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EVIDENCE OF SEXUALITY IN EUROPEAN *RUBUS* (ROSACEAE) SPECIES BASED ON AFLP AND ALLOZYME ANALYSIS[1]

JOHANNES KOLLMANN,[2] THOMAS STEINGER, AND BARBARA A. ROY

Geobotanisches Institut, ETH Zürich, Zürichbergstrasse 38, 8044 Zürich, Switzerland

Reproduction of polyploid *Rubus* species is described as facultatively apomictic. Pollination is needed for seed set, but most seedlings are produced asexually by pseudogamy. Although sexual processes may occur, clonal diversity can be extremely low. We performed a pollination experiment to investigate the breeding system and used allozyme and AFLP markers to analyze genetic variation among and within seed families in *R. armeniacus* and *R. bifrons.* Pollination either with self or outcross pollen was necessary to trigger seed set. Outbreeding marginally increased the number and quality of seeds compared with selfing. The enzyme PGI revealed some genetic variation within seed families. Seven other enzyme systems were monomorphic. The more detailed AFLP analyses with five primer pairs detected the same rate of genetic variation (14–17% of seedlings were genetically distinct) and confirmed the allozyme results for the same individuals. No genetic variation was found between the seed families from within a species collected in widely separated populations, but clear species-specific differences were observed. The results support the view that polyploid *Rubus* species are pseudogamous apomicts with low genetic diversity among and within seed families. However, sexual reproduction occasionally occurs and contributes to the maintenance of genetic variation within natural populations.

Key words: AFLP; allozymes; clonal uniformity; pollination experiment; pseudogamy; Rosaceae; *Rubus.*

The subgenus *Rubus* in central Europe consists of numerous polyploid apomictic species (Weber, 1995). The exceptions are a few diploids ($2n = 14$) that show normal sexual reproduction, for example, *R. canescens* and *R. ulmifolius.* The polyploid species ($2n = 21$–84) need pollination to trigger seed set and endosperm development, but the ovule is not fertilized and thus most seedlings are produced asexually. This mode of reproduction (''pseudogamy''), and the resulting embryogenesis, are well understood (Christen, 1950; Berger, 1953; Czapik, 1981), although the genetic basis is not clear. As early as 1881, Focke (1881) and Gustafsson (1930) demonstrated for some *Rubus* species that no seeds are set in emasculated flowers that are protected from pollination. Although occasional sexuality and interspecific hybridization have been observed in many *Rubus* species (Crane and Darlington, 1927; Berger, 1953; Nybom, 1988, 1995; Antonius and Nybom, 1995), clonal diversity of the apomictic species can be extremely low (Kraft and Nybom, 1995; Kraft, Nybom, and Werlemark,1996), and based on morphology hybrids are rarely found in the field (Weber, 1995, p. 294). This result contrasts with a much higher genetic variation in sexual *Rubus* species (Nybom and Schaal, 1990; Antonius and Nybom, 1994).

Although the breeding system of some *Rubus* species is well investigated (Gustafsson, 1930; Dowrick, 1961, 1966), and several experimental hybrids have been produced (Crane and Darlington, 1927; Haskell, 1960; Jennings, Craig, and Topham, 1967; Nybom, 1988, 1995; Antonius and Nybom, 1995), the natural degree of sexuality and hybrid formation has to our knowledge never been studied on a genetic level under field conditions. To study breeding systems, it is necessary to investigate variation among and within seed families. Genetic variation of plant populations is largely determined by the breeding system (Loveless and Hamrick, 1984; Roy, 1995); and genetic variation within the numerous *Rubus* species might be important for resistance to pathogens (Williamson, Breese, and Shattock, 1995; Teifion-Jones and McGavin-Wendy, 1998), and for variation in fruit and seed size that might affect seed dispersal (Jordano, 1984). Most *Rubus* species in Europe are probably of relatively young origin and therefore are confined to rather small areas of distribution (Matzke-Hajek, 1997). Dispersal of such species and their habitat specificity may be at least partly controlled by genetic variation of the populations. Therefore, it is important to investigate genetic variation of *Rubus* populations to challenge the current belief that these species are genetically uniform.

