

Patterns of repeated hybridization between dewberry, *Rubus caesius* (Rosaceae), and blackberries within *Rubus* sect. *Corylifolii*

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Abstract

We have studied the genetic relations among blackberries, *Rubus* sect. *Corylifolii*, and dewberries, *R. caesius*, by comparing presumed hybrids with their parental taxa. To be able to determine the status of the hybrids we have observed absence or presence of nuclear microsatellite alleles in electrophoresis, and calculated the different relative genetic distances between the relevant taxa. Interpreting the results has allowed us to get a better view the taxonomical relations among the studied taxa.

In this study *R. cyclomorphus* comes out as a hybrid between *R. caesius* and *R. norvegicus*. Similarly, *R. tiliaster* appears to be a hybrid between *R. caesius* and *R. camptostachys*. We also believe to have found individuals of *R. fasciculatus* x *R. caesius* and *R. gothicus* x *R. caesius*.

We also studied the genetic structuring of *R. caesius* population. Samples taken from as close as 10 meters apart from each other mostly belonged to different genotypes, showing sexual propagation to be common, and possibly also a high degree of intermixing and long persistence of clones in this species.

Introduction

Background

The genus *Rubus* (Rosaceae) presents us with great taxonomical complexity (Gustafsson 1942). The section *Corylifolii*, blackberries, seems to have emerged from repeated crosses between species in sect. *Rubus*, blackberries, and *R. caesius*, dewberry, within sect. *Caesii*. Additionally, members of *Corylifolii* and *R. caesius* can also produce hybrids as a result of secondary hybridization events. Except for a few sexual species in sect. *Rubus*, *R. caesius* and the mentioned taxa are pseudogamous agamosperms. Most of *R. caesius* and sect. *Rubus* agamosperms are tetraploid. Inter-crosses and back-crosses can produce local *Rubus* communities. Interspecific crosses seem to trigger the sexual reproduction complex in hybrids so that they reproduce sexually (Gustafsson 1942). Sexual hybrids then stabilize into agamospermus lines of hybrid clones when recessive apomixis becomes fixed in the successful segregate lines as new apomictic recombination variants. Moreover, Lindfors (1914; cited in Gustafsson 1942) also reported that recombination can occur in unreduced cells as a result of auto-segregation in unreduced egg-cells. Most of the apomicts are tetraploid, and none is diploid. *Rubus caesius* is believed to have an allopolyploid origin (Gustafsson 1942).

In 1942, Gustafsson had not yet observed that *R. caesius* could serve as maternal parent to hybrids in crossing experiments. However, in a recent study from 2015 (Sochor et. al.), plastid genomes from both *R. caesius* and diploid *Rubus* sect. *Rubus* chloroplasts were found in apomictic species. This implies that both these taxa can act as maternal species in hybridization events, since we assume the plastid genome maternally inherited in *Rubus*.

Gustafsson (1933) argued that even with only 3000 taxa worldwide within *Rubus*, local variants in smaller areas would not be feasible to be recognized as formal taxa, as they often show superficial similarity to variants in other areas. This brings importance to genetic studies of *Rubus*, which then better could provide knowledge of the number of genuine species.

Aim

This study tries to resolve some of the complexity resulting from hybridization between members of *Rubus* sect. *Corylifolii* and *R. caesius* (Fig. 1) in Sweden. For several populations of hybridogenous origins, we wanted to establish whether a putative taxon should be regarded as a species, i.e. a genetically and morphologically uniform group of individuals with a common origin that forms an independent distribution, or a group of genetically unrelated primary hybrids that have arisen independently on repeated occasions. Specifically, we wanted to decide whether *R. cyclomorphus* (Fig. 1), is a proper species or a collection of unrelated, but similar, hybrids between *R. caesius* and *R. norvegicus* (Fig. 1). Likewise we wanted to settle whether *R. tiliaster* (Fig. 1), is a uniform species or a collection of *R. camptostachys* (Fig. 1) x *R. caesius* hybrids. Conversely, we also wanted to study whether the *R. caesius* x *fasciculatus* (Fig. 1) hybrid, which is unusually frequent in northern Halland, and the *R. caesius* x *gothicus* (Fig. 1) hybrid, which has many localities in NW Skåne, have formed a locally spreading species, or if these hybrids are genetically unrelated in these areas.

In addition, we also wanted to address some related questions, such as: Is *R. glauciformis* (Fig. 1) a proper species or a collection of *R. Corylifolii* x *R. caesius* hybrids? Which sect. *Corylifolii* species are involved in this species or in its hybrids? Which sect. *Corylifolii* parent is involved in other putative *R. caesius* hybrids and which species of *R. sect. Rubus* has been involved in the formation of the various Sect. *Corylifolii* species? We also would like to test the suggestion of Hylander (1958) that there are two different species in the *R. fasciculatus* complex in Sweden, which he separated as *R. fasciculatus* and *R. ambifarius*, respectively. However, the latter is what we today call *R. fasciculatus*; accordingly we will treat them as *R. fasciculatus* sensu Hylander and sensu Weber, respectively, in the following. Finally, we would also like to estimate the degree of sexual reproduction in *R. caesius*. Our hypothesis is that if outcrossing is predominant, a single population should contain several genetically different individuals, not correlating in geographical distance among genets.

Molecular differentiation patterns were examined by using nuclear microsatellites with dinucleotide repeat regions. The four loci 117B, 105B (Graham et al., 2004), 2A8 (Ansellem et al., 2001) and 275A (Graham et al., 2004) were chosen for analysis.

In the formation of hybrids between *R. caesius* and members of sect. *Corylifolii*, we assume that a reduced pollen from *R. caesius* has fertilized an unreduced sect. *Corylifolii* egg cell, since we assume that *R. caesius* has a stable number of gamete chromosomes. This would result in hybrids with four blackberry genomes and two dewberry genomes in accordance with the observation that most hybrids are hexaploids.



Fig. 1. Upper side of leaves from the studied species. Top row from the left: *R. caesius*, *R. norvegicus*, *R. cyclomorphus*, and *R. camptostachys*. Bottom row from the left: *R. tiliaster*, *R. fasciculatus*, *R. gothicus*, and *R. glauciformis* (Ryde 2005 A-H)

Method

Sampling

Most samples were already collected when the study was initiated. Additional *R. caesius* samples were collected at sites where other taxa, and hybrids between other taxa and *R. caesius* had been collected. *Rubus caesius* was also sampled at a locality with a continuous distribution of plants, where 36 samples were collected along a transect of 518 meters. To avoid resampling the same genet, sampling sites were at least ten meters apart. At the other locations there was no continuous distribution of *R. caesius* in the vegetation and the samples were collected as they could be achieved. Samples were collected as young leaves and shoots and were stored in a freezer in -80°C prior to DNA extraction.

Extraction

Samples were extracted by grinding a piece of a leaf in preheated mortars by aid of sand and preheated CTAB extraction buffer (Doyle & Doyle 1990) at ca 70°C .

The homogenate was poured into 2 ml Eppendorf tubes, filling 1/2 to 2/3 of the tubes. The tubes were then incubated at 70°C for minimum of half an hour, while occasionally turning them gently. Then, the tubes were filled with SEVAG [24:1

chloroform:isoamyl alcohol] and were then agitated lying on their side for 20 minutes on a tilting table. Next, the tubes were centrifuged for ten minutes at 10 000 rpm, after which 0.6 ml of the upper phase was removed and the remainder was transferred to clean 1.5 ml Eppendorf tubes. DNA was precipitated by adding two thirds of 95% ethanol at -20°C , after which the samples were put into a freezer for a minimum 16 hours.

After DNA had been collected by centrifuging the tubes for ten minutes at 13 000 rpm the ethanol was poured off, 1 ml wash buffer [0.5 M NaAc in 70% ethanol] was added and the tubes was put in the freezer for a minimum of 20 minutes. Then, the tubes were centrifuged at 13000 rpm for one minute and the wash buffer was poured off and was replaced by 1 ml 70% ethanol. The 70% ethanol was poured off after centrifuging for one minute at 13 000 rpm and was replaced by 1 ml 95% ethanol. After centrifuging and pouring off the ethanol, the tubes were left to air-dry. The remaining dried pellet was resuspended in 100 or 150 μl $1\times$ TE, depending on the size of the pellets.

$1\times$ TE:

10 mM Tris-HCl, pH 8.0

0.11 mM Na₄EDTA

Amplification of microsatellite markers

The samples were diluted to a concentration of about 14 ng DNA per μl water. 5.8-5.6 μl PCR mix were added to 0.6 μl DNA solution.

