Genetic background of resistance to gall mite in *Ribes* species

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Resistance to gall mite is an important genetic trait of *Ribes*. *P* and *Ce* genes, responsible for gall mite resistance, were established in *Ribes* species and interspecific hybrids using molecular markers. Resistance in *R. americanum* is determined by *P* gene and in *R. sanguineum* by *Ce* gene. Both molecular markers were absent in *R. dikuscha* genome. Molecular markers related to *P* and *Ce* genes were identified in the genome of *R. aureum.* Resistance to gall mite in the field conditions in *R. nigrum* x *R. americanum, R. nigrum* x *R. aureum* and *R. nigrum* x *R. sanguineum* F_3 hybrids fitted an expected Mendelian segregation ratio of 1:1, 3:1 and 1:1, respectively. 75.0% of hybrids with a pyramidal resistance to gall mite carrying markers related to *Ce* and *P* genes were obtained in the cross combination *R. nigrum* x *R. aureum* and will be included in the future breeding programs.

Key words: Cecidophyopsis ribis, *Ce* gene, *P* gene, resistance.

Introduction

Blackcurrant (*Ribes nigrum* L.) is one of the most important fruit crops in Europe, including Lithuania. Blackcurrant gall mite (*Cecidophyopsis ribis* Westw.) damages floral buds and is a vector of blackcurrant reversion virus (BRV) (Jones 2000). Gall mite infection causes heavy losses in blackcurrant production (Šutic et al. 1999).

Several sources of resistance to gall mite have been identified in genus *Ribes*. The most studied gall mite resistance gene, designated *Ce*, has been identified in gooseberry (*R. uva-crispa*) (Knight et al. 1974, Keep et al. 1982, Brennan et al. 1993). The resistance determined by this gene is monogenic and ensures effective protection against gall mite and BRV. However, application of the gene in the development of black currants resistant to gall mite is limited. F_1 hybrids of interspecific crosses of gooseberry and blackcurrant are weakly developed and have low agronomic value. Their further use in breeding programmes is limited due to low viability and sterility (Stanys et al. 1994). As a consequence, the *Ce* gene is not common in economically important blackcurrant cultivars.

P is another gene responsible for blackcurrant resistance to gall mite. It was identified in *R. nigrum* ssp. *sibiricum* (Anderson 1971, Jones et al. 1998, Kniazev and Ogolcova 2004). This resistance gene was determined in the cultivar 'Dainiai' bred at the Institute of Horticulture, Lithuanian Research Centre for Agriculture and Forestry (Sasnauskas et al. 2009).

Molecular markers for *Ce* gene, governing gall mite resistance, were developed using interspecific hybrids of gooseberry and blackcurrant by Brennan and colleagues (2009). We have identified a molecular marker related to *P* gene in blackcurrant hybrids derived from cultivars with *R. nigrum* ssp. *sibiricum* (Mazeikiene et al. 2012).

Interspecific hybridisation resulting in black currant cultivars with natural resistance to gall mite could be one of the most effective tools to solve the problem of gall mite infection in black currant plantations. Species from genus *Ribes* - *R. americanum*, *R. nigrum* spp. *sibiricum*, *R. uva-crispa*, *R. aureum, R. sanguineum*, have been used as donors of resistance to various *R. nigrum* diseases and pests (Stanys et al. 2004, Siksnianas et al. 2005, Rubauskis et al. 2006, Brennan et al. 2009). However, genetic background of resistance to gall mite in species *R. americanum, R. aureum, R. dikusha* and *R. sanguineum* still remains unknown.

Therefore, the current study was focused on the application of molecular markers related to *P* and *Ce* genes to define the origin of resistance to gall mite in *R. americanum, R. aureum, R. dikusha* and *R. sanguineum* species and to assess inheritance of gall mite resistance in the genotypes obtained by interspecific hybridization.

