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RESEARCH ARTICLE

SSR fingerprinting of a German *Rubus* collection and pedigree based evaluation on trueness-to-type

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Abstract Eighty-two genotypes of *Rubus* available in germplasm collections, nurseries and home gardens were collected and evaluated using a set of 16 simple sequence repeat (SSR) markers to estimate the level of genetic diversity and relatedness of the germplasm and for testing them on trueness-to-type. Each of the 16 SSRs was successful in amplifying alleles from most genotypes. Fifteen of the markers produced polymorphic bands, whereas marker RhM023 was monomorphic. The polymorphic information content among genotypes varied from 0.056 to 0.83 with an average of 0.348. A neighbor-joining analysis allocated the genotypes to four major clusters containing 11, 24, 39 and eight genotypes, respectively. Cluster I consists of floricane-fruiting cultivars originating from the Scottish and/or British breeding programs or cultivars which have those cultivars in their pedigree. Cluster II included cultivars that have 'Autumn Bliss' or 'Tulameen' in their pedigree. Cluster III consists of summer-bearing raspberry cultivars, some primocane-fruiting cultivars, and a few intermediate summer-fall-bearing types. Cluster IV consists of the blackberry 'Navaho' (*R. fruticosus* L.), the interspecific hybrid 'Dorman Red' and a few other raspberry varieties. A number of yellow fruited varieties was dispersed on three different clusters suggesting a convergent evolution of this trait. The pedigree of several genotypes could be confirmed using a Pedimap based approach, whereas other cultivars were found to be genetically identical. The results disclose the alarming narrow genetic base of *Rubus* resources in Germany. Broadening of this base is urgently needed.

Keywords DNA fingerprinting · Genotyping · Raspberry · *Rubus* · SSR

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Introduction

Red raspberry (*Rubus idaeus* L.), like many other temperate fruit crops, belongs to the Rosaceae family. The genus *Rubus* is comprised by several hundreds of species and hybrids. Most of the cultivated types belong to one of the two largest subgenera: *Rubus* (blackberries) and *Idaeobatus* (raspberries). Plants of the raspberry subgenera are diploid ($2n = 2x = 14$) and comprise the European red raspberry (*R. idaeus* L.), the North American red raspberry (*R. strigosus* Michx.), the black raspberry (*R. occidentalis* L.) and their hybrid aptly known as the purple raspberry (*R.* × *neglectus* Peck). In contrast, blackberries species vary greatly in ploidy (Thompson 1995, 1997; Jennings 1988; Meng and Finn 2002; Castillo et al. 2010; Fernández-Fernández et al. 2011).

According to the statistics of the Food and Agriculture Organization of the United Nations (FAO), the global production of raspberries yielded around 578,233 metric tons (MT) in 2013 (FAOSTAT http://faostat3.fao.org/browse/Q/QC/E 2013). Europe is with 74.9 % the main raspberry producer in the world. The most significant parts are produced in Russia (143,000 MT), Poland (121,040 MT) and Serbia (68,458 MT). In Germany, the production of red raspberries was about 5,564 MT in 2014 (https://www.destatis.de), with 4,230 MT produced in the open field and 1,334 MT produced in glasshouses, foil tents or tunnels.

Raspberries are favorite berries for consumers because of their excellent taste and the high content of biologically active compounds. Beside strawberry and blueberry, which are the economically most important small fruits in the German market, the current situation for raspberries is promising. In 2013, the German inland consumption was about 73,000 MT (BMEL 2014) and in productive years profit contributions of >15,000 € per hectare are feasible. However, about 94 % of the raspberries sold in Germany are imported from other countries (BMEL 2014). The commercial raspberry production is limited due to the high cost of establishing new plantations and the insufficient ability of raspberry fruit for long term storage and long distance transport. It is further hampered by the small number of appropriate cultivars with fruit of excellent quality and taste which additionally are highly resistant to biotic stress factors. Most raspberry cultivars successfully grown in Germany are susceptible to a range of diseases which lead to reductions in yield and fruit quality, and in extreme cases to the loss of the whole plantation (Weber and Entrop 2008). Especially root and cane diseases like cane blight (*Leptosphaera coniothyrium* [Fuckel] Sacc.), spur blight (*Didymella applanata* [Niessl] Sacc.), anthracnose (*Colletotrichum gloeosporioides* [Penz.] Penz. et Sacc., *Elsinoe veneta* [Burkh.] Jenkins), and cane botrytis (*Botrytis cinerea* Pers.), can lead to drastic yield reductions (Ellis et al. 1991). In Germany, *Fusarium avenaceum* (Fr.) Sacc. is the major pathogen for cane diseases (Weber and Entrop 2007, 2008; Weber et al. 2008). Currently there are only a few indirect measurements (e.g. cane management) available allowing a reduction of infection pressure. Breeding of resistant cultivars could be a promising solution. However, for several decades, no raspberry breeding program with a focus on commercial fruit quality was established.

Outside Germany there are about 30 *Rubus* breeding programs running in 19 countries, almost all of which are in Europe or North America (Graham and Jennings 2008). In Europe, there has been a large important breeding program since the 1950's at the Scottish Crop Research Institute (SCRI). This program has been phenomenally successful and is perhaps best known for its 'Glen' series of cultivars which are grown throughout the world.

Efficiency in raspberry breeding is low, but can be improved by application and development of molecular markers for germplasm assessement as well as for the selection of optimal parents in hybridization programs. Domestication in raspberry has resulted in a reduction of both morphological and genetic diversity in red raspberry with modern cultivars being genetically very similar (Graham and McNicol 1995). This narrow genetic diversity is a serious problem for future *Rubus* breeding aimed at the introduction of important agricultural traits, like disease resistance. In this respect, molecular markers can help to estimate genetic distances between genetic resources to be utilized in future breeding programs.

In 2012, the JKI's Institute for Breeding Research on Fruit Crops in Dresden (Germany) began to re-establish a raspberry breeding program which is aimed at breeding of new cultivars with excellent fruit quality and a good level of resistance to cane diseases. As a first step, genetic raspberry resources available in small German genebank collections, in nurseries and at the private sector were collected to establish a German *Rubus* collection comprising of 82 genotypes. Subsequently, this collection was evaluated using a set of simple sequence repeat (SSR) markers to estimate the genetic diversity and intraspecific relationships, and to identify accessions with identical genotype which will then be eliminated from the collection for improving the effectiveness of preservation.

Materials and methods

Plant material

Seventy-nine raspberry and three blackberry cultivars from different origins, including both primocane and floricane fruiting types, were chosen for SSR marker

analysis. The materials were collected from still existing *Rubus* genebank collections, from private nurseries, breeders and from the private sector (Table 1).