PCR mix for 96 samples:

440 μl ddH₂O

66 μl Taq buffert with MgCl₂

56 μl dNTPs

25 μl Cy5 primer

10 μl complementary primer

3.0 μl Taq polymerase

The PCR were run in 35 cycles with an annealing temperature of 59°C for primer 117B and 52°C for the other three primers. The samples were coloured with 8-5 μl dye solution containing one or two known size fragments, and run on acrylamide gels. The fragment lengths were registered, measured and recalculated digitally. On all gels we used two slots with appropriate size markers and we also included two *R. Corylifolii* samples as nucleotide pair size references.

Processing data

The registered data were synchronized and amalgamated with already existing datasets (Mikael Hedrén and Ulf Ryde unpublished data) compiled in other projects to create matrices for testing the various hypotheses presented in the introduction. Based on presence or absence data for alleles identified in individual samples, pairwise comparisons between all pairs of samples were calculated as Jaccard coefficients (Jaccard 1908). Resulting similarity matrices were subjected to Principal Coordinates

Analysis, and differentiation patterns given by the first two principle coordinates were inspected and interpreted in a Principal Coordinate Orientation (PCO). Based on the same similarity matrices, minimum spanning trees (MST) and neighbor-joining trees (NJ) were also calculated for some of the subprojects. Calculations were performed in NTSYSpc v.2.1q (Rohlf 1994).

The material selected for this study included the following species and hybrid groups with their respective numbers of samples: *R. caesius* [n 47], *R. norvegicus* [n 12], *R. cyclomorphus* [n 18], *R. camptostachys* [n 6], *R. tiliaster* [n 7], *R. fasciculatus* [n 14], potential hybrids between *R. fasciculatus* and other species [n 15], *R. gothicus* [n 21], potential hybrids between *R. gothicus* and other species [n 7] and *R. glauciformis* [n 26].

The individuals from the *R. caesius* transect were evaluated with respect to the geographical and genetic distances by means of a Mantel test running 10 000 random permutations.

We also calculated average pairwise differences between the individuals within certain taxa (Nei & Li 1979) in order to obtain absolute measures of genetic diversity within taxa based on allele presence/absence data. These estimates were obtained using the computer program Arlequin ver. 3.5.1.2 (Excoffier et al. 2005). We chose to add a reference for the genetic diversity values of a sexual diploid [*R. idaeus*, n=24], a representative of sect. *Corylifolii* [*R. eluxatus*, n=13], their stabilized hybrid [*R. cordatiformis*, n=5] and their spontaneous hybrid [n=2].

Results

Rubus cyclomorphus

When performing a PCO for all samples of *R. caesius*, *norvegicus*, and *cyclomorphus* and plotting the two first coordinates in a diagram, the *R. cyclomorphus* samples are located in between the samples of *R. caesius* and *R. norvegicus*, as shown in Fig. 2.

In the corresponding neighbor joining tree, shown in Fig. 3, most of the *R. cyclomorphus* samples are found in between *R. norvegicus* and *R. caesius*. However a few samples are found within the *R. norvegicus* cluster, two samples are found in the upper part of the *R. caesius* cluster and two samples are found together with one *R. caesius* sample in a separate cluster, essentially unrelated to the other samples. The latter may indicate that these three samples are not *R. norvegicus* x *caesius* hybrids, but instead involve another *Corylifolii* parent. The samples given as *R. cyclomorphus* samples are very diverse, both morphologically and molecularly, and we interpret them to be too diverse to be recognized as a distinct species. Instead it seems to be a collection of independently arisen hybrids, and should be regarded as such. The genetic distance to both parental taxa indicates that we have found the true parental taxa of *R. cyclomorphus*.

The genetic diversity is greater in *R. cyclomorphus* than in the parental taxa, as shown in Fig. 7, again supporting the interpretation of *R. cyclomorphus* as a collection of independently derived hybrids.

Table 1. Distribution of alleles present in all *R. cyclomorphus* individuals among putative parental taxa.

Locus	<i>R. caesius</i>	<i>Corylifolii</i>	Both	Not found
117B	9	2	3	2
105B	5	-	3	1
2A8	6	2	3	3
275A	4	1	6	2

In Table 1, we report the total number of observed bands identified in *R. cyclomorphus* and their distribution in its parental taxa. At locus 117B two alleles present in both parental taxa were infrequent in *R. caesius* and common in *R. norvegicus*. The two alleles that were not found in the parental taxa differed by less than one dinucleotide repeat from a *R. norvegicus* band. At locus 105B, one allele was uncommon in *R. caesius* and very common in *R. norvegicus*. Similar to the situation at locus 117B, a common allele in *R. cyclomorphus* that was not found in the parental taxa differed by just one repeat from an allele present in all *R. norvegicus*. At locus 2A8, two alleles were uncommon in *R. caesius* and common in *R. norvegicus*. At locus 275, two alleles were uncommon in *R. caesius* and common in *R. norvegicus*. Moreover, the allele restricted to *R. norvegicus* was only a weak band.

Fig. 4 compares a sexual species, a *Rubus* sect. *Corylifolii*, their stabilized hybrid and their spontaneous hybrids. The sexual species have the highest diversity and the stabilized hybrid has a higher genetic diversity than its *Corylifolii* parent. The primary hybrids, comprising of only two samples do not express much diversity.

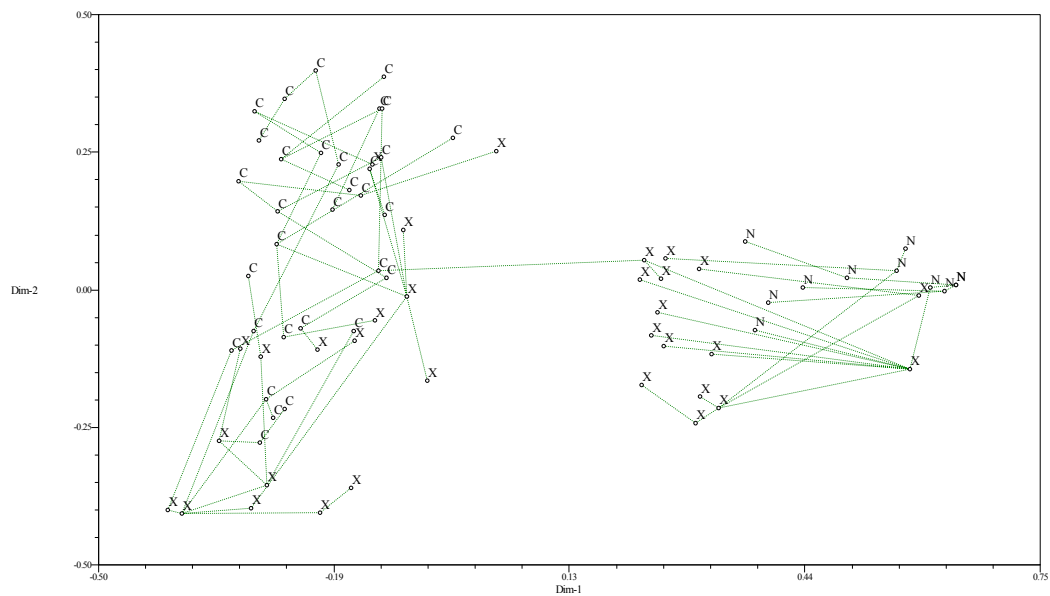
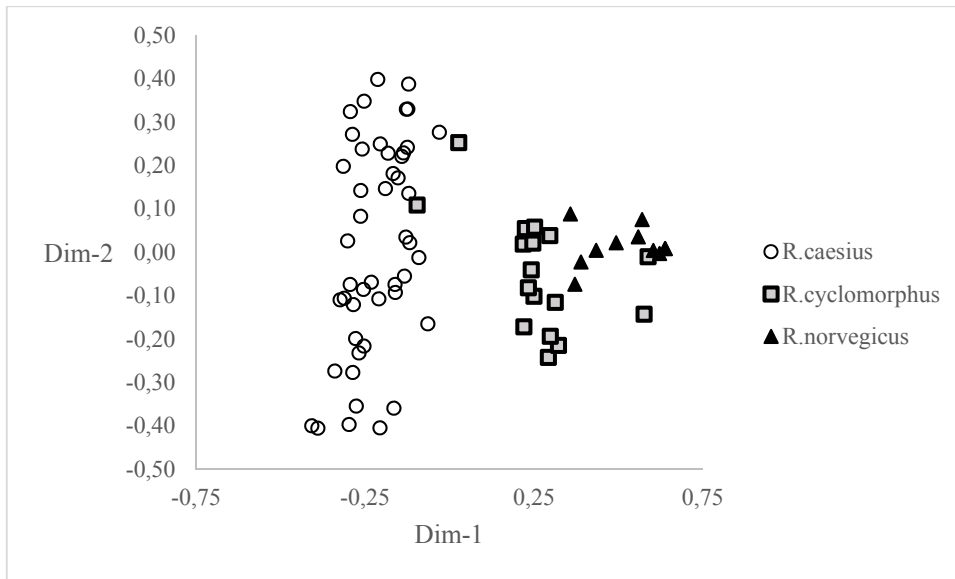


