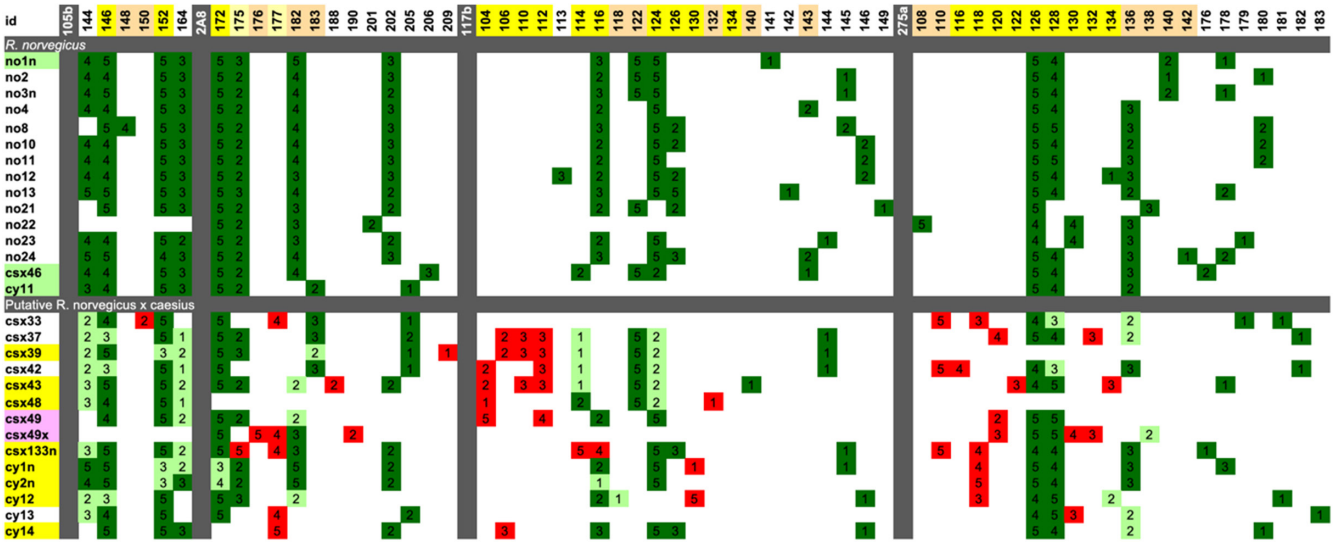


Figure 4. MSDA results for *R. raduloides* and the putative *R. raduloides* × *caesius* hybrids (corresponding to what has been called *R. cyclomorphus*). The upper 15 samples are *R. raduloides* and other samples are putative *R. raduloides* × *caesius* hybrids. The figure shows the various alleles observed in MSDA, together with the relative intensities (1–5) of the PCR products. The lengths in bp are shown in the first row. Those shown in yellow are frequently observed for *R. caesius*, whereas those in orange are only found for a few samples. The first column shows a sample code, which is coloured according to the ploidy level: light green – tetraploid, magenta – pentaploid, yellow – hexaploid and white – chromosome number not determined. The alleles are also colour coded: dark green – allele probably from *R. raduloides*, light green – allele probably from *R. raduloides*, but with a lowered intensity and red – allele probably from *R. caesius*.

R. cyclomorphus samples show a large variation that overlaps with the *R. raduloides* cluster. Therefore, we conclude that *R. cyclomorphus* is not a proper species but rather a collection of genetically unrelated *R. raduloides* × *caesius* hybrids. This is strongly supported by the morphology, which shows a very large variation. Consequently, the name *R. cyclomorphus* should be avoided. All hybrids were collected within the distribution range of *R. raduloides.*

Rubus tiliaster and *R. camptostachys* × *caesius*

Rubus tiliaster H.E.Weber was originally described as *R. corylifolius* subsp. *dumetorum* var. *tileaceus* Lange (Areschoug 1885), but it was mixed up with a taxon known from Denmark (Weber 1981). It occurs in north-western Skåne, Schleswig–Holstein and a few scattered localities in Denmark (Weber 1981, Pedersen and Schou 1989, Tyler et al. 2007). It is characterised by linden-shaped leaves with finely serrate margin, long straight prickles and hairy anthers. However, besides these characters, it is morphologically very diverse. Moreover, the chromosome number, 2n = 42 (Gustafsson 1943), indicates that it may have arisen from hybrids with *R. caesius*, most likely with *R. camptostachys*, which is the only *Corylifolii* species in Sweden with hairy anthers and which is common in the part of Skåne where *R. tiliaster* occurs.

Six samples of *R. camptostachys* and 18 samples of putative *R. tiliaster* were studied by MSDA. Six of them were also studied with cytometry, which indicated that they are all hexaploids. The MSDA confirm that all samples of *R. tiliaster* originated from *R. camptostachys* × *caesius* hybrids: they have all or most of the alleles from *R. camptostachys* (Fig. 5) and some have 1–3 extra alleles, presumably from *R. caesius* (further discussed in the Supporting information). However, the extra alleles are at different positions for all the samples, showing that they are all genetically distinct. This clearly shows that *R. tiliaster* is not a proper apomictic species in Sweden but a collection of primary *R. camptostachys* × *caesius* hybrids. Therefore, the name *R. tiliaster* should be avoided.

Rubus glauciformis

Rubus glauciformis was suggested by Gustafsson to represent a form of the hybrid *R. caesius* × *wahlbergii* (Gustafsson 1938). However, in the provinces of Blekinge, Småland and Öland in eastern Sweden, it seems to be a characteristic and uniform species (Holmgren 1942, Hylander 1958b, Burén 2009). On the other hand, in the province of Skåne, it has rather few and scattered localities (Tyler et al. 2007) and it is sometimes hard to discern it from other taxa and hybrids.