DNA isolation and SSR marker analysis

Genomic DNA was extracted from 0.1 g of young leaves using the DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA was re-suspended in 50 µL AE buffer (Qiagen, Hilden, Germany) and diluted to 10 ng/µL for use in PCR. For DNA analysis, we used 16 SSR markers (Table 2) taken on the basis of former reports from Castillo et al. (2010) and Fernández-Fernández et al. (2011). Multiplex PCR was performed using the Type-it Microsatellite PCR Kit (Qiagen, Hilden, Germany) in 10 µL volume with up to three different primer pairs and 20 ng template DNA. Concentrations of primers were 0.2 µM for forward primers labeled with Dye-751, 0.1 µM for primers labeled with BMN-6 and 0.05 µM for primers labeled with BMN-5. Reverse primers had the same concentration as the forward primers. All primers were ordered using biomers.net GmbH (Ulm, Germany). The optimum annealing temperature for each primer pair is given in Table 2.

For all markers labeled with I (Table 2), the PCR reaction was performed by denaturation at 95 °C for 5 min, followed by 28 cycles of 1 min denaturation at 95 °C, 90 s annealing and 30 s extension at 72 °C. A final extension was performed 30 min at 60 °C. The PCR cycling regime for markers labeled with II consisted of an initial denaturation step for 3 min at 94 °C, ten touch-down cycles comprising a 30 s denaturation at 94 °C, followed by 90 s of annealing starting at 60 °C and decreasing 1 °C per cycle, down to 51 °C, and 60 s of extension at 72 °C. Subsequently, 25 identical cycles were conducted with an annealing temperature of 50 °C followed by a final 30 min extension step at 60 °C. All PCR products were diluted to 1:10 using ddH_2O and the plate for capillary electrophoresis was prepared using 29.9 µL SLS buffer, 0.1 µL 400 bp size standard and 2 µL of diluted PCR product for each sample, adding a final drop of mineral oil to avoid evaporation. The evaluation was carried out on the CEQ 8800 Genetic Analysis System sequencer; fluorescent labeled products were analyzed using CEQ 8800 software (both Beckman Coulter, Krefeld, Germany) and checked visually by at least two researchers. Failed reactions were repeated taking care to include samples that worked in the original screen to ensure consistency of scoring.

Data analyses and marker scoring

DNA fingerprints were calculated for each of the 82 *Rubus* accessions by scoring the size of each marker peak in bp using the GenomeLab™ GeXP genetic analysis system (Beckman Coulter, Krefeld, Germany). These fingerprints were used for genotype comparison. For estimating the quality of the chosen markers, a data matrix was created where each cultivar was checked for the presence/absence of each marker allele. The presence of a given marker allele was scored as 1, whereas the absence was scored as 0. This data matrix was used for calculating the polymorphism information content value ($PIC = 1 - \sum_{i=1}^{l} P_i^2 - \sum_{i=1}^{l-1} \sum_{j=i+1}^{l} 2P_i^2 P_j^2$; Nagy et al. 2012), the expected heterozygosity ($H_e = 1 - \sum_{i=1}^{l} P_i^2$ averaged over all loci), the observed heterozygosity ($H_o = \sum_{i=0}^{n} (1 \text{ if } ai1 - ai2)/n$; Berg and Hamrick 1997; Lui 1998), the number of alleles per marker, and the size range of the peaks.

A neighbor-joining phylogenetic tree was constructed with 1000 bootstrap replicates using the Orchiai's coefficient and genotype data produced by fifteen SSR markers. The tree was constructed using Darwin 5.0 (Version: 5.0.158 [2009-07-06], Marchese et al. 2005).

Evaluation on trueness-to-type using pedigree information

Pedigree information was retrieved from literature, the internet or directly from the breeders and are summarized in Table 1. Pedigrees for selected genotypes were visualized using the Pedimap software (Voorrips et al. 2012) and used for evaluation of trueness-to-type by comparing genotypic data for each of the genotypes of the pedigree.

Results

Polymorphism and estimation of genetic diversity

Using 15 out of the 16 SSR markers, a total of 224 different fragments were amplified. The size of the

Table 1 *Rubus* genotypes analyzed in this study

Cultivar	Parentage	Fruiting type	Fruit color	Spines yes/no	Origin	Genotype proven on		
						Female parent	Male parent	Progeny
Raspberry								
Alyonushka	Kaliningradskaya × Lloyd George[3]	SF	Red	n	BY[3]	–	n.c.	–
Andenken an Paul Camenzind	Preußen × Lloyd George[7]	SF	Red	y	CH[7]	–	–	–
Aroma Queen	Autumn Bliss open pollinated	PF	Red	y	DE	c.	–	–
Autumn Best	Autumn Bliss × Tulameen[3]	PF	Red	y	CH[3]	c.	c.	–
Autumn Bliss	EM 2806/36 × EM 2335/47 (Complex parentage includes Lloyd George, *R. ideaus* var. *strigosus* Michx., *R. arcticus* L., *R. occidentalis* L.)[2,4]	PF	Red	y	GB[1]	–	–	c.
Autumn First	Autumn Bliss × Tulameen[3]	PF	Red	y	CH[3]	c.	c.	–
Black Jewel	(Bristol × Dundee) × Dundee[6]	SF	Black	y	US[6]	–	–	–
Cascade Delight	Chilliwack × WSU994 (derv. of Meeker and Haida)[6]	SF	Red	y	US[6]	–	–	–
Deutschland 1	Unknown	SF	Red	y	DE	–	–	–
Deutschland 43	Unknown	SF	Red	y	DE	–	–	–
Dorman Red	*Rubus parviflorus* Nutt. × Dorsett[2]	SF	Red	y	US[1]	–	–	–
Driscoll Maravilla	Q491.1 × Q480.3[8]	PF	Red	y	US[8]	–	–	–
Erika	Polka × Tulameen[5] Tulameen × Autumn Bliss[6]	PF	Red	y	IT[5,6]	c.	c.	–
Evraziya	Unknown seedling[3]	PF	Red	y	RU[3]	–	–	–
Fall Gold	NH-R7 × (Taylor × *R. pungens* var. *oldhamii*)	PF	Yellow	y	USA[1]	–	–	–
Gelbe Antwerpener syn. Jaune de Hollande	Unknown origin[4,7]	SF	Yellow	y	EU[7]	–	–	–
Gelbe Siebenkugel	Unknown	PF	Yellow	y	?	–	–	–
Gelbe Sugana	Mutation of Twotimer Sugana[3]	SF/PF	Yellow	y	CH[3]	c.	–	–
Glen Ample	SCRI 7326E1 × SCRI 7412H16 (Derivative of G. Prosen, Meeker, Preußen, M. Promise)[4,6]	SF	Red	n	SC[4,6]	–	–	c.
Glen Coe	SCRI 7751C4 × Glen Prosen	SF	Red	n	SC	–	c.	–
Glen Moy	SCRI 688/12 × SCRI 6815/113 (Complex SCRI-Hybrid of *Rubus occidentalis* L. (Glen Clova, Lloyd George) × Malling Landmark)[2,4]	SF	Red	n	SC[1,4]	–	–	–
Glen Prosen	SCRI 6531/84 × SCRI 6549/1 (SCRI-Hybrid of *Rubus occidentalis* L. (Malling Jewel, Burnetholm, Lloyd) × Malling Landmark)[2,4]	SF	Red	n	SC[1,4]	–	–	c.
Golden Bliss	Yellow sport of Autumn Bliss[3,4]	PF	Yellow	y	GB	c.	–	–
Golden Everest	Yellow sport of Autumn Bliss	PF	Yellow	y	?	c.	–	–

