Original article

SSR fingerprinting of raspberry cultivars traded in Germany clearly showed that certainty about the genotype authenticity is a prerequisite for any horticultural experiment

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Summary

Raspberry (*Rubus idaeus* L.) cultivars are propagated vegetatively, as many other fruit species from the *Rosaceae* family. During propagation, mistaken identities of cultivars can be caused by mislabelling plants for various reasons. This poses a problem not only for growers who buy cultivars because of specific characteristics (e.g., resistance) but also for breeders who are being deprived of licensing fees for cultivars under Plant Variety Protection (PVP). In this study, six raspberry cultivars of up to six different origins were tested for trueness-to-type by fingerprinting with 16 SSR markers. Nine out of 33 samples turned out not to be true-to-type, seven from online shops and two from nurseries.

Keywords

genotyping, horticulture, Plant Variety Protection, *Rosaceae*, small fruit, trueness-to-type

Significance of this study

What is already known on this subject?

- Red raspberry cultivars are different in their resistance, adaptation to local climate and fruit qualities, hence the importance of trueness-to-type.

What are the new findings?

- The extent of mislabelled plants is higher than previously thought.

What is the expected impact on horticulture?

- Genotyping newly acquired cultivars may be necessary to ensure the validity of horticultural experiments and with planting commercial plots.

Introduction

The evaluation of cultivars on economically important traits represents one of the standard experimental approaches in horticultural science. Results of such evaluation trials are commonly used to recommend cultivars for cultivation in certain regions, for selected cultivation systems or to answer scientific questions in horticultural and breeding research. The benefit of those experiments strongly depends on the experimental design, the methods applied, and the certainty on trueness-to-type of all genotypes used for evaluation. Mix-ups of genotypes which can unintentionally occur and which often remain undetected will inevitably lead to wrong conclusions and, in the worst-case scenario, to fatal consequences.

In practice, nobody would doubt the trueness-to-type of plant material, which is provided by genebanks or commercial plant retailers. However, the results of the present study, which was performed on red raspberry, clearly show that trueness-to-type is never guaranteed and needs to be tested before starting any horticultural experiment.

Red raspberry (*Rubus idaeus* L.), is a temperate small fruit grown both commercially and in private gardens. For commercial fruit production, raspberry plants are usually sold in large quantities by specialized nurseries and plant retailers. The number of those companies is rather small in Europe, as red raspberry does not belong to the leading crops in commercial fruit production. Further reasons for the com-

paratively small number of plant retailers are the limited production area, the low number of cultivars successfully grown in commercial raspberry fruit production, and the expenses that have to be paid to breeders for plant propagation licenses. For private gardens, a wider spectrum of cultivars is sold, including new, but also traditional and locally adapted cultivars. As with many other crops, new alternatives to traditional acquisition from nurseries are appearing in the form of online sources. This is especially the case for raspberry plants sold in small quantities for private use. The latter outlets obtain their propagated plant material from their own nursery or act as re-sellers of material obtained from large nurseries.

Like most fruit crops from the *Rosaceae* plant family, raspberries are propagated vegetatively. Vegetative propagation of raspberries in the field is commonly realised through root cuttings or by root suckers. For root cuttings new canes emerge from shoot buds of root pieces that have been cut off from so-called mother plants and planted. For suckering, canes have already emerged from the shoot buds at the time of propagation (Rieger, 2007). As some raspberry cultivars can generate a prolific number of suckers on their sprawling root system, great care is to be taken to prevent cultivar mix-ups (Janick, 2009).

Trueness-to-type in raspberry cultivation is important for several reasons, including plant health and overgrowth as well as fruit quality and shelf life. For example, plant health can be severely compromised if the acquired genotype's resistance or climate requirements deviate from the intended cultivar's attributes. Additionally, cultivars with a high propensity for suckering can take over genotypes that make less suckers. There were instances of cultivar mix-ups negative-

ly impacting raspberry plant production, for example when 'Glen Shee' was mistaken as 'Glen Ample' in the UK. Another mix-up happened when a 'Meeker' stock was contaminated with 'Willamette' in the Pacific Northwest. Even though the contamination was only 1% it resulted in no certification until it was completely eliminated (Janick, 2009). Finally, as shelf-life can differ amongst cultivars (Haffner et al., 2002), fruit from a mixture of cultivars in one container will limit the storage ability to the fruit with the shortest shelf life. Identification of possible mix-ups by phenotype alone is often difficult since fruits appear on plants months after they have been planted.