We have considered 29 samples of putative *R. glauciformis* from Skåne, Blekinge, Småland, Öland and Denmark. Of these, 23 were also examined by cytometry, showing that 16 of them are tetraploids, the reported ploidy level for *R. glauciformis* (Gustafsson 1933). The results are shown in Fig. 6. Four samples from Skåne and one from Denmark are hexaploids, which indicate that they instead are *R. caesius* hybrids. Two are pentaploids, indicating that they may be *R. idaeus* hybrids. Taking the MSDA, cytometry and morphological analysis together, we suggest (discussed in the Supporting information) that 21 of the samples represent *R. glauciformis* (from all four Swedish provinces) and six samples probably represent *R. glauciformis* × *caesius* hybrids (the hexaploids).

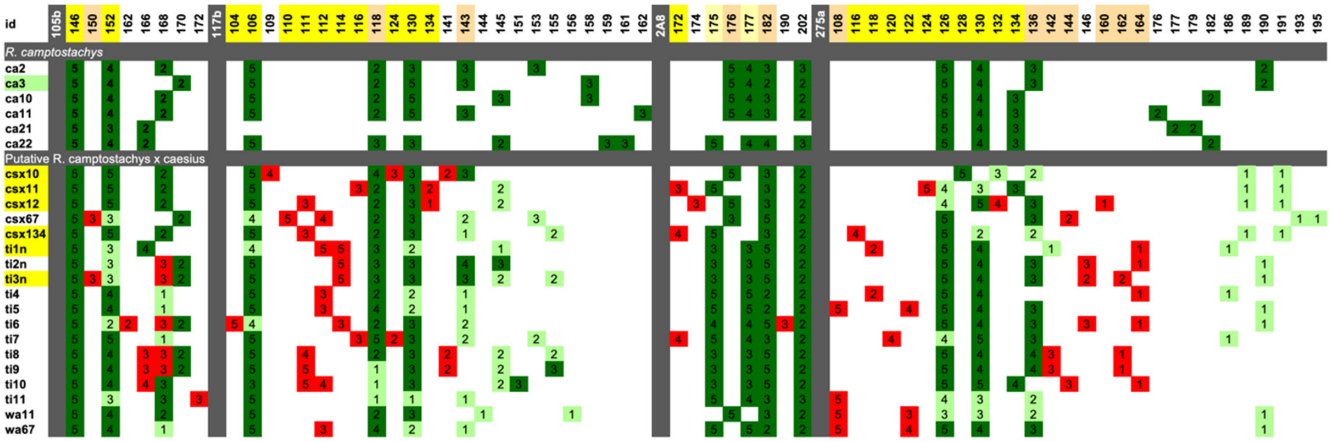


Figure 5. MSDA results for *R. camptostachys* and the putative *R. camptostachys* × *caesius* hybrids (corresponding to what has been called *R. tiliaster*). The upper six samples are *R. camptostachys* and other samples are putative *R. camptostachys* × *caesius* hybrids. The figure shows the various alleles observed in MSDA, together with the relative intensities (1–5) of the PCR products (1–5). The lengths in bp are shown in the first row. Those shown in yellow are frequently observed for *R. caesius*, whereas those in orange are only found for a few samples. The first column shows a sample code, which is coloured according to the ploidy level: light green – tetraploid, magenta – pentaploid, yellow – hexaploid and white – chromosome number not determined. The alleles are also colour coded: dark green – allele probably from *R. camptostachys*, light green – allele probably from *R. camptostachys*, but with a lowered intensity and red – allele probably from *R. caesius*.

Clearly, *R. glauciformis* from Blekinge, Småland and Öland show reasonably uniform MSDA results. However, the material from Skåne is somewhat more variable in the MSDA. Still, we interpret them as *R. glauciformis* because they have a morphology that agrees with the material from Blekinge, Småland and Öland.

There are two samples from Skåne that are pentaploids. They differ significantly from *R. glauciformis* in morphological