Table 1 continued

Cultivar	Parentage	Fruiting type	Fruit color	Spines yes/no	Origin	Genotype proven on		
						Female parent	Male parent	Progeny
Golden Queen syn. Goldkönigin	Yellow seedling of Cuthbert[7]	SF, PF	Yellow	y	US[7]	–	–	–
Gradina	Malling Exploit × Rubin[4]	SF	Red	y	YU[1,4]	–	–	–
Heritage	(Milton × Cuthbert) × Durham[2,6]	PF	Red	y	US[1,6]	–	–	c.
High Noon	Autumn Bliss × Tulameen[3]	PF	Red	y	CH[3]	c.	c.	–
Himbo Star (Rafzelsa)	Rote Wädenswiler open pollinated[4]	SF	Red	y	CH[1,4]	–	–	–
Immertragende von Feldbrunnen syn. Perpetuelle de Billard	Chance seedling possibly from Hornet[7]	PF	Red	y	F[7]	–	–	–
Korbfüller	Chance seedling of unknown origin[4]	PF	Red	y	DE[4]	–	–	–
Kozachka	Blagorodna × Shtambovyi-19	SF	Red	n	UA	–	–	–
Latham	King × Loudon[2,6]	SF	Red	y	US[6]	–	–	n.c.
Lloyd George	Selection from wild raspberry in Dorset[2]	PF	Red	y	GB	–	–	–
Lowden	Parentage unknown, but possibly from Bristol Black × Sodus Purple[9]	SF	Red	y	CA[9]	–	–	–
Lumina	Autumn Bliss[3]	PF	Yellow	y	CH[3]	c.	–	–
Madavaska	Lloyd George × Newman 23	SF	Red	y	CA	n.c.	–	–
Malahat	Meeker × BC/SCRI 7853/116[2,4,6]	SF	Red	y	CA[4,6]	n.c.	–	–
Malling Jewel	Preussen × EM 23/50 (EM 23/50 = Pyne's Royal × Lloyd George)[6]	SF	Red	y	GB[6]	n.c.	–	–
Malling Minerva	EM 5030/3 × SCRI 7269/67 (Complex parentage including M. Promise, *R. crataegifolius* Bunge, *R. occidentalis* L., *R. idaeus* var. *strigosus* Michx. × SCRI 7269/67)[6]	SF	Red	y	GB[6]	–	–	–
Malling Promise	Newburgh × EM 30/8 (EM 30/8 = Lloyd George × Pyne's Royal)	SF	Red	y	GB	–	–	–
Meeker	Willamette × Cuthbert[2,4]	SF	Red	y	US[1,4]	n.c.	–	–
Multiraspa	Preußen open pollination[4]	SF	Red	y	DE[4]	c.	–	–
Nootka	Carnival × Willamette	SF	Red	y	CA[1]	–	c.	c.
Octavia	Glen Ample × EM5928/114[4] Malling Hestia × Glen Ample[6]	SF	Red	y	GB[4,6]	c.	c.	–
Orange Marie	(Autumn Bliss × Fallgold) × Fallgold[4]	PF	Orange	y	NL[4]	c.	n.c.	–
Patiritsiya	Maroseika × Donor 102[3]	SF	Red	n	RU	–	–	–
Pingvin	Autumn Bliss	PF	Red	y	RU	c.	–	–
Polana	Heritage × Zeva Herbsternte[4,6]	PF	Red	y	PL[4,6]	c.	–	–
Preussen	Superlative × Marlborough[2,4]	SF	Red	y	DE[4]	–	–	–

Table 1 continued

Cultivar	Parentage	Fruiting type	Fruit color	Spines yes/no	Origin	Genotype proven on		
						Female parent	Male parent	Progeny
Proma	Malling Promise × Schönemann[4]	SF	Red	y	DE[4]	n.c.	c.	–
Prospera	Autumn Bliss × Tulameen[3]	PF	Red	y	CH[3]	c.	c.	–
Rafzmach (Elida)	Malling M. × Chilcotin[4,7]	SF	Red	y	CH[4,7]	–	–	–
Reflamba	unknown	SF	Red	y	?	–	–	–
Resa (Lucana)	Chance seedling of unknown origin[4]	SF, PF	Red	n	DE[4]	–	–	–
Royalty	N.Y. 252 × N.Y. 17861 (Cumberland × Newburgh) × (Newburgh × Indian Summer)[4]	SF	Red	y	US[1,4]	–	–	–
Rubaca (Niniane)	Rutrago × Latham[4]	SF	Red	y	DE[4]	c.	n.c.	–
Rubin Bryansky	Kostinbrodskaya × Malling Promise	PF	Red	y	RU	–	n.c.	–
Rucami	Klon 4a × Andenken an Paul Camenzind[4]	SF	Red	y	DE[1,4]	–	n.c.	–
Rucanta (Rutrago)	Klon 4a × Tragilo[4,6]	SF	Red	y	DE[1,4,6]	–	–	–
Rumiloba	Promiloy × Klon 4a[4]	SF	Red	y	DE[1,4]	–	–	–
Rumla	Unknown origin[4]	SF	Red	y	DE[4]	–	–	–
Sanibelle	Unknown	SF	Red	y	CH	–	–	–
Saxa Bliss	Autumn Bliss open pollinated[4]	PF	Red	y	DE[4]	c.	–	–
Saxa Record	Autumn Bliss open pollinated[4]	PF	Red	y	DE[4]	c.	–	–
Schönemann	Lloyd George × Preußen[4]	SF	Red	y	DE[4]	c.	n.c.	–
September	Marcy × Ranere[2]	PF	Red	y	US	–	–	–
Stuttgart	unknown	SF	Red	y	DE	–	–	–
Sugana	Tulameen × Autumn Bliss[3,6]	PF	Red	y	CH[3,6]	c.	c.	c.
Tarusa	Shtambovyi-1 × Stolichnaya[3]	SF	Red	n	RU	–	–	–
Tula Magic	Autumn Bliss × Tulameen[5]	SF	Red	y	CH[5]	c.	c.	–
Tulameen	Nootka × Glen Prosen[2,4,6]	SF	Red	y	CA[1,4,6]	c.	c.	c.
Valentina	EM6225/11 × EM5588/81[6]	SF	Orange	y	GB[6]	–	–	–
Wei-Rula	Rutrago × Latham[4]	SF	Red	y	DE[4]	n.c.	n.c.	–
Willamette	Newburgh × Lloyd George[2,4]	SF	Red	y	US[4]	–	n.c.	–
Winklers Sämling	Chance seedling[7]	SF	Red	y	N/DE[7]	–	–	–
Zefa 3	(Romy × Indian Summer) × Romy[4]	PF	Red	y	CH[4]	–	–	–
Zhar Ptitsa	7-73-2 open pollinated[3]	PF	Red	y	RU	–	–	–
Zheltyi Gigant	Maroseika × Ivanovskaya[3]	SF/PF	Yellow	y	RU	–	–	–