Molecular markers are suitable for detecting genetic diversity in *Rubus* species, as demonstrated for allozymes (Cousineau and Donnelly, 1992), RFLPs (restriction fragment length polymorphisms; Waugh et al., 1990), RAPDs (randomly amplified polymorphic DNAs; Graham and McNicol, 1995), and minisatellites (Nybom, 1995). In the present study, AFLPs (amplified fragment length polymorphisms) are used for the first time within the genus *Rubus*, and this method will be compared with results from an allozyme study. The AFLP analysis supplies a large number of dominant markers, which are more reliable than, for example, RAPD markers (Barker et al., 1999). We analyzed genetic diversity in seed families from two common European blackberry species, *R. armeniacus* Focke and the closely related *R. bifrons* Vest. In addition, we investigated the breeding system of *R. armeniacus* with a pollination experiment. This experiment was necessary to substantiate the observations of Bammi and Olmo (1966) and Nybom (1986) who describe that *R. armeniacus* is a facultative pseudogamous apomict. Further, this experiment allows us to detect potential effects of pollen origin (self vs. outcross) on seed number, germination, and seedling growth.

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[2] Author for reprint requests (Department of Ecology, Royal Veterinary and Agricultural University, Rolighedsvej 21, 1958 Frederiksberg Copenhagen, Denmark).

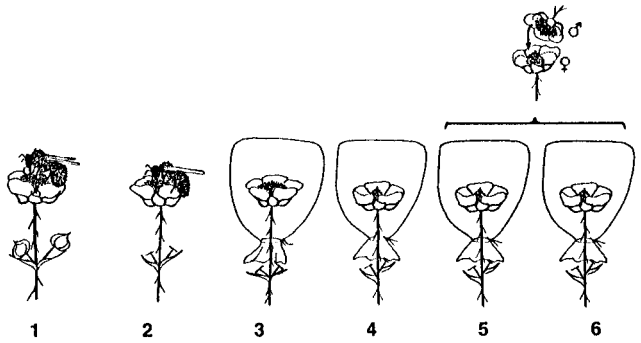

Fig. 1. Pollination experiment on *Rubus armeniacus* with the following treatments: (1) ''Open pollination I,'' the terminal bud exposed to natural pollination, sister buds intact; (2) ''open pollination II,'' as (1) but the sister buds removed (similar 3–6); (3) ''autogamy,'' terminal bud permanently bagged; (4) ''apogamy,'' terminal bud emasculated, bagged, no pollination; (5) ''experimental selfing,'' bud emasculated, bagged, pollen from the same plant; and (6) ''experimental outbreeding,'' bud emasculated, bagged, pollen from a shrub 10–100 m in distance (drawings by Sophie Karrenberg).

The study focuses on the following questions: (1) Is pollination needed for seed set in the study species? (2) Does outbreeding lead to higher quality and quantity of seed set than selfing? (3) Is there genetic variation within seed families of the two apomictic species, suggesting sexual reproduction?

MATERIALS AND METHODS

Study species—We investigated the breeding system and genetic variation in two putatively apomictic *Rubus* species in northern Switzerland: *R. armeniacus* Focke (tetraploid, $2n = 28$) and the closely related *R. bifrons* Vest ($2n = 48$, both series Discolores; Weber, 1995). The species are among the commonest blackberries in Switzerland; they grow on calcareous soils along forest edges and in shrublands in numerous places in the lowlands (Weber, 1987). *Rubus armeniacus* was introduced to central Europe probably from the Caucasus as a fruit plant about two centuries ago; today the species is widely naturalized. *Rubus bifrons* is an old native species (Weber, 1995).

Pollination experiment—A pollination experiment to investigate the breeding system was performed with *R. armeniacus*, because this species is more common and time constraints did not allow the inclusion of *R. bifrons.* For the experiment, 24 shrubs of *R. armeniacus* were chosen at a site near Waldshut (Southwest-Germany; 345 m a.s.l.; 47°38′ N, 8°16′ E). All study plants grew in sunny, south-facing locations on nutrient-rich calcareous soil; the distance between the study plants was 10–100 m. Each shrub covered an area of ~5–10 m^2 and produced numerous flowers and fruits.

In early June 1998, six well-developed racemes per plant were selected on different branches at 1–2 m height. For each raceme, one terminal bud with two smaller subordinate buds was labelled (Fig. 1). Six treatments were applied to each shrub following Dafni (1992) and Roy (1995): (1) ''open pollination I,'' the terminal bud exposed to natural pollination, sister buds intact; (2) ''open pollination II,'' as (1) but the sister buds removed (these were also removed in treatments 3–6); (3) ''autogamy,'' terminal bud permanently bagged; (4) ''apogamy,'' terminal bud emasculated, bagged, no pollination; (5) ''experimental selfing,'' bud emasculated, bagged, pollen from the same plant; and (6) ''experimental outbreeding,'' bud emasculated, bagged, pollen from a shrub 10–30 m away. Shortly before flowering the labelled buds were carefully opened and all anthers were removed; the buds were then protected by a bag (6×4 cm^2) of fine nylon tissue. Four days later the flowers were receptive and those of the treatments ''experimental selfing'' and ''experimental outbreeding'' were hand pollinated with a picked flower in full anthesis. The weather was dry during the day of experimental pollination. One week after pollination, the flowers of the treatments ''open pollination I and II'' were also bagged to achieve similar conditions for the developing fruits and to avoid losses of ripe fruits.

