The development of molecular genetic tools to improve efficiency in raspberry breeding

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Abstract

Raspberry growing and trade has expanded over the last 20 years, fueled by the increasing demand for this healthy fruit. At the same time, raspberry breeding is challenged by changes in economics, climate and growing technology. In the following thesis, traits important for raspberry breeding were explored through molecular genetic tools, subsequently using this knowledge to develop molecular genetic tools to aid breeding.

As trueness-to-type is very important for growing and breeding clonally propagated crops, the state of the German raspberry trade was explored by genotyping six cultivars from up to six sources with 16 SSR markers for cultivar mismatches. Out of the 33 samples, nine were not true-to-type, indicating an issue of cultivar mix-ups in the German raspberry trade.

Subsequently, a trait relevant to fruit size and quality, self-compatibility, was studied. For this purpose, the progeny of 16 open pollinated cultivars were genotyped with up to 16 SSR markers to determine if they were self- or cross-fertilized. One cultivar, 'Rumla', was found to be highly self-incompatible in both this, and subsequent hand-pollination and topcross experiments. The other 15 cultivars showed more flexibility in tolerating self-fertilization. The existence of self-incompatible cultivars in raspberry, usually considered self-fertile, cautions from using cultivars with unknown self-fertility status as a monoculture in protected growing.

Furthermore, a trait relevant for fungal resistance, waxy bloom, was investigated in an interspecific *Rubus occidentalis* × *R. idaeus* population. The trait was mapped to linkage group 2, corresponding to chromosome 2, in *R. occidentalis* by using SNP markers obtained from Genotyping-by-Sequencing, SSR markers and phenotyping data.

Additionally, the molecular genetic basis of floral development, which is relevant for fruit size and quality traits in raspberry, was studied. First, 82 MADS-box gene candidates were identified in *R. occidentalis* by Hidden Markov Model search. These results were used in primer development to identify and sequence genes in *R. idaeus*. First two *PISITLLATA* homologues, the paralogous genes *RidPI1* and *RidPI2*, then a *LEAFY* homologue with two alleles, *RidLFY-1* and *RidLFY-2*, were found. *RidLFY-2* has a 3.7 Mb transposon inserted into their first intron compared to *RidLFY-1*, and it correlates with a sepaloid flower mutation occurring in a raspberry population.

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Abbreviations

AG	- *AGAMOUS*
AGL24	- *AGAMOUS-LIKE24*
AP1	- *APETALA1*
AP2	- *APETALA2*
AP3	- *APETALA3*
ASK1	- *ARABIDOPSIS SKP-LIKE1*
C-terminal DBD	- C-terminal DNA-binding domain
CI	- competitive interaction
FD	- bZIP transcription factor
FT	- *FLOWERING LOCUS T*
FUL	- *FRUITFULL*
GA	- gibberellin
GBS	- Genotyping-by-Sequencing
GO	- gene ontology
GSI	- gametophytic self-incompatibility
LFY	- *LEAFY*
LG	- linkage group
MAS	- marker assisted selection
miR156	- microRNA156
miR172	- microRNA172
PI	- *PISTILLATA*
pI	- isoelectric point
PoI	- probability of identity
RiSEP	- RiSEP_Him15K7 population
SAM	- Sterile Alpha Motif domain
SC	- self-compatibility
SFB	- *S HAPLOTYPE-SPECIFIC F-BOX*

SFBB	- *S LOCUS F-BOX BROTHER*
SEP1	- *SEPALLATA1*
SEP2	- *SEPALLATA2*
SEP3	- *SEPALLATA3*
SEP4	- *SEPALLATA4*
SI	- self-incompatibility
S-locus	- self-incompatibility locus
SHP1	- *SHATTERPROOF1*
SHP2	- *SHATTERPROOF2*
SLFL	- *S-LOCUS F-BOX-LIKE*
SOC1	- *SUPPRESSOR OF CO-OVEREXPRESSION 1*
SPL3	- *SPOROCYTELESS3*
SPL4	- *SPOROCYTELESS4*
SPL5	- *SPOROCYTELESS5*
SRF-TF	- Serum Response Factor-type transcription factor
STK	- *SEEDSTICK*
T6P	- trehalose-6-phosphate
TAIR	- The Arabidopsis Information Resource
UFO	- *UNUSUAL FLORAL ORGANS*
VIGS	- virus induced gene silencing

1. General introduction

1.1. Economics of raspberry; importance and its influence on breeding

The European red raspberry, *Rubus idaeus* L., is a temperate fruit crop belonging to the Rosaceae family. The Rosaceae family contains many other fruit crops of economic importance in the temperate regions of the world: apple, pear, sweet and sour cherry, peach, plum and strawberry. The global production of raspberry was 822,493 tonnes in 2019 (Fig. 1), with the highest producers being Russia, Mexico and Serbia.

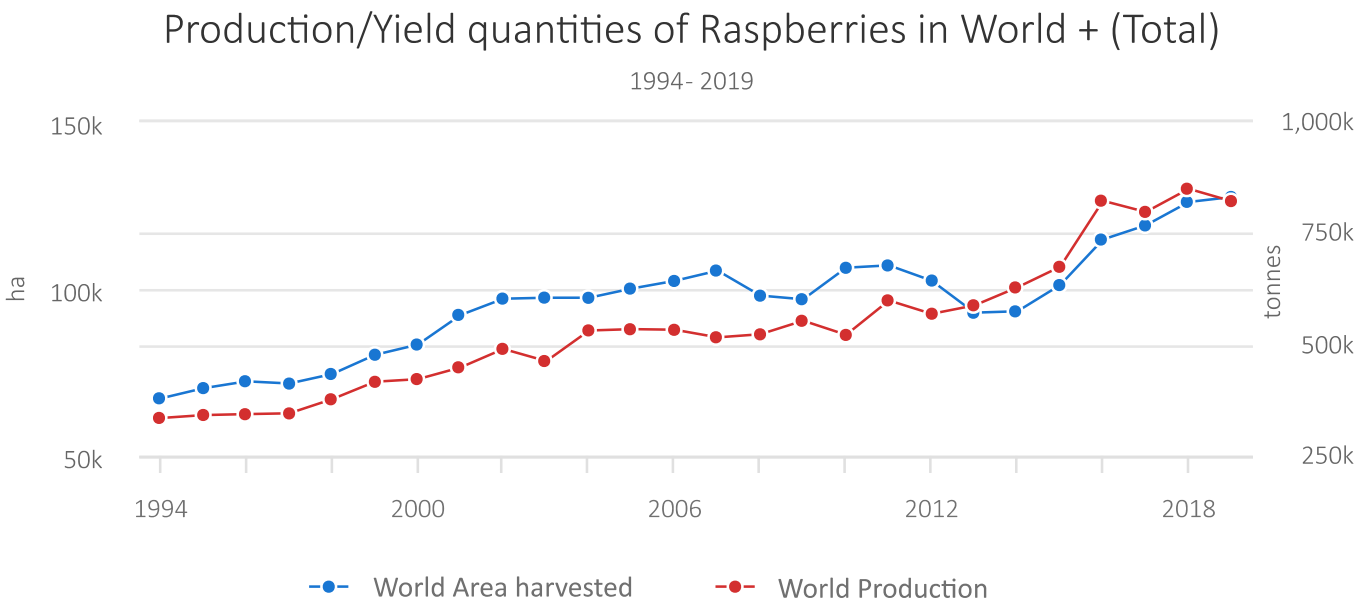

Figure 1.1: Worldwide raspberry production statistics. Harvested area and production between 1994 and 2019, modified from FAOSTAT (2020).

While this may not reach the over 87 million tonnes of apples produced in the same year, raspberries are an important crop worldwide, with production more than doubling in the last three decades (FAOSTAT, 2020). Although raspberry harvesting is more laborious than apple due to the size and fragility of the fruit, its approximately tenfold product price makes it a profitable crop. Germany's raspberry yield was 7540 tonnes in 2019, which made it the 13th country in production worldwide and the sixth country in Europe in that year (FAOSTAT, 2020). Europe, thanks to its favorable climate and lower labor costs in its eastern countries, had

73,8% of the world's raspberry production in the last 25 years, although this has decreased to 66,8% in 2019 (FAOSTAT, 2020).

The change of Europe's leading position in raspberry growing is due to several factors based on changes both in economics and climate (Schmidt and Maack, 2003). Global trade, while opening up new markets and growing regions (Finn and Knight, 2001; Marchi et al., 2019; Oliveira et al., 2001), causes the spread of pests and diseases to previously unaffected regions (Bragard et al., 2018; Calabria et al., 2012; Seemüller et al., 1986; Steffen et al., 2015). At the same time, growing concern from consumers places a large importance on reducing or even completely avoiding chemical plant protection for fresh fruit (Gullino and Kuijpers, 1994; Lehberger and Becker, 2020; Werner and Alvensleben, 1984; Williamson, 2003). The combination of these two factors places a high importance on the development of new cultivars with durable genetic resistances (Ellis, 2002). Climate change and the appearance of new cultivation techniques (Linnemannstöns, 2020; Oliveira et al., 1996), like protected growing or out-of-soil cultivation, are opening up new regions for cultivation. This affects a push for development of cultivars with new, different adaptations. Rising wages make fruit size especially important, as raspberry harvesters are paid an hourly wage, making larger fruit more economically viable, as a higher weight can be harvested in the same time frame. These global developments present increasingly complex requirements for the development of new cultivars.

Many traits have to be present concurrently for a successful new raspberry cultivar: fruit size, taste, good shelf life, plant health and resistance to diseases, the right habitus and sufficient number of young canes, just to name a few. As cultivars are adapted to different growing conditions, climates and disease and pest resistances, planting the right cultivar is of the utmost importance for growers. Similarly, breeders need specific genotypes in directed crossings to obtain progeny with the desired traits. Thus, the most important prerequisite in combining new traits in breeding is to first make sure one has the right genotype. Unfortunately, raspberry cultivars are very difficult to discern on phenotype alone, especially without fruit present. Cultivars can be easily mixed-up during propagation or shipping, which has caused a known issue of not true-to-type cultivars in both red and black raspberry trade

(Bassil et al., 2012; Dossett et al., 2012). Therefore, an exploration of the situation on the German market would be a valuable information to growers and breeders alike.

1.2. The origins of raspberry

The main catalysts for phenotypical similarity among cultivars both relate to raspberry's domestication: its comparatively recent occurrence and specific circumstances. Raspberries are grown as a horticultural crop for only around 500 years. This is a short time compared to wheat or maize, which were traced back to 10,000-6,000 years, or even apples, at least to 3,000 years (Cornille et al., 2019; Doebley et al., 2006). Earlier domestication combined with larger dispersion of species to different parts of the world can cause specialization and the keeping of allele richness through distinct land races with regional adaptations. However, the influence of domestication is a complex issue: it all depends on the size of the founding population and the length of the domestication event itself, as earlier domestication alone can not prevent loss of allelic diversity caused by domestication bottlenecks. For example maize, while very distinct from its wild relatives, has a high genetic diversity, which can explained by a bottleneck of short duration and small size (Eyre-Walker et al., 1998). From the origin and history of raspberry we can infer the duration and size of its bottleneck.

Raspberries were first mentioned by the Greeks around 370 BCE, its area of origin is thought to be in Turkey (Hummer and Janick, 2007). This is reflected by its binomial name, idaeus, referring to Mount Ida in Balikesir province. The name doesn't necessarily mean an exact origin, but rather a general area, as no raspberries were found on Mount Ida by modern botanists (Jennings, 1988). Raspberries were most likely spread across Europe by Roman soldiers; for example, their seeds were found in Roman forts throughout Britain (Jennings, 1988). Dioscorides, a physician in the Roman army wrote about raspberry as a medicinal plant around 65 CE (Hummer and Janick, 2007). From the 16th century onwards, more sources start to mention cultivation in gardens, not just woodland collection of fruit, showing a comparatively recent domestication of the crop (Jennings, 1988). In a 1656 catalogue of plants in Britain, four different raspberry cultivars were mentioned, which indicates that selection has started for the crop (Tradescant, 1656). Spiny and spineless forms were first mentioned by Dioscorides in about 65 CE (Hummer and Janick, 2007), the twice-bearing trait in 1780 by

Richard Weston in Britain (Jennings, 1988). After being brought overseas in the 1700s, raspberry cultivars were enriched by crossings with *Rubus* species native in North America, *R. strigosus*, American red raspberry, and *R. occidentalis*, black raspberry, resulting in hybrid cultivars in both America and Europe (Darrow, 1920; Jennings, 1988).