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Materials and methods

Plant material

Plants of different *Ribes* species maintained at a collection of Institute of Horticulture, LRCAF (55°60' N, 23°48' E) - *R. americanum*, *R. sanguineum*, *R. dikuscha*, *R. aureum* 'Corona'; *R. nigrum* ssp. *sibiricum* as *P* gene donor*, R. uva-crispa* 'Bedford Yellow' as *Ce* gene donor, interspecific F_3 hybrids of *R. nigrum* x *R. americanum*, *R. nigrum* x *R. aureum*, *R. nigrum* x *R. sanguineum;* were used in the study. Fertility of interspecific F_1 hybrids was weak, a small number of F_2 hybrids were planted in the field. Fertile interspecific F_3 hybrids, obtained after open pollination in the field conditions, with economically important traits were chosen for analysis of molecular markers of gall mite resistance. In 2014 and 2015, the extent of bush damage caused by *C. ribis* was evaluated on 5–6-year-old plants in points using 0 to 3 scale, with score 0 indicating only undamaged buds per bush, score 1 indicating one to three damaged buds, score 2 indicating four to ten damaged buds, score 3 indicating more than ten damaged buds.

Genomic DNA was extracted from 0.2 mg of fresh leaf tissue using the CTAB-based extraction protocol by Doyle and Doyle (1990).

Gene identification

Ce gene

Ce gene was identified using a molecular marker based on sequence of AFLP fragment (Brennan et al. 2009). Primer set 5'TTGAGACCTCCAAGTCCTGCT3' and 5'CTTGGCTTCGTTGTTAGATGC3' for PCR fragment in 180 bp length was used. PCR was performed in a 20 µl reaction volume containing 50 ng of total DNA, 1U Taq DNA polymerase (ThermoScientific Ltd.), 1 Taq DNA polymerase reaction buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.5 µM each of forward and reverse primers. DNA amplification reactions were performed under the following conditions: initialization step of 5 min at 94 °C, 30 cycles of 30 s at 94 °C, 30 s at 55 °C, 30 s at 72 °C and final elongation step of 10 min at 72 °C. The amplification products were separated on 1.5% agarose gel and stained with ethidium bromide. GeneRulerTM DNA Ladder Mix (Thermo Scientific Ltd.) was used as size standard. Fragments in the length of 180 bp of *Ce* gene were sequenced. These 180 bp fragments were extracted from agarose gel using a NucleoSpin Extract II kit (Macherey–Nagel Ltd.), according to the manufacturer's protocol and sequenced at the DNA Sequencing Centre (Institute of Biotechnology, Vilnius University). Genamics Expression software was used for multiple sequence alignment (Corpet 1988, Thompson et al. 1994).

P gene

AFLP analysis was performed according to Vos et al. (1995) method. AFLP Plant Fingerprinting Kit (Applied Biosystems Ltd.) was used for sample preparation; all procedures were performed according to the manufacturer's protocol. Two hundred ng of genomic DNA was digested with restriction endonucleases *EcoRI* and *Tru1I* (*MseI*) (Thermo Scientific Ltd.) and corresponding adaptors were ligated. Preamplification was carried out with standard primers *EcoRI* A and *MseI* C (205 nM each) in a 20 µl reaction volume containing 4 µl of diluted restriction-ligation mix and 15 µl of AFLP Amplification Core Mix (Applied Biosystems Ltd.). Preamplification conditions were as follows: 94 °C hold for 2 min followed by 20 cycles of 20 s at 94 °C, 30 s at 56 °C and 120 s at 72 °C, followed by final steps of 2 min at 72 °C and 30 min at 60 °C.

PCR

Selective PCR amplification was carried out under the same reaction conditions. The difference was that 2 µl of diluted pre-amplification template was added and *EcoRI* and *MseI* primers were used at 50 nM and 250 nM concentration, respectively. Selective amplification was performed using the following programme: an initial cycle of 30 s at 94 °C, 30 s at 65 °C and 80 s at 72 °C, followed by 10 cycles of 30 s at 94 °C 30 s at 65 to 56 °C, 1 °C per cycle, 80 s at 72 °C; followed by 23 cycles of 30 s at 94 °C, 30 s at 55 °C and 80 s at 72 °C, final step of 5 min at 72 °C. Samples were prepared for capillary electrophoresis by mixing 1 µl of the PCR product with 8.88 µl of Hi-Di formamide and 0.12µl of Gene Scan 500 LIZ ladder (Applied Biosystems, Ltd.), and analysed using a 3130 Genetic Analyzer (Applied Biosystems Ltd.). Heterogeneity between recombination frequencies (AFLP marker and resistance to gall mite in the field condition) in the four populations was examined using the chi-squared test in JoinMap 4 (Van Ooijen 2011).