Some of the emasculated flowers were aborted shortly after pollination and subsequently dried up. Branches of one individual were buried by a fallen tree. Because the fruits of this plant were not fully ripe, only the number of seeds could be determined and the seeds were excluded from the germination experiment. In the other shrubs no losses occurred and the fruits were fully ripe after 2 mo. They were harvested in the last week of July. The number of seeds per fruit were counted after cleaning in cold water.

The seeds were dried at room temperature and stored at 4°C. In mid-December 1998, the dry seeds were treated with cold 98% H_2SO_4 for 1 h to break the innate dormancy (protocol adapted from Nybom, 1980). Afterwards the pH was neutralized twice with distilled water and once with 0.25 mo L $NaHCO_3$. In flower pots of 10 cm diameter all seeds of a single fruit (1–53) were placed on 5 cm peat-based soil covered with 1 cm of quartz sand. The pots were protected against seed predation by rodents and remained in the garden of the institute in a randomized order over winter and spring. Germination of seedlings occurred in April and May. In early July 1999, all seedlings were harvested and the mean dry mass was determined for the seedlings of each fruit.

Plant material for the genetic studies—In early September 1996, we collected 20–30 ripe fruits of *R. armeniacus* and *R. bifrons* in the Swiss Jura near Olten. To avoid mixed collections from different clones, we strictly took fruits only from a single branch per species and site. The two species were sampled along two forest edges that were separated by a distance of 9 km (''Hägendorf 2'' and ''Losdorf 8''); for coordinates, altitude, and climate see Kollmann and Schneider (1997). The seeds were cleaned and stratified as described above. In April 1997, the emerging seedlings were transplanted to individual pots and kept in the institute's experimental garden as a source of fresh plant material for the genetic studies.

Allozyme method—Fresh, young leaf material (~0.25 cm^2) was homogenized in the grinding buffer for ''moderate levels of interfering substances'' as described by Wendel and Weeden (1989, p. 9). The extracts were then immediately absorbed on paper wicks and loaded onto starch gels (12.8%). Horizontal electrophoresis was performed. Gel and electrode buffer system number 3 gave the best results for PGI, the only enzyme with polymorphic bands (Tris-citrate buffer pH 8.5, after Soltis et al., 1983). We investigated 62/21 seedlings of the two seed families in *R. armeniacus,* and 11 of one seed family in *R. bifrons.*

AFLP method—Genomic DNA was extracted from freeze-dried leaf tissue (~50 mg) which was previously ground to a fine powder with the aid of a shaking mill (see Steinger, Körner, and Schmid, 1996). A modified CTAB (cetyl trimethyl ammonium bromide) method was used for DNA extraction (Steinger, Körner, and Schmid, 1996). Typical yields were 3–6 μg DNA as determined with agarose gel electrophoresis. Due to high concentrations of phenolic compounds, DNA extracts had to be further purified with Qiagen spin columns to achieve complete digestion with restriction enzymes. The AFLP analysis was done as described in Vos et al. (1995) with some minor modifications. In short, the purified genomic DNA (~0.5 μg per sample) was digested with the restriction enzymes *EcoRI* and *MseI.* Two adapters with known sequences were then ligated to the sticky ends of the digested DNA fragments. The resulting products were diluted (20-fold) and subsequently pre-amplified by PCR (polymerase chain reaction) with primers that match the adapter sequence but contain an additional (selective) base at the 3′ end. PCR amplifications were performed in a Genius thermocycler (Techne Ltd., Duxford, Cambridge, UK) programmed with the following temperature profile: one cycle of 2 min at 72°C, followed by 23 cycles of 30 sec at 94°C, 30 sec at 56°C, and 2 min at 72°C, followed by one final cycle of 30 min at 60°C.