Table 1 continued

Cultivar	Parentage	Fruiting type	Fruit color	Spines yes/no	Origin	Genotype proven on		
						Female parent	Male parent	Progeny
Blackberry								
Buckingham Tayberry	Sports Tayberry		Black	n	GB	–	–	–
Navaho	Ark. 583 (Thornfree × Brazos) × Ark. 631 (Ark. 550 × Cherokee)		Black	n	US	–	–	–
Tayberry	Aurora (8*x*) × SHRI 626/67 (4*x*)[6]	SF	Black	y	GB[6]	–	–	–

[1] Dale et al. (1993); [2] Castillo et al. (2010); [3] Information was obtained directly from the breeder (e.g. phone, e-mail, homepage); [4] Bundessortenamt (2006), [5] Dillmann et al. (2011), [6] Fernández-Fernández et al. (2011), [7] Szalatnay et al. (2011), [8] Fear et al. (2004), [9] Lowden (1961). *PF* primocane fruiting; *SF* floricane fruiting; *y* yes; *n* no; *c.* confirmed; *n.c.* not confirmed; *BY* Belarus; *DE* Germany; *CH* Switzerland; *CA* Canada; *UA* Ukraine; *RU* Russia; *SC* Scotland; *YU* Yugoslavia; *GB* Greatbritian; *N* Norway; *F* France; *EU* Europe; *US* United States of America; *IT* Italy; *NL* Netherlands; *PL* Poland

Cond allele

amplified fragments ranged between 112 and 381 bp with 4 (RiG001) to 28 (Rubus285a) alleles per locus. The average number of alleles per locus was 14 (Table 3). Marker RhM023 was found to be monomorphic with a unique allele of 197 bp in size, which was present in all cultivars investigated. Therefore, as this marker was not informative, it was eliminated from further studies. Markers RhM011, RiM017, RhM021, RhM003 and RiM015, Rubus123a, Rubus285a, Rubus270a amplified fragments for all cultivars. Other markers were only successful with some cultivars. For example, with marker RhM043 no fragment was amplified in 'Dorman Red' and 'Navaho' and marker RiM019 was not successful in 'Navaho' and 'Lowden'. The absence of dectable alleles using those markers was regarded as a "null allele" condition.

The observed heterozygosity (H_o) for individual loci ranged from 0.26 for markers RiG001 and RhM021 to 0.96 for markers Rubus275a and RhM011with an average respective value of 0.6. The expected heterozygosity (H_e) for individual loci ranged from 0.44 to 0.91 with an average respective value of 0.67 (Table 3). The polymorphic information content (PIC) among genotypes varied from 0 (RhM023, RhM023 and RhM015) to 0.7 (Rubus123a) with an average of 0.3. The best SSR loci, based on high observed H_o and polymorphism information content were RhM011, RiM019, RhM003, RiG001, Rubus123a, Rubus285a, Rubus223a, Rubus270a and Rubus275a.

Adjusting SSR fragments on reference genotypes

For cross-comparison of results obtained in this study with other international studies, eight (RhM011, RiM019, RhM003, Rubus123, Rubus285a, Rubus223a, Rubus270a, and Rubus275a) out of the 16 markers were validated using a set of standard cultivars consisting of 'Autumn Bliss', 'Glen Ample', 'Heritage', 'Latham', 'Malling Jewel', and 'Tulameen' (Table 4), which were also tested by (Fernández-Fernández et al. 2011) using the same SSRs. With these markers the genotype of 'Tulameen', 'Malling Jewel' and 'Heritage' could be confirmed (Table 4). It could also be confirmed for 'Autumn Bliss' and 'Malling Jewel' for most of the markers/alleles. In 'Autumn Bliss' an additional allele of 141 bp was amplified using the marker Rubus270a

Table 2 List of 16 simple sequence repeat (SSR) primer pairs evaluated in raspberry and blackberry genotypes

Locus	Primer sequences (5′-3′)[a]	No. products	Annealing temperature in °C	Multiplex (Dye)	PCR program
RhM043[1]	Fwd: GGACACGGTTCTAACTATGGCT	7	56	MP_A (BMN-5)	I
	Rev: ATTGTCGCTCCAACGAAGATT				
RiM017[1]	Fwd: GAAACAGGTGGAAAGAAACCTG	8	59	MP_A (BMN-6)	I
	Rev: CATTGTGCTTATGATGGTTTCG				
RhM011[1]	Fwd: AAAGACAAGGCGTCCACAAC	20	56	MP_A (Dye-751)	I
	Rev: GGTTATGCTTTGATTAGGCTGG				
RiM019[1]	Fwd: ATTCAAGAGCTTAACTGTGGGC	19	52	MP_B (BMN-5)	I
	Rev: CAATATGCCATCCACAGAGAAA				
RhM001[1]	Fwd: GGTTCGGATAGTTAATCCTCCC	14	51	MP_B (BMN-6)	I
	Rev: CCAACTGTTGTAAATGCAGGAA				
RhM021[1]	Fwd: CAGTCCCTTATAGGATCCAACG	15	50	MP_B (Dye-751)	I
	Rev: GAACTCCACCATCTCCTCGTAG				
RiM036[1]	Fwd: AGCAACCACCACCTCAACTAAT	8	51	MP_C (BMN-5)	I
	Rev: CTAGCAGAATCACCTGAGGCTT				
RhM023[1]	Fwd:CGACAACGACAATTCTCACATT	1	53	MP_C (BMN-6)	I
	Rev: GTTATCAAGCGATCCTGCAGTT				
RhM003[1]	Fwd:CCATCTCCAATTCAGTTCTTCC	10	50	MP_C (Dye-751)	I
	Rev: AGCAGAATCGGTTCTTACAAGC				
RiM015[1]	Fwd:CGACACCGATCAGAGCTAATTC	5	62	MP_D (BMN-5)	I
	Rev: ATAGTTGCATTGGCAGGCTTAT				
RiG001[1]	Fwd:TGTCCGATCCTTTTCTTTGG	4	55	MP_D (BMN-6)	I
	Rev: CGCTTCTTGATCCTTGACTTGT				
Rubus123a[2]	Fwd:CAGCAGCTAGCATTTTACTGGA	25	52	MP_E (BMN-6)	II
	Rev: GCACTCTCCACCCATTTCAT				
Rubus285a[2]	Fwd:TCGAGAAGCTTGCTATGCTG	28	52	MP_E (BMN-5)	II
	Rev: GGATACCTCAATGGCTTTCTTG				
Rubus223a[2]	Fwd:TCTCTTGCATGTTGAGATTCTATT	15	51	MP_F (BMN-5)	II
	Rev: TTAAGGCGTCGTGGATAAGG				
Rubus270a[2]	Fwd:GCATCAGCCATTGAATTTCC	23	51	MP_F (BMN-6)	II
	Rev: CCCACCTCCATTACCAACTC				
Rubus275a[2]	Fwd:CACAACCAGTCCCGAGAAAT	23	51	MP_F (Dye-751)	II
	Rev: CATTTCATCCAAATGCAACC				