DNA-fingerprinting allows the unambiguous identification of cultivars at the vegetative stage by employing molecular markers (Rongwen et al., 1995). Simple sequence repeat (SSR) markers make use of tandem repeat DNA patterns, which are most frequently found in the untranslated regions of eukaryotic genomes and which are prone to mutations (Levinson and Gutman, 1987; Litt and Luty, 1989; Vieira et al., 2016). Therefore, the polymorphic marker patterns of SSRs are ideally suited for genotyping on the cultivar level. DNA fingerprinting of raspberry cultivars using SSR markers was successfully done by Bassil et al. (2012), Bradish et al. (2016), Dossett et al. (2012), and Girichev et al. (2015). Girichev et al. (2015) established DNA fingerprints for 82 *Rubus* genotypes available in German germplasm collections, nurseries and home gardens using a set of 16 commonly used SSR markers. As most of the commercially sold raspberry cultivars were also among the 82 *Rubus* genotypes, the data provided by Girichev et al. (2015) represent a great baseline for cultivar identification and evaluations on trueness-to-type.

To verify the level of cultivars that are sold true-to-type in Germany, samples of six different raspberry cultivars were ordered from a total of 14 different nurseries and online suppliers. These samples were tested using the set of 16 SSR-markers. The DNA fingerprint profiles were compared among the samples, but also with samples, which are known to be true-to-type to prove their cultivar authenticity.

Materials and methods

Plant material

Plants of the six raspberry cultivars 'Glen Ample', 'Meeker', 'Polka', 'Preußen', 'Schönemann', and 'Tulameen' were acquired as potted single plants. A total of five to six samples per cultivar were ordered from seven different nurseries (designated by capital letters: B, E, F, H, J, K, and L) and sev-

en online shops (designated by lowercase letters: a, c, d, g, i, m, and n), respectively. All plants were grown in pots at the experimental field of the Julius Kühn-Institut (JKI), Dresden Pillnitz (Germany). Leaf material of each plant was harvested and used for the isolation of genomic DNA. Leaf samples of plants grown at the German Federal Plant Variety Office in Wurzen, which are known to be true-to-type, were used as positive controls (P). An overview about the plant material used in this study is given in Table 1.

DNA isolation and SSR marker analysis

Young leaf material (0.1 g) was processed with the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol to extract genomic DNA, which was then re-suspended in 50 µL of the elution buffer included in the kit. A working solution was diluted with ddH₂O to 10 ng µL⁻¹ for the use in the polymerase chain reaction (PCR). Sixteen simple sequence repeat (SSR) markers chosen from Castillo et al. (2010) and Fernandez-Fernandez et al. (2011) were divided into six multiplexes (Table 2). Ten ng of genomic DNA was used as template and amplified with the Type-it Microsatellite PCR Kit (Qiagen, Hilden, Germany) in a reaction volume of 10 µL per sample. The concentration for both forward and reverse primers was 0.2 µM for primers with Dye-751, 0.1 µM for primers labelled with BMN-6 and 0.05 µM for primers with BMN-5 fluorescent labelling. All primers were ordered at biomers.net GmbH (Ulm, Germany). The annealing temperature of the multiplexes was A: 57°C, B: 51°C, C: 52°C, D: 57°C, E: 52°C and F: 51°C. The PCR program was as follows: initial denaturation at 95°C for 5 min, 28 cycles of 95°C for 1 min, annealing for 90 s and 72°C for 30 s, with a final extension step of 60°C for 30 min.

The PCR product was diluted 1:20 with ddH₂O and 1 µL was added to 24.9 µL SLS buffer and 0.1 µL 400 bp size standard per reaction in the capillary electrophoresis plate. A drop of mineral oil was added to prevent evaporation. All samples were evaluated with the CEQ 8800 Genetic Analysis capillary electrophoresis system (Beckman Coulter, Krefeld, Germany). The resulting marker profiles were inspected visually through the CEQ 8800 software of the same supplier. Failed reactions were repeated with samples that worked in previous runs as positive controls.

Statistical analysis was conducted with GenAlEx 6.5 (Peakall and Smouse, 2012). Probability of Identity was calculated with 15 marker data from the positive controls, samples with marker profile not fitting their expected fingerprints and 75 cultivars genotyped by Girichev et al. (2015),

TABLE 1. Samples of different raspberry cultivars used in this study. As positive controls, samples of each cultivar were obtained from the Federal Plant Variety Office Wurzen (P). Different nurseries are indicated by capital letters, whereas lowercase letters indicate different online suppliers. The real names of nurseries and online suppliers are not provided to avoid any damage to business. Samples that were found to be wrongly labelled are shown in bold.