Although these interspecific crossings created a wider genetic base, only a few cultivars dominated breeding for the last century. Dale et al. (1993) found that of 137 cultivars, the founding clones of the majority can be lead back to 20 cultivars, four of them progenitors for around hundred cultivars each.
One of the most important founding clone, 'Lloyd George', originating from early 20th century Britain, was a donor for multiple traits like fruit quality, yield, primocane fruiting and an aphid resistance (Janick, 2009). The widespread use of a small number of successful cultivars resulted in the establishment of many good traits across all resulting cultivars, but also an overall homogeneity for many other traits. This, which was initially set off by the recent domestication of the species, is the main reason for the long and tight domestication bottleneck for raspberries. Modern raspberry cultivars still have a small genetic diversity (Dale et al., 1993; Girichev et al., 2015).

1.3. The role of self-incompatibility in raspberry breeding and growing

Another factor encouraging the use of the same cultivars in breeding is the high heterozygosity of the species. Raspberry flowers look like typical outbreeding species, their abundant nectar and showy petals attracting pollinators. These facts already hint at raspberries being a typical Rosaceae crop with a self-incompatibility system. Wild *R. idaeus* is self-incompatible (SI), while raspberry cultivars are considered self-compatible (SC), though with cultivar differences reported (Keep, 1968a). Due to the self-compatibility of cultivars and thus no horticultural necessity, *Rubus* self-incompatibility has not been heavily researched. However, even with its SI system inactivated, *Rubus* still carries other methods for impeding multiple inbreeding generations. Thus, raspberries suffer from inbreeding depression, often displaying reduced vigor after only one inbreeding generation (Janick, 2009; Jennings, 1962; Keep, 1968a). The viability of seed from self-pollinations reduces drastically compared to cross-pollination (Crane and Lawrence, 1931). There are several lethal factors that are linked to homozygous

traits, like to the homozygous form of the hairy cane (gene *H*), spininess (gene *S*) and fruit color (gene *T*) as well (Crane and Lawrence, 1931; Jennings, 1967; Lewis, 1939). As a result, raspberry plants are highly heterozygous. This genetic trait carries consequences for the breeding of the species, as crossing two plants with high heterozygosity will deliver a large variety of trait combinations. This necessitates a large number of progeny to obtain a few genotypes with the desired trait combinations, making breeding more complicated and lengthy than species with a higher tolerance for inbreeding and lower heterozygosity.

To understand the underlying processes of inbreeding depression and self-incompatibility in raspberry, it can be placed in context with other species in the subgenus *Idaeobatus* (Tab. 1.1). Wild *R. strigosus*, like wild *R*. *idaeus* is self-incompatible (Keep, 1968a). In contrast, *R. occidentalis* has been most likely self-compatible for many generations in the wild (Jennings, 1988). Raspberry can be easily crossed with *R. strigosus* and *R. occidentalis*, producing fertile progeny (Darrow, 1920). However, *R. occidentalis* has to be used as mother plant in crossings, as there is a unilateral incompatibility present (Keep, 1968a), which can be led back to the self-incompatibility system of Rosaceae.

Table 1.1: Taxonomic classification of *Rubus idaeus* L.

Taxa	***Rubus idaeus***
Kingdom	Plantae
Clade	Magnoliophyta
Clade	Eudicots
Family	Rosaceae
Subfamily	Rosoideae
Genus	*Rubus*
Subgenus	*Idaeobatus*

Self-incompatibility can be categorized as sporophytic, gametophytic and ovarian SI systems. Gametophytic self-incompatibility (GSI) occurs in Solanaceae, Papaveraceae, Poaceae and Rosaceae. Although SI prevents self-fertilization, SI systems recognize incompatibility types

rather than plants recognizing their own pollen. These chemical recognition systems are genetically determined (Charlesworth, 2010).

The basis of function of GSI in Rosaceae are the two different products of a single self-incompatibility locus (S-locus). These products affect one of the two organs in the reproductive system, pollen and the pistil. The pollen specificity is created by one or more F-box genes (Rea and Nasrallah, 2008). The pistil S-locus encodes cytotoxic proteins, ribonucleases, called S-RNases (Golz et al., 1995). These S-RNases degrade pollen tubes in the style germinating from incompatible pollen after pollination.

Many Rosaceae crops have active SI systems, like apples and cherries. Similar to the cultivars of these species, raspberries are propagated clonally; therefore, a functioning SI would mean pollen incompatibility not only from self-pollination, but also from the same cultivar. In general, the S-haplotype of the pollen has to differ from both S-haplotypes of the diploid pistil for a successful fertilization (Franklin-Tong and Franklin, 2003). As such, pollen from different cultivars with the same allele combination of the S-locus would result in incompatible pollination as well. Although the characteristics of the genetic control of SI of *Rubus* remains unclear, the unilateral incompatibility with *R. occidentalis* follows the SI x SC pattern true for most GSI interspecies crossings (Keep, 1968a; Lewis and Crowe, 1958). For this reason it has been presumed that the self-compatibility of *R. idaeus* occurred at a different and more recent time from that of *R. occidentalis* (Keep, 1968a; Lewis and Crowe, 1958). It has also been already suggested that the loss of functionality in raspberry affects only the F-box gene(s), but not the one(s) controlling S-RNase function (Keep, 1968a). Crossing studies done by Keep (1985) on 'Lloyd George' showed that it is heterozygous for self-compatibility. As mentioned above, 'Lloyd George' was used extensively as a crossing partner, therefore it makes sense that most cultivars are self-fertile.

Self-fertility is an important factor in growing as well. Raspberry, although called a berry, is botanically an aggregate fruit, consisting of multiple single fruitlets. The fruitlets themselves are drupes, each single drupelet developing from a carpel. Like many other Rosaceae drupes or achenetums, the fruitlets can only develop from a fertilized carpel, as fruit set is induced by fertilization (Liu et al., 2020). This aggregate fruit structure establishes the two factors

affecting the size of raspberry fruit: the number and size of the individual drupelets. As such, promoting the growth of as many drupelets as possible is essential for raspberry growing. This makes fertilization an important factor for reaching the full fruit size potential of a cultivar. Fertilization is simpler to plan for with self-compatible crops, as flowers can be fertilized by their own pollen without the need of another cultivar as pollen donor. This makes a fully fertilized flower possible even in environments with limited pollen accessibility. As protected growing is increasingly prevalent in raspberry production, the differences in self-compatibility of individual cultivars is crucial information for growers and breeders alike.

1.4. Disease and pest resistance in raspberries

Another ever-evolving trait required from new cultivars is the resistance to diseases. As noted briefly before, globalized trade, although making selling fruit easier, enables the spread of previously unknown diseases and pests to new areas. Concurrently, more and more consumers wish for the use of less chemical plant protection. This puts even more importance on the development of genetic disease and pest resistances.

As mentioned above, many traits have to be present concurrently for a successful new raspberry cultivar. For disease resistance, stacking multiple resistance genes, pyramiding, has the lowest probability of resistance breakdown (Delmotte et al., 2016). As breeding for even one desired trait is difficult due to the heterozygosity of raspberry plants, combining traits presents even more of a challenge.

Common disease resistance mechanisms in plants can be an inducible post-infection plant defense (Andersen et al., 2018), or pre-formed structures and compounds acting as mechanical barriers or natural defense compounds. Cane pubescence, spininess, and waxy bloom on canes all belong to the latter mechanism of resistance. These morphological traits facilitate the water run-off on the cane thus changing the conditions needed for infection of several fungi. Cane pubescence and spininess has been already mapped (Graham et al., 2004, 2006), among other important resistance genes (Table 1.2).

Table 1.2: Overview of resistance genes identified in *Rubus idaeus*. Modified from Janick (2009).

Gene symbol	Gene effect	Reference first description	Reference mapping	Mapped to
B	Waxy cane/lack of bloom on cane; *Elsinoe* avoidance	Jennings, 1962; Keep, 1964, 1968b	Not mapped until now	-
H	Hairy / pubescent cane (*Botrytis, Didymella* avoidance/resistance, susceptibility to cane spot, yellow rust, powdery mildew	Crane and Lawrence, 1931	Graham et al., 2004, 2006	Linkage group 2 (LG2)
Ls	Dominant gene for expression of leaf spot virus angular lesion symptoms	Jones and Jennings, 1980	Raluca et al., 2006	LG2, LG8
Lm	Dominant gene for expression of leaf mottle virus angular lesion symptoms	Jones and Jennings, 1980	Not mapped until now	-
S/s	Spiny, glandular cotyledons/spineless, eglandular cotyledons, confers reduced *Botrytis*, cane blight, and spur blight in 'Glen Moy', 'Glen Prosen'	Haskell, 1960; Jennings, 1962	Graham et al., 2004; Khadgi and Weber, 2021	Gene *H* pleiotropic effects: LG2 – spines LG8 –density/ diameter Gene S: LG4
AB/A_{K4a}	Resistance to *Amphorophora rubi*	Baumeister, 1961; Keep et al., 1980	Not mapped until now	-
A_1-A_{10}/ A_{cor1-2}	Resistance to *A. rubi* strains	Keep, 1989; Keep and Parker, 1976; Knight et al., 1960	Fernández-Fernández et al., 2013; Sargent et al., 2007	LG3 – A_1 LG7 – A_{10}
A_{g1}-A_{g4}	Resistance to *Amphorophora agathonica*	Daubeny, 1966; Keep, 1989	Bushakra et al., 2015	RLG6 – Ag_4
Bu	Resistance to the common strain of RBDV	Jennings, 1980, 1987	Stephens et al., 2016	Putative Bu
I_{am}	Immunity to Arabis mosaic virus	Jennings, 1964	Not mapped until now	-
I_{rr}	Immunity to raspberry ringspot virus	Jennings, 1964	Not mapped until now	-
I_{tb}	Immunity to tomato black ring virus	Jennings, 1964	Not mapped until now	-
Pa	Resistance to *Pucciniastrum americanum*	Jamieson and Nickerson, 1998	Not mapped until now	-
Sp_1Sp_2	Resistance to powdery mildew: *Sphaerotheca macularis*	Keep, 1968b; Keep et al., 1977	Not mapped until now	-
sp_3	Resistance to powdery mildew	Keep, 1968b; Ourecky, 1975a	Not mapped until now	-
Yr	Resistance to yellow rust, *Phragmidium rubi-idaei*	Keep, 1989	Not mapped until now	-

As mapping these important and breeding relevant resistance genes is paramount for easing and shortening breeding through for example marker assisted selection (MAS), adding to the list of already mapped resistance genes is of the utmost importance. This leads us to the best strategy for shortening and simplifying the breeding cycle: to reduce the number of plants to be phenotyped and cared for by pre-screening a large number of seedlings with trait-relevant markers. For the development of such markers, it is necessary to widen our knowledge about the genetics of important traits in raspberry.

1.5. Genetics of floral development

One of the most important trait in raspberry is fruit quality. As floral development directly influences the outcome of fruit growth, understanding flowering, fertility and reproduction is a critical part of breeding research. However, compared to self-incompatible rosaceous crops, the genetics of flowering in raspberries is a neglected area in the field of breeding research.

The first step towards floral development in higher plants is the floral transition that marks the end of the vegetative and the beginning of the reproductive state. To ensure that the plant flowers only at favorable conditions, the required environmental, developmental and physiological cues converge into floral integrator genes like *FLOWERING LOCUS T* (*FT*), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), *APETALA1* (*AP1*) and *LEAFY* (*LFY*) for floral induction (Fig. 1.2) (Blázquez et al., 2006; Wellmer and Riechmann, 2010; Wigge et al., 2005). These genes, in addition to other endogenous signals, then regulate the floral initiation (Quiroz et al., 2021). The subsequent step, flower development itself, is controlled by the interaction of meristem identity genes, cadastral genes and organ identity genes (Weigel and Meyerowitz, 1994).