After a 20-fold dilution step, the PCR products were amplified in a second round with primers containing three selective bases. The temperature profile for this step was the following: one cycle of 2 min at 94°C, followed by 12 cycles of 30 sec at 94°C, 30 sec at 65°C (for the first cycle, subsequently reduced each cycle by 0.7°C for the next nine cycles), and 2 min at 72°C, followed by 23 cycles of 30 sec at 94°C, 30 sec at 56°C, and 2 min at 72°C. The PCR was terminated with a final incubation step of 30 min at 60°C. The

TABLE 1. The breeding system of *Rubus armeniacus* as indicated by results of a pollination experiment. For description of the experiment see Fig. 1; the treatments were performed on 24 plants. Since some plants produced no mature seeds or the seeds failed to germinate, the exact numbers of replicates are added in brackets (*N*). Given are means (± 1 SE) and the results of a Kruskal-Wallis test.

Pollination treatment	Seed number	Germination (%)	Seedling dry mass (mg)
Open pollination I	28.0 ± 2.2 (*24*)	43.0 ± 4.5 (*22*)	12.3 ± 1.4(*22*)
Open pollination II	32.3 ± 2.1 (*24*)	41.3 ± 3.6 (*23*)	13.4 ± 2.3 (*23*)
Autogamy	28.2 ± 1.8 (*24*)	33.3 ± 3.9 (*22*)	13.8 ± 1.6 (*22*)
Apogamy	2.0 ± 0.6 (*24*)	10.6 ± 7.1 (*4*)	22.0 ± 6.3 (*3*)
Experimental selfing	8.8 ± 2.3 (*24*)	33.3 ± 6.9 (*9*)	13.1 ± 2.8 (*12*)
Experimental outbreeding	14.5 ± 2.3 (*24*)	41.2 ± 4.4 (*15*)	16.0 ± 1.7 (*17*)
H (P)	85.5 (<0.001)	11.2 (0.048)	8.1 (0.15)

following primer combinations were used for selective amplification: E-AGG plus either M-CAA, M-CAC, M-CAG, or M-CAT. The *EcoRI*-Primer was labelled with a fluorescent dye (FAM), and the amplified fragments were separated with capillary electrophoresis on an ABI Prism 310 Genetic Analyzer (Perkin Elmer Corp., Norwalk, Connecticut, USA). Fragments in the range of 50–400 bases were scored as either present or absent. All fragments with an intensity above a threshold of 50 were included (see Fig. 3).

For the AFLP study, 12 seedlings from the two seed families of both species were randomly selected, i.e., a total of 48 seedlings. In 75 of 240 possible plant–primer pair combinations the selective amplification failed partly due to insufficient DNA quality or concentration, and these samples had to be excluded from the analysis.

Statistical analysis—In the pollination experiment, seed number per fruit, germination rate, and mean dry mass per seedling were analyzed with the nonparametric Kruskal–Wallis test, because the residuals were not normally distributed even after log or arcsine square-root transformation (program JMP®, SAS Institute). The germination rate was only calculated for fruits with more than seven seeds.

Differences between AFLP fragment profiles were quantified with a commonly used similarity index $G_s = 2\,N_{AB}/(N_A + N_B)$, where N_{AB} is the number of scored bands common in the AFLP profiles A and B, N_A is the number of scored bands in A, and N_B is the number of scored bands in B (Nei and Li, 1979; Nybom, 1993; Sanchez et al., 1999).

RESULTS

Effects of experimental pollination on seed set, germination, and seedling mass—The pollination experiment indicated that pollination is necessary for normal seed production in *R. armeniacus* (Table 1), as is expected for either a pseudogamous or a selfing species. The fruits were smaller and the number of seeds significantly lower in flowers that were emasculated and not pollinated (apogamy treatment) as compared with open pollination. However, some flowers of this treatment developed 1–10 seeds.

Clearly, the flowers were able to produce numerous seeds only when self pollen was available in bagged flowers (autogamy treatment). However, the average number of seeds in this treatment was slightly lower than for open pollination II, which allowed for outbreeding. The same result was indicated by the two treatments with hand pollination, since here seed set was again slightly lower for experimental selfing than for outbreeding. Thus, the quality of pollen seems to matter for seed set, although, in general, hand pollination gave poorer results than open pollination. The comparison of the treatments open pollination I and open pollination II revealed allocation costs, since the number of seeds was slightly higher for flowers where the two sister buds had been removed.