Cultivar	Origin			Number of samples ¹
	Wurzen	Nursery	Online shop	
Glen Ample	P	B, F	a, c, d, m	6
Meeker	P	B, E, L	a, c	5
Polka	P	B, H	a, c, n	5
Preußen	P	B , K	a, c, i	5
Schönemann	P	B , J	a, c, g, m	6
Tulameen	P	B, F	a, c, d, m	6
Total				33

¹ Number of individual plant samples obtained from nurseries and online shops (positive controls are not included).

TABLE 2. SSR markers used in this study. The table contains information on the name, the forward and reverse sequences of the primers used for each marker, the linkage group where the marker was genetically mapped and the fluorescent label of each SSR marker used in the multiplex PCR reactions.

Multiplex	Primer	Sequence (5' → 3')	Linkage group	Label
A	RhM043	GGACACGGTTCTAACTATGGCT ATTGTCGCTCCAACGAAGATT	4 ²	BMN-5
	RiM017	GAAACAGGTGGAAGAAACCTG CATTGTGCTTATGATGGTTTCG	7 ²	BMN-6
	RhM011	AAAGACAAGGCGTCCACAAC GGTTATGCTTTGATTAGGCTGG	7 ²	Dye-751
B	RiM019	ATTCAAGAGCTTAACTGTGGGC CAATATGCCATCCACAGAGAAA	5 ²	BMN-5
	RhM001	GGTTCGGATAGTTAATCCTCCC CCAACCTGTTGTAATGCAGGAA	2 ²	BMN-6
	RhM021	CAGTCCCTTATAGGATCCAACG GAACTCCACCATCTCCTCGTAG	5 ²	Dye-751
C	RiM036	AGCAACCACCACCTCAACTAAT CTAGCAGAATCACCTGAGGCTT		BMN-5
	RhM023	CGACAACGACAATTCTCACATT GTTATCAAGCGATCCTGCAGTT		BMN-6
	RhM003	CCATCTCCAATTCAGTTCTTCC AGCAGAATCGGTTCTTACAAGC	2 ²	Dye-751
D	RiM015	CGACACCGATCAGAGCTAATTC ATAGTTGCATTGGCAGGCTTAT	3 ²	BMN-5
	RiG001	TGTCCGATCCTTTTCTTTGG CGCTTCTTGATCCTTGACTTGT		BMN-6
E	Rubus123a	CAGCAGCTAGCATTTTACTGGA GCACTCTCCACCCATTTCAT	6 ¹	BMN-6
	Rubus285a	TCGAGAAGCTTGCTATGCTG GGATACCTCAATGGCTTTCTTG	1 ¹	BMN-5
F	Rubus223a	TCTCTTGCATGTTGAGATTCTATT TTAAGGCGTCGTGGATAAGG	3 ¹	BMN-5
	Rubus270a	GCATCAGCCATTGAATTTCC CCCACCTCCATTACCAACTC	2 ¹	BMN-6
	Rubus275a	CACAACCAGTCCCGAGAAAT CATTTCAATCCAAATGCAACC	5 ¹	Dye-751

¹ Castillo et al., 2010.² Fernandez-Fernandez et al., 2011.

90 samples in all. The neighbour joining phylogenetic tree was calculated with DARwin 6.0.21 (Perrier et al., 2003) with 1,000 bootstrap replicates and the genetic distance data from all 39 samples used in this study.

Results

Using the 16 SSR markers a total of 69 different alleles were detected in this study. The amplified allele sizes ranged between 112 and 377 bp with up to eight alleles per locus (Table 3). Marker RhM023 was monomorphic for all six cultivars. Fourteen markers amplified at least two different alleles within the tested cultivars. Marker RiG001 amplified no fragment for the cultivar 'Meeker', whereas marker RiM036 delivered no fragments with any of the cultivars.