[1] Castillo et al. (2010); [2] Fernández-Fernández et al. (2011); [a] *Fwd* forward; *Rev* reverse

(Table 4), whereas in 'Malling Jewel' the 150 bp allele of marker Rubus123a described by Fernández-Fernández et al. (2011) could not be detected (Table 4). The sample of 'Glen Ample' tested in the present study was genetically different from the sample tested by Fernández-Fernández et al. (2011). Differences were found using the markers Rubus123a and Rubus270a (Table 4).

Several SSR markers amplified fragments of very less intensity, which were only hardly detectable. Therefore, these fragments were not counted in this study.

Neighbor joining cluster analysis

Genetic relatedness among the 82 *Rubus* genotypes was examined based on SSR markers. The dendrogram generated from the neighbor-joining cluster analysis enabled us to identify four major groups (Fig. 1).

Table 3 Allelic diversity, expected heterozygosity (H_e), observed heterozygosity (H_o), and polymorphism information content (PIC) for 16 *Rubus* SSR primer pairs in 82 *Rubus* cultivars

Locus	No. of homozygous plants	H_o	H_e	PIC value	No. of alleles	Product range (bp)
RhM043	56	0.293	0.441	0.83	7	345–381
RiM017	44	0.463	0.485	0.056	8	185–206
RhM011	9	0.89	0.873	0.497	20	252–320
RiM019	16	0.78	0.868	0.571	19	162–220
RhM001	42	0.476	0.637	0.129	14	168–264
RhM021	58	0.293	0.542	0.175	15	253–314
RiM036	37	0.549	0.565	0	8	296–316
RhM023	82	0	0	0	1	197
RhM003	9	0.878	0.792	0.199	10	190–219
RiM015	49	0.402	0.509	0	5	348–362
RiG001	61	0.207	0.577	0.444	4	347–350
Rubus123a	14	0.829	0.925	0.728	25	136–257
Rubus285a	11	0.866	0.91	0.656	28	146–236
Rubus223a	29	0.634	0.735	0.404	15	139–176
Rubus270a	9	0.89	0.82	0.308	23	138–234
Rubus275a	4	0.927	0.893	0.575	23	112–182
Average	33.13	0.59	0.66	0.348	14.06	

The first cluster (I) consists of cultivars originating from the Scottish and/or British breeding programs or cultivars which have cultivars of these two breeding programs in their parentage. All cultivars of cluster I are summer-bearing (floricane-fruiting) cultivars labeled with SF. In this cluster a range of cultivars bred in Russia ('Tarusa', 'Patritsiya', 'Zheltyi Gigant'), Ukraine ('Kozachka') and Belarus ('Alyonushka') can be found. However, they all are progenies derived from donors obtained by the Russian breeder Kichina in the 1970's from the East Malling Research Station (Kichina 2005; Kichina et al. 2012). 'Kozachka' and 'Tarusa' are two cultivars of cluster I which are characterized by a dwarf and very compact phenotype, which could become interesting for home gardens and for future breeding activities aiming at mechanical harvesting.

The second cluster (II) included cultivars of the fall-bearing (primocane-fruiting) type (labeled with PF) with some exceptions of summer-bearing cultivars, like 'Tula Magic', 'Tulameen', 'Black Jewel', and 'Meeker'. Many cultivars of this cluster have 'Autumn Bliss' or 'Tulameen' in their parentage.

The third cluster (III) was revealed to be the largest and consists of summer-bearing raspberry cultivars, some primocane-fruiting cultivars like 'Lloyd George', 'Gelbe Siebenkugel', 'September', 'Evrasiya', 'Korbfüller', 'Zefa 3', 'Polana', and 'Immertragende von Feldbrunnen' and a few intermediate summer-fall-bearing types, like 'Lucana' and 'Golden Queen'. It is difficult to divide cluster III into small sub-groups due to less information about the origin of a range of cultivars. However, some relatedness has been shown, i.e. between 'Proma' and 'Schönemann' being the father of 'Proma'; 'Octavia' and 'Glen Ample' being the father of 'Octavia'; 'Multiraspa' and 'Preussen' being the mother of 'Multiraspa'; 'Polana' and 'Zefa 3' being the mother of 'Polana'; and 'Niniane' having 'Rutrago' as mother. It is interesting to note that cluster II also contains the two tayberry (*R. fruticosus* L. × *R. idaeus* L.) cultivars 'Tayberry' and 'Buckingham Tayberry' which represent hybrids between blackberry and raspberry.

The fourth cluster (IV) consists of the blackberry 'Navaho', the interspecific hybrid 'Dorman Red' and the cultivars 'Deutschland 43', 'Latham', 'Royalty',

Table 4 Allele sizes of a set of six red raspberry cultivars obtained with fifteen SSR markers