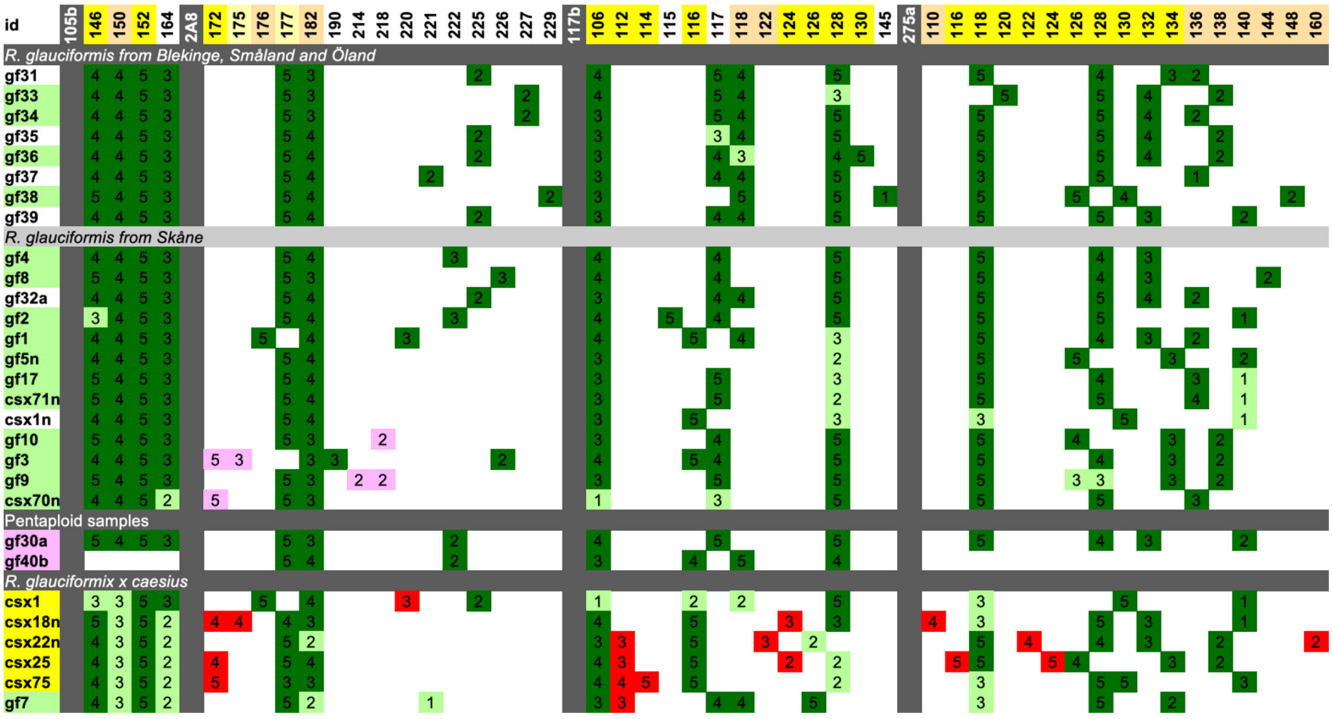


Figure 6. MSDA results for *R. glauciformis* and putative *R. glauciformis* × *caesius* hybrids. The figure shows the various alleles observed in MSDA, together with the relative intensities (1–5) of the PCR products (1–5). The lengths in bp are shown in the first row. Those shown in yellow are frequently observed for *R. caesius*, whereas those in orange are only found for a few samples. The first column shows a sample code, which is coloured according to the ploidy level: light green – tetraploid, magenta – pentaploid, yellow – hexaploid and white – chromosome number not determined. The alleles are also colour coded: dark green – allele probably from *R. glauciformis*, light green – allele probably from *R. glauciformis*, but with a lowered intensity (in this figure also shown for the *R. glauciformis* samples), red – allele probably from *R. caesius* and magenta alleles only observed for samples from Skåne.

characters. However, in the MSDA, they fall well within the variation observed for Swedish *R. glauciformis*, with no extra alleles and no alleles with a decreased intensity. Still, based on the ploidy level and the differing morphology, we interpret them as *R. idaeus* hybrids.

A natural question is how *R. glauciformis* has arisen and which *R. caesius* hybrids give similar results. A clustering analysis places *R. mortensenii* Frid. & Gelert ex H.H.L. Krause closest to *R. glauciformis*. It has exactly the same alleles as *R. glauciformis* at the 105b locus. However, at the other loci, the overlap is much lower: three out of four alleles overlap at locus 275a, two at 117b and only one at 2A8. *Rubus mortensenii* is fairly common in the same parts of Blekinge, Småland and Öland, where *R. glauciformis* occurs (cf. Fig. 7). Therefore, we consider it as a possible ancestor of *R. glauciformis* and also for hybrids with a similar appearance, even if the overlap of the distributions is less conspicuous in Skåne.

Rubus slesvicensis and *R. firmus*

Rubus slesvicensis is a species that is known from Denmark (Jylland) and Schleswig–Holstein. It has also been reported from one locality in Norway, probably introduced by ballast (Elven et al. 2018), and from two disjunct areas in Skåne in Sweden, Ljunghusen and V. Karaby (Oredsson 2004, 2006, Tyler et al. 2007). Yet, the Swedish material differs morphologically from the Danish and German material, in particular by white flowers with distinctly red styles and rarely 7-nate leaves (Fig. 8). The cytometry measurements show that both the Swedish and Danish–German material are hexaploids.

The MSDA clearly shows that the Swedish samples are genetically distinct from the other samples, forming a uniform and well-separated group in the principal component analysis plot (Fig. 9). In clustering analysis, the material from Skåne comes closest to *R. placidus* H.E.Weber from Germany (but it is clearly distinct from this species). Considering that the Swedish material is uniform and distinct morphologically, that it occurs in two areas 45 km apart, and that it shows a tendency to spread locally in both areas, we have decided to describe it as a new species with the name *Rubus ruboculus*.

On the other hand, the samples from Denmark, Germany and Norway are more varying. Three samples from the area of Viborg (central Jylland, 14 km apart) and one sample from Schleswig form another uniform group (although with the latter at some distance; light green in Fig. 9). Morphologically, these samples differ somewhat from the description of *R. slesvicensis* (based on material from Schleswig). In particular, they show no tendency to form 7-nate leaves. The remaining four samples from Schleswig and Norway are scattered in the plot (red in Fig. 9), indicating that they may represent varying and genetically unrelated *R. caesius* primary hybrids. This suggests that *R. slesvicensis* requires further investigations, but our material from Schleswig is too restricted to support any firm conclusions. It should be noted that Weber points out that the species is variable (Weber 1981).