The DNA-fingerprint pattern of all six samples of 'Glen Ample' and 'Meeker' was identical to the pattern of the positive controls. All samples of these two cultivars were true-to-type. Of the remaining 22 samples of 'Polka', 'Preußen', 'Schönemann', and 'Tulameen' only 13 were identical to sam-

ples used as control. Nine samples (Table 3) expressed marker pattern that differed from the pattern expected for the respective cultivar. They were found to be not true-to-type. Seven of these samples were obtained from five different online shops (a, c, d, m, and n), whereas two samples originated from nursery B (Table 3). One sample that was sold as 'Polka' expressed a DNA-marker profile fitting to 'Meeker'. Two samples sold as 'Preußen' expressed DNA-marker profiles fitting to 'Meeker' and 'Tulameen', respectively. Two samples sold as 'Schönemann' showed a DNA-marker profile that is very similar, but not identical to 'Schönemann'. The remaining samples that were found to be not true-to-type had an unknown DNA fingerprint profile.

Probability of Identity is under 0.05 after application of the first four markers (Figure 1). The Neighbour joining cluster analysis shows 'Meeker' having greater genetic distance to the other five cultivars, with these five cultivars being in a cluster together (Figure 2).

TABLE 3. Fragment sizes of the positive control plants (P) and 33 sample plants. Alleles of positive control plants are in bold; fragments not fitting to the pattern of the positive controls have grey background.

Multiplex		MP-A			MP-B			MP-C			MP-D			MP-E			MP-F		
Origin	Cultivar	RhM011	RhM043	RiM017	RiM019	RhM021	RhM001	RhM023	RhM003	RiM036	RiM015	RiG001	Rubus123a	Rubus285a	Rubus223a	Rubus270a	Rubus275a		
P	Glen Ample	287 291	377	195	180 281	241	197	198 202	350	348	148	161	172 175	154	156	164	130 182		
a		287 291	374 377	195	180 281	241	197	198 202	350	348	148	161	172 175	154	156	164	130 182		
B		287 291	374 377	195	180 281	241	197	198 202	350	348	148	161	172 175	154	156	164	130 182		
c		287 291	374 377	195	180 281	241	197	198 202	350	348	148	161	172 175	154	156	164	130 182		
d		287 291	374 377	195	180 281	241	197	198 202	350	348	148	161	172 175	154	156	164	130 182		
F		287 291	374 377	195	180 281	241	197	198 202	350	348	148	161	172 175	154	156	164	130 182		
m		287 291	374 377	195	180 281	241	197	198 202	350	348	148	161	172 175	154	156	164	130 182		
P	Meeker	287	377	195 168	180 281	239	197	202 210	350	362	148	149	172 197	154	177	156	207 114 182		
a		287	377	195 168	180 281	239	197	202 210	350	362	148	149	172 197	154	177	156	207 114 182		
B		287	377	195 168	180 281	239	197	202 210	350	362	148	149	172 197	154	177	156	207 114 182		
c		287	377	195 168	180 281	239	197	202 210	350	362	148	149	172 197	154	177	156	207 114 182		
E		287	377	195 168	180 281	239	197	202 210	350	362	148	149	172 197	154	177	156	207 114 182		
L		287	377	195 168	180 281	239	197	202 210	350	362	148	149	172 197	154	177	156	207 114 182		
P	Polka	291 293 374	377 194	195 182	184 281	239 241	197 198	198	353 348	349 144	148 172	178 152	148 152	148 152	156 181	114 174	174		
a		291 293 374	377 194	195 182	184 281	239 241	197 198	198	353 348	349 144	148 172	178 152	148 152	148 152	156 181	114 174	174		
B		291 293 374	377 194	195 182	184 281	239 241	197 198	198	353 348	349 144	148 172	178 152	148 152	148 152	156 181	114 174	174		
c		287	377	195 168	180 281	239	197	202 210	350	362	148	149	172 197	154	177	156	207 114 182		
H		291 293 347	377 194	195 182	184 281	239 241	197 198	198	353 348	349 144	148 172	178 152	148 152	148 152	156 181	114 174	174		
n		291 310 375	377	195 182	198 281	239	197	198	350	353 348	149 207	175 197	148 168	156	112	176	176		
P	Preußen	287 289	377	195 176	184 281	239 241	197 198	206 217	350	359 348	350 148	255 172	181 152	164	207	124 174	174		
a		283 287	377	195 168	182 281	286	197	202 217	350	349 350	148 172	199 152	156	207	182	182	182		
B		287	377	195 168	180 281	239	197	202 210	350	362	148	149	172 197	154	177	156	207 114 182		
c		283 291	377	195 168	184 281	239 241	197 198	217	350	348 349	144 178	197 152	156	148	182	182	182		
i		287 289	377	195 176	184 281	239 241	197 206	217	350	359 348	350 148	255 172	181 152	164	207	124 174	174		
K		287 289	377	195 176	184 281	239 241	197 206	217	350	359 348	350 148	255 172	181 152	164	207	124 174	174		
P	Schönemann	287 316	377	195 168	184 281	239 241	197 202	206	350	348 350	350 144	167 178	190 152	156	207	130 174	174		
a		287 316	377	195 168	184 281	239 241	197 202	206	350	348 350	350 144	167 178	190 152	156	207	130 174	174		
B		287	377	195 168	184 281	239	197	202 210	350	353	350 353	148 149	172 197	152 154	156	207	124 182		
c		287 316	377	195 168	184 281	239 241	197 202	206	350	348 350	350 144	167 178	190 152	156	207	130 174	174		
g		287 316	377	195 168	184 281	239 241	197 202	206	350	348 350	350 144	167 178	190 152	156	207	130 174	174		
J		287 316	377	195 168	184 281	239 241	197 202	206	350	348 350	350 144	167 178	190 152	156	207	130 174	174		
m		283 287	374 377	195 172	180 281	239 241	197 198	202	350	349 349	144 175	178 152	154	164	207	122 182	182		
P	Tulameen	283 291	377	195 168	184 281	239 241	197 198	217	350	348 349	144 178	197 152	148 168	156	148	182	182		
a		291 310 374	377	195 182	198 281	239	197	198	350	353	348 149	207 175	197 148	156	112	174	174		
B		283 291	377	195 168	184 281	239 241	197 198	217	350	348 349	144 178	197 152	148 168	156	148	182	182		
c		283 291	377	195 168	184 281	239 241	197 198	217	350	348 349	144 178	197 152	148 168	156	148	182	182		
d		283 287	374 377	195 172	180 281	239 241	197 198	202	350	349 350	144 178	175 152	154 164	122	182	182	182		
F		283 291	377	195 168	184 281	239 241	197 198	217	350	348 349	144 178	197 152	148 168	156	148	182	182		
m		283 291	377	195 168	184 281	239 241	197 198	217	350	348 349	144 178	197 152	148 168	156	148	182	182		