The pollination treatments had also significant effects on germination rate. Germination rate was 41–43% for open pollination I and II and for experimental outbreeding, and it was ~33% for autogamy; markedly lower germination was observed for the few seeds that resulted from the apogamy treatment. The latter result must be caused by lower quality pollination because competition among the low number of seeds must have been less important than in the other treatments. Actually, there was a slight positive correlation between the number of seeds per fruit and their germination rate (Spearman rank correlation: $r_s = 0.19$, $P = 0.069$). This result may indicate that outbreeding and open pollination had positive effects not only on seed set but also on seed quality.

The average dry mass of the seedlings after 3 mo of growth was not affected by the pollination treatments. However, seedling mass was negatively correlated with seed number per fruit ($r_s = -0.20$, $P = 0.042$), and this could reflect higher allocation to embryos in fruits with fewer seeds. Unfortunately, we failed to measure seed mass, and seeds should have been sown in constant number per flower pot to avoid density effects on growth. However, no correlation was found between seedling number per flower pot and seedling mass after 2 mo of growth in a common garden ($r_s = -0.12$, $P = 0.25$), indicating that competition among seedlings was not severe at the sown densities of 1–29 seedlings per pot.

Allozyme analysis and genetic variation—Eight enzymes out of 26 tested gave reliable bands. The enzymes EST, LAP, MDH, and 6-PGD showed no variation among and within species; ME, PGM and TPI revealed only variation among species, and polymorphic bands with differences among and within species were only found in PGI, a dimeric enzyme. Among 62 offspring from the *R. armeniacus* plant from site Lostorf, nine seedlings showed a missing band in the second locus of PGI (Fig. 2). No variation in PGI was found for 21 seedlings from the *R. armeniacus* plant in the second site (Hägendorf). Thus, ~11% of all seedlings were polymorphic for this locus. A version of the Simpson index corrected for finite samples (Peet, 1974) indicated moderate genetic diversity, based on the PGI results ($D = 0.20$). Mean number of alleles per locus and observed vs. expected heterozygosity were not calculated, because few seed families were sampled, the number of polymorphic loci was low, and polyploidy and apomixis make these calculations difficult.

The banding pattern in PGI was different among the two species, but resolution was less clear in *R. bifrons* and thus allowed no intraspecific comparison of the species. *Rubus bifrons* had consistently weaker bands in all enzyme systems tested.

AFLP analysis and genetic variation—Between 69 and 93 bands were scored for each of the five selective primer pairs, resulting in a total of 411 reliable bands (Table 2). The patterns

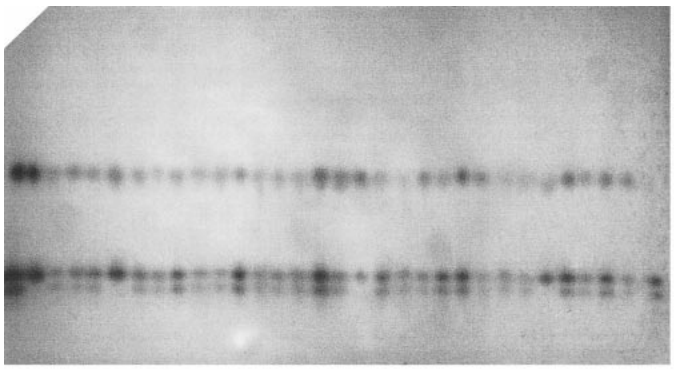

Fig. 2. Polymorphic bands in the allozyme PGI for 32 offspring of one seed family in *Rubus armeniacus.* The plants 2, 6, 17, and 27 show only a single band for the second locus and are probably the result of sexual reproduction by hybridization or automixis.

in the two species were clearly different, and 24% of the bands were polymorphic across species (for an example see Fig. 3). In contrast, no polymorphisms were found among the seed families from the two sites, suggesting that both *Rubus* species were of clonal origin. The similarity in size and intensity of the fragments was striking for seedlings from mother plants collected far from each other.

However, for both seed families pooled per species, deviations of the common banding pattern were found in three seedlings of *R. armeniacus* (14%) and four seedlings of *R. bifrons* (17%), indicating sexual reproduction. This percentage of sexual deviation is similar to the results of the allozyme study, and the likely sexual individuals in the AFLP analysis were the same that had different banding patterns in the enzyme PGI. The sexual offspring had new or missing bands for several primers. Overall, 14.3% polymorphic loci were found in *R. armeniacus* and 7.6% in *R. bifrons.* The average similarity indices for sexual vs. apomictic seedlings (*R. armeniacus*, $G_s = 0.92$; *R. bifrons*, $G_s = 0.95$) were higher than for a comparison of the two *Rubus* species ($G_s = 0.86$). It is possible that at least some of the sexual seedlings were hybrids between the two species, or that the two species have a common ancester.