Fig. 2. PCO on genetic distances between samples of *R. caesius*, *R. cyclomorphus* and *R. norvegicus*. a) Positions of *R. caesius*, in circles, *R. cyclomorphus* in squares, and *R. norvegicus* in triangles. b) The same PCO superimposed by a minimum spanning tree. N=75

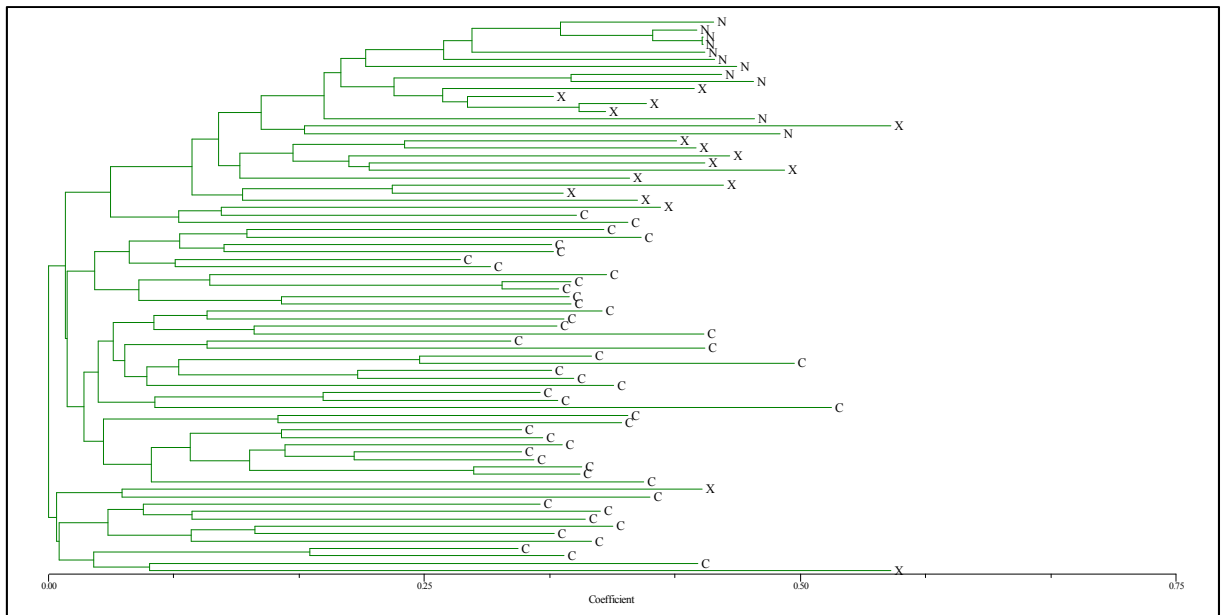


Fig. 3. Neighbor joining tree of 75 samples of *R. caesius* (C), *R. cyclomorphus* (X) and *R. norvegicus* (N). *R. cyclomorphus* samples group with each other or with *R. norvegicus*.

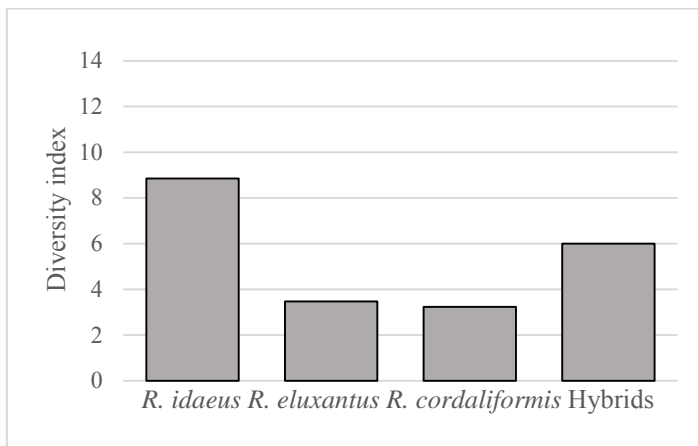


Fig. 4. Total diversity values *R. idaeus*, *R. eluxatus*, *R. cordatiformis*, and two primary hybrids.

Rubus tiliaster

Also *R. tiliaster* seems to have arisen independently several times, and appears to have originated from hybridization between *R. caesius* and *R. camptostachys*. As shown in Fig. 5, *R. camptostachys* is a genetically well-characterized and composed of samples of very similar allele composition [n=6].

The patterns shown in Fig. 6 indicates that three samples of *R. tiliaster* might be of multiple independent origins, from *R. caesius* and another member of sect. *Corylifolii*. The remaining individuals group with *R. camptostachys*. The genetic diversity is greater in *R. tiliaster* than in *R. camptostachys* and *R. caesius*, as shown in Fig. 7.

Table 2. Distribution of alleles present in all *R. tiliaster* individuals among putative parental taxa.

Locus	<i>R. caesius</i>	<i>Corylifolii</i>	Both	Not found
117B	9	3	3	2
105B	3	2	2	3
2A8	2	1	3	2
275A	6	1	3	3

At locus 117B, two of the alleles were less than a dinucleotide from alleles present in *R. camptostachys*.

At locus 2A8, two of the *R. tiliaster* alleles found also in both parental taxa were uncommon in *R. caesius*, but present in all *R. camptostachys*. Moreover, one allele that was present in all the hybrids was also present in all *R. camptostachys*. The two alleles absent from the parental taxa were less than one dinucleotide off from any of the two *R. caesius* alleles. At locus 275A, two alleles found in both parents were uncommon in *R. caesius*, but present in all *R. camptostachys*.

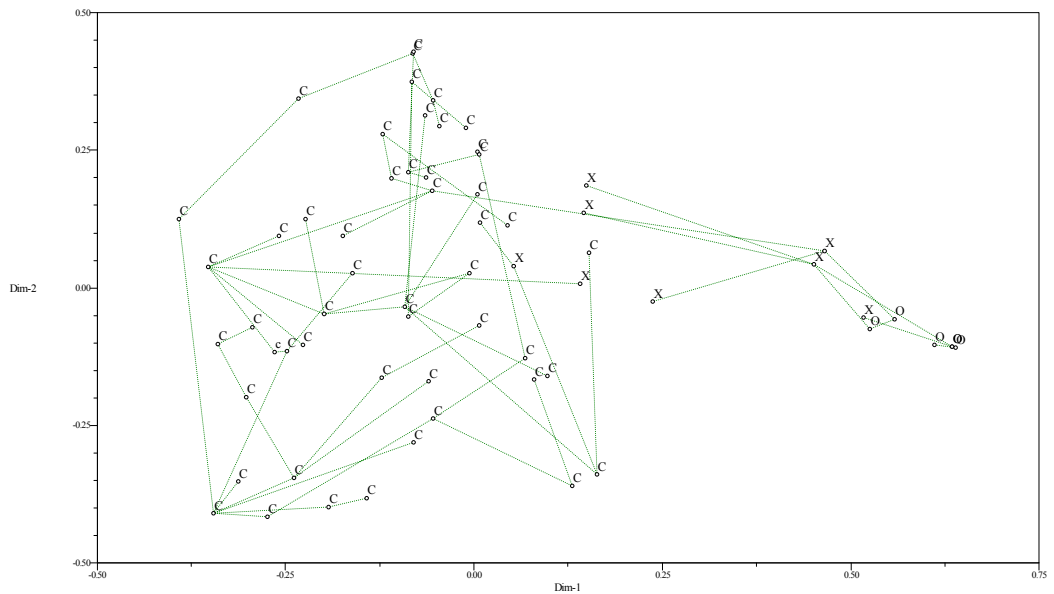
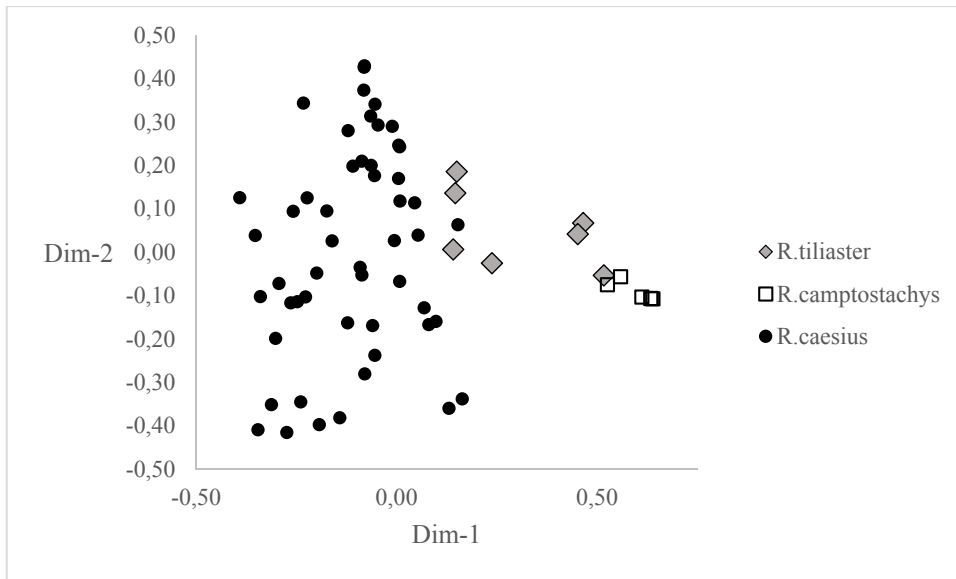