Meristem identity genes induce the genes responsible for organ identity specification, cadastral genes regulate the spatial expression of organ identity genes, latter in turn directly control organ identity (Weigel and Meyerowitz, 1994). Coen and Meyerowitz (1991) used a series of homeotic mutations in *Arabidopsis thaliana* and *Antirrhinum majus* to develop the ABC model, which describes the interplay of floral organ identity genes necessary for wild-

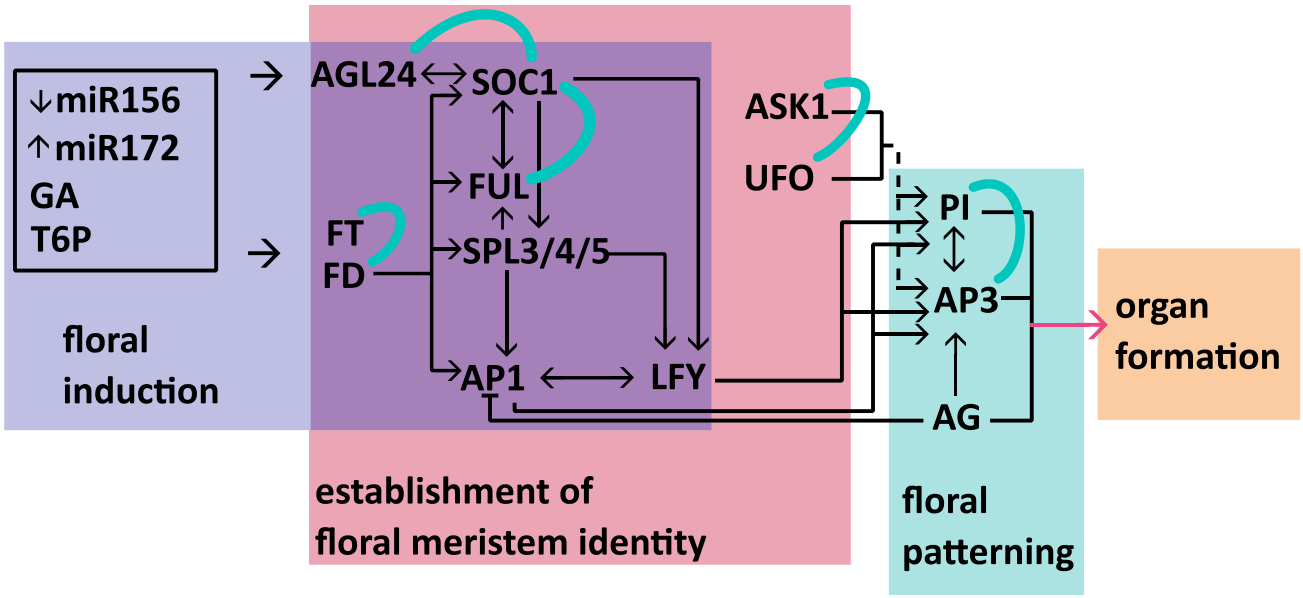

Figure 1.2: Excerpt of the gene regulatory network controlling early floral development. The diagram represents a selection of the key actors involved in floral induction and meristem identity and then focuses on genes involved in or interacting with class B organ identity function. ASK1 and UFO are part of a ubiquitine ligase complex. Black lines represent transcriptional regulation, dashed lines non-transcriptional regulation, arrows positive, squared off lines negative regulation, blue curved lines protein interactions and magenta arrow indicates an interaction with several organ formation genes. The vertical arrows in front of the miRNAs indicate their increase/decrease as signal. Abbreviations: *AG: AGAMOUS, AGL24: AGAMOUS-LIKE24, AP1: APETALA1, AP3: APETALA3, ASK1: ARABIDOPSIS SKP-LIKE1,* FD: bZIP transcription factor*, FT: FLOWERING LOCUS T, FUL: FRUITFULL,* GA: gibberellin*, LFY: LEAFY*, miR156: microRNA156, miR172: microRNA172, *PI: PISTILLATA, SOC1: SUPPRESSOR OF CO-OVEREXPRESSION 1, SPL3/4/5: SPOROCYTELESS3/4/5*, T6P: trehalose-6-phosphate and *UFO*: *UNUSUAL FLORAL ORGANS*. Figure modified from Quiroz et al. (2021) and Wellmer et al. (2006)**.**

type flower development. According to this model, the four whorl regions of a floral primordium have three regions, each for the gene classes A, B, or C. Genes belonging to class A [*AP1, APETALA2* (*AP2*)] are responsible for sepal development alone, class A and B [*PISTILLATA* (*PI*)*, APETALA3* (*AP3*)] genes combined for petal development, class B and class C [*AGAMOUS* (*AG*)] genes together for stamen development and class C gene for carpel development alone (Haughn and Somerville, 1988; Weigel and Meyerowitz, 1994). This model was later extended by homeotic genes belonging to classes D and E (Colombo et al., 1995; Theißen, 2001). Class D genes, *SHATTERPROOF1/2* (*SHP1*, *SHP2*), *SEEDSTICK* (*STK*), are responsible for ovule development (Favaro et al., 2003; Pinyopich et al., 2003). Class E organ identity genes *SEPALLATA1/2/3/4* (*SEP1*, *SEP2*, *SEP3*, *SEP4*) are necessary for flower formation, although they do exhibit functional redundancy among each other (Pelaz et al., 2000). Most

floral development genes belong to the MADS-box gene family, i.e. they all share a conserved DNA binding and dimerization domain (Schwarz-Sommer et al., 1990). Through this domain, MADS-box proteins can influence the transcription of target genes by binding to specific DNA motifs, in form of multimeric protein complexes (Riechmann et al., 1996; Shore and Sharrocks, 1995). More recent evidence reveals that these transcription complexes provide the molecular basis for the combinatorial interaction of the floral homeotic genes (Theißen et al., 2016; Theißen and Saedler, 2001). According to this model of floral quartets, the identity of the five floral organs is determined by organ-specific tetrameric complexes formed of SEP proteins in combination with their respective proteins of the classes A, B, C and/or D (Immink et al., 2009; Melzer et al., 2009; Theißen et al., 2016; Theißen and Saedler, 2001).

Meristem identity genes control the genes responsible for organ identity, for example class B genes are induced by the interaction of *LFY*, *ARABIDOPSIS SKP-LIKE1* (*ASK1*) and *UNUSUAL FLORAL ORGANS* (*UFO*) (Weigel and Meyerowitz, 1994; Wellmer et al., 2006). A mutation in any of these genes results in a changed floral phenotype as well (Bowman et al., 1989, 1991). For example, a flower phenotype without petals or stamens would occur if the function of a class B gene or one of its regulators was disturbed. A sepaloid mutation like this was shown to be caused by the loss of function mutation of the class B gene *PI* in apple (Yao et al., 2001). *PI* is a single copy gene in *A. thaliana*, apple and peach (Goto and Meyerowitz, 1994; Yao et al., 2018; Zhang et al., 2008), while *Fragaria vesca* has two and *Rosa chinensis* has multiple homologues (Davis et al., 2010; Liu et al., 2018). All species above, except for *A. thaliana*, belong to *Rosaceae*. At this time, no floral homeotic genes have been identified in *R. idaeus* as well as no genomic information is available online from a peer-reviewed source. However, its close relative, *R. occidentalis* presents a similar genome (VanBuren et al., 2016). As such, black raspberry can be explored for analogous genes for the European red raspberry, as its genomic information is accessible online.

1.6. Objectives

The manuscripts in the following chapter present results on the development of molecular genetic tools to address the issues in modern raspberry breeding discussed above. As such,

they focus on traits which are important in the changing landscape of raspberry growing and trade: adaptability to new growing techniques, resistance to diseases and pests, fruit quality and yield.

As the most important requirement in breeding is the combining of specific traits, using the right genotype is essential. Previous studies found cultivar mix-ups in otherwise phenotypically similar plants. A large extent of cultivar mismatches would warrant extra caution for breeders and growers alike, as required traits could not be guaranteed without cultivar identification testing. The trait of adaptability to new growing techniques is gaining importance due to the appearance and expansion of protected growing in Europe. Because of the restricted pollen availability in this cultivation form and due to the connection between fruit quality, yield and full fertilization, the previously unexamined self-fertility status of raspberry cultivars is coming into focus. Resistance traits have always been important for raspberry, with several resistance genes already mapped for both post-infection plant defenses and morphological traits. Waxy bloom changes the water run off on canes, disrupting ideal infection conditions for some fungal diseases. Gene *B*, responsible for this trait, has not been mapped until now. Finally, similarly to other Rosaceae crops grown for their fruit, the traits of yield and fruit quality are inexorably connected to floral development in raspberry. Despite this interest, this fundamental area in the field of raspberry breeding, the genetic basis of the floral development have not been dealt with in depth. With these facts in mind, this thesis aims to answer the questions below to advance raspberry breeding.

1. What is the extent of cultivar mismatches present in raspberry trade in Germany?
2. Are there self-incompatible raspberry cultivars that would pose a problem in protected growing?
3. What is the map location of gene *B*, which is responsible for waxy bloom, and can it be mapped in a *R. occidentalis* x *R. idaeus* interspecific hybrid population with the use of Genotyping-by-Sequencing (GBS) data?
4. Which gene is responsible for a sepaloid flower mutation found in a raspberry population and can *R. occidentalis* floral development genes be identified and then used for finding the mutated gene in *R. idaeus*?

2. Manuscripts and publications

2.1. 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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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 comparatively 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 seven 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 ddH_2O 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 ddH_2O 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	4[2]	BMN-5
		ATTGTCGCTCCAACGAAGATT		
	RiM017	GAAACAGGTGGAAAGAAACCTG	7[2]	BMN-6
		CATTGTGCTTATGATGGTTTCG		
	RhM011	AAAGACAAGGCGTCCACAAC	7[2]	Dye-751
		GGTTATGCTTTGATTAGGCTGG		
B	RiM019	ATTCAAGAGCTTAACTGTGGGC	5[2]	BMN-5
		CAATATGCCATCCACAGAGAAA		
	RhM001	GGTTCGGATAGTTAATCCTCCC	2[2]	BMN-6
		CCAACTGTTGTAAATGCAGGAA		
	RhM021	CAGTCCCTTATAGGATCCAACG	5[2]	Dye-751
		GAACTCCACCATCTCCTCGTAG		
C	RiM036	AGCAACCACCACCTCAACTAAT		BMN-5
		CTAGCAGAATCACCTGAGGCTT		
	RhM023	CGACAACGACAATTCTCACATT		BMN-6
		GTTATCAAGCGATCCTGCAGTT		
	RhM003	CCATCTCCAATTCAGTTCTTCC	2[2]	Dye-751
		AGCAGAATCGGTTCTTACAAGC		
D	RiM015	CGACACCGATCAGAGCTAATTC	3[2]	BMN-5
		ATAGTTGCATTGGCAGGCTTAT		
	RiG001	TGTCCGATCCTTTTCTTTGG		BMN-6
		CGCTTCTTGATCCTTGACTTGT		
E	Rubus123a	CAGCAGCTAGCATTTTACTGGA	6[1]	BMN-6
		GCACTCTCCACCCATTTCAT		
	Rubus285a	TCGAGAAGCTTGCTATGCTG	1[1]	BMN-5
		GGATACCTCAATGGCTTTCTTG		
F	Rubus223a	TCTCTTGCATGTTGAGATTCTATT	3[1]	BMN-5
		TTAAGGCGTCGTGGATAAGG		
	Rubus270a	GCATCAGCCATTGAATTTCC	2[1]	BMN-6
		CCCACCTCCATTACCAACTC		
	Rubus275a	CACAACCAGTCCCGAGAAAT	5[1]	Dye-751
		CATTTCATCCAAATGCAACC		

[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 samples 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**				**353**	**348**	**349**	**144**	**148**	**172**	**178**	**148**	**152**	**156**	**181**	**114**	**174**
a		291	293	374	377	194	195	182	184	281		239	241	197	198				353	348	349	144	148	172	178	148	152	156	181	114	174
B		291	293	374	377	194	195	182	184	281		239	241	197	198				353	348	349	144	148	172	178	148	152	156	181	114	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				353	348	349	144	148	172	178	148	152	156	181	114	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
P	Preußen	**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**
a		283	287		377		195	168	182	281	286	239		197	202	217		350		349	350	148	149	172	199	152		156	207		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	149	178	197	152		156		148	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
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
P	Schönemann	**287**	**316**		**377**		**195**	**168**	**184**	**281**		**239**	**241**	**197**	**202**	**206**		**350**		**348**	**350**	**144**	**167**	**178**	**190**	**152**	**154**	**156**	**207**	**130**	**174**
a		287	316		377		195	168	184	281		239	241	197	202	206		350		348	350	144	167	178	190	152	154	156	207	130	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	144	167	178	190	152	154	156	207	130	174
g		287	316		377		195	168	184	281		239	241	197	202	206		350		348	350	144	167	178	190	152	154	156	207	130	174
J		287	316		377		195	168	184	281		239	241	197	202	206		350		348	350	144	167	178	190	152	154	156	207	130	174
m		283	287	374	377		195	172	180	281		239	241	197	198	202		350			349	144	161	175	178	152	154	164	207	122	182
P	Tulameen	**283**	**291**		**377**		**195**	**168**	**184**	**281**		**239**	**241**	**197**	**198**	**217**		**350**		**348**	**349**	**144**	**149**	**178**	**197**	**152**		**156**		**148**	**182**
a		291	310	374	377		195	182	198	281		239		197	198			350	353	348		149	207	175	197	148	168	156		112	174
B		283	291		377		195	168	184	281		239	241	197	198	217		350		348	349	144	149	178	197	152		156		148	182
c		283	291		377		195	168	184	281		239	241	197	198	217		350		348	349	144	149	178	197	152		156		148	182
d		283	287	374	377		195	172	180	281		239	241	197	198	202		350		349	350	144	161	178	175	152	154	164		122	182
F		283	291		377		195	168	184	281		239	241	197	198	217		350		348	349	144	149	178	197	152		156		148	182
m		283	291		377		195	168	184	281		239	241	197	198	217		350		348	349	144	149	178	197	152		156		148	182