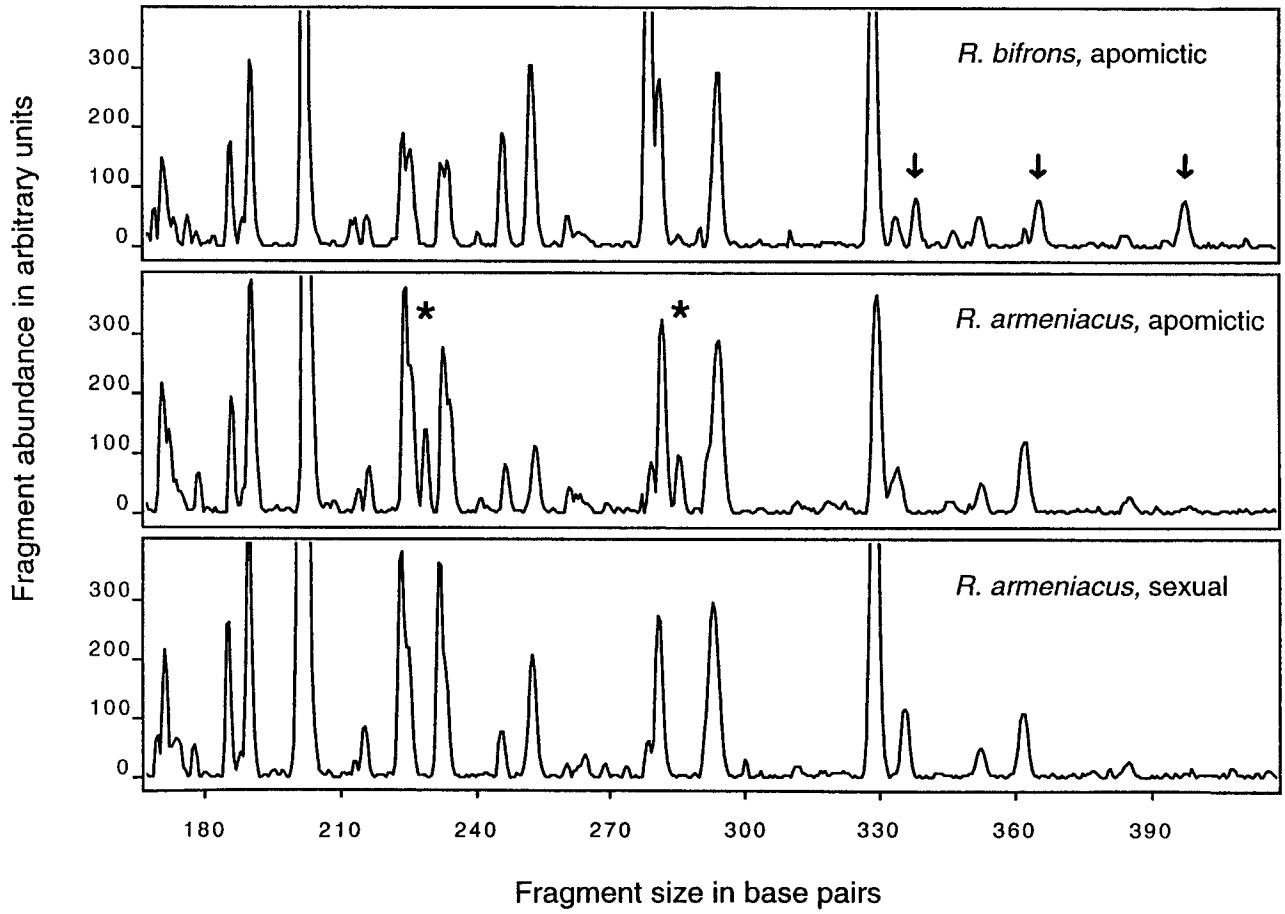

Fig. 3. Representative AFLP profiles of the two *Rubus* species, which show the high degree of reproducibility in shape and size of the peaks. Size of the fragments in base pairs on the *x*-axis and intensity of the bands in relative fluorescent units on the *y*-axis (primer set 1, M-CAA). Significant variation in the AFLP profiles is indicated for a comparison of the apomictic *R. bifrons* and two plants of one seed family of *R. armeniacus* (the sexual seedling is ''plant 2'' in Fig. 2; apomictic seedling, ''plant 3''). Arrows indicate deviating fragments in *R. bifrons* compared with the apomictic and the sexual *R. armeniacus*; asterisks indicate fragments of the apomictic *R. armeniacus* seedlings that were missing in sexual *R. armeniacus* and in *R. bifrons.*