Fig. 5 a) PCO genetic distance between *R. tiliaster*, in diamonds, *R. camptostachys*, in squares, and *R. caesius* in circles. b) The same PCO superimposed by a minimum spanning tree of the same individuals. N=65

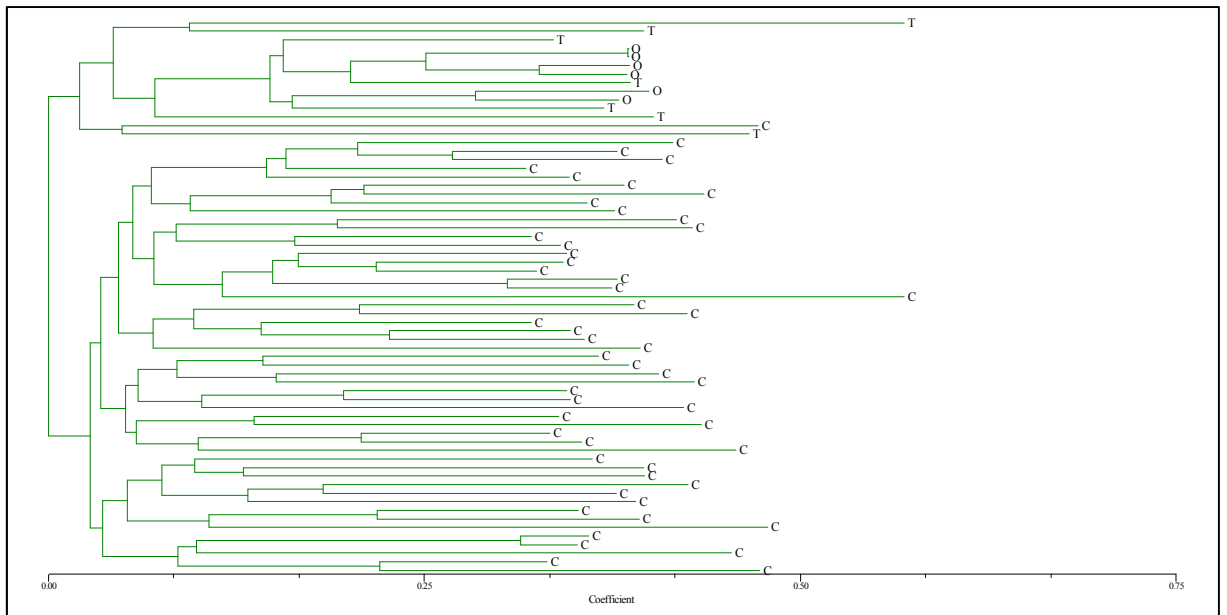


Fig. 6. Neighbor joining tree including samples of *R. tiliaster* (T), *R. camptostachys* (O) and *R. caesius* (C). N=65

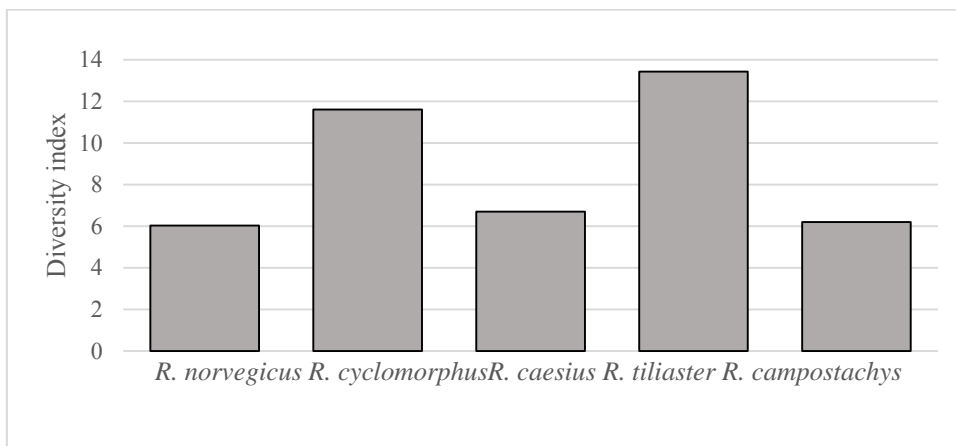


Fig. 7. Genetic diversity, given as mean number of pair-wise differences between individuals, of *R. cyclomorphus* and *R. tiliaster* and their putative taxa.

Rubus fasciculatus

When examining the genetic distances within *R. fasciculatus*, it became evident that the taxon actually consists of two species. In Fig. 8, one can see how the two clusters are more genetically similar to each other than to other individuals. The smaller cluster is *R. fasciculatus* sensu Hylander and the larger, clustering with the hybrids, is *R. fasciculatus* sensu Weber. In Fig. 11 the genetic diversity index of *R. fasciculatus* and the hybrids is shown.

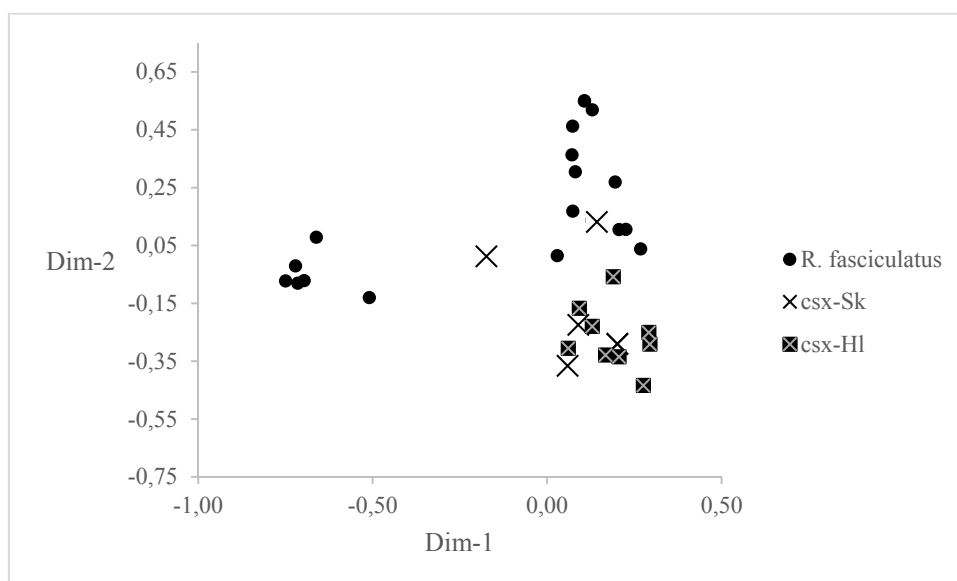


Fig. 8. PCO on genetic distance within *R. fasciculatus*, in circles, and compared to *R. caesius* x *fasciculatus* hybrids From Skåne and Halland, as crosses. N=35

When comparing *R. fasciculatus* sensu Weber and the hybrids with *R. caesius* we can see that the hybrids have arisen independently multiple times, as shown in Fig. 9. Fig. 10 shows that most of the hybrids cluster close to *R. fasciculatus* sensu Weber.

Table 3. Distribution of alleles present in all *R. caesius* x *fasciculatus* individuals among putative parental taxa.