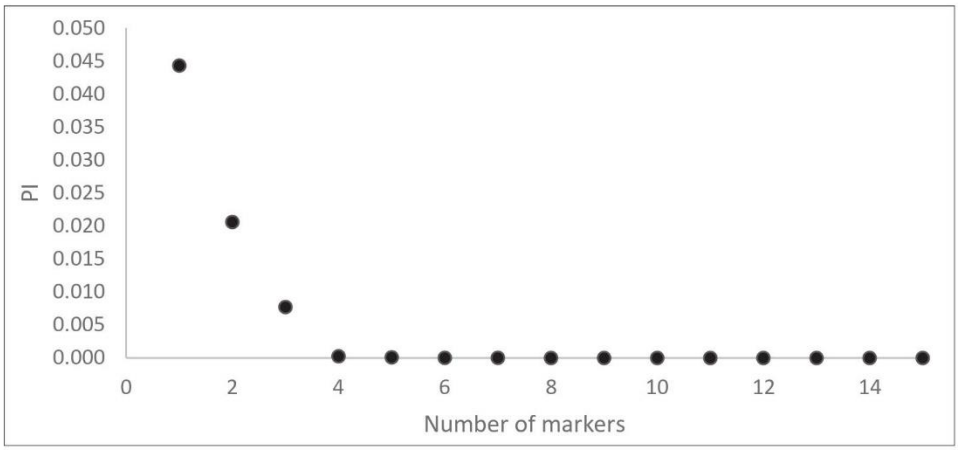

Figure 1. Probability of Identity (PI) of markers applied on positive control plants, plants that were not true-to-type and 75 cultivars, published by Girichev et al. (2015). PI was calculated from the allele frequency of 15 markers on 90 samples.

Discussion

As cultivar identity is an important issue for nurserymen, plant retailers, fruit growers, and horticultural scientists, 33 samples of six different raspberry cultivars were ordered from different nurseries and online shops and tested using a set of 16 SSR-markers on trueness-to-type. In parallel samples of five cultivars which originated from the collection of the Federal Plant Variety Office were also tested. As plants of this collection are assumed to be true-to-type, these samples were used as control. DNA fingerprints of the control samples were compared to fingerprints published recently by Girichev et al. (2015). Surprisingly, only three out of the five control samples amplified marker profiles that were identical to the fingerprints of the published study. For the cultivar 'Polka' no published fingerprint data was available for comparison. 'Glen Ample', 'Schönemann', and 'Tulameen' were fitting the published fingerprints, although some few marker alleles were shifted by 1 bp. The differences found for 'Meeker' and 'Preußen' suggest that the samples genotyped by Girichev et al. (2015) were not true-to-type. This is supported by the fact that Girichev et al. (2015) failed to confirm the fingerprints of these two cultivars by proving their parent-offspring relationships.

Subsequently, all 33 samples of the commercially sold plants were tested. Whereas 24 samples appeared to be true-to-type, nine samples were found to be wrongly labelled. Their DNA-marker profiles differed from the profiles expected for the cultivars whose names were mentioned on the label provided along with the given plant samples. Those differences in the DNA-marker profile can stem from cultivar mix-ups, spontaneous mutations or technical errors during analysis. As all of the differing marker profiles detected in this study had more than one marker disparity, spontaneous mutation is unlikely the source of mismatch. As for technical errors, results could be interpreted wrong when a primer has lost its fluorescence, as it happened with RiM036 in this study. However, as the differences to the expected alleles are not of this nature, technical error as cause can be excluded as well.

On this account an unwanted mix-up that has possibly occurred during propagation or shipping is the most likely reason. A mix-up with a completely different cultivar could occur during both stages. Canes of a different cultivar could grow through from a neighboring row and thus be collected with the wrong cultivar during propagation. Mistakes during propagation in vitro are also possible. A mix-up during shipping could stem from mislabelling of packed plant material. The remaining unknown profiles, together with the 'Schönemann'-like profiles did not match any of the cultivars with available SSR-marker fingerprints (Girichev et al., 2015). They could originate from a cultivar not in that list or a progeny of a crossing. In the case of the two plants which

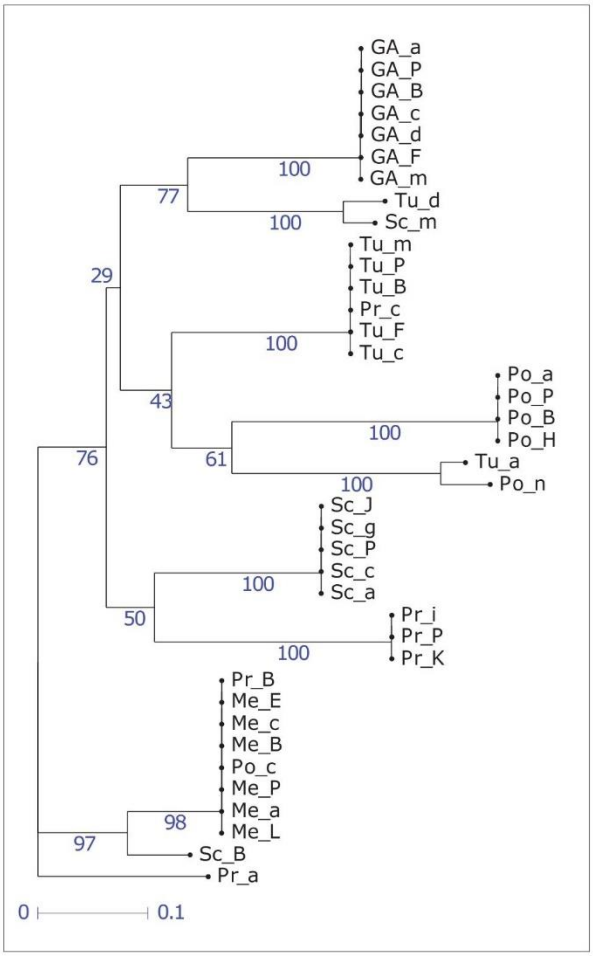

Figure 2. Neighbour joining phylogenetic tree of positive control plants (P) and 33 sample plants. The dendrogram was calculated from the genetic distance of 39 samples using allele sizes of 15 loci. GA: 'Glen Ample', Me: 'Meeker', Po: 'Polka', Pr: 'Preußen', Sc: 'Schönemann', Tu: 'Tulameen'.

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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Manuscript II

2.2. Self-incompatibility of raspberry cultivars assessed by SSR markers

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Contribution to the publication	Planned and performed all experiments, material preparation, data collection and analysis, wrote the manuscript and prepared all tables and figures.
Contribution of the other authors	Marcel von Reth collected plant material and supervised parts of the research. Henryk Flachowsky and Magda-Viola Hanke contributed in conception and planning of the experimental design, Henryk Flachowsky in revision of the manuscript.

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Self-incompatibility of raspberry cultivars assessed by SSR markers

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ABSTRACT

Self-incompatibility in European red raspberry (*Rubus idaeus* L.) is less studied compared to other horticulturally relevant rosaceous crops, although cultivars of this species show differences in self-fertility. In this study, we genotyped progenies of 16 open pollinated raspberry cultivars with SSR markers to determine their natural propensity for self- and cross-fertilization. In further experiments, we genotyped progenies of selected cultivars from a topcross environment and studied seed set after hand pollination. A wide spectrum of cross-fertilized progeny ratio was discovered among the cultivars ranging from 5% of 'Dorman Red' progeny to 100% of 'Rumla' progeny derived by cross-fertilization. This was consistent with results obtained by hand pollination, where a significantly higher number of seed was produced in self-pollinated fruit of 'Dorman Red' and cross-pollinated fruit of 'Rumla'. The difference was particularly large in 'Rumla'; its self-pollinated fruit developed 10.95 drupelets per fruit on average, almost seven times less than its cross-pollinated fruit. The cultivar 'Rumla' showed 100% cross-fertilized progeny in a topcross environment as well, in contrast to the cultivars 'Lucana' and 'Preußen', which both had no cross-fertilized progeny. The results of this study show that there are differences in fertilization behavior between raspberry cultivars. Such information on the fertilization behavior of selected cultivars is useful in planning for cultivar selection in protected growing, where pollination is of special consideration.

1. Introduction

European red raspberry, *Rubus idaeus* L., is an important small fruit crop traditionally grown in temperate regions. Raspberry production worldwide had an increasing trend from 370,000 t in 1998 to over 870,000 t in 2018 (FAOSTAT, 2020). Protection from pests and diseases, the expansion to previously unsuitable climates and a desire to further increase yield has introduced new growing environments and technologies, for instance protected growing (Darnell et al., 2006; Marchi et al., 2019; Palonen et al., 2017). Fertilization has always been an important factor in raspberry fruit production. Drupelets of the raspberry aggregate fruit only develop if their corresponding carpel is pollinated. However, incomplete fertilization results in quality flaws e.g. crumbly fruit, and ultimately low yield. The recent expansion of protected growing has increased the importance of fertilization in raspberry production especially as these environments come with restricted pollen availability. Thus, self-incompatibility of cultivars will pose a serious concern in protected environments.

Self-incompatibility is well known in different species of the Rosaceae plant family. However, raspberry cultivars are believed to have gained self-fertility during their domestication (Jennings, 1988). Nevertheless, modern raspberry cultivars have a complex ancestry with one or more wild raspberry species (e.g. *R. idaeus, R. strigosus* and *R. occidentalis*) in their pedigree. Wild *R. idaeus* and *R. strigosus* are self-incompatible (Keep, 1968), whereas *R. occidentalis* in the wild is most likely self-fertile (Jennings, 1988). *R. arcticus*, which is also a wild relative of *R. idaeus* that is native to North Eurasia and North America, has partially self-fertile populations (Tammisola and Ryynänen, 1970). It is therefore expected that modern cultivars from such a complex ancestry may differ in self-fertility. However, previous works focused on only a few cultivars (Daubeny, 1971; Keep, 1968). Although most raspberry cultivars are considered self-fertile (Keep, 1968), several traits associated with self-incompatibility could still be found in some cultivars. Furthermore, many cultivars suffer from inbreeding depression (Jennings, 1962; Keep, 1968). Nevertheless, these cultivars look like typical outcrossing species, with prominent flowers producing ample nectar that attracts pollinators. The floral structure of raspberries allows for both self- and cross-pollination, with the androecium surrounding the gynoecium and many stamens directly touching carpels (Delaplane and Mayer, 2000).

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Beside fruit production, knowledge about the predominant type of fertilization of raspberry cultivars is also of importance for breeding. Breeders aiming to establish bi-parental populations require information on incompatibility between cultivars in order to determine the number of crosses to reach a sufficient amount of seed. Using SSR markers to assess the progeny of open pollinated cultivars as a method for fertilization type preference has other advantages as well. This genotyping dataset could also be used to discover possible apomicts among the progeny. Apomixis occurs regularly in many species of the genus *Rubus*, although it is rare in the subgenus *Idaeobatus* (Weber, 1996). These rare occurrences has been reported in polyploid *R. strigosus* and a progeny from an interspecific pollination of *R. idaeus*, but not in diploid *R. idaeus* (Antonius and Nybom, 2004; Pratt et al., 1958). Apomictic progeny could be an alternative and economic means of propagation instead of root cuttings or suckers.

Until recently, the effect of the type of pollination and fertilization on fruit quality had been unclear. A few studies explored this issue through the analysis of fruit set and fruit size differences in self- and cross-pollination by hand (Żurawicz, 2016; Żurawicz et al., 2018). These studies showed that cross-pollination increases raspberry fruit size and the number of drupelets. Furthermore, the studies described differences in cross-pollinating efficiency between different cultivars. The authors concluded that cross-fertilization is essential for high yield and improved mass of each berry. As raspberry cultivars are clonally propagated, fertilization between different plants of the same cultivar is in fact self-fertilization. This becomes especially important with cultivation under high tunnels for early harvest, where predominantly single cultivars are grown (Żurawicz et al., 2018).