Locus	Autumn Bliss	Glen Ample	Heritage	Latham	Malling Jewel	Tulameen
RhM043	377	374/377	345/374	374/377	377	377
RiM017	194/195	195	195	194/198	195	195
RhM011*	282/320	287/291	285/293	287/289	287/289	283/291
	277/316	**282/286**	**280/288**	**282/284**	**282/284**	**278/286**
RiM019*	184/220	180	182/184	184/191	180/184	168/184
	185/220	**167/181**	**183/185**	**183/192**	**181/185**	**169/185**
RhM001	239/241	241	239	239	235/237	239/241
RhM021	281	281	281/291	281/285	281	281
RiM036	301/316	314	316	301/314	314	314/316
RhM003*	198/200	198/202	200/206	198/217	198/217	198/217
	196/198	**196/200**	**198/204**	**196/214**	**196/214**	**196/214**
RiM015	350/353	350	350/353	350/353	350	350
RiG001	347	348	348	350	347	347/348
Rubus123a	144/183	148/161	147	169/(253)	149/(253)	144/149
	142/183	**142/146**	**146**	**150/169**	**148**	**142/148**
Rubus285a*	178/186	172/175	172/181	180/184	180/196	178/197
	175/183	**169/171**	**169/177**	**177/181**	**177/193**	**175/193**
Rubus223a*	152/156	155	147/154	139/147	152	152
	148/152	**150**	**143/150**	**134/143**	**148**	**148**
Rubus270a	141/156	156/164	180/(207)	180/(207)	156/186	156
	156	**186**	**181/207**	**181/209**	**156/188**	**156**
Rubus275a*	114/124	130/(182)	114/144	128/130	140/(174)	148/(182)
	116/126	**131/184**	**116/146**	**129/131**	**142/178**	**150/184**

Underlined markers were also used by Fernández-Fernández et al. (2011). Alleles of these markers written in bold were obtained by Fernández-Fernández et al. (2011) using an ABI 3100 prism genetic analyzer (applied biosystems), whereas alleles written in standard style were obtained in this study using an CEQ 8800 Genetic Analysis System sequencer (Beckman Coulter). Alleles written in brackets are sometimes hardly detectable. Their peak intensity is significantly lower compared to the peak of the second allele

* More or less comparable differences were obtained for all alleles of these markers for each cultivar tested except of 'Glen Ample', which seems to be different from the genotype tested by Fernández-Fernández et al. (2011)

'Glen Coe', 'Lowden' and 'Driscoll Maravilla'. All genotypes of this cluster are summer fruiting ones except of 'Driscoll Maravilla'.

Cultivars with yellow and orange fruit color are dispersed in different clusters

Cultivars with yellow fruits are dispersed in three out of the four clusters with 'Zheltyi Gigant' belonging to cluster I, 'Fall Gold', 'Golden Bliss', 'Golden Everest', 'Lumina', and 'Gelbe Sugana' all belonging to cluster II and 'Gelbe Siebenkugel', 'Gelbe Antwerpener', and 'Golden Queen' which are located in cluster III. The two cultivars 'Valentina' and 'Orange Marie' with orange fruit color belong to the clusters I and II, respectively.

Pedigree based evaluation on trueness-to-type

Thirty-nine cultivars were tested on trueness-to-type using pedigree information and the SSR fingerprints of either both parents, the female parent only, the male parent only, and/or some progeny (if available). The results of this investigation are shown in Table 1. Based on DNA fingerprint information, the female parent could be confirmed for 22 cultivars. Six cultivars were found for which the female parent could not be confirmed. The male parent could be

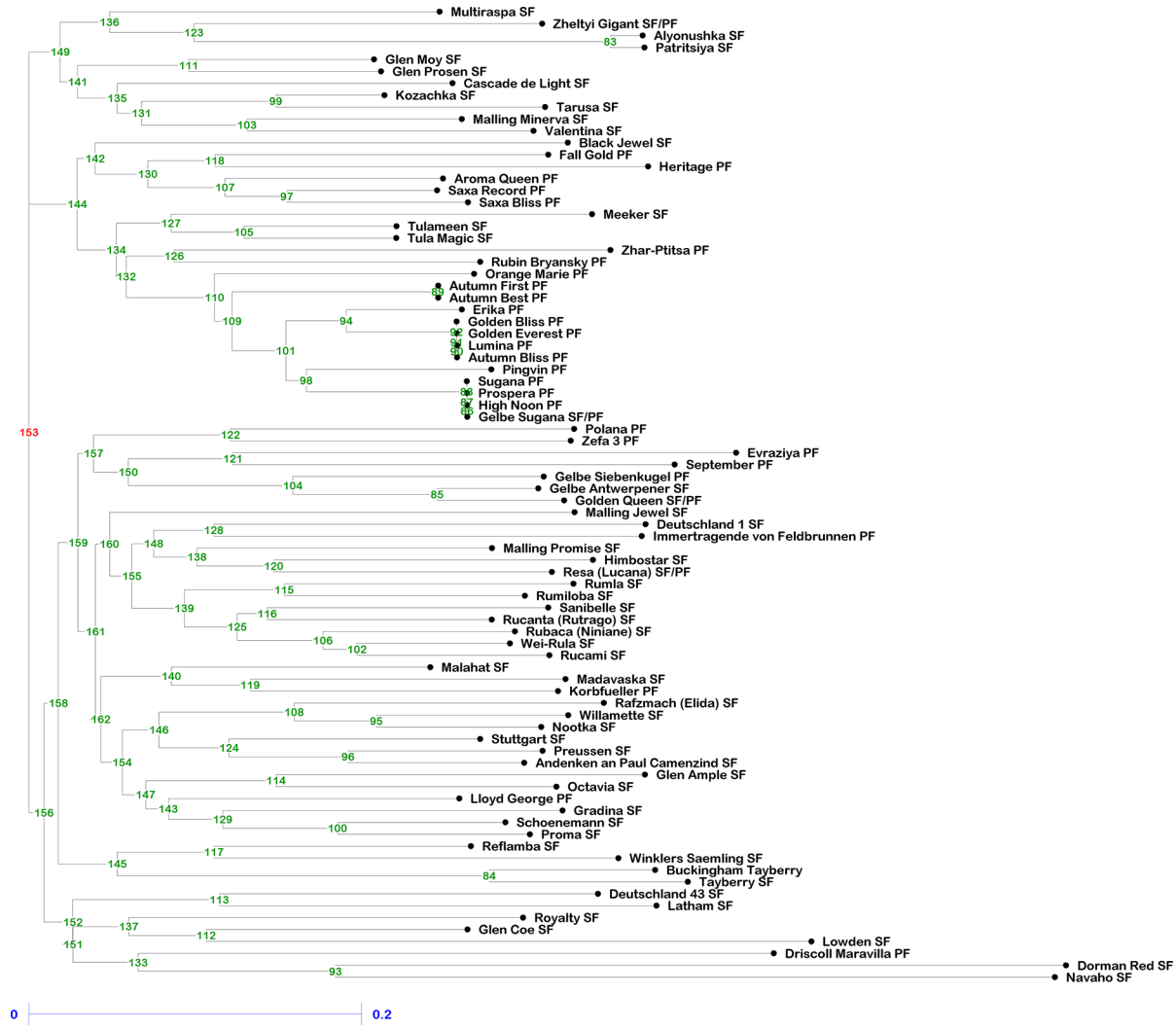

Fig. 1 Neighbor-joining phylogenetic tree of 79 raspberry and 3 blackberry genotypes. The phylogenetic tree was constructed with 1000 bootstrap replicates using the Orchiai's coefficient and genotype data produced by fifteen SSR markers

confirmed for 12 cultivars. For eight cultivars the male parent could not be confirmed. For nine cultivars, both parents could be confirmed. For seven cultivars the fingerprint could be confirmed based on information about some progenies. An example of this investigation is shown in Fig. 2.