Locus	<i>R. caesius</i>	<i>Corylifolii</i>	Both	Not found
117B	5	-	4	-
105B	-	1	4	-
2A8	-	-	2	1
275A	7	1	3	1

At locus 105B, one of the four alleles present in both parental taxa were found in one *R. caesius* individual, but was common in *R. fasciculatus*. At locus 2A8 the single allele not matching any parental alleles was displaced by just once dinucleotide repeat from a common allele present in both parental taxa. At locus 275A, the single allele not matching the parental alleles was closely similar to two parental alleles.

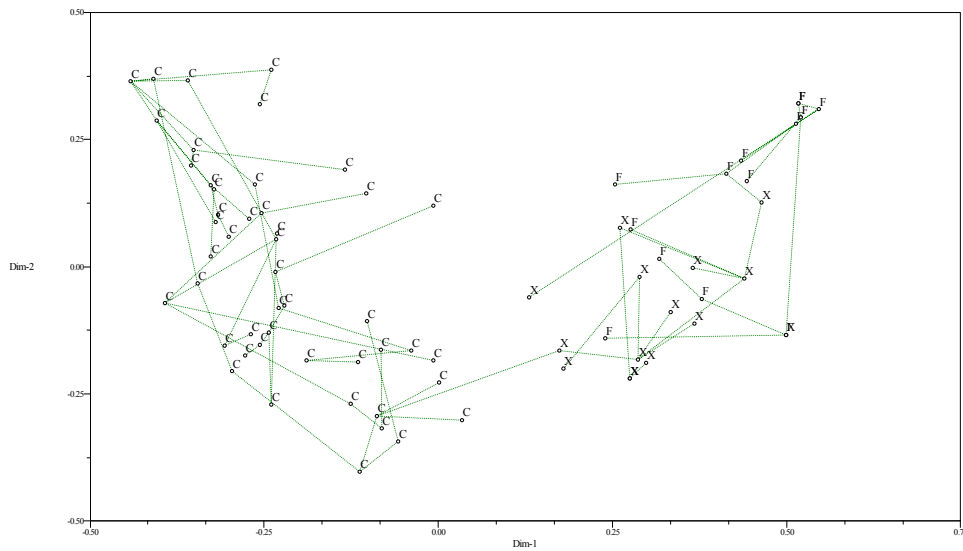
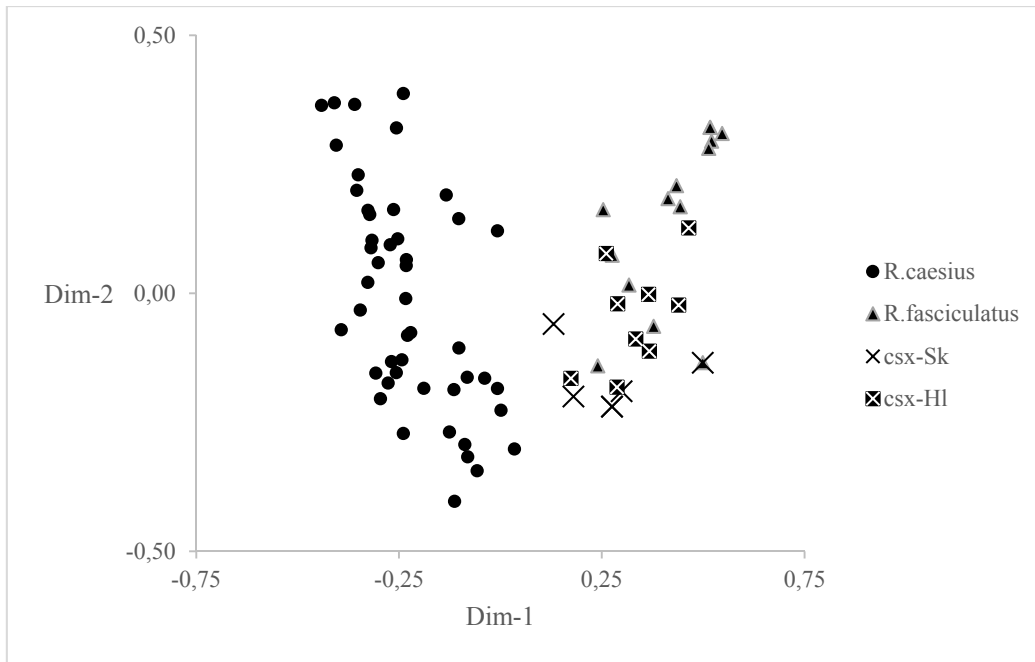


Fig. 9 a) PCO on genetic similarity between samples of *R. caesius*, in circles, hybrids from Skåne, in black crosses, hybrids from Halland, in white crosses, and *R. fasciculatus* in triangles. b) The same PCO superimposed by a minimum spanning tree of the same individuals. N=76

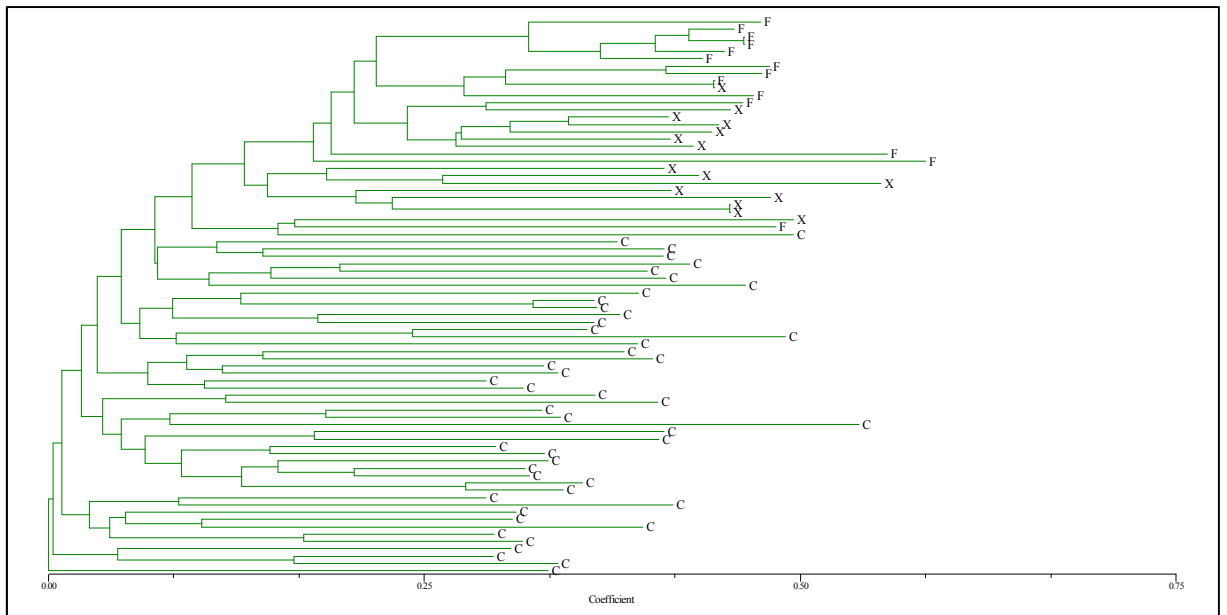


Fig. 10. Neighbor joining tree of *R. fasciculatus* (F), *R. caesius* (C), and their hybrids from Skåne and Halland (X). N=76

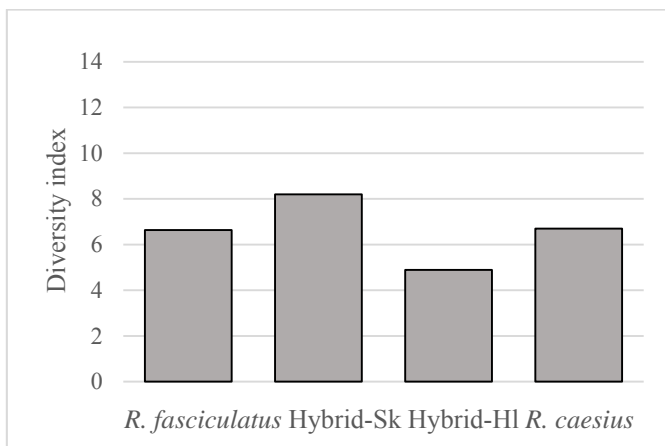


Fig. 11. Genetic diversity, given as mean number of pair-wise differences between individuals, of *R. fasciculatus*, *R. caesius*, and their hybrids from Skåne- and Halland.

Rubus gothicus

By using microsatellite markers, we can confirm that several samples analyzed are *R. caesius* x *gothicus* hybrids. It seems that the hybrids have arisen independently at least four times [n=7]. However, Fig. 12 indicates that four of the hybrids may involve another *Corylifolii* parent than *R. gothicus*.

Table 4. Distribution of all alleles present in all *R. caesius* x *gothicus* individuals among putative parental taxa.