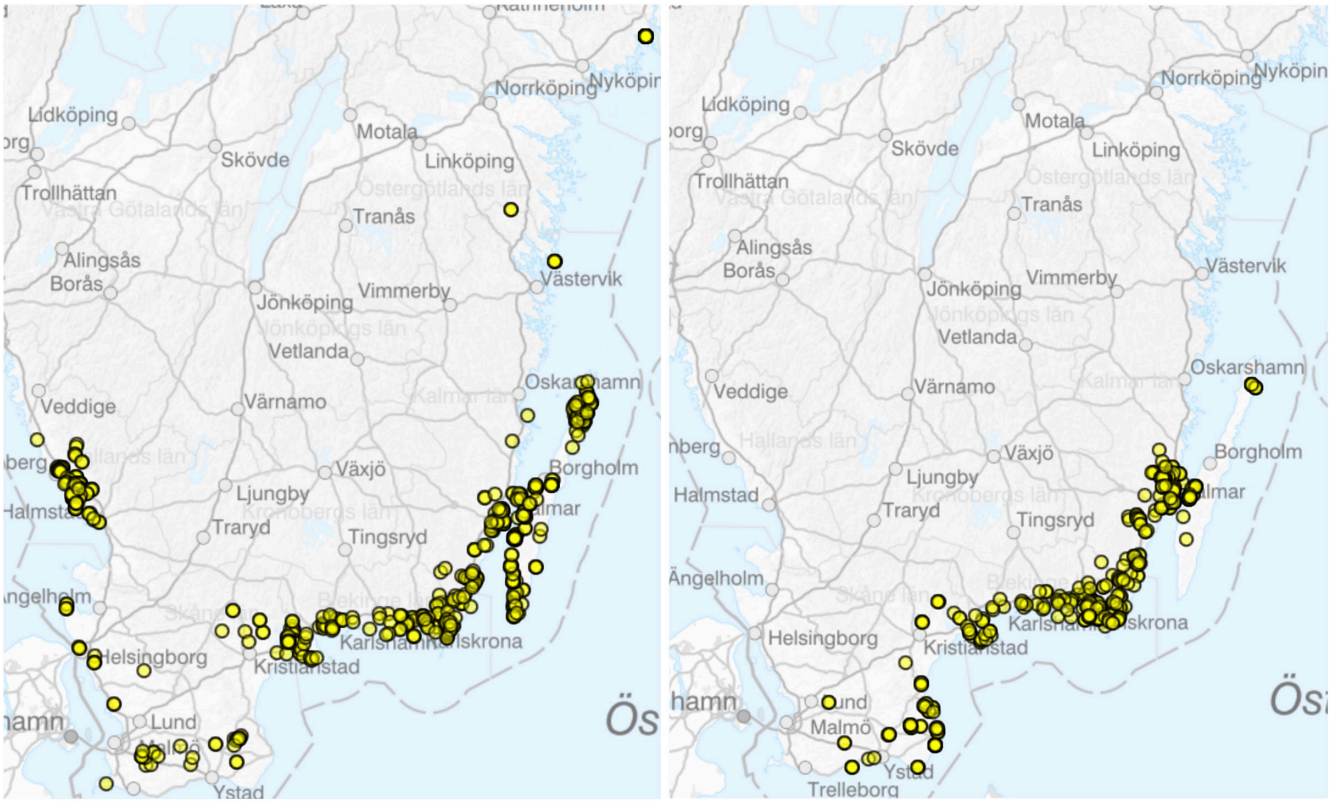


Figure 7. Distribution maps of *R. mortensenii* and *R. glauciformis* according to the Swedish Species Observation System (2022-01-29).



Figure 8. Pictures of *R. ruboculus*, upper and lower side of leaves, inflorescence, flower, flower bud, dissected flower, young fruit, ripe fruit, upper and lower side of upper leaves, unusual example of a 7-nate leave, primocane with detail and transect, inflorescence axis with detail.

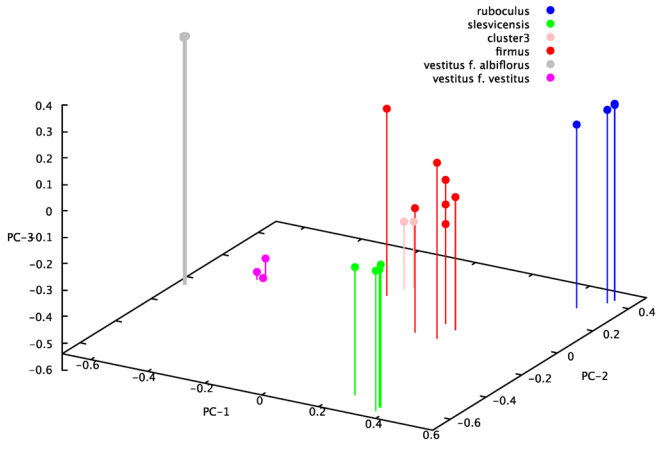


Figure 9. PCA results for *R. ruboculus*, *R. slesvicensis s.l.*, *R. firmus*, *R. vestitus f. albiflorus* and *f. vestitus*.

Rubus firmus is a species that is usually considered to be related to *R. slesvicensis* – these two species constitute *R.* series *Vestitiusculi* (Weber 1981) and they occur in the same area in Schleswig–Holstein, Jylland and some of the Danish islands (Weber 1981, Pedersen and Schou 1989, Oredsson 2004). However, it has also been reported from two localities in Sweden: Lund (Tyler et al. 2007) and Stockholm (Rydberg and Wanntorp 2001). We have collected samples from both Swedish localities, but it is doubtful whether the original plant is still present in Lund. At the locality, which is a private garden, there is plenty of *R. caesius* and some plants with hybrid character, but none with the long-stalked red glands that are the hallmark of *R. firmus* (Weber 1981). The samples were pentaploid (Lund) and hexaploid (Stockholm). To obtain reference material for *R. firmus*, four samples were collected in Denmark and Schleswig–Holstein. All four samples turned out to be tetraploids.

Interestingly, MSDA shows that all six samples are genetically distinct, with no similarity between any pairs of the samples and also no similarity to any other species. In the PCA analysis, they are scattered (Fig. 9; in red) together with the four unclear samples of *R. slesvicensis s.l.* mentioned above, between the two well-defined groups identified for the *R. slesvicensis* samples (*R. ruboculus* and the samples from Viborg). The only exception is a sample from Langeland, which was essentially identical to a sample of *R. slesvicensis s.l.* from Gross-Quern in Schleswig (Fig. 9; light pink).

Thus, our results call into doubt that *R. firmus* is a well-defined species, instead indicating that it is a collection of *R. caesius* hybrids. However, considering the limited material studied from Denmark and Germany, we cannot exclude that there actually is a genetically uniform species in that area. In any case, our study does not support the suggestion that *R. firmus* occurs in Sweden.