Results

We analysed the presence of *Ce* gene in plants of 5 *Ribes* species (Fig. 1 A), using a molecular marker developed by Brennan et al. (2009). The analysis established that PCR product of expected size was identified in gooseberry 'Bedford Yellow' characteristic of the marker of gene *Ce*. *R. aureum* 'Corona' and *R. sanguineum* plants had marker of the gene *Ce* that was at the identical position as in the case of *R. uva-crispa* plants (Fig. 1 A, lane 2, 5 and 6). *Ce* marker was absent in genomes of *R. americanum* and *R. dikuscha* and in *R. nigrum* ssp. *sibiricum* (Fig.1 A, lane 1, 3 and 4). Identification of molecular marker related to the *Ce* gene in wild type *R. sanguineum* and *R. aureum* enabled us to assume the presence of similar genomic region including resistance gene homologous to *Ce* gene of *R. uva-crispa*. Homology of molecular marker sequence was verified by sequencing the specific 180 bp size fragments, similar to molecular marker for *Ce* gene in *R. sanguineum, R. aureum and R. uva-crispa* 'Bedford Yellow' (Fig. 1 B). Nucleotide sequences of *R. uva-crispa* 'Bedford Yellow' from our collection were 100% identical, indicating the inheritance of the marker specific to gooseberry *Ce* gene by the interspecific F_1 hybrid. In addition, strong nucleotide sequence homology 90.0% and 88.3% was found between the sequences of the *Ce* molecular marker of gooseberry and *R. sanguineum* or gooseberry and *R. aureum,* respectively (Fig. 1 B). Therefore, it is possible that resistance to gall mite in *R. aureum* and *R. sanguineum* is determined by the *Ce* gene like in gooseberry.

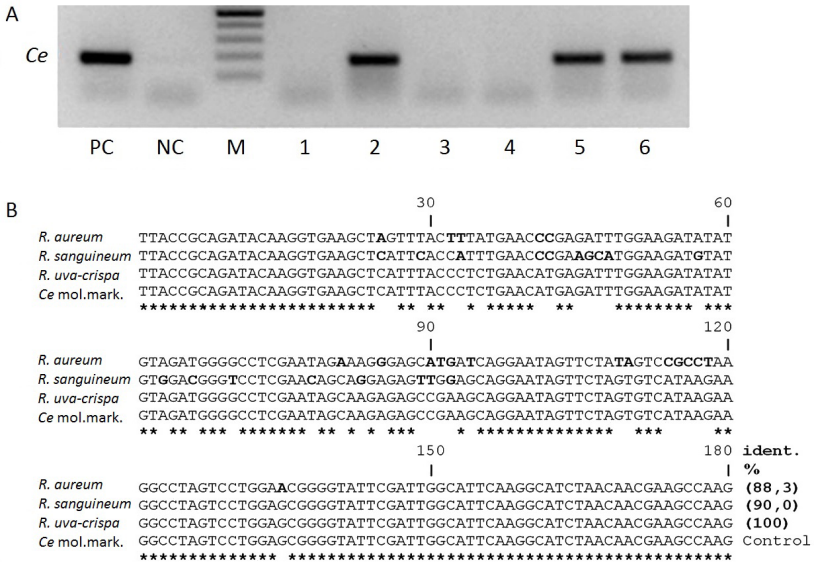

Fig. 1. Molecular marker for gene *Ce* in different *Ribes* species. A. Amplified PCR fragment in 180 bp length; B. *Ce* molecular marker sequences in *Ribes* species, compared to Brennan et al. 2009. PC = positive gene control (PCR with DNA of plasmids with insert of *Ce* marker sequence), NC = negative control (PCR mix without DNA template), M = size standard 100–500 bp (O'GeneRuler SM1173, Thermo Scientific Ltd.), 1 = *R. americanum*, 2 = *R. aureum*, 3 = *R. dikuscha* 4 = *R. nigrum* ssp. *sibiricum*, 5 = *R. sanguineum*, 6 = *R. uva-crispa* 'Bedford Yellow'

Electropherograms of the AFLP fragments with the marker related to *P* gene are shown in Fig. 2. This marker was developed at our laboratory (Mazeikiene et al. 2012). The fragment 107 bp in length demonstrates that the marker was present in *R. nigrum* spp. s*ibiricum, R. americanum* and *R. aureum* 'Corona'. The molecular marker for gene *P* was not identified in species of *R. sanguineum, R. dikuscha* and *R. uva-crispa* 'Bedford Yellow' (Fig. 2).