Locus	<i>R. caesius</i>	<i>Corylifolii</i>	Both	Not found
117B	6	-	4	-
105B	2	-	4	1
2A8	2	-	3	-
275A	4	1	6	1

At locus 105B, two of the four alleles found in both parental taxa were present in most of the hybrids and were uncommon in *R. caesius* but present in all the *R. gothicus* samples, while the other two alleles were very common in *R. caesius* and present in all *R. gothicus*, but only found in one hybrid individual.

At locus 2A8, two of the alleles present in the parental taxa were present in all the hybrid individuals, uncommon in *R. caesius* and present in all of the *R. gothicus* samples. The third allele at this locus was frequent in *R. caesius* and always present in *R. gothicus*.

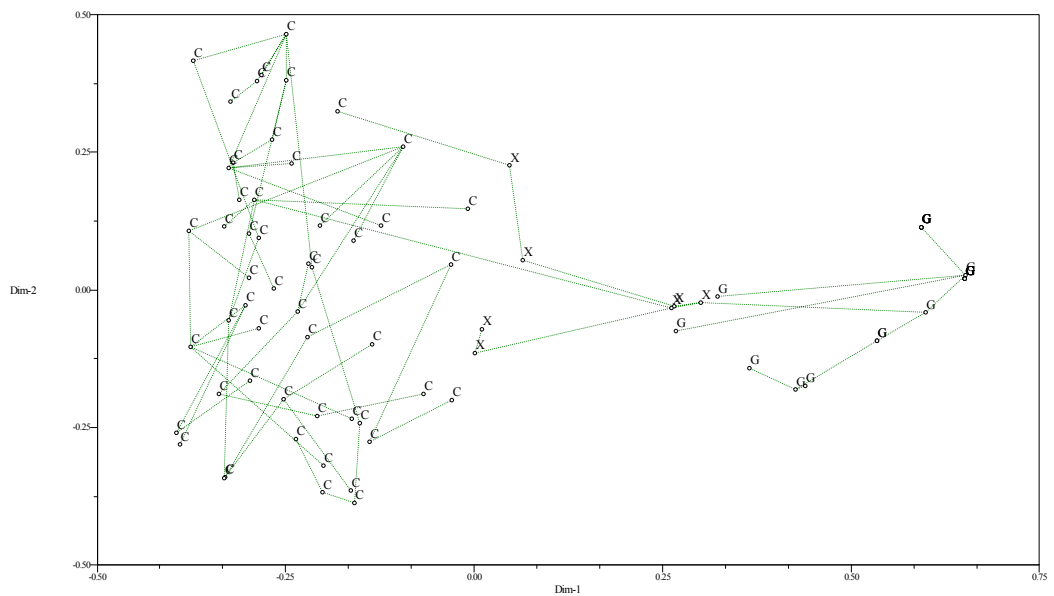
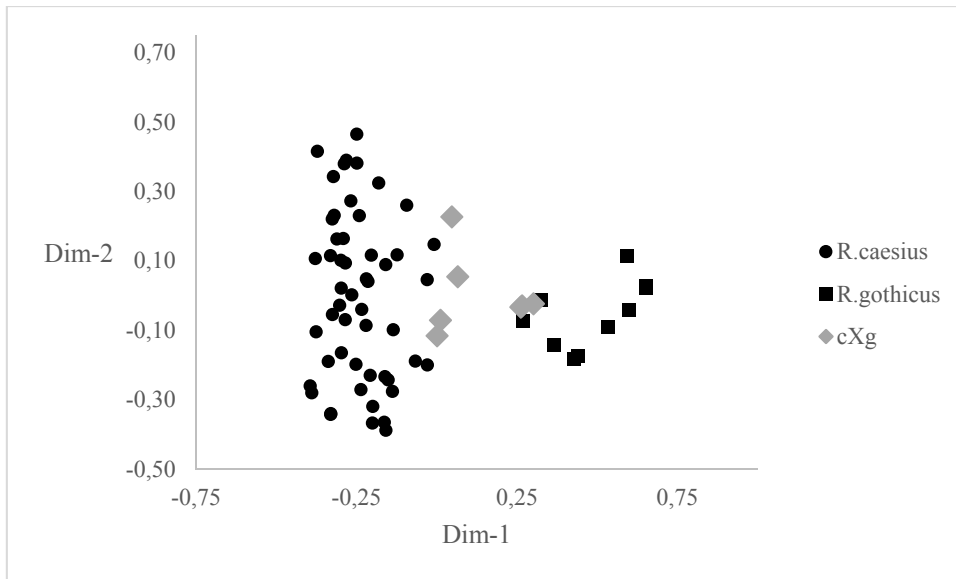


Fig. 12 a PCO on genetic distances between samples of *R. caesius* in circles, *R. gothicus* in squares, and their hybrids in diamonds. 5b. The same PCO superimposed by a minimum spanning tree. N=80

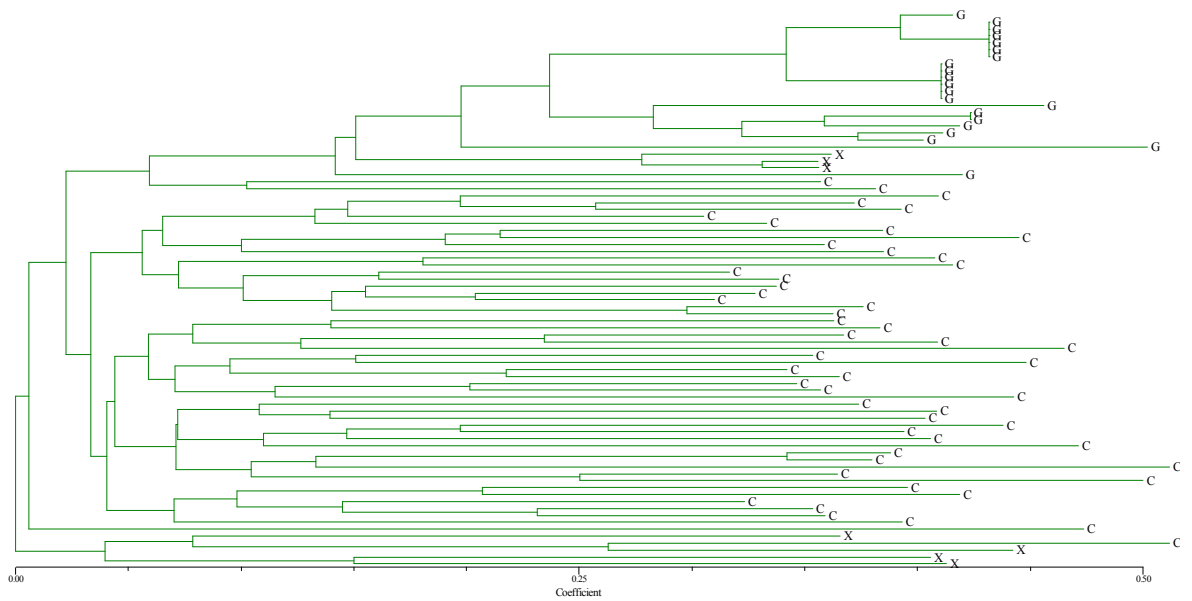


Fig. 13. A neighbor joining phylogenetic tree where the various hybrids are grouped with both *R. caesius* and *R. gothicus*. The x-axis shows a diversification coefficient. N=80.

Rubus glauciformis

We cannot settle how *R. glauciformis* has arisen, i.e. from which *Corylifolii* species. But when comparing it against *R. gothicus* and its *R. caesius* hybrids, we see that *R. glauciformis* is not a uniform species, as shown in Fig. 14. With *R. glauciformis* individuals from Småland, Skåne and Blekinge have clearly have arisen independently, and may not even have originated from the same parental species. Most of the samples cluster in one group, in the upper right of Fig. 14, but six samples cluster together with the *R. gothicus* x *caesius* hybrids instead, and might represent such hybrids.