It has been suggested that both *R. slesvicensis* and *R. firmus* may have arisen from hybrids between *R. caesius* and *R. vestitus* Weihe (belonging to *R.* sect. *Rubus*) (Weber 1981). To test this hypothesis, we also included *R. vestitus* in our investigation. There are two forms of *R. vestitus* that differ in the colour of the flowers: *R. vestitus f. vestitus* with pink flowers and *f. albiflorus* G.Braun ex Kretzer. with white flowers (Pedersen and Schou 1989, Oredsson 2003). We have collected 3–4 samples of each variety from Sweden and Denmark. Interestingly, they are very distinct in MSDA (Fig. 9 and 10; discussed in the Supporting information). This indicates that the two formae are genetically quite distinct and probably correspond to two separate species. They also show disjunct distributions in both Sweden and Denmark (Pedersen and Schou 1989, Oredsson 2003). Anyway, we find no strong support for the suggestion that *R. slesvicensis s.l.* and *R. firmus* have arisen from hybrids between *R. caesius* and *R. vestitus* (further discussed in the Supporting information), although it is possible that the *R. caesius* hybrids with blackberries from *R.* sect. *Rubus* may behave somewhat differently from those with *Corylifolii*.

Other hybrids

We have studied several other *R. caesius* hybrids and most of them were confirmed by the MSDA. These include *R. eluxatus* × *caesius* from two nearby localities in the province of Halland (one pentaploid and one hexaploid), *R. antecedens* Ryde × *caesius* from one locality in Halland (previously interpreted as *R. polybrachteatus* Ryde × *caesius* (Ryde 2009, 2011)), *R. hallandicus* (Gabr. Ex. F.Aresch.) Neuman × *caesius* from one place each in Skåne (hexaploid) and in Halland (tetraploid; tentatively suggested to be *R. wahlbergii* × *caesius* (Ryde 2009, 2011); found at a distance of 12 km from the closest known locality of *R. hallandicus*), *R. lidforssii* (Gelert) Lange × *caesius* from three nearby localities in southeast Skåne (all genetically distinct), *R. wendtii* Ryde × *caesius* from two nearby localities in northwest Skåne (genetically distinct), *R. oredssonii* T.Mattsson × *caesius* from one locality in Bohuslän and *R. dissimulans* Lindeb. × *caesius* (or rather the Danish taxon of *R. dissimulans s.l.* that will be described as a separate species in a forthcoming publication) from central Jylland in Denmark. All of them are shortly discussed and described by figures in the Supporting information.

A few samples were originally interpreted to represent the *R. wahlbergii* × *caesius* hybrid. However, MSDA does not give any support to those suggestions. *R. wahlbergii* is pentaploid, so such hybrids would be expected to beheptaploids. No heptaploid sample has been found so far. Yet, considering

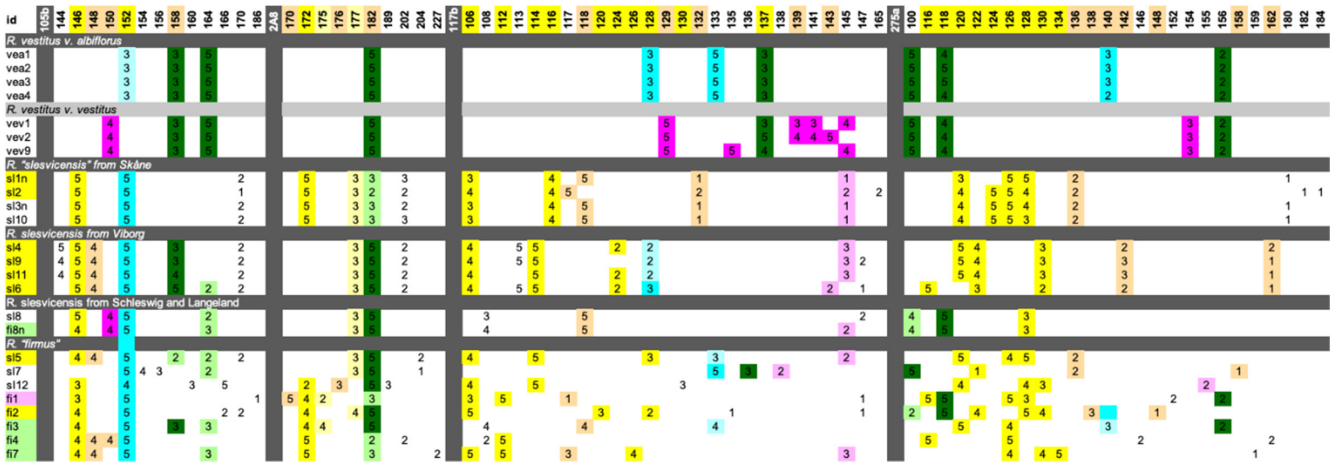


Figure 10. MSDA results for the two varieties of *R. vestitus* and the various putative *R. slesvicensis s.l.* and *R. firmus s.l.* The figure shows the various alleles observed in MSDA, together with the relative intensities of the PCR products (1–5). The lengths in bp are shown in the first row. Those shown in yellow are frequently observed for *R. caesius*, whereas those in orange are only found for a few samples. The first column shows an identifier of each sample, which is are coloured according to the ploidy level: light green – tetraploid, magenta – pentaploid, yellow – hexaploid and white – chromosome number not determined. The alleles are also colour coded: green – allele observed for both varieties of *R. vestitus*, magenta – allele common with *R. vestitus v. vestitus*, cyan – allele common with *R. vestitus v. albiflorus*. In all three cases, a lighter colour indicates a lowered intensity. Yellow, light yellow and white indicate alleles observed, frequently, less frequently and rarely for *R. caesius*.

that the *R. gothicus* × *caesius* hybrids sometimes come quite close to *R. wahlbergii* in the MSDA, some of these hybrids may have arisen from *R. wahlbergii* instead, after a loss of one set of chromosomes, resulting in a hexaploid and therefore lacking alleles.

One sample from Brunnby in northwest Skåne comes from a large and vigorous bush that is a hexaploid. In clustering of the MSDA data, it comes closest to *R. lagerbergii* Lindeb. This is somewhat unexpected because the latter species is pentaploid and the closest locality is ~15 km away, across the bay of Skälderviken. However, this interpretation is at least partly supported by the MSDA (Fig. 11). At the 275a locus, the four alleles of the putative hybrid are also found in *R. lagerbergii* and one has a slightly decreased intensity. One allele from *R. lagerbergii* is lacking, which is expected because the hybrid is hexaploid and *R. lagerbergii* is pentaploid, so one set of genes should be lost. At locus 2A8, two alleles from *R. lagerbergii* are lacking, but three samples of *R. lagerbergii* also lack one or two alleles. One allele has a decreased intensity. However, at 105b, three alleles from *R. lagerbergii* are lacking, which is harder to explain. There is also an extra allele, but at a length not observed in *R. caesius*. Unfortunately, the MSDA failed for locus 117b. Still, the similarity between the putative hybrid and *R. lagerbergii* is so conspicuous that we think the interpretation as *R. lagerbergii* × *caesius* is attractive.