The present study is a preliminary attempt to examine if there are self-incompatible raspberry cultivars that could pose an issue for growers in a protected environment. We used molecular markers to prescreen 16 raspberry cultivars to ascertain raspberry cultivars with full or partial self-incompatibility. To determine if there is a difference in the extent of self- and cross-fertilization, fruits of these 16 raspberry cultivars grown in an experimental field trial were collected after open pollination. A range of variability was observed between the self-fertilization ratios of cultivars. In an effort to investigate whether the two cultivars on the opposite spectrum of self-fertilization ratio retained their observed differences, their seed set was evaluated after hand-pollination. Finally, a topcross environment was set up in the greenhouse with bumblebees as pollinators to examine if cultivars maintain their low self-fertilization ratio in a restricted pollen environment.

2. Material and methods

2.1. Plant material

Fruits were collected from 16 raspberry cultivars (Table 1) that were open pollinated in an experimental field in Borthen, Germany (lat 50.968778, long 13.826466), where pollinators had access to a wide range of different *Rubus* genotypes. The experimental field was established for cultivar evaluation. Fifty-six raspberry genotypes (cultivars and breeding clones) were grown in a randomized block design consisting of at least two blocks per cultivar with 20 plants per block. The plants were planted 0.3 m from each other initially, with no additional space between blocks and 3 m distance between rows. This field trial was surrounded by a commercial raspberry production field where different standard varieties (mainly 'Tulameen' and 'Glen Ample') were grown. Additionally, the commercial field consisted of a few blocks of different blackberry cultivars and in close distance to the field were hedges where wild raspberries and blackberries were present. The fruits were collected randomly through a cultivar block and from the plant itself and then pooled between the two blocks. Seeds were extracted with a household hand blender and dried over calcium chloride. 200 seeds per cultivar were scarified and stratified according to the protocol of Jennings and Tulloch (1965). After the appearance of the first leaves, 20 seedlings per cultivar were selected randomly from the germination tray. Additional 20 seedlings were selected for the cultivars 'Dorman Red' and 'Rumla', making it a total of 360 progenies. Leaf samples of each plant were sampled for DNA fingerprint analysis. Leaf samples of plants grown at the German Federal Plant Variety Office (Wurzen, Germany), which are known to be true-to-type, were used as positive controls for the female parents. These plants were chosen for positive control as a concurrent test of the mother cultivar plants for trueness-to-type.

2.2. Seed set experiment in the greenhouse

'Dorman Red' and 'Rumla' plants grown in 25 l pots were placed in the greenhouse. Their flower buds were emasculated by removing the anthers using a scalpel before flowers opened. The pollen from these anthers was harvested. Two days after emasculation, open flowers were hand pollinated with pollen of their own or the cultivar 'Tulameen'. Pollinations were repeated until carpels turned brown. Drupelets were counted on harvested ripe fruit.

2.3. Topcross experiment

One plant each of the cultivars 'Lucana', 'Preußen' and 'Rumla', growing in 25 l pots, were placed at 1 m distance from each other in the greenhouse pre-bloom with a commercial box of bumblebees (*Bombus terrestris* L.) mini hive for 100 m^2 for cross-pollination (Katz Biotech AG, Baruth, Germany). Fruits were harvested 33 days after start of bloom. Seeds were treated as described above to germinate progenies. Leaf samples of 20 randomly selected seedlings per cultivar were collected for DNA analysis.

2.4. DNA isolation and SSR marker analysis

DNA was extracted from 0.05 g of young leaf materials using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. For marker analysis, 18 simple sequence repeat (SSR) markers (Castillo et al., 2010; Graham et al., 2002, 2004) were used in this study. Of these 18 markers, 15 were previously used by Girichev et al. (2015) and Pinczinger et al. (2020a) as well as five by Pinczinger et al. (2020b). The remaining three SSR markers, Rub244a, Rubus2a and Rubus12a were developed by Graham et al. (2004, 2006). To optimize marker information output, multiplexes were arranged individually for the progeny group of every cultivar with markers that were polymorphic for the cultivar in previous experiments by Girichev et al. (2015) and Pinczinger et al. (2020a). Two to four markers were used per multiplex in one PCR reaction (File S1). All samples were visualized and evaluated with the CEQ 8800 Genetic Analysis capillary electrophoresis system (Beckman Coulter, Krefeld, Germany) and the results were analyzed through the CEQ 8800 software of the same supplier. The resulting peaks were assessed visually. Failed reactions were repeated with samples that worked in previous runs as positive controls.

2.5. Data analysis and statistical analysis

Results of the extent of cross-fertilization of the 16 cultivars were presented with the software R (R Core Team, 2017). Statistical significance of the seed set experiment was analyzed by one-way ANOVA followed by a Tukey HSD multiple pairwise-comparison using the software R (R Core Team, 2017). For a descriptive statistical analysis, allele and genotype frequencies as well as Hardy-Weinberg distribution of 38 self-fertilized 'Dorman Red' progenies was investigated with Genepop version 4.7.5 (Raymond and Rousset, 1995; Rousset, 2008). Chi square test was calculated with the following equation:

Table 1
Allele sizes of the 16 raspberry cultivars used as positive controls for the SSR marker analysis of open pollinated progenies obtained with up to 18 SSR markers. Markers with no allele listed were not tested on the cultivar or progeny group in this study, as they delivered monomorphic results in previous studies for the cultivar. Underlined markers did not fit the progenies' allele sizes. gray markers were tested on the female parent, but not used in the evaluation of the progenies because of weak signal strength in samples. The SSR markers were developed by: [1]Castillo et al. (2010), [2]Graham et al. (2004), [3]Graham et al. (2002) and [4]Graham et al. (2006).

	RhM011[1]	RhM043[1]	RiM017[1]	RiM019[1]	RhM021[1]	RhM001[1]	RhM003[1]	RiM036[1]	RiM015[1]	RiG001[1]	Rubus 123a[3]	Rubus 285a[2]	Rubus 223a[3]	Rubus 270a[2]	Rubus 275a[2]	Rubus 2a[2]	Rubus 12a[2]	Rub 244a[4]
Dorman Red	280 284		204 206	172 212	283 295		198 202	306 309	350 356		169 181	181 206	139 145	176 234	134 171			
Elida	283 287	377	195	168 182	281 285	239	202 217		350	349	148 150	172 199	149 152	156 207	176	159 166	143 148	148 170
Glen Ample	287 291	374 377	195	180	281	241	198 202		350	348	148 161	172 175	155	156 164	130 182	157 166	102 148	157
Lucana	289 291	374 377	195	180 182	281	239 241	206 217		350	347	144 148	172 178	154 156	142 180	114 144			
Malling Promise			195	180 184	281	239 241	206 210	314 316	350 353		149 253	180 197	154 170	156 164	130 182			
Meeker	287	377	195	168 180	281	239	202 210	314 316	350 362		147 149	172 196	154 176	156	114 182	146 184	130 143	156
Oktavia	283 287	374 377		172 180	281	239 241	198 202	314 316		348	144 161	174 178	152 154	164 207	122 182			
Preußen	287 289		195	176 184		239 241	198 217		350 359		144 149	172 180	152 154	164 207	148 174	166 182	130 143	147 168
Royalty	287 291	377	192 195	184	281	239 241	210 219	314 316	350	347 350				156 180		164 166	102 125	154 170
Rumla				168 195	281	239 241	198 206			348 350	147	172 213	154	156 164	124 130	159 177	125	150 157
Rutrago			194 195	168 184	281 285	239	200 206	234 316	350		149 245	189 197	147 154	156 207	130	157 162	102 125	148 155
Sanibelle			194 195	195 197	281 285	239	198 206	314 316	350		149 245	188 196	152 154	156 160	130	159 165	102 125	149 155
Schönemann	287 316	377		168 184	281	239 241	202 206			347	144 167	178 190	152 154	156 207	130 174			
Tulameen	283 291	377		168 184	281	239 241	198 217	314 316		347 348	144 149	178 197		156	148 182	165	130 143	147 170
Valentina	282 289	374 377	195 198	180	281 291	239 241	198	314	350	348		172 178	139 152	186 207	114 182			
Willamette			195	168 184			202 210		350 353		148 149	172 197	154 156	156 207	124 182	157 159	143 148	148 156

$$\chi^2 = \sum_{i=1}^{n} \frac{(O_i - E_i)^2}{E_i}$$

Where O_i is the number of observations of type i, E_i the expected count of type i and n the number of genotypes.

The SSR fingerprints and familial relationships of the 'Rumla' progenies originating from the topcross experiment were visualized with Pedimap version 1.2 (Voorrips, 2004) and visually enhanced with Inkscape version 0.92 (Inkscape Project, 2017) and Adobe Illustrator 25.0.1 (Adobe Inc., 2020).

3. Results

3.1. Pre-screening to identify genotypes differing in their fertilization propensity

Genotyping the 320 progenies originating from open pollination of 16 different raspberry cultivars (20 progenies per cultivar) resulted in large differences for cross-fertilization ratio between the cultivars. The cross-fertilization ratio varied between 100% obtained for 'Rumla' and 5% obtained for 'Dorman Red'. To confirm these differences, 20 additional seedlings of 'Rumla' and 'Dorman Red' were genotyped. The genotyping results of all 360 progenies are shown in Fig. 1. Even after adding more progenies, 'Dorman Red' had the lowest cross-fertilization ratio with all but two progenies out of 40 (5%) resulting from self-fertilization. 'Rumla' retained its high cross-fertilization ratio as well with all 40 progenies resulting from cross-fertilization (100%). There were 213 out of 360 progeny classified as resulting from cross-fertilization. These progenies had between 1 and 10 marker alleles originating from their paternal parent, on average 4.11 paternal alleles per plant. Although the marker allele patterns were analyzed for possible apomicts, no apomictic seedling was found among any of the 360 progenies. A plant was determined to be an apomictic candidate if all its marker alleles matched its maternal parent. The self-fertilized progeny had between one and nine alleles missing compared to their maternal parent, representing 4.9 alleles per progeny on average.

3.2. Association between seed set and pollen parent

The highly differing cross-fertilization propensity of 'Dorman Red' and 'Rumla' was further analyzed by hand pollination in a controlled environment with their own pollen and pollen of the cultivar 'Tulameen'. Plants of each cultivar ('Dorman Red' and 'Rumla') were grown in the greenhouse. The flowers opened incrementally on the mother plants, accumulating to 40 flowers on 'Dorman Red' and 164 flowers on 'Rumla'. Hand pollination was done with the aim to obtain an equal number of self- and cross-pollinated flowers for each cultivar. The seed set was determined by counting the number of drupelets per fruit. The seed set of the fruit among all progeny groups was significantly different following ANOVA (Table 2). After performing a post-hoc Tukey

Table 2
Seed set of two hand pollinated raspberry cultivars. The first cultivar name denotes the female parent, whereas the second cultivar name denotes the male parent. The arithmetic mean was calculated from single drupelets on n number of hand-pollinated flowers. The software R was used to calculate the one-way ANOVA.