'Nootka' and 'Glen Prosen' could be confirmed as parents of 'Tulameen', whereas 'Tulameen' and 'Autumn Bliss' were most likely the parents of 'Erika', 'Sugana', 'Gelbe Sugana', 'Prospera', 'Autumn Best', 'Autumn First', 'High Noon', and 'Tula-magic'. 'Autumn Bliss' could be furthermore confirmed as one parent of 'Golden Bliss', 'Golden Everest', 'Lumina', 'Saxa Bliss', 'Saxa Record', 'Aroma Queen', and 'Pingvin'. Surprisingly, the SSR fingerprints of 'Autumn First' and 'Autumn Best' were identical. Separate fingerprints were also found in 'Sugana', 'Prospera', 'High Noon', and 'Gelbe Sugana'. The three cultivars 'Golden Bliss', 'Golden Everest' and 'Lumina' with yellow fruits showed fingerprints which were identical to that of 'Autumn Bliss' (Fig. 2).

Discussion

In view of re-establishing a raspberry breeding program in Germany, genetic resources of *Rubus*

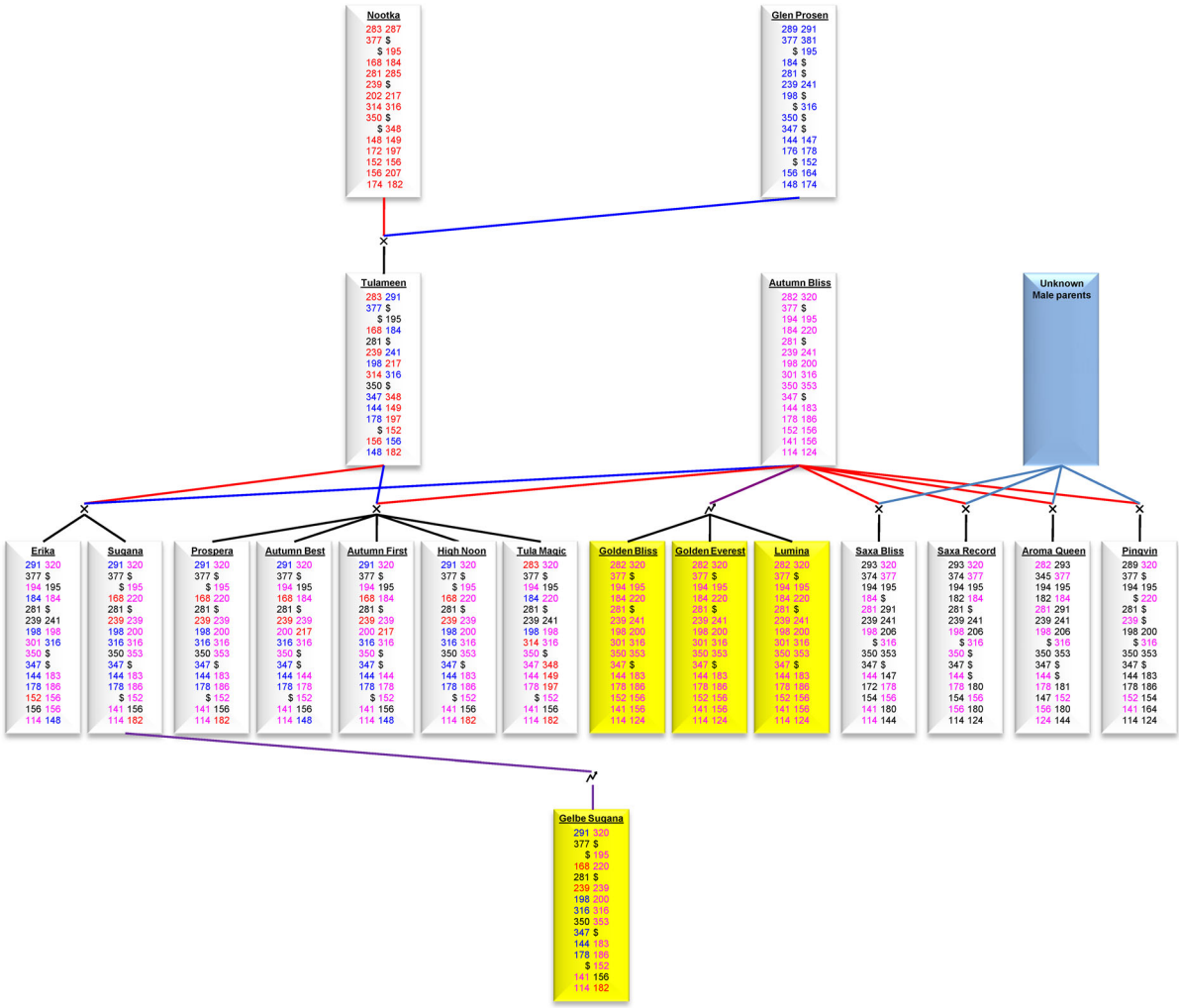

Fig. 2 Pedigree of selected raspberry cultivars validated using SSR fingerprints. Cultivars shown in *white boxes* have *red fruit* and were derived from crosses with known parents or after open pollination. Cultivars presented in *yellow boxes* have *yellow fruit* and seem to be mutants derived from *red fruited* cultivars. Alleles written in the same color (*red*, *blue*, *pink*) originate from the same parent. For alleles written in black it isn't clear yet from which parent they were inherited. The male parent of 'Saxa Bliss', 'Saxa Record', 'Aroma Queen' and 'Pingvin' is unknown (*blue box*). They were derived from open pollination. The cross symbol is used for crosses. The inverted flash is used for selected mutants. The $ symbol is used in cases where it isn't clear whether the allele is in a homozygous state or heterozygous with a null allele. *Red* and *dark blue lines* were used for the female and male parent of crosses, respectively. *Violet lines* were used for cultivars from which the mutants were definitively or most likely derived from. *Light blue lines* were used for the unknown male parent of cultivars derived from open pollination. (Color figure online)

were collected from genebank collections, from nurseries and private home gardeners. Subsequently, all 82 genotypes were evaluated for genetic diversity and trueness-to-type using a set of 16 SSR markers. Eight out of these markers were validated for cross-comparison of our results with those obtained in other studies using a set of six standard genotypes suggested by Fernández-Fernández et al. (2011). Three out of the six reference genotypes could fully be confirmed, two genotypes showed only differences in one allele for one of the eight markers, but one cultivar was completely different. The three genotypes which were true-to-type suggested that the method was successful. However, some problems occurred with the other three genotypes. The occurrence of differences in one allele of a single marker, as found for two genotypes,

could be related to the marker itself or to some technical issues (e.g., different sequencers used, different fluorescence dyes used). These small differences are not problematic as long as the researcher is aware of their existence from the beginning of the study. More problematic are the differences that occurred with 'Glen Ample'. This sample used in our study was completely different from the sample used by Fernández-Fernández et al. (2011). This is unexpected, because different samples of 'Glen Ample' were used in our studies that were retrieved from different international plant retailing companies. Using this type of 'Glen Ample' its parentage could be confirmed in 'Octavia' (Table 1). However, such problems are not surprising since mistakes (e.g. a mix up of the plant material) could occur in these types of studies. Therefore, a validation of markers for cross comparison should always been done using a set of reference genotypes originating from a single source.