One sample has been interpreted as *R. insularis* × *caesius*, because it grows together with *R. insularis* (belonging to *R.* sect. *Rubus*) and is strongly angiocarp. The MSDA supports this suggestion, as can be seen in Fig. 12, especially as it is found to be a tetraploid and therefore should have lost half of the alleles from *R. insularis*. In fact, Lidforss reported this hybrid from another place in southern Skåne (Lidforss 1899), but he also stated that he had failed to produce this hybrid by crossing experiments (Lidforss 1914). This is the only hybrid between *R. caesius* and a species in *Rubus* sect. *Rubus* confirmed in this investigation.

After these cytometry and MSDA investigations, only two samples remain for which we have not been able to reach any convincing interpretation. Both clearly show the morphology of *R. caesius* hybrids. One sample is a hexaploid from southeast Skåne, where it grows together with *R. armeniacus* Focke. It is large, vigorous and similar to *R. armeniacus*, so it seemed quite natural to interpret it as *R. armeniacus* × *caesius*. However, the MSDA does not give any support to such a suggestion. On the contrary, there is only minor similarity between this hybrid and *R. armeniacus* (0, 2, 2 and 1 overlapping alleles for the four loci; cf. Supporting information).

The other sample comes from Valinge in central Halland, with no other *Corylifolii* found in the neighbourhood. It is a tetraploid and therefore harder to interpret. In MSDA, it comes closest to *R. polybrachteatus* which occurs only in

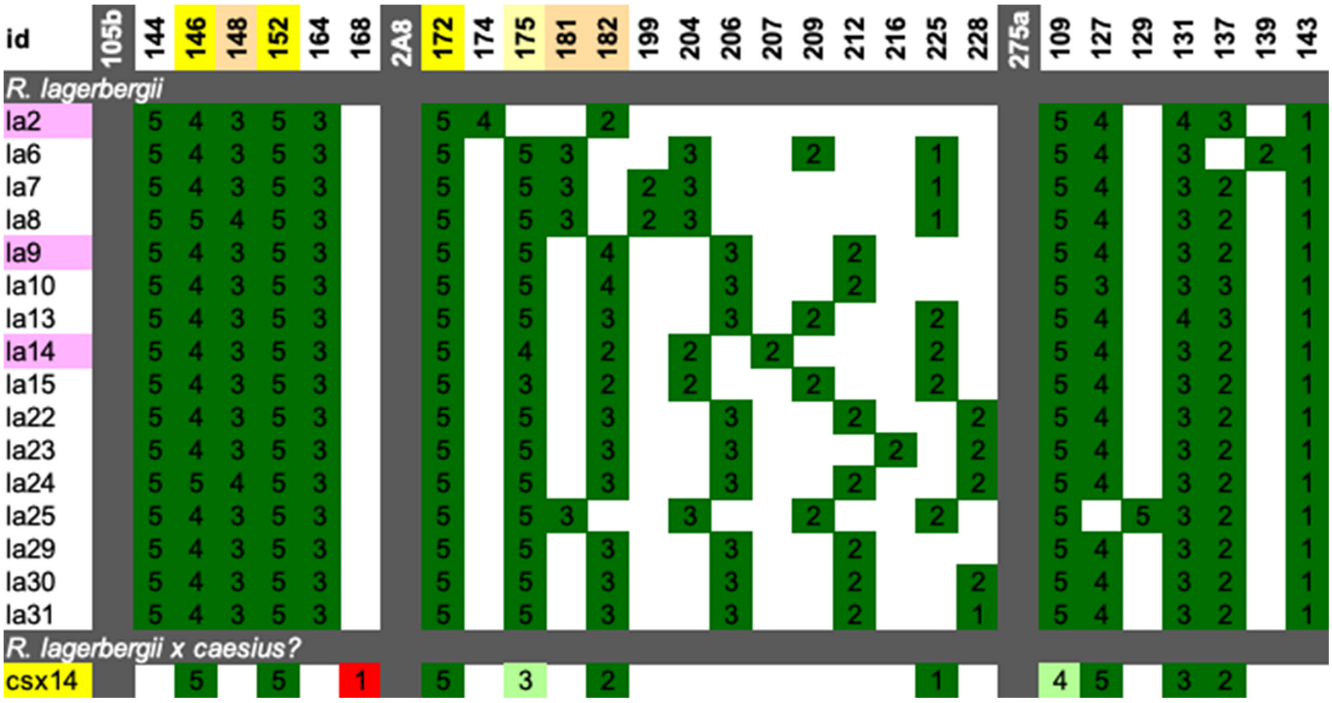


Figure 11. MSDA results for *R. lagerbergii* and the putative *R. lagerbergii* × *caesius* hybrid. The figure shows the various alleles observed in MSDA, together with the relative intensities of the PCR products (1–5). The lengths in bp are shown in the first row. Those shown in yellow are frequently observed for *R. caesius*, whereas those in orange are only found for a few samples. The first column shows a sample code, which is coloured according to the ploidy level: magenta – pentaploid, yellow – hexaploid and white – chromosome number not determined. The alleles are also colour coded: dark green – allele probably from *R. lagerbergii*, light green – allele probably from *R. lagerbergii*, but with a lowered intensity and red – allele probably from *R. caesius*. Unfortunately, the MSDA failed for the 117b locus.