TABLE 2. Summary result of the AFLP analysis with 12 seedlings of two seed families of *Rubus armeniacus* and *R. bifrons*, respectively. The *Rubus* species grew side by side in two sites separated by 9 km (*N*, total number of bands; *n*, number of specific bands of the two species). For sexual deviants the number of additional/missing bands is given in comparison with the apomictic seedlings. Included are similarity indices (G_s) for comparisons between (column 3) and within species (apomictic vs. sexual seedlings, columns 6 and 9; for calculation see Materials and Methods).

Primers E-AGG+	Number of bands and similarity indices							
			R. armeniacus seedlings			*R. bifrons* seedlings		
	N	G_s	*n*	deviants	G_s	*n*	deviants	G_s
M-CAA	82	0.84	11	4/9	0.89	11	4/2	0.96
M-CAC	69	0.90	6	0/7	0.94	6	0/0	—
M-CAG	78	0.86	17	3/6	0.93	7	4/4	0.93
M-CAT	93	0.91	5	3/8	0.94	10	5/1	0.96
M-CTA	89	0.79	22	5/10	0.90	16	7/0	0.95
Total	411	0.86	61	15/40	0.92	50	20/7	0.95

DISCUSSION

Importance of pollination for seed set—This study has shown that pollination is necessary for seed set in *R. armeniacus*, as reported by Bammi and Olmo (1966) and Nybom (1986). The results of the pollination experiment suggest that *R. armeniacus* is a facultative pseudogamous apomict, as has been found for many other members of the subgenus *Rubus* (Weber, 1995). For such species one would expect that no seeds are produced in emasculated flowers without pollination (Gustafsson, 1930; Roy, 1995, for *Arabis*). The experiment supports this hypothesis, although some seeds resulted in the emasculation treatment. Most likely some hidden anthers were overlooked at the bases of these flowers. Another explanation could be that *Rubus* pollen slipped through the nylon bags or that apomixis without pollination occurred. This result is similar to that of Finn (1996), who demonstrated that blackberries set some seeds if they were not carefully protected from cross pollination.

We observed lower seed set for hand-pollinated flowers compared with open pollination, which is a common result in pollination experiments (Guitián and Fuentes, 1992; Holderegger, 1996). We pollinated each flower only once, and not all stigmas may have been receptive at the same time. Repeated hand pollination may have increased seed set. However, it has been recommended that during crossing experiments it is best to avoid potential sire effects by avoiding multiple pollinations (Nybom, 1995)

Outbreeding effects on seed set—The second question set out in the introduction was whether outbreeding in polyploid *Rubus* species leads to higher (quality) seed set than selfing. Kerr (1954) and Haskell (1960) showed that pollination is not only needed to trigger endosperm development, but that species identity of pollen may influence the number and size of seeds and drupelets, seed germination and seedling vigor, with positive effects of increasing ploidy and taxonomic relatedness. We now have some evidence that intraspecific effects exist as well, although the genotypes of the pollen sources used for experimental outcrossing were unknown. Outbreeding, both natural and by hand pollination, tended to produce more seeds with a slightly higher germination rate than selfing; similar effects on the number of seeds were reported by Nybom (1986). In contrast, no significant effects were found for dry mass of seedlings. We expected a positive correlation between seed number per fruit and seedling mass, if both traits reflect quality of pollination. However, allocation trade-offs between seed number and single seed mass, and the different densities of seedlings in the germination experiment could have led to the observed negative relationship between seed number and seedling mass.

Our explanation for the higher seed set in outbred flowers is that pollen of a different clone may cause positive heterosis during endosperm formation. A heterotic endosperm might have a competitive advantage during embryogenesis and seedling establishment as shown by Haskell (1960) for interspecific crosses with different ploidy levels. This observation led him to coin the term ''pseudogamous heterosis.'' However, the higher seed number in the outbreeding treatment could also be due to a higher percentage of sexual embryos, and we performed a genetic analysis to determine the natural level of sexuality in the predominately apomictic study species.