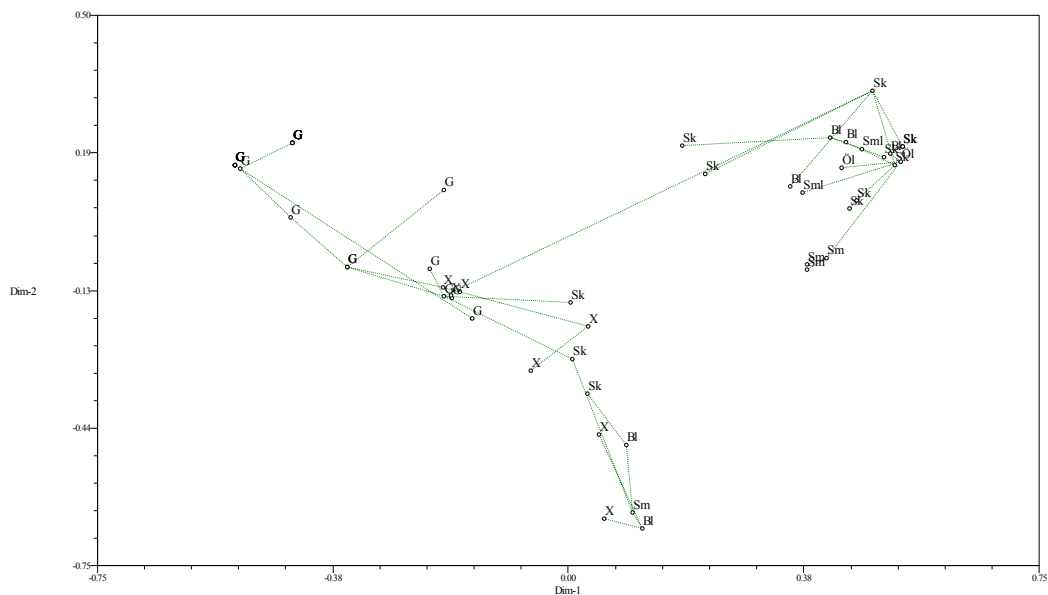
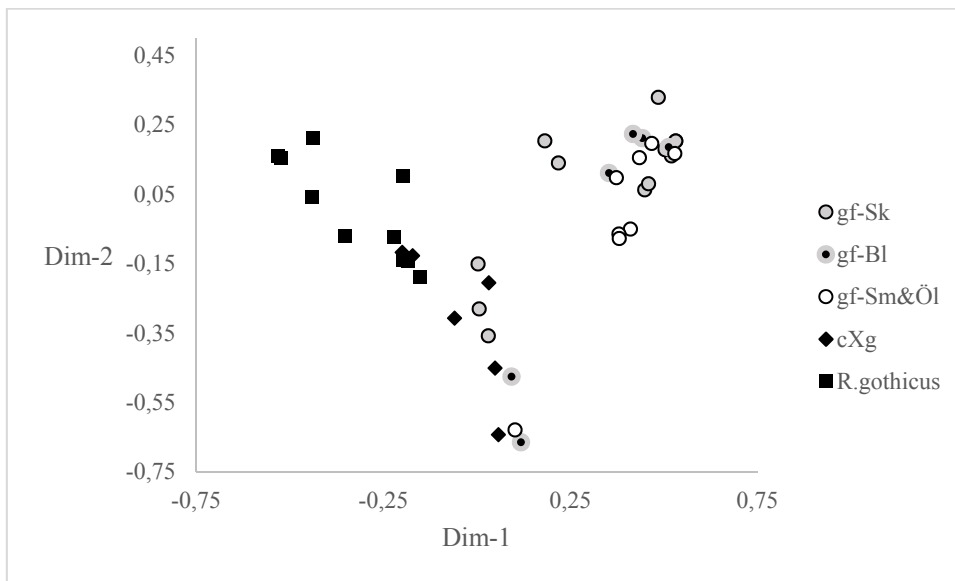


Fig. 14 a) PCO on genetic distances between samples of *R. glauciformis* in circles, *R. glauciformis* from Halland, in black, from Skåne, in gray with black outline, Blekinge, in black circles with gray outline, and Småland and Öland, in white circles. *R. gothicus* hybrids are in diamonds and *R. gothicus* in squares. b) The same PCO superimposed by a minimum spanning tree. N=54

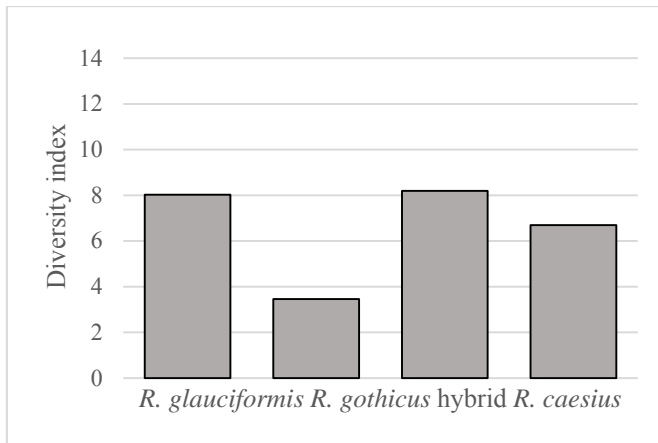


Fig. 15. Genetic diversity, given as mean number of pair-wise differences between individuals, of *R. glauciformis*, *R. gothicus*, the *R. gothicus* hybrid and *R. caesius*.

When analyzing the small cluster of *R. glauciformis* [n=6] from Fig. 7 together with *R. gothicus* and *R. caesius* in a common PCO, *R. glauciformis* is located at an intermediate position between the other two taxa, as in Fig. 16. When analyzed together with *R. gothicus*, the putative *R. gothicus* x *caesius* hybrids [n=7] and *R. caesius*, *R. glauciformis* groups with the hybrids, in between the two other taxa, as in Fig. 17. If only *R. glauciformis* and the *R. gothicus* x *caesius* hybrid are considered in the analysis, they form two relatively distinct groups, as in Fig. 18.

When analyzing all *R. glauciformis* samples together with *R. gothicus* and *R. caesius* in a common PCO, the larger cluster of *R. glauciformis* identified in Fig. 7 [n=20] is more genetically diverse than *R. gothicus* and *R. caesius*, and than the two latter are to each other, as can be seen in Fig. 19.

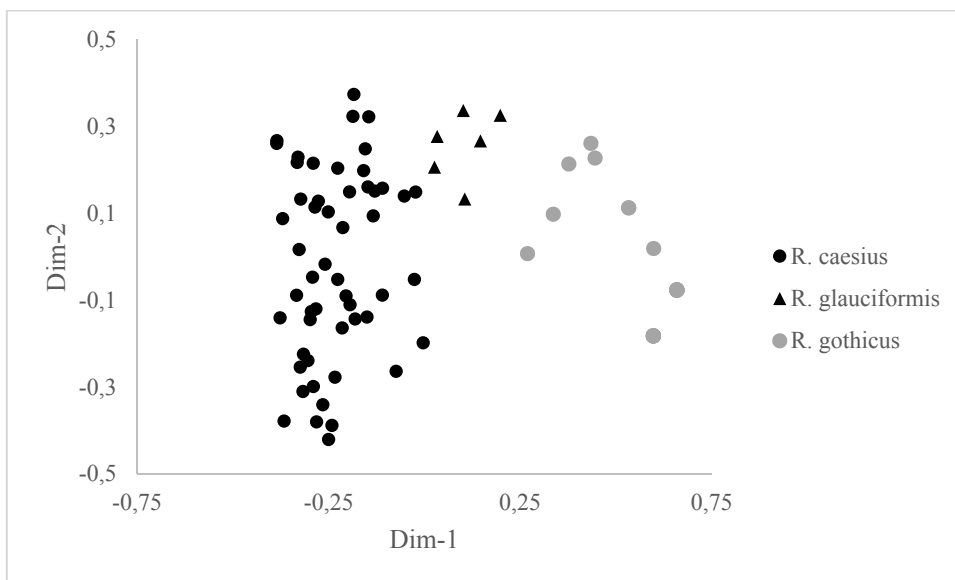


Fig. 16. PCO on genetic distances between samples of *R. caesius* in black circles, *R. glauciformis* in black triangles and *R. gothicus* in gray circles.

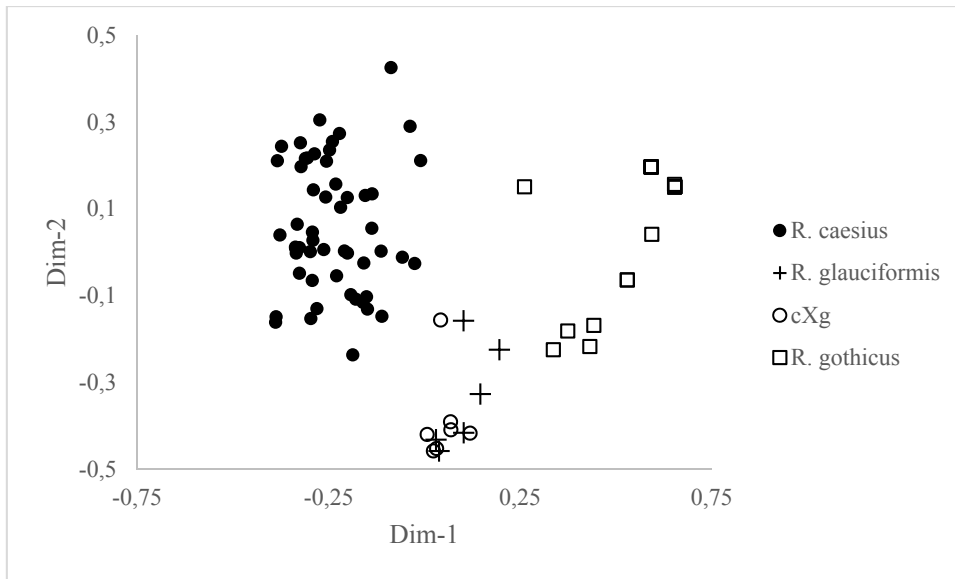


Fig. 17. PCO on genetic distances between samples of *R. caesius* in black circles, *R. glauciformis* in black crosses, the *R. gothicus* hybrids in white circles and *R. gothicus* in white squares.