are very similar to 'Schönemann', although their marker profile is similar to the positive control, they cannot be a direct progeny due to some of their markers not containing any of 'Schönemann's alleles. The genetic distance of the not true-to-type plants with unknown profiles points to a mix-up with a different cultivar or unknown crossing as well, as they are all not in the near vicinity to their supposed cultivar.

Fourteen polymorphic markers were used for differentiation between cultivars. All seven linkage groups were represented with at least one marker (Castillo et al., 2010; Fernandez-Fernandez et al., 2011). Other cultivar identification studies were conducted with 14 markers in hazelnut (Akin et al., 2016) and in pistachio (Ahmad et al., 2003), in grapevine even 9, although in the latter combined with description and photos (Maul et al., 2015). Although the focus of these aforementioned studies was not the testing of trueness-to-type, sample discrepancies within cultivars were still found in the studies of Maul et al. (2015) and Akin et al. (2016). As red raspberry cultivars have a small genetic diversity (Girichev et al., 2015), not only the number but the extent of polymorphism of the SSR markers is important. As the Probability of Identity is under 0.05 after using only four markers, the 15 markers used in this study should be more than sufficient to ensure reliable cultivar identification.

Dossett et al. (2012) found in their study that several black raspberry cultivars had differing SSR marker profiles even among the same source. Additionally, cultivars 'Jewel' and 'Allen' did not match their reported pedigree. Bassil et al. (2012) reported similar issues with red raspberry cultivars, concluding the different SSR marker profiles stem from somaclonal variation with 'Meeker' and a cultivar mix-up with 'Cuthbert'.

The present study raises awareness of a problem that could be of a bigger extent than previously assumed. At least in some cases Plant Variety Protection Rights could become violated. However, the sampling size of this study is limited and demands further study of the extent of mix-ups in the sale of raspberry cultivars. The tendency of mix-ups was higher in the case of online shops than with the direct sourcing from nurseries. In nurseries, trained personnel can more easily spot a difference in the appearance and thus figuring out a mix-up before a sale can take place. Additionally, the time constraint that a purely online business puts on the shipping process is conducive to errors.

This information could be particularly useful for growers who find trueness-to-type important enough for the higher cost of direct nursery sourcing, where the corresponding training and diligence could prevent more mix-ups than with online shop personnel. Furthermore, this study underlines the necessity of DNA fingerprinting for growers, breeders and propagators alike.

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