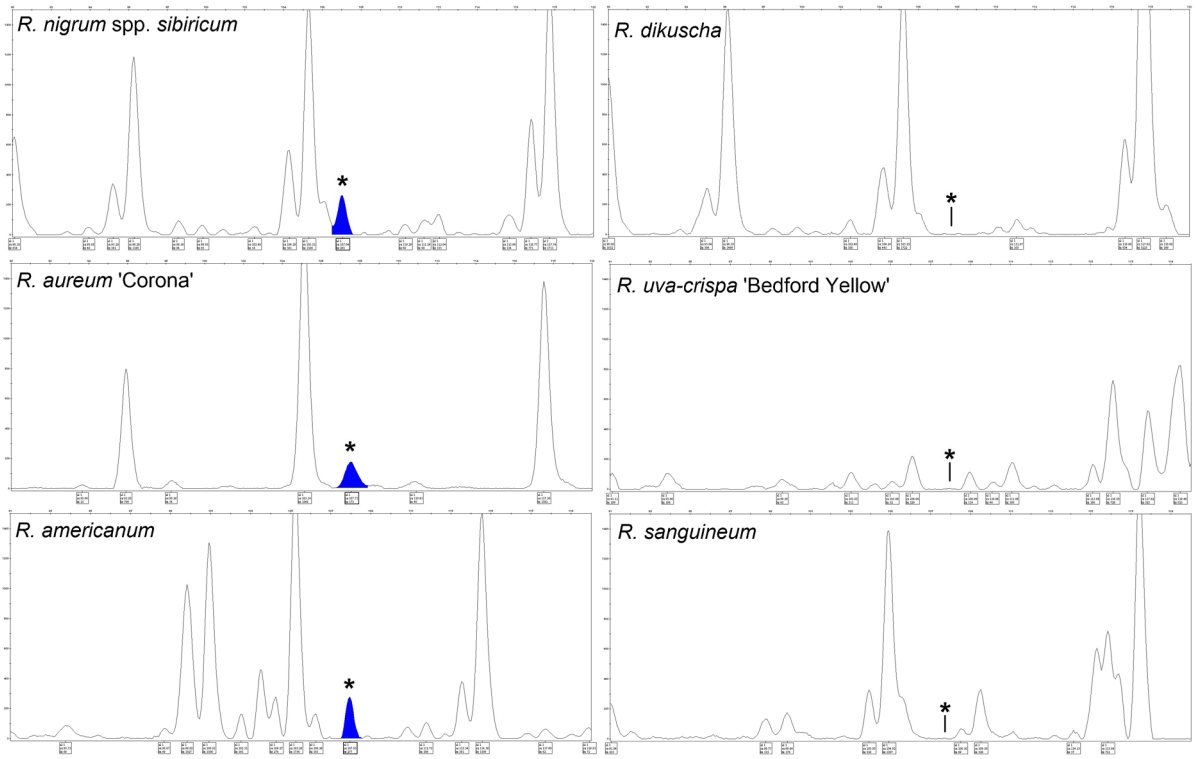

Fig. 2. Electropherogram of 107 bp AFLP marker related to dominant *P* gene in different *Ribes* species. * = location of molecular marker, related to *P* gene

Interspecific F_3 hybrids were obtained after open pollination of F_1 and F_2 hybrids in the field conditions. Field resistance to gall mite of interspecific F_3 hybrids from families *R. nigrum* x *R. sanguineum, R. nigrum* x *R. aureum* and *R. nigrum* x *R. americanum* was evaluated. Hybrids with score 0 were considered as resistant, and those with scores 1, 2 or 3 were considered as susceptible. Hybrids resistant to gall mite segregated at 1:1 ratio in F_3 generation of *R. nigrum* x *R. americanum* and *R. nigrum* x *R. sanguineum*. Interspecific F_3 hybrids obtained from the cross *R. nigrum* x *R. aureum* segregated at 3:1 ratio (Table 1).

Table 1. Resistance of interspecific F_3 hybrids of *Ribes* genus to gall mite in the field conditions

Cross combination*	Number of hybrids	Resistance to gall mite in field condition Resistant, no.	Susceptible, no.	χ2 (expected segregation ratio)	*p*
R. nigrum x *R. sanguineum*	45	26	19	1.09 (1:1)	>0.25
R. nigrum x *R. aureum*	40	27	13	1.20 (3:1)	>0.25
R. nigrum x *R. americanum*	33	15	18	0.27 (1:1)	>0.50

* F_3 progenies obtained after open pollination

The results of genetic analysis of gall mite resistance in *R. nigrum* x *R. americanum, R. nigrum* x *R. aureum* and *R. nigrum* x *R. sanguineum* interspecific hybrids based on the presence of the 107 bp AFLP and of the 180 bp PCR molecular markers are shown in the Fig. 3.