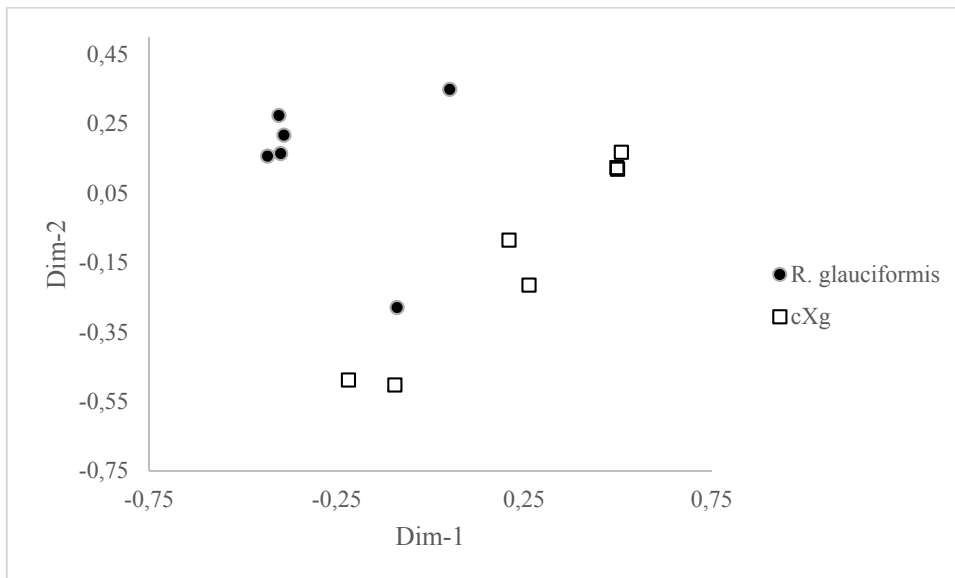


Fig. 18. PCO on genetic distances between samples of *R. glauciformis* in black circles, and the *R. gothicus* hybrids in white squares.

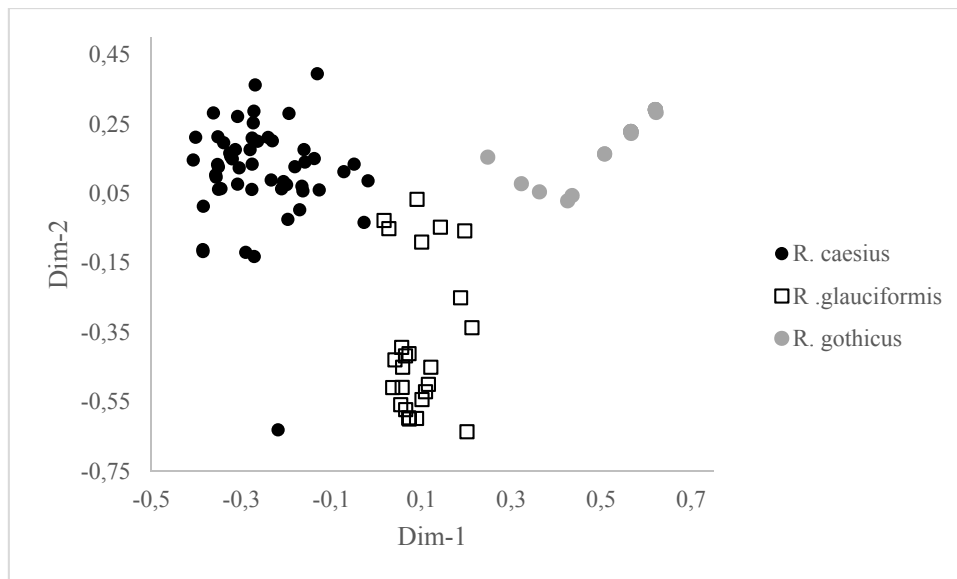


Fig. 19. PCO on genetic distances between samples of *R. caesius* in black circles, *R. glauciformis* in white squares and *R. gothicus* in gray circles.

Rubus caesius

Rubus caesius is more diverse than the apomicts recognized as good species included in this study, as shown by Figs. 2, 5, 9 and 12. But it is rather uniform at the microsatellite locus 105B compared to the other taxa.

In the population of *R. caesius* in which samples were collected along a transect, no significant correlation between the geographical and genetic distances between plants were found in a Mantel test ($r=0.16$, $p=0.01$).

Discussion

Rubus Sect. *Corylifolii* species are considered to be difficult to determine based on morphological characters. We believe that some of the confusion is caused by the fact that hybrids between *R. caesius* and various *Corylifolii* are quite common in nature and are morphologically very variable. Still, most of the hybrids are characterized by a number of conspicuous characters, e.g. sepals that tightly enclose the fruits, rich and early fruits, many glands both on the stem and the inflorescence, and broad stipules. Specimens with these characters, combined with hair-like terminal leaflets and pink styles have been called *R. cyclomorphus* in Bohuslän and in combination with hairy anthers they have been called *R. tiliaster* in NW Skåne. Our hypothesis is that these are not proper species, but rather a collection of *R. norvegicus* \times *caesius* or *R. camptostachys* \times *caesius* hybrids, respectively, which have arisen independently many times and therefore show a larger morphological variation. Sometimes, other hybrids with some similar character combinations have been given the same names, e.g. *R. fasciculatus* \times *caesius* from Skåne. In our mind, it would be much simpler and biologically more correct if these are considered as hybrids and not ill-defined species.

Therefore, based on the results of this study, we suggest that both *R. cyclomorphus* and *R. tiliaster* are rejected as species, and instead are considered as temporary, independent primary hybrids.

We have also considered the possibility that new species might have arisen from two hybrids, *R. caesius* x *R. fasciculatus* and *R. caesius* x *gothicus*, which are conspicuously common in two parts of Sweden, namely north Halland and northwestern Skåne, respectively. However, our microsatellite study does not give any support to such a suggestion. There are some clustering of the hybrids, e.g. seen in Figs. 2, 3 and 6, but these patterns do not correlate with the geographical distribution and are therefore probably better explained by random similarity and perhaps vegetative propagation.

For *R. fasciculatus* our investigation confirms that there are two independent species, one with a western distribution, *R. fasciculatus* sensu Weber, and one with a more eastern distribution, *R. fasciculatus* sensu Hylander. Further studies of material from Central Europe are needed to decide the correct names of these species.

For *R. glauciformis*, we also identify two clusters. However, in this case, one cluster seems to represent the proper *R. glauciformis*, whereas the other seems to be a collection of primary hybrids, most likely *R. gothicus* x *caesius*. For the samples from Småland and Blekinge, this was unexpected, based on morphological characters. We need to study the species in nature to determine whether it is possible to distinguish *R. glauciformis* and the primary hybrids on basis of morphological characters.

Alleles that differ one base pair in a dinucleotide repeat could pose the practical problem to decide the exact lengths of the microsatellite fragments. Alleles that are present in hybrids but not in the parental species may be due to that such individual exist but have not been sampled, problems in correlating results obtained in different gels, or to somatic mutations.

After the hybridization event, recombination and loss of chromosomes may make it hard to determine exactly which species have contributed with the major genetic mass. Also revoking of methylation thus of hybridization could make new microsatellites appear by exposing silenced alleles. Furthermore, changes in methylation patterns associated with hybridization could result in the amplification of previously inaccessible microsatellite loci.

Over a distance of more than half a kilometer, there was no correlation between distance and the establishment of clones in *R. caesius*. This could indicate a small production of asexual seeds and might therefore reflect the proportion of apomictic reproduction relative to the sexual reproduction, contradicting what Gustafsson wrote 1942. Unless there is a wide spread in distance of the seeds by the animals consuming its fruit.

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