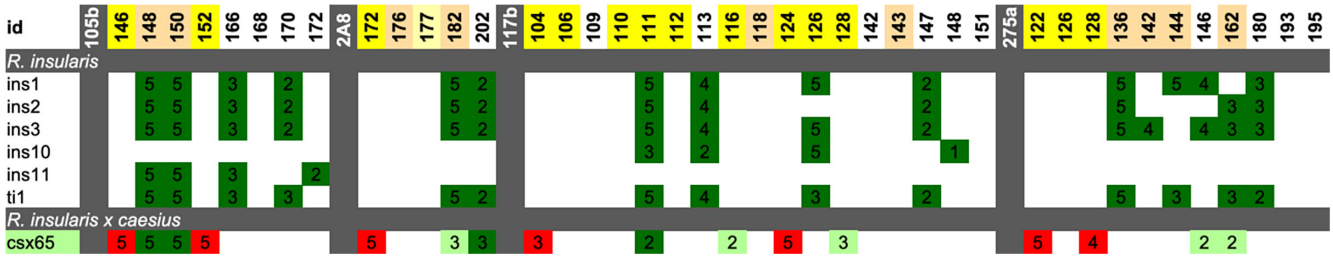


Figure 12. MSDA results for *R. insularis* and the putative *R. insularis* × *caesius* hybrid. The figure shows the various alleles observed in MSDA, together with the relative intensities of the PCR products (1–5). The lengths in bp are shown in the first row. Those shown in yellow are frequently observed for *R. caesius*, whereas those in orange are only found for a few samples. The first column shows a sample code, which is coloured according to the ploidy level: light green – tetraploid, magenta – pentaploid, yellow – hexaploid and white – chromosome number not determined. The alleles are also colour coded: dark green – allele probably from *R. insularis*, light green – allele probably from *R. insularis*, but with a lowered intensity and red – allele probably from *R. caesius*.

northern Halland, at a distance of ~35 km. At three of the loci, one or two alleles from *R. polybrachteatus* are lacking and at the fourth, the lengths of two of the alleles differ by 2–4 bp (Supporting information, csx26). At 105b and 117b, there are 2 or 1 extra alleles, but at the former locus, it is not of a length observed for *R. caesius*, making the interpretation as the *R. polybrachteatus* × *caesius* hybrid less attractive.

Conclusions

In this investigation, we have studied hybrids between dewberry and blackberries, primarily in Sweden, using cytometry and MSDA. We have obtained several results.

- In the MSDA, *R. caesius* shows a level of variation intermediate between that of *R. idaeus* (sexual) and the blackberries (apomictic), although the level of variation varies much between the four loci studied.
- *Rubus caesius* hybrids can typically be identified by morphological characters, ploidy level and MSDA. However, the interpretation of the latter is harder than for the *R. idaeus* hybrids (Ryde et al. 2021), because *R. caesius* has fewer alleles, and they often overlap with those of the blackberry parent (hybridisation may sometimes only be indicated by a change in the intensity pattern) and the hybrids almost always show some deviations from the expected pattern (all alleles from the *Corylifolii* parent and two extra alleles from *R. caesius*). This gives the impression that hybridisation with *R. caesius* gives rise to some irregularity of the genetic processes.
- We have not found any evidence that the hybrids spread apomictically, not even in areas where certain hybrids are conspicuously common.
- Our results indicate that what has been called *R. cyclomorphus* in Sweden is a collection of genetically unrelated primary hybrids (*R. raduloides* × *caesius* in Bohuslän, *R. fasciculatus* × *caesius* in Skåne). Likewise, what has been called *R. tiliaster* in Sweden are genetically unrelated *R. camptostachys* × *caesius* primary hybrids. Therefore, these two names should be avoided.
- *Rubus slesvicensis* and *R. firmus* are also problematic species. The MSDA identifies two or three distinct clusters and a collection of scattered samples, which probably are unrelated primary hybrids. One of the clusters is described here as *R. ruboculus* because it is a well-defined and uniform taxon, occurring in two disjunct areas in Sweden, 45 km apart, showing a clear tendency to spread in both areas. The second cluster comes from Viborg in Denmark and in Schleswig, but it is probably not identical to *R. slesvicensis s.s.* We find no support for *R. firmus* as a valid species in Sweden, Denmark or northern Germany. The material is morphologically and genetically inhomogeneous, and it is represented by three ploidy levels.
- *Rubus glauciformis* is a distinct species in eastern Sweden. However, in the province of Skåne, the material is harder to interpret and some samples are difficult to identify by morphology, MSDA or ploidy level.
- Most *Corylifolii* species can form hybrids with *R. caesius* and such hybrids are quite frequent in areas where *R. caesius* is common. We have identified hybrids with 12 *Corylifolii* species and with *R. insularis* from section *Rubus*. We have not found convincing evidence for hybrids with the pentaploid *R. wahlbergii*, but one sample may represent the hybrid with the pentaploid *R. lagerbergii*.
- Our analysis supports the suggestion that all *Corylifolii* species have *R. caesius* as an ancestor, indicated by a few invariant alleles common with *R. caesius*. However, the MSDA has not allowed us to identify any likely *R.* sect. *Corylifolii* or *Rubus* parents to the Swedish *Corylifolii* species. The reason for this is probably that the hybrids are somewhat genetically unstable, possibly due to automixis.
- We have not found any clear evidence that *R. caesius* has been the maternal parent to any of the hybrids, and therefore no case in which it has contributed four sets of chromosomes and the *Corylifolii* parent only two. Our data thus agree with previous hybridisation studies where such hybrids have not been possible to produce (Lidforss 1914, Dowrick 1966). In contrast to these data, it has been observed that many *Corylifolii* species share a plastid haplotype with *R. caesius* (Sochor et al. 2015).

Artificial hybridisation studies by Lidforss (1899, 1901, 1907, 1914) indicated that primary hybrids are fertile and that in the second and later generations, a very large variation in morphology is observed, including characters not observed in any of the parents. In nature, no such extensive variation has been observed. These observations indicate that the later generations have low viability. This conclusion is in line with the observation by Lidforss that seedlings of the hybrids were conspicuously weaker than those of the pure species (Lidforss 1907).