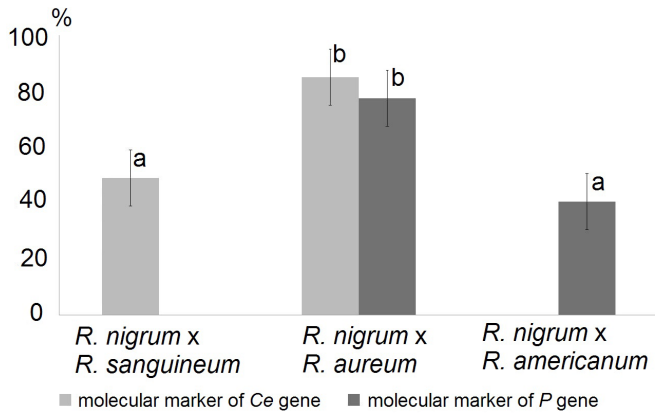

Fig. 3. Molecular markers related to resistance to gall mite in interspecific F_3 *Ribes* hybrids

Molecular marker of *P* gene was found in the genome of interspecific F_3 hybrids obtained after open pollination of *R. nigrum* x *R. americanum* and *R. nigrum* x *R. aureum*. The presence of the molecular marker of *Ce* gene in *R. nigrum* x *R. sanguineum* and *R. nigrum* x *R. aureum* F_3 hybrids was confirmed in the field conditions. Resistance to gall mite was determined by *Ce* gene in 48.9% hybrids in *R. nigrum* x *R. sanguineum* family. The molecular marker of *P* gene was present in 40.5% genotypes of *R. nigrum* x *R. americanum.* Molecular markers of *P* and *Ce* genes were present in *R. nigrum* x *R. aureum* hybrids (77.5 and 85.0%, respectively). 75.0% promising hybrids with a pyramidal resistance to gall mite obtained in cross combination *R. nigrum* x *R. aureum* are a valuable source of resistance for future breeding.

Discussion

Two genes responsible for resistance to *C. ribis* have been known to date: *P* gene has been identified in *R. nigrum* spp*. sibiricum* (Anderson 1971) and *Ce* gene is characteristic of *R. uva-crispa* (Knight et al. 1974).

Resistance of *R. sanguineum* to gall mite and mildew has been reported by Keep (1986). This species was used as a donor of resistance in heredity research (Keep 1981, Goodman et al. 1987, Siksnianas et al. 2008). However, genetic background of the resistance of *R. sanguineum* has not been assessed previously. Identification of molecular marker for the *Ce* gene in wild type *R. sanguineum* and *R. aureum* in our analysis enabled us to assume the presence of similar genomic region including resistance gene homologous to *Ce* gene of *R. uva-crispa*. It was shown earlier, that *R. dikuscha* is immune to reversion virus (Adams and Tresh 1987); however, we did not establish *Ce* marker in its genome. *P* resistance is specific to *R. nigrum* ssp. *sibiricum* and *R. nigrum* cultivars 'Rus', 'Dainiai', 'Ben Lomond' and 'Titania' (Anderson 1971, Mazeikiene et al. 2012), therefore marker for *Ce* gene was not established in these plants. In gooseberry, the resistance to gall mite is controlled by a single *Ce* gene (Knight et al. 1974). Interspecific *R. nigrum* x *R. uva-crispa* hybrids inherit *Ce* gene at a 1:1 ratio in F_1. The main problem is that hybrids are mostly infertile and the majority of the *R. uva-crispa*, *R. sanguineum* or *R. aureum* genome is lost during the earliest stages of backcrossing (Keep 1986, Siksnianas et al. 2008). F_1 hybrids of interspecific crosses of gooseberry and blackcurrant are weakly developed and have low agronomic value. Their further use in breeding programmes is limited due to infertility and low viability (Stanys et al. 1994). Interspecific hybrids can improve fertility after open pollination in the field conditions in F_2 generation, and economically important traits improve in F_3. As a result, we used promising F_3 hybrids in our study. Early diagnostics of valuable traits in F_1 progeny is crucial in the breeding process.