	'Dorman Red' x 'Dorman Red'	'Dorman Red' x 'Tulameen'	'Rumla' x 'Rumla'	'Rumla' x 'Tulameen'
Drupelets per fruit, mean	32.11	25	10.95	76.45
N	19	21	98	66
Standard deviation	10.08	9.08	15.84	23.92
One-way ANOVA		$p < 0.0001$		

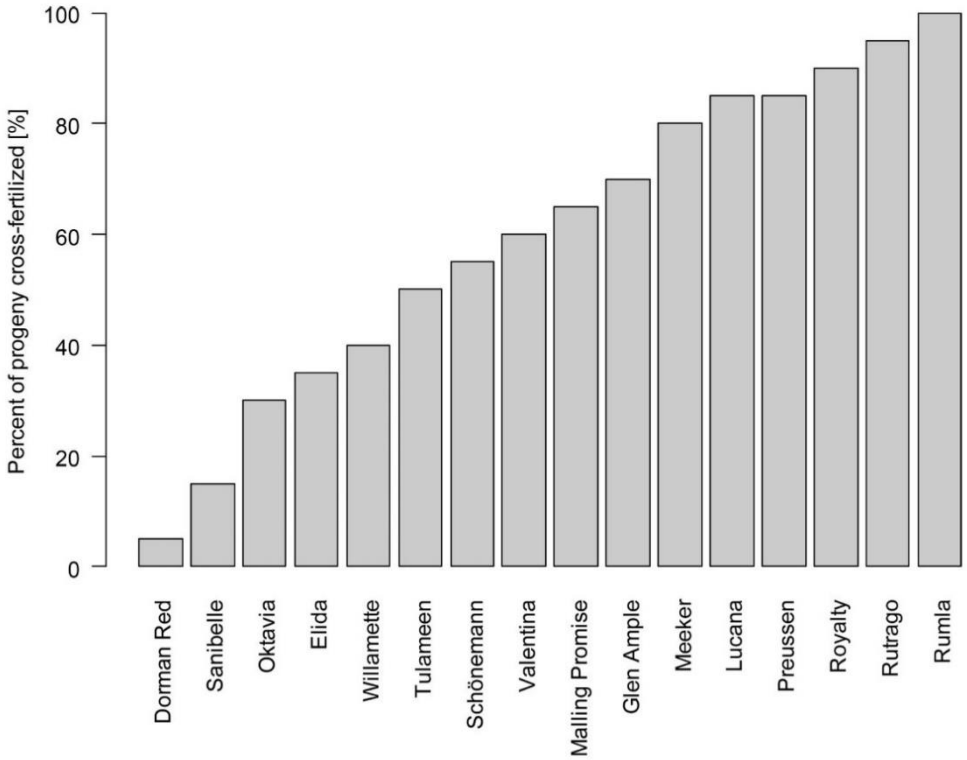

Fig. 1. The ratio of cross-fertilized progeny of 16 open pollinated raspberry cultivars identified by SSR fingerprinting. The percentage was calculated from 20 progenies per cultivar except for 'Dorman Red' and 'Rumla'. For these two cultivars, 40 progenies each were genotyped. Progenies with marker alleles exclusively from its female parent were classified as self-fertilized.

HSD multiple-comparison, the seed set of 'Rumla' was significantly higher in cross-pollinated than in self-pollinated fruit ($p < 0.01$), confirming the results from the field experiment that the cultivar 'Rumla' is self-incompatible. Such cultivars are only suitable for protected cultivation to a limited extent, as suitable pollinator cultivars have to be planted as well. In contrast, differences between the seed set of 'Dorman Red' fruit was not significant (Table 2). This shows that the cultivar 'Dorman Red' accepts foreign pollen but can also be self-pollinated in shortage situations. Such varieties are very suitable for protected cultivation. No seed set or fruit development was obtained for 18 flowers of 'Rumla', of which 16 flowers were self-pollinated and two flowers were cross-pollinated. No failure in seed set and fruit development was found for 'Dorman Red' irrespective of the pollen source.

3.3. Heterozygote deficiencies calculated from genotype frequencies of self-fertilized progenies

Hardy-Weinberg equilibrium (HWE) test was used to assess allele frequencies of the 11 polymorphic SSR markers in the 38 'Dorman Red' progenies originating from self-fertilization in the field. Two markers, RiM017 and RhM021, showed a statistically significant deviation from HWE, with both markers showing heterozygote deficiency (Table 3). However, the remaining markers fit HWE. When allele distribution fits the Hardy-Weinberg equilibrium, an undisturbed inheritance of the alleles is assumed, as was the case for most of the markers used.

3.4. Verifying the fertilization behavior of selected genotypes in a topcross environment

The cultivars 'Lucana', 'Preußen' and 'Rumla' were chosen for the topcross experiment since all three showed high cross-fertilization rate in the open pollination environment. However, their cross-fertilization rate in the artificial topcross environment only partly corresponded to the results obtained after open pollination. All 'Rumla' progenies were cross-fertilized. This is consistent with the results obtained for progenies originating from open pollination. Based on the SSR marker data, 19 out of the 20 'Rumla' topcross progenies matched to the SSR marker data of 'Preußen' and one to 'Lucana' as the male parent (Fig. 2).

In contrast, 'Lucana' and 'Preußen', which both had 85% cross-fertilized progenies after open pollination, had no progenies resulting from cross-fertilization in the topcross environment. It is unclear whether the lack of cross-pollination of these two varieties is due to preferences of the bumblebees or other reasons. However, it is certain that the cultivars 'Preußen' and 'Lucana' are not self-incompatible.

4. Discussion

There is little known about the specifics of self-incompatibility (SI) in *Rubus idaeus* cultivars. However, it is known that some cultivars are able to self-fertilize and seed set is often improved by cross-pollination (Keep, 1968; Żurawicz, 2016). The results of this study provide evidence for the existence of a mechanism for self-incompatibility in red raspberry. At least the cultivar 'Rumla' was found to be self-incompatible to a large extent. The self-incompatibility of this cultivar was demonstrated in three independent experiments with (i) seedlings obtained after open pollination in the open field, (ii) seed set obtserved after hand pollination in the greenhouse, and (iii) seedlings derived from a topcross experiment. The other 15 cultivars used in this study showed a variance in their self-fertilization ratios after open pollination in the field. The causes for this variance can be genetic or purely coincidental. The approach used for pre-screening, especially the limited number of seedlings tested, is not suitable for making statistically significant statements about fertilization behavior of different cultivars. However, this pre-screening was shown to be suitable to identify genotypes that may differ strongly in this trait. The existence of self-incompatibility in red raspberry is important, as a sufficient amount of self-fertility is necessary in modern raspberry production, where protected growing limits possible pollen sources and thus requires planning for cultivar compatibility.

Reports about the self-fertility of raspberry cultivars differ, with Keep (1968) seeing it as a rule, whereas Daubeny (1971) found a lack of full self-fertility among cultivar groups from certain origins. There is quite a few evidence of gametophytic self-incompatibility mechanism in raspberry consisting of a pollen S-gene and a stylar S-RNase (Franklin-Tong and Franklin, 2003; Keep, 1968). Studies on the inheritance of self-compatibility posited that 'Lloyd George', a main founder for many cultivars and ancestor of 87% of all European and American raspberry cultivars (Dale et al., 1993), is heterozygous for a mutated pollen S-allele (Keep, 1968; Lewis, 1940). It is therefore plausible that this mutated S-allele is widespread in raspberry cultivars.

Our findings reinforce the results of Żurawicz et al. (2018) on self-fertilization. The authors reported a mean number of seeds per fruit in self-pollinations of 19.43 (39%), 26.40 (52%) and 33.70 (57%) for 'Glen Ample', 'Schönemann' and 'Willamette', respectively. The ratio of self-fertilized progeny of these cultivars in our study was 30%, 45% and 60%, respectively. Although the percentages do not match perfectly, the order of cultivars remains the same. Since the number of progenies tested was comparably low and the seeds were retrieved from open pollination in the field, other factors (pollinator behavior, weather conditions and differences in flowering time) could also have influenced the amount of self- and cross-fertilization. In order to minimize these factors, a follow-up hand-pollination and topcross experiment was done on selected cultivars.

In all three pollination experiments, the cultivar 'Rumla' consistently showed a significant impediment in self-fertilization and preference to cross-fertilization. Although there were some fertilized drupelets in the seed set experiment, pseudo-self-compatibility has been proposed to be the cause of seed set in self-incompatible pairings (Keep, 1968). Surprisingly, only one out of 20 'Rumla' progeny had 'Lucana' as the male parent in the topcross experiment. Further work would be needed to

Table 3
Allele and genotype frequencies, Hardy-Weinberg distribution and chi square test of 38 'Dorman Red' progenies originating from self-fertilization in the open field.* significant effect, Hardy-Weinberg frequency does not fit at 0.05 significance level for 1 df if chi square test is over 3.84.

	Allele frequency		Genotype frequency							
Marker	A	B	AA observed	AA expected	AB observed	AB expected	BB observed	BB expected	Hardy-Weinberg distribution	X^2 test
RhM011	0.645	0.355	18	15.680	13	17.640	7	4.680	0.154	2.7138
RiM017	0.408	0.592	13	6.200	5	18.600	20	13.200	0.000	20.9052*
RiM019	0.592	0.408	14	13.200	17	18.600	7	6.200	0.738	0.2893
RhM021	0.776	0.224	28	22.813	3	13.373	7	1.813	0.000	24.0614*
RhM003	0.474	0.526	7	8.400	22	19.200	9	10.400	0.514	0.8301
RiM015	0.461	0.540	6	7.933	23	19.133	9	10.933	0.325	1.5944
Rubus123a	0.500	0.500	9	9.123	19	18.753	9	9.123	1.000	0.0066
Rubus285a	0.500	0.500	9	9.373	20	19.253	9	9.373	1.000	0.0587
Rubus223a	0.697	0,.303	17	18.373	19	16.253	2	3.373	0.443	1.1259
Rubus270a	0.461	0.540	6	7.933	23	19.133	9	10.933	0.325	1.5944
Rubus275a	0.526	0.474	11	10.400	18	19.200	9	8.400	0.752	0.1525

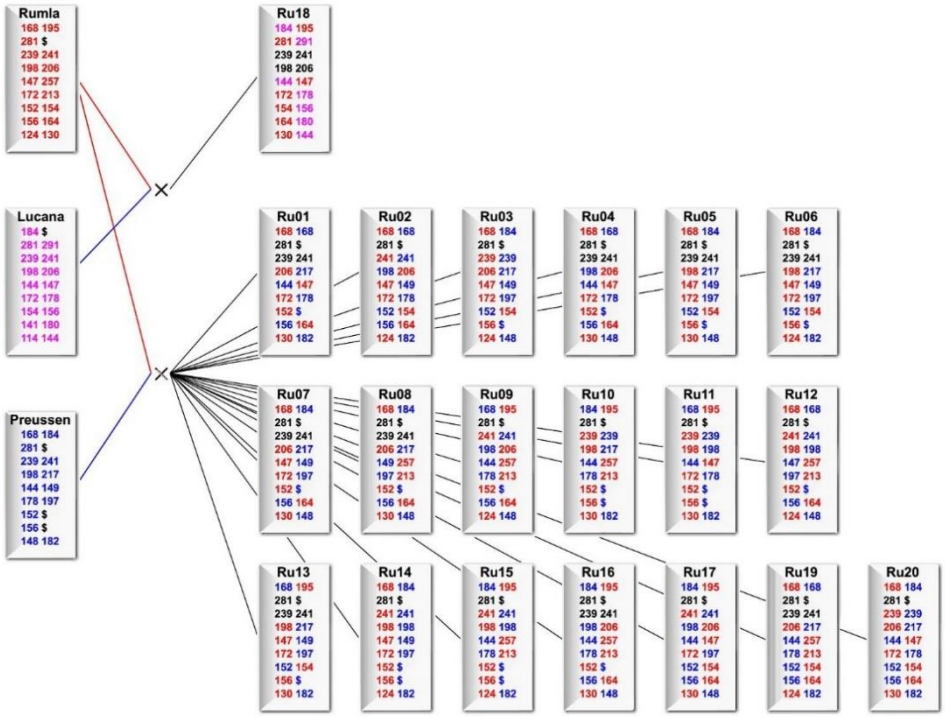

Fig. 2. SSR fingerprints of the 'Rumla' progenies originating from the topcross experiment. The alleles of the progenies are indicated with different colors according to the parent the progeny originates from. The alleles inherited from the female parent, 'Rumla', are depicted in red, the alleles from the male parents 'Lucana' and 'Preußen' in pink and blue color, respectively. The alleles where the parental source is undetermined are depicted in black. The '$' symbol indicates an allele combination that can be homozygous or heterozygous with a null allele. The crosses depict a crossing event between parents, where the red lines connect to the female parent, the blue lines to the male parent and the black lines to the progenies.

determine if this is due to the small sample size or a pollen incompatibility between 'Rumla' and 'Lucana'. Hand-pollination experiments with different raspberry cultivars combined with a pollen tube growth analysis could further elucidate the incompatibility of 'Rumla'.

'Lucana' and 'Preußen' did not retain their high cross-fertilizing ratio in the topcross experiment. This suggests that only 'Rumla' has a genetic cause for its high cross-fertilizing ratio. The other two cultivars can self-fertilize in a pollen-restricted environment. Interestingly, genotyping 'Lucana' and 'Preußen' progeny revealed no cross-fertilized progeny at all. There can be various reasons for this, including cultivar-specific incompatibility, random occurrence of this distribution due to an insufficient sample size, or cleistogamy.

Results of the seed set experiment suggest that 'Dorman Red' has no significant preference to self-fertilization. 'Dorman Red' has a floral morphology conducive for self-pollination, as it has small flowers half the size of other raspberry cultivars with petals curved inwards. The petals close off the flower to foreign pollen from pollinators for days while containing ripe pollen. This could explain why all but two out of 40 of 'Dorman Red's progenies were self-fertilized.