Undoubtedly, *R. caesius* hybrids have been a major source of confusion and taxonomical problems among the Swedish *Corylifolii*. The hybrids are common and conspicuous, and have therefore often led to suggestions of new taxa. For example, the cultivation studies of Lidforss showed that *R. progenerans* (Lidf.) Lidf., *R. nemoralis* P.J.Müll *v. rudensis*, *R. maximus v. silvestris*, *R. maximus v. rotundifolius* and *R. acutus* are inconstant and therefore likely hybrids (Lidforss 1905). Here, we add *R. cyclomorphus*, *R. tiliaster*, *R. slesvicensis* and *R. firmus* to this list. If *Corylifolii* hybrids with either *R. caesius* or *R. idaeus* are properly recognized as hybrids, very few taxonomical problems remain in Sweden among the *Corylifolii*, and essentially every stand can be identified (although the *Corylifolii* parent of the hybrids is not always obvious), once a few species with local distributions are discerned (Ryde 2011, Ryde et al. 2021). Currently, we know only a few stands in Sweden with unknown taxa. Two of them are most likely adventitious and probably occur in other areas in Europe. Others give the impression of being locally arisen new taxa, including the one from Kläppa in Halland, mentioned above and one from Ransvik in Skåne (*R. eluxatus* Neum. *v. ransvikensis* Torg. Hj.Hyl. (Hylander 1958b)). However, as long as they do not show any tendency to spread, we see no reason to give them formal scientific names.

Thus, by a proper recognition of *R. caesius* and *R. idaeus* hybrids, essentially all taxonomical problems within *Rubus* can be solved in Sweden. It is our hope that this and our former investigation on *R. idaeus* hybrids (Ryde et al. 2021) will inspire similar studies in other parts of the world and that it may solve some of the taxonomical problems for the genus *Rubus* there also.

Rubus ruboculus Ryde sp. nov. (Fig. 8)

Type: Sweden, Skåne, Räng: Ljunghusen, east of Stjärndalavägen 18, RT90 6145814 1320545 (WGS84 55.409245, 12.971723), leg. U. Ryde. 20 Jun 2022 (holotype: LD, isotypes: GB, S, UPS).

Stem procumbent, mainly rounded, 4–6 mm in diameter, light red, with pruinose patches, sparsely hairy and with occasional–few stipitate glands. Internodes 8–15 cm. Prickles recurved, with the same colour as the stem, 10–40 per 5 cm, 4.5–6.5 mm long, with a base of 2.5–4.0 mm, often interspersed with smaller prickles. Stipules 2.5–4.0 mm wide. Leaves 17–25 cm long, mostly 5-foliolate, but occasionally with 3–4 leaflets, yellowish green, thin. Petiole 6–11 cm, with 8–16 prickles, 2.7–3.4 mm long recurved or with a slightly curved tip. Terminal leaflets narrowly ovate with a rounded or slightly cordate base, 8.0–12.0 × 6.5–9.5 cm, widest at the base, sparsely hairy at both sides (mainly on the veins on the green lower side). Petiolule of terminal leaflet 1.2–3.5 cm, with 3–7 prickles. Leaf margin biserrate and periodic with teeth of unequal size, ~2 mm. Tip of the terminal leaflet acuminate, 1.3–2.3 cm long. Lateral leaflets 8–11 cm long with a petiolule of 2–8 mm. Basal leaflets 4.5–7.5 cm long with a petiolule of 0–1 mm. Inflorescence typically rather short and sparse, with a densely hairy rachis and many stalked glands. Prickles in the inflorescence straight to recurved and 4–6 mm long, 5–10 per 5 cm. Pedicels 10–16 mm with 2–5 almost straight prickles of length 2–3 mm. Upper leaves 2–5, mostly three-lobed, green to slightly grey beneath. Flowers 2.1–3.5 cm wide. Petals white, but slightly pink in bud. Filaments slightly pink and styles conspicuously pink to almost red. Sepals green, densely hairy, with white borders, many stipitate glands and several prickles, with long tips, patent or partly embracing the fruit. Receptacle sparsely hairy. Carpels mainly glabrous. Stamens exceeding the styles in length; anthers glabrous. Fruits aborted or with 1–3 drupelets. The species is illustrated in Fig. 8 and in the Supporting information.

In short, the species is characteristic with its large, almost white flowers with conspicuously red styles (no other *Rubus* species in Sweden have such flowers). Moreover, the prickles are many, long and almost straight on all axes (notably on the petiole and the pedicels). The stem is more rounded and hairier than in most other *Corylifolii* species. The stipules are wide and the upper leaves are green below. The sepals partly embrace the fruits and have plenty of glands and several prickles. The fruits are aborted or have only 1–3 drupelets. The scientific epithet alludes to the flowers with white petals and brightly red styles.

The species has been recorded from two areas in Skåne in South Sweden, 45 km apart: in Västra Karaby on a forest edge and along a road, and from Ljunghusen in Räng on several nearby localities on roadsides and paths in a residential area. The DNA-ploidy level is hexaploid (2n = 42).

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Investigation (supporting); Writing – review and editing (supporting). **Tomas Burén**: Formal analysis (supporting); Investigation (supporting); Writing – review and editing (supporting). **Mikael Hedrén**: Data curation (lead); Formal analysis (equal); Investigation (supporting); Methodology (lead); Resources (lead); Software (lead); Supervision (lead); Validation (supporting); Writing – review and editing (supporting). **Ulf Ryde**: Conceptualization (lead); Data curation (lead); Formal analysis (lead); Funding acquisition (lead); Investigation (supporting); Methodology (supporting); Project administration (lead); Supervision (supporting); Visualization (lead); Writing – original draft (lead); Writing – review and editing (lead).

Data availability statement

Data are available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.6q573n62f> (Lewin et al. 2022).

Supporting information

The Supporting information associated with this article is available with the online version.

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