In this study, the presence of the molecular marker for dominant *P* gene was assessed in *Ribes* species and F_3 interspecific hybrids. Previously, traits of resistance to gall mite were identified in *R. nigrum* spp. *sibiricum* (Anderson 1971, Jones et al. 1998), *R. americanum* and *R. aureum* (Barney and Hummer 2005, Rubauskis et al. 2006); however, genetic background of the resistance was unknown. Our results suggest that resistance to gall mite in *R. nigrum* spp*. sibiricum*, *R. americanum* and *R. aureum* is determined by the dominant *P* gene. Molecular markers

linked to both *P* and *Ce* genes are found in the genome of *R. aureum.* The molecular marker for *P* gene was not observed in *R. dikuscha*, *R. sanguineum* and *R. uva-crispa.* Plants of *R. dikuscha* are resistant to gall mite in the field conditions (Trajkovski and Pääsuke 1976); however, in our study molecular markers for *Ce* and *P* genes were absent.

It was established that *P* gene, derived from *R. nigrum* spp. *sibiricum*, responsible for blackcurrant resistance to gall mite was dominant (Anderson 1971) and 1:1 segregation ratio of resistance to gall mite in hybrids was observed (Mazeikiene et al. 2012). In our study, inheritance of gall mite resistance trait derived from *R. americanum* fitted the expected 1:1 ratio in F_3 hybrids *R. nigrum x R. americanum*, as well. Therefore, we may conclude that *P* gene is in homozygous state in *R. americanum*. *R. americanum* is resistant to diseases and it flowers later compared to other *Ribes* species. In temperate climate zone, late flowering reduces the risk of currant flower damage during spring frosts. It was shown that early flowering and adaptivity in *Ribes* species are determined by cytoplasm, and therefore interspecific *R. nigrum* x *R. americanum* F_1 hybrids and their progeny were open pollinated in the field conditions with pollen from *R. nigrum* cultivars with low frequency of *P* gene (Keep 1986, Siksnianas et al. 2005). Inheritance of resistance to gall mite remained at 1:1 ratio in F_3 generation derived from this cross combination. Similar data were observed while studying inheritance of *Ce* gene. Inheritance of resistance to gall mite derived from *R. sanguineum* fitted the expected 1:1 ratio in *R. nigrum* x *R. sanguineum* F_3 hybrids. Blackcurrant hybrids with *R. nigrum* cytoplasm flowered earlier, and their progeny was open pollinated by *R. nigrum* hybrids or species. In our research, *R. aureum* species is characterized by the pyramidal (*P* and *Ce* genes) resistance to gall mite. In *R. nigrum* x *R. aureum* cross combination, gall mite resistance genes *P* and *Ce* remained in heterozygous state in F_3 progeny. Gall mite resistance ratio remained at 3:1 in *R. nigrum* x *R. aureum* F_3 hybrids, such ratio is favourable for the creation of resistant cultivars with other economically important traits.

Gall mite resistance remains a high priority for most currant breeding programmes. Distant hybridisation between different species provides qualitatively new material for currant breeding. *R. uva-crispa*, *R. sanguineum* and *R. aureum* as donors of *Ce* gene and *R. sibiricum*, *R. americanum* and *R. aureum* as donors of *P* gene may be effectively used as source material for gall mite resistance in the future breeding programmes. Early diagnosis of these genes allows selecting promising hybrids, and enables creating novel varieties with complex resistance to gall mite.

Our results suggest that resistance to gall mite in *R. sanguineum* is determined by *Ce* gene. Resistance to gall mite in *R. americanum* is determined by the dominant *P* gene as in *R. nigrum* spp. *sibiricum*. Resistance to gall mite in *R. aureum* is controlled by two genes *Ce* and *P*. The presence of molecular markers and resistance to gall mite in *R. nigrum* x *R. americanum* and *R. nigrum* x *R. sanguineum* F_3 hybrids, obtained after open pollination in the field conditions of F_1 and F_2 hybrids, fitted the expected segregation ratio 1:1. *R. americanum* x *R. aureum* F_3 hybrids, obtained after open pollination in the field conditions of F_1 and F_2 hybrids, fitted 3:1 ratio. Pyramiding of the *Ce* and *P* genes in breeding programs will produce hybrids with natural resistance to gall mite, thus resulting in unique breeding material.

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