Subsequently, all 82 genotypes were tested with the 16 SSR markers of which 15 were polymorphic. Using the data obtained with these 15 markers, a neighbor-joining phylogenetic tree was constructed. The cluster analyses revealed a narrow genetic base of the raspberry genetic resources still present in Germany which is also reflected by the low average PIC value (Table 3) and the low bootstrap values (Fig. 1). This was expected and is in agreement with Dale et al. (1993) who investigated the genetic diversity of 137 red raspberry varieties released throughout the world since 1960. These 137 varieties originated from only 50 founding clones with 'Lloyed George' being in the parentage of 79 % out of these 137 varieties. The missing genetic diversity is expected to deny plant breeders of the possibility to react to future problems, which are predicted as a consequence of the global climate change (Dale 2009; Tuovinen 2009; Krüger 2009). To avoid this problematic situation Dale et al. (1993) suggested four strategies, which include (1) the increase of the number of parents per generation, (2) the introduction of unrelated germplasm from improved sources, (3) the introduction of *R. idaeus* germplasm from wild, and (4) the introduction of germplasm from other *Rubus* species. These four strategies should be adopted by each red raspberry breeder throughout the world (Dale et al. 1993).

Within the 79 raspberry cultivars there were nine cultivars with yellow fruits which were dispersed in three different phylogenetic clusters. Yellow fruited cultivars are frequently obtained as sports from red fruited cultivars or as seedlings from crosses. Selected sports are cultivars like 'Lisa' obtained from 'Meeker', (Nikolić and Milivojević 2008), 'Golden Queen' found in 'Cuthbert' (Szalatnay et al. 2011), 'Golden Bliss' selected from 'Autumn Bliss' (Bundessortenamt 2006), as well as 'Kiwigold' and 'Graton Gold' retrieved from 'Heritage' (Thomas 2000; Dixon 1991). Yellow cultivars originating from crosses, are for example, 'Anne' ('Amity' × 'Glen Gerry', Swartz et al. 1998) and 'Fall Gold' (NH-R7 × ['Taylor' × *R. pungens* var. *oldhamii* Mig.], Dale et al. 1993). Although, the yellow fruit color trait has been frequently described in literature, less is known about the genetic factors controlling this trait. In several studies a recessive allele of a gene called *T* was assumed to be responsible for the yellow fruit color if *T* is homozygous recessive *tt*, or for apricot fruit color in the case of *tt*, but in the presence of a second gene called *P* (Crane and Lawrence 1931). In other studies, a dominant gene called *Y* was described for the yellow fruit character (Jennings and Carmichael 1975). These authors agreed that *T* has a fundamental role in anthocyanin synthesis, whereas *Y* acts at a later stage and cannot be affected by genes which modify the action of gene *T*. Recently a loss-of-function mutation in the anthocyanidin synthase gene was found as the reason for the lack of the red fruit color in some yellow fruited varieties (Rafique et al. 2014). This loss-of-function mutation is in agreement with the findings of a recessive gene which leads to yellow fruits in its homozygous state and with the occurrence of spontaneous sport mutants in red fruited varieties. Three yellow mutants ('Golden Bliss', 'Golden Everest' and 'Lumina') of 'Autumn Bliss' with identical SSR fingerprints were detected in this study. 'Golden Bliss' and 'Golden Everest' were selected as sports, but based on information given by the breeder, 'Lumina' was selected from a cross of 'Autumn Bliss' by 'Tulameen'. However, the fingerprint of 'Lumina' is identical to that of 'Autumn Bliss'. This leads to the conclusion that 'Lumina' is an apomictic offspring of 'Autumn Bliss'. This is not surprising because automixis and apomixes were described severally in *Rubus* (Nybom 1988; Antonius and Nybom 1995; Clark and Jasieniuk 2012). Whether the yellow fruit character of all three cultivars originated from different mutation events in the anthocyanidin synthase gene or not needs to be tested now.

Using a pedigree based evaluation a number of identical genotypes could be identified within the investigated *Rubus* varieties. Examples are the two Swiss primocane-fruiting raspberry cultivars 'Autumn First' and 'Autumn Best'. Based on information directly retrieved from the breeder, these cultivars were selected as full-sib sister seedlings from a large crossbreed population 'Autumn Bliss' × 'Tulameen'. However, both cultivars have identical fingerprints for all of the 16 SSR markers tested in this study. Excluding a mix up of the plant material by the breeder from whom the plants were purchased directly, it seems rather to be the case that one cultivar was selected vegetatively as a best-performer or sport from the other one. This is also assumed for 'Sugana', 'High Noon', and 'Prospera', which also have identical SSR fingerprints. Our hypothesis is supported by the results obtained by a pair-wise comparison of the fingerprints of 'Tulamagic', 'Erika', and 'Sugana', which are all shown to be true full-sib progenies of 'Autumn Bliss' and 'Tulameen'. The fingerprints of these cultivars differ in at least nine to ten alleles for the 16 SSR markers from each of the other fingerprints (Fig. 2). Similar rates (seven to nine different alleles) of different alleles were also found by comparing the fingerprints of the half-sib progenies 'Saxa Bliss', 'Saxa Record', and 'Aroma Queen', which were all retrieved from 'Autumn Bliss' as female parent after open pollination. However, the genetic differences of all these cultivars will now be studied in more detail by using a genotyping-by-sequencing approach, which is a promising strategy for exploring genetic diversity on a genome-wide scale (He et al. 2014).

The results obtained in the present study disclosed the alarming situation of the narrow genetic base of *Rubus* resources still available in Germany. Broadening of this base by introducing new and genetically unrelated breeding material seems to be a necessary prerequisite for the successful re-establishment of a raspberry breeding program.

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Compliance with ethical standards

Conflict of interest The authors certify that they have no conflict of interest with any financial organization regarding the subject matter or materials discussed in this manuscript.

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