Genetic variation among and within seed families—The third research question of this study concerned the degree of genetic variation among and within seed families of the two apomictic species. The genetic diversity as measured with the Simpson index ($D = 0.20$) was below the average of 0.59 reported for clonal plants in general by Ellstrand and Roose (1987), but well within the range of 0.10–0.99 for 21 plant species reviewed by these authors. However, a comparison with the literature is complicated because we investigated only a few seed families, and only one enzyme revealed polymorphic bands. However, this result of the allozyme analysis was confirmed by the AFLP study, since the similarity indices of the AFLP profiles were rather high among the two species (band sharing value $G_s = 0.86$) and also within seed families (0.92–0.95). These values are higher than those reported by Nybom (1993) for unrelated conspecific individuals (0.30–0.50, based on minisatellite probes) or seed families (0.80).

Although the genetic analyses revealed high levels of genetic similarity among and within seed families of the two *Rubus* species, some seedlings showed genotypic deviations and were most likely the product of sexual recombination. The aberrant seedlings may have been produced by natural hybridization or automixis, i.e., the fusion and subsequent parthenogenetic development of two egg nuclei in a reduced embryo sac (Crane, 1940; Antonius and Nybom, 1995). Our study is the first to demonstrate genetic variation within seed families of apomictic blackberries under field conditions, with ~14% putative sexual progeny in *R. armeniacus* and 17% in *R. bifrons.* However, we may have underestimated the percentage of sexual seedlings as we cannot differentiate heterozygotes resulting from sexual processes from heterozygotes as a result of asexual reproduction. In addition, the experimental design did not allow us to investigate whether or not sexually derived seedlings appear more often after selfing, or after intraspecific or interspecific pollination.

The rates of sex that we found are typical for facultative apomicts, and they are within the range suggested by Nybom (1988, 1995) based on common garden experiments in southern Sweden. Nybom (1988, 1995) described ~70 seedlings from interspecific crosses among *R. nessensis*, *R. scissus*, and *R. sulcatus.* Hybrid blackberries seemed to have improved meiosis and were often fully sexual, whereas an average level of 10% sexuality was indicated by DNA fingerprinting for non-hybridogenous parents. Nybom (1995) hypothesized that

sexual reproduction in apomictic *Rubus* species is more common in central and southern Europe than in Sweden, as shown by Asker and Jerling (1992) for other plant species. Our results indicate that apomixis may be more common in central Europe than previously believed.

Consequences for dispersal and reproduction—This study suggests that outbreeding in apomictic *Rubus* species affects seed number and fruit size. European blackberries are mainly dispersed by frugivorous passerines (see Jordano, 1982; Snow and Snow, 1988). Variation in fruit and seed size has potential effects on seed dispersal since frugivory of many passerines is limited by gape width as shown by Herrera (1984) and Snow and Snow (1988, p. 190). Jordano (1984) reported considerable variation in seed mass among and within clones of the sexual *R. ulmifolius.* Jordano (1995) found also a negative correlation between fruit size (and seed mass) and dispersal efficiency for the fleshy-fruited species *Prunus mahaleb,* whereas Sallabanks (1993) reported a positive relationship between size and attractiveness of *Crataegus monogyna* fruits in North America (similar to Piper, 1986). If outbreeding leads to bigger fruits (and seeds) due to improved endosperm performance (Kerr, 1954; Haskell, 1960), this could become a disadvantage when the avian dispersers are not able to swallow the fruit. Birds also prefer fruits with low seed to fruit ratio (Herrera, 1987; Murray et al., 1993), and Jordano (1984) reported a positive relationship between seed size and this ratio in *R. ulmifolius.* Moreover, large seeds are often regurgitated after a relatively short time, whereas small seeds pass through the gut and are defecated after longer periods (Levey, 1986). Therefore, small fruits and small seeds are more likely to be dispersed over large distances.

Although we found some sexual seedlings in both study species, genetic variation within apomictic *Rubus* species is remarkably low as has also been reported by Kraft and Nybom (1995) and Kraft, Nybom, and Werlemark (1996). It is well established that plant populations with low genetic variation should suffer particularly from high mortality and reduced growth due to pathogens and herbivores (but see Roy, 1993). On the other hand, even low levels of sexual recruitment can be frequent enough to maintain genetic variation in a predominantly apomictic population (Watkinson and Powell, 1993). However, little is known from the literature about these effects in blackberries. Future studies should map the distribution of genotypes of *Rubus* species in the field (Nybom and Schaal, 1990; Eriksson and Bremer, 1993; Hamrick, Murawski, and Nason, 1993) and investigate the consequences of genetic variation on population dynamics and fitness of *Rubus* populations, as discussed by Jordano (1984) for seed dispersal.

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