During marker analysis, a progeny sample was identified as cross-fertilized if one of their two alleles was different from their female parent cultivar's in at least one marker. However, there were samples with discrepancies in both alleles. As results stayed the same in replications, technical error can be excluded. There were two types of these unexpected marker results, the first where the marker alleles of the whole progeny group do not fit both of the female parent cultivar's. This occurred with five markers in 'Preußen' and 'Lucana' (Table 1). As the samples used for positive controls were taken from the Federal Plant Variety Office in Wurzen and not from the original field the fruit was collected from, a cultivar mix-up, spontaneous mutation or somaclonal variation could have occurred with the maternal parent in the field. This is a documented occurrence in raspberry, with several publications reporting that commercially sold cultivars are not true-to-type (Bassil et al., 2012; Pinczinger et al., 2020a). Furthermore, there are raspberry cultivars with multiple well-known types, like 'Schönemann' with types 'Kraege', 'Meyer' and 'Penkhues', which originated from different propagators.

The second marker scenario we observed involved one individual progeny not having alleles of the female parent cultivar. This was the case in seven progenies; one in 'Dorman Red', two in 'Rumla', one in 'Rutrago', one in 'Tulameen' and two in 'Willamette'. This type of discrepancy could be explained with a spontaneous mutation, which is common in raspberry, with an occurrence of visible mutation of 0,05% in a planted field reported by Janick (2009). As SSR markers are in untranslated regions of tandem repeats, slippage is easier to occur than in translated regions, causing a size difference in the marker (Kalia et al., 2011). The seven marker discrepancies we report here represent 0.129% of the 5440 marker alleles evaluated. This can still fall under the species-specific high mutation rate if the properties of SSR markers are considered. Nevertheless, the presence of these mutation discrepancies should be noted for further studies on raspberry progeny pools.

As more of raspberry production is moving into protected growing in Europe, the tolerance for self-fertilization of individual cultivars becomes an economically relevant question. According to the findings of this study, there are raspberry cultivars with higher self-compatibility, which could be used as mono-cultivars in protected growing environments. If cultivars with lower self-compatibility were chosen based on other traits, a mix of cultivars would be advised to guarantee better seed set.

Availability of data and material: File S1: table of the different multiplex PCRs with their individual SSR marker combinations used for each 16 cultivar progeny groups. Any other specific data not in the article is available from the corresponding author on reasonable request.

Author contribution statement

HF, M-VH and MvR contributed to the study conception and design. MvR collected plant material. Material preparation, data collection and analysis were performed by DP. The first draft of the manuscript was written by DP and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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2.3. Mapping of the Waxy Bloom Gene in 'Black Jewel' in a Parental Linkage Map of 'Black Jewel' × 'Glen Ample' (*Rubus*) Interspecific Population

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Type of authorship: First author

Type of article: Research article

Contribution to the publication: Planned and performed all phenotyping, planned and performed genotyping with SSRs, wrote parts of the manuscript and prepared some of the figures.

Contribution of the other authors: Marcel von Reth supervised part of the research and performed some analyses of the population. Jens Keilwagen and Thomas Berner extracted putative genes in the waxy bloom physical interval on the *Rubus* V3.0 genome. Andreas Peil performed SSR and marker analyses. Henryk Flachowsky contributed in conception and planning of the experimental design, supervised the research and revised the manuscript. Ofere Francis Emeriewen performed SSR genotyping, analyses, mapping and wrote the manuscript.

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Mapping of the Waxy Bloom Gene in 'Black Jewel' in a Parental Linkage Map of 'Black Jewel' × 'Glen Ample' (*Rubus*) Interspecific Population

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Abstract: Black and red raspberries (*Rubus occidentalis* L. and *Rubus idaeus* L.) are the prominent members of the genus *Rubus* (Rosaceae family). Breeding programs coupled with the low costs of high-throughput sequencing have led to a reservoir of data that have improved our understanding of various characteristics of *Rubus* and facilitated the mapping of different traits. Gene *B* controls the waxy bloom, a clearly visible epicuticular wax on canes. The potential effects of this trait on resistance/susceptibility to cane diseases in conjunction with other morphological factors are not fully studied. Previous studies suggested that gene *H*, which controls cane pubescence, is closely associated with gene *B*. Here, we used tunable genotyping-by-sequencing technology to identify the *de novo* SNPs of *R. occidentalis* and *R. idaeus* using an interspecific population that segregates for the waxy bloom phenotype. We created linkage maps of both species and mapped the identified SNPs to the seven chromosomes (Ro01–Ro07) of *Rubus*. Importantly, we report, for the first time, the mapping of gene *B* to chromosome 2 of *R. occidentalis* using a genetic map consisting of 443 markers spanning 479.76 cM. We observed the poor transferability of *R. idaeus* SSRs to *R. occidentalis* and discrepancies in their previously reported chromosome locations.

Keywords: black raspberry; gene *B*; SNPs; *Rubus* maps

1. Introduction

Rubus, with approximately 750 species, is one of the genera of the Rosaceae family of plants with domesticated subgenera comprising of raspberries and blackberries [1]. The European red raspberry (*Rubus idaeus* L. subsp. *idaeus*), the North American red raspberry (*Rubus idaeus* L. subsp. *strigosus* (Michx.) Focke), and the black raspberry (*R. occidentalis* L.) are the most popular raspberries in this subgenus (*Idaeobatus*) and are diploid species with 2n = 2× = 14 chromosomes. Other members of the domesticated subgenera include flowering raspberries, blackberries, and arctic fruits [1]. Red and black raspberries are cross compatible and produce hybrid purple raspberries [2]. The canes of *Rubus* species are the shoots, which are perennials due to their biennial growth habit. Summer raspberries (floricanes) bear fruit in the second year on two-year-old canes; however, primocane raspberries bear fruit in the autumn of the first year on current-year shoots.

Worldwide, raspberries are important fruit crops consumed fresh or as processed products. Raspberries have a high content of phenolic compounds with antioxidant activity [3], making them a popular choice with health-conscious consumers. The production of red raspberries, grown in a temperate climate, has doubled over the last 20 years [4]. Raspberry breeding has not always been easy due to the long seed dormancy phase, limited seed germination, as well as the relatively long juvenility period of this perennial crop. Breeding challenges such as a lack of diversity and availability of genetic resources [2] are part of the reason why *Rubus* breeding has lagged behind. However, the development and use of traditional molecular markers [5–9] and, recently, the use of robust DNA sequencing technology [10–13] have facilitated breeding and genetic mapping studies in raspberry [14]. Nevertheless, one limiting factor in raspberry production in field plantations is its susceptibility to several fungal diseases, which often decrease the yield or destroy plantings. The importance of these diseases often varies depending on the specific location of planting, as the local climate, particularly precipitation, influences their occurrence and severity. As chemical protection is often not economical or effective, coupled with its ban in Europe, breeding new cultivars with resistance or tolerance against these diseases could present a way to aid production. The influence of various cane morphology traits on fungal diseases has been studied previously in raspberries. Jennings [15] studied cane hairiness, spines, and the presence of waxy bloom in relation to spur blight (*Didimella applanata*), grey mold (*Botrytis cinerea*), and cane spot (*Elsinoe veneta*). The interaction of these morphological traits resulting in better water run-off on canes was proposed to be the cause of reduced disease incidence with spur blight and grey mold, but not with cane spot, where an intrinsic tissue resistance was proposed.

The gene responsible for hairy canes (pubescence), gene *H*, has been widely investigated as a major factor in disease resistance, and it has been finally mapped to linkage group 2 in 'Glen Moy' [7,16]. Markers associated with the trait spininess (gene *S*) were also mapped to linkage groups 2 and 3 [6,7]. In contrast, only few studies on the waxy bloom trait have been published since it was initially described in raspberry. The gene for waxy bloom was first described in *R. idaeus* as gene *B* [17], with waxy bloom being the wild-type trait and plants with bloomless canes being homozygous recessives for the gene. The visual appearance of the bloom depends on both the amount and chemical composition of the epicuticular wax. Bloomless canes had four times less amount of total wax compared to canes with dense bloom on 'Latham', while having a similar proportion of paraffins to esters [18]. In contrast, the canes with a sparse bloom of 'Malling Exploit' had a greater total amount of wax than the canes with an intermediate bloom of 'Norfolk Giant', while having a marked difference in their composition [18]. The correlation between the chemical composition of wax structures and their micromorphology [19], the latter in turn affecting the light refraction and thus the appearance of bloom can explain this. The influence of waxy bloom on fungal diseases is complex and depends on the infection process of the disease and other morphological factors of the cane. Waxy bloom on canes prevents the complete wetting of the cane surface, thus creating water droplets that run off canes but collect at the nodes, where spur blight can infect the plant even in the presence of cane hairs, which otherwise promote water run-off from nodes [15]. Waxy bloom acts as a mechanical barrier and fungistatic with grey mold and cane spot, making plants with a dense waxy bloom less susceptible to these diseases [15]. Waxy bloom also confers a protection against winter chill injury in canes [15,20]. The trait is prevalent in *R. occidentalis*. Waxy glaucous coating is typical for canes of this species [21].

Interestingly, all three morphological traits mentioned above have been shown to be linked based on their segregation, with their order proposed as locus *B* between locus *H* and locus *T* (which is responsible for fruit and cane pigmentation) and being distal to locus *S* [22]. To date, the waxy bloom trait (gene *B*) as well as the genes *T* and *S* have not been mapped in any raspberry population. Graham et al. [7] mapped gene *H* in a 'Glen Moy' × 'Latham' population, a cross between the European red raspberry and the North American red raspberry [6]. Here, we report for the first time the mapping of the waxy bloom trait in a population. A population derived from 'Black Jewel' (*R. occidentalis*) × 'Glen Ample' (*R. idaeus*) was used for the development of genetic maps using genotyping-by-sequencing technology, thus facilitating the mapping of the waxy bloom trait on linkage

group 2 of 'Black Jewel'. The predicted genes in the *Rubus* whole genome V3.0 assembly within the waxy bloom interval as well as the mapping of a few *Rubus* SSR markers are presented.

2. Materials and Methods

2.1. Plant Material and Phenotyping

The population was comprised of 145 progeny from a cross between 'Black Jewel' (*R. occidentalis*) and 'Glen Ample' (*R. idaeus*). The cross was performed by hand on emasculated flowers in the greenhouse. The seeds were scarified according to [23], stratified for six weeks at 4 °C, and germinated in a tray on planting soil with a thin layer of sand cover. The resulting seedlings were hardened in the greenhouse for 24 weeks and then taken to the experimental field of the Julius Kühn-Institut in Dresden-Pillnitz (Germany), where they were cultivated in single pots with drip irrigation. The progeny were screened for the waxy bloom trait in late fall and early winter between the beginning of leaf coloration and the vegetative pause. Waxy bloom is a visible epicuticular wax on canes (Figure 1). The phenotype was scored as present (1) or absent (0) in the progeny in two consecutive years, in the second and third year after planting on primocanes. Phenotypic data were converted to marker data for mapping.

Figure 1. Two individuals from the population showing different waxy cane phenotypes: waxy bloom cane (whitish-colored cane, **left**) and no waxy bloom (**right**) on one-year-old canes in late autumn. There is a strong visible difference between the two categories.

2.2. DNA Isolation and SSR Marker Analysis

For genomic DNA isolation, 0.1 g of young leaf material was taken from the plants. Genomic DNA was extracted using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol and resuspended in 50 µL of elution buffer of the same kit. The resulting DNA template was diluted to 10 ng/µL with ddH_2O for the PCRs. The trueness of the cross was verified in the progeny of the population with five SSR markers (Table 1) [6,24,25].

Table 1. SSR markers applied to the population and their allele sizes in base pairs (bp).

SSR	LG	Source	'Black Jewel' Alleles	'Glen Ample' Alleles
‡ RhM011 [1]	LG7	Castillo et al. [24]	280, 282	288, 292
‡ RhM043 [2]	LG4	Castillo et al. [24]	–	374, 377
‡ RiM017 [3]	LG7	Castillo et al. [24]	192, 194	196
‡ Rub123a [2]	LG6	Graham et al. [7]	139	148, 162
‡ Rub285a [2]	LG1	Graham et al. [7]	167	172, 174
† Rub107a [2]	LG2	Graham et al. [7]	173	166, 168
† Rub210a [2]	LG1	Graham et al. [7]	103	117, 123
† Rub124a [4]	LG1	Graham et al. [7]	–	163
† Rub270a [2]	LG2	Graham et al. [7]	183	175, 183
† Rub56a [5]	LG2	Graham et al. [7]	–	–
† Rub76b [4]	LG2	Graham et al. [7]	217	211, 217
† Rub4a [4]	LG2	Graham et al. [7]	–	154 [6]
† Rub163a [5]	LG2	Graham et al. [7]	–	–
† Rub293b [2]	LG2	Graham et al. [7]	–	162, 164, 200, 202
† Rub284a [1]	LG2	Graham et al. [7]	114, 116, 122 [6], 124, 126	156, null
† Rubnebp2O23 [4]	LG2	Graham et al. [7]	234	238, 240

‡ Markers analyzed on the CEQ 8800 Genetic Analysis capillary electrophoresis system (Beckman Coulter, Krefeld, Germany), and used to analyze the trueness of the cross; † markers analyzed on the ABI Genetic Analyzer 3500 XL (Applied Biosystems, ThermoFisher Scientific, Darmstadt, Germany) with allele sizes according to Schuelke (2000), nine of which were chosen from linkage group 2 (LG2); [1] polymorphic in both parents; [2] polymorphic in only 'Glen Ample'; [3] polymorphic in only 'Black Jewel'; [4] monomorphic in both parents; [5] failed to amplify in both parents; [6] fragment failed to amplify or was monomorphic in the progeny.

The PCRs were conducted using the Type-it Microsatellite PCR Kit (Qiagen, Hilden, Germany) in a 10 µL reaction multiplex with 10 ng of genomic DNA as template and primer concentrations of 0.2 µM for Dye-751, 0.1 µM for BMN-6, and 0.05 µM for BMN-5 labeled fluorescent markers. The PCR program was the following: initial denaturation at 95 °C for 5 min; 28 cycles of 95 °C for 1 min; annealing at 51 °C, 52 °C, or 57 °C 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 with ddH_2O 1:20 and 1 µL was added to 24.9 µL of SLS (sample loading solution) buffer and 0.1 µL 400 bp size standard for use in the CEQ 8800 Genetic Analysis capillary electrophoresis system (Beckman Coulter, Krefeld, Germany). A drop of mineral oil was added to every sample in the capillary electrophoresis plate to prevent evaporation. The resulting fragment size profiles of the markers were visually assessed through the CEQ 8800 software of the same supplier.

2.3. Tunable Genotyping-by-Sequencing (tGBS)

Lyophilized leaf materials of 146 progeny individuals and both parents were sent to Data2Bio (Ames, IA, USA) for DNA isolation and tunable genotyping-by-sequencing (tGBS) analyses. Briefly, the genomic DNA were digested with two restriction enzymes. NspI leaves a 3′ overhang and BfuCI/Sau3AI leaves a 5′ overhang. Thereafter, two single-strand oligos were ligated to the complementary 3′ and 5′ overhangs. The oligo matching the 3′ overhang contains a sample-specific internal barcode sequence for sample identification. The oligo matching the 5′ overhang is universal and present in every reaction for later amplification. Target sites were then selected using a selective primer with variable selective bases ("CA") that match selective sites in the digested genome fragments and a nonselective primer. When properly amplified, the selective site is complementary to the selective bases. Finally, the primers matching the amplification primer and the selective primer, which contain

the full Proton adapter sequences, were used for the amplification of the final library. The final sequence contains the 5′ Proton adapter sequence, an internal barcode, the NspI restriction enzyme site, the target molecule, selective bases, the BfuCI/Sau3AI restriction enzyme site, and the 3′ Proton adapter sequence.

2.4. Trimming of Sequencing Reads and Alignment to the Reference Genome

The nucleotides of each raw read were first scanned for low-quality bases. Bases with a PHRED quality value ≤15 out of 40 [26,27] were removed by the trimming pipeline. Each read was examined in two phases. In the first phase, the reads were scanned starting at each end and nucleotides with quality values lower than those of the threshold were removed. The remaining nucleotides were then scanned using overlapping windows of 10 bp and sequences beyond the last window with an average quality value less than the specified threshold were truncated. The trimming parameters were referred to the trimming software, Lucy [28]. Trimmed reads were aligned to the *Rubus occidentalis* V3.0 reference genome [29], available at http://www.rosaceae.org, using GSNAP [30], and confidently mapped reads were filtered if mapped uniquely (≤2 mismatches every 36 bp and less than 5 bases for every 75 bp as tails) and used for subsequent analyses.

2.5. Discovery of Polymorphic Sites

The polymorphisms at each potential SNP site were carefully examined and putative homozygous and heterozygous SNPs were identified in each sample separately. For homozygous SNP calling, the most common allele was supported by at least 80% of all the aligned reads covering that position. For heterozygous SNP calling, each of the two most common alleles was supported by at least 30% of all aligned reads covering that position. For both criteria, the polymorphisms in the first and last 3 bp of each read were ignored and each polymorphic base had at least a PHRED base quality value of 20 (≤1% error rate). Any site that was deemed to be polymorphic (homozygous or heterozygous) as compared to the reference genome sequence in at least one sample was included in the set of polymorphic sites.

2.6. Criteria for Homozygous and Heterozygous Calls

A SNP site was called homozygous in a given diploid sample if at least 5 reads supported the major common allele at that site and at least 90% of all aligned reads covering that site shared the same nucleotide at that site. A SNP was called heterozygous in a given diploid sample if at least 2 reads supported each of at least two different alleles and each of the two allele types separately comprised more than 20% of the reads aligning to that site and when the sum of the reads supporting those two alleles was at least equal to 5 and comprised at least 90% of all reads covering the site. The SNP sets were further filtered to define the MCR50 (minimum call rate) SNP set (i.e., the SNP minimum call rate, each of which was genotyped in at least 50% of samples).

2.7. Rubus Linkage Group 2 (LG2) SSR Genotyping

Since the gene *H* maps on Linkage Group 2 (LG2) [7] and a previous publication suggest that gene *B* (waxy bloom) is close to gene *H* [22], nine microsatellites (SSR markers) mapping on chromosome 2 in the same population where gene *H* was mapped [6,7] were chosen (Table 1). The SSRs were tested for polymorphism on 'Black Jewel', 'Glen Ample', and a subset of six progenies. The PCR conditions were as described for SSR marker analyses above. However, this time the forward primers included the M13 elongation primer (5′-TGTAAAACGACGGCCAGT-3′) and the PCR reaction contained the M13 primer [31], with labelled dyes suitable for the ABI Genetic Analyzer for fragment detection and analyses (i.e., FAM, ATTO532, and ATTO550). For analyses on the ABI Genetic Analyzer 3500 xL (Applied Biosystems, ThermoFisher Scientific, Darmstadt, Germany), the PCR products were diluted 1:100 and 1 µL of the dilution was mixed with 8.95 µL of HiDi formamide (Applied Biosystems) and 0.05 µL of Liz 600 size standard (Applied Biosystems) in a total volume of 10 µL. The mixture was denatured in a thermocycler at 94 °C for 5 min before loading onto the ABI. The SSR fragments were

analyzed using the GeneMapper™ software version 6 (ThermoFisher Scientific, Darmstadt, Germany). Polymorphic SSRs were then used to genotype the population.

2.8. Genetic Map Construction

The SSR data and the converted marker data of the waxy bloom phenotypic trait were integrated into the SNP data. The genetic map was constructed using JoinMap 4.0 version [32]. Regression mapping algorithm was used to create the linkage map of both parents using the Kosambi function at a LOD (logarithm of odds) grouping of 12–18. Markers showing segregation distortions were excluded after the first genotype frequency calculation. Only groups with at least 20 markers were used to generate the linkage maps at a chosen LOD of 15. More markers were excluded in the respective groups when linkage maps could not be created due to poor linkages. The linkage groups were manually renamed, and some of them were flipped in order to be consistent with the physical map.

2.9. Predicted Gene Search in the Rubus Reference Genome

The structural and functional gene annotation were extracted from Rubus occidentalis V3.0 reference (available at ftp://ftp.bioinfo.wsu.edu/www.rosaceae.org/Rubus_occidentalis/Rubus_occidentalis-genome.v3.0) within the physical interval that contains SNPs, which flank the waxy trait in the 'Black Jewel' genetic map.

3. Results

3.1. Waxy Bloom Phenotyping

Of the 145 individuals scored in both years, 66 individuals consistently showed presence while 69 individuals consistently showed absence of the phenotype. Ten individuals showed inconsistent phenotypes in both years (i.e., present in one year and absent in the other year or vice versa). If these 10 individuals with inconsistent phenotypes are excluded, the ratio of segregation is 1:1. We transformed the phenotypic data of all individuals into marker data for mapping purposes. Data for the respective phenotypic years as well as the consistent phenotype data excluding the 10 inconsistent individuals were used for mapping.

3.2. SSR Marker Analyses

In total, 16 *Rubus* SSR markers already published in the literature were tested on the mapping population. Nine of these SSR markers were chosen from LG2, since gene *H* (pubescence) known to be associated with gene *B* (waxy bloom), was previously mapped on LG2 in a 'Glen Moy' × 'Latham' population [7]. The rationale therefore was to ascertain their genetic proximity by genetic mapping using this mapping population. Table 1 lists the SSRs tested in this study including the alleles amplified in 'Black Jewel' and 'Glen Ample'. Whilst only three of these SSRs were polymorphic in 'Black Jewel', nine were polymorphic in 'Glen Ample'. Six primer pairs were either monomorphic in both or failed to amplify in both parents and the progeny (Table 1). Two SSRs, Rub284a and Rub293b, amplified multiple alleles in 'Black Jewel' and 'Glen Ample', respectively. Only six polymorphic SSRs (Rub284a, RiM017, RhM011, Rub123a, Rub285a, RhM043) could be mapped in both parental maps. Subsequently, the primer sequences of the six mapped SSRs were used in a BLAST search against the *Rubus* V1 [33,34] and V3.0 [29] assembled genomes to ascertain their positions relative to the genetic positions in our developed map.

3.3. tGBS SNPs Identification and Genotyping

Tunable genotyping-by-sequencing (1-bp selection) of the 148 samples submitted to Data2Bio resulted in 370,664,239 reads. The sequence reads were first scanned for low-quality sequences and then aligned to the *Rubus occidentalis* V3.0 reference genome [29]. Of 320,186,444 quality trimmed reads, an average of 2,163,421 were identified per sample. Of the total quality reads, 82.5% (264,078,189)

aligned in more than one location and 47.3% (151,400,902) aligned in a single location in the reference genome. The physical positions of the SNPs in the *Rubus* V3.0 genome are included to identity the SNPs. Only uniquely aligned reads were used for further analyses. A total of 86,982 polymorphic sites were identified from 3,088,267 bases that had ≥5 reads in at least 50% of samples. The initial set of SNPs identified was 47,794 (File S1). Based on further filtering parameters (SNP minimum call rate, each of which was genotyped in at least 50% of samples: MCR50), the number of SNPs was reduced to 18,700. Finally, the SNPs were filtered according to the parental genotype information—i.e., heterozygous in one parent and homozygous in the other parent, as well as heterozygous in both parents. Finally, 1059 and 3498 SNPs found to be heterozygous in 'Black Jewel' and 'Glen Ample', respectively, were used for mapping. Eighty SNPs were heterozygous in both parents. One individual, found not to be an offspring of the 'Black Jewel' × 'Glen Ample' cross, was excluded from the mapping.

3.4. Linkage Map Construction

One hundred and forty-five (145) individuals were used to construct the linkage groups of the respective parental maps. To construct the linkage map of 'Black Jewel', 1059 heterozygous SNPs, 3 SSRs, and the waxy bloom phenotypic marker data were used. Although Rub284a produced five alleles in 'Black Jewel', it was treated as four loci since the 122 bp allele was monomorphic in the progeny. In total, 1066 loci were imputed into JoinMap 4.0 for regression mapping calculation. After the first locus genotype frequency calculation, 187 SNP markers that showed the highest level of segregation distortion (≥*** i.e., $p = 0.01$ to 0.0001) were excluded from further calculations. Neither the SSR loci nor the waxy bloom phenotypic marker data showed segregation distortion. At a LOD of 15 and the selection of groups with >20 loci, seven groups were formed that represented all seven chromosomes of raspberry. Rub284a SSR marker was found to be a multi-loci marker and was mapped on LGs 5, 6, and 7 in 'Black Jewel'. One SSR marker, RhM011, mapped on LG4 at a LOD of 14 but was ungrouped at a LOD of 15 (Table 2). Table 2 summarizes the characteristics of the linkage maps of 'Black Jewel' and 'Glen Ample'. In general, the genetic map of 'Black Jewel' consists of 443 markers spanning 479.76 cM (Figure 2 and File S2a). LG6 is the longest, with a genetic length of 101.96 cM, and LG4 is the shortest, with a genetic length of 47.72 cM.

For the creation of the 'Glen Ample' linkage map, 3503 markers including five SSRs were imputed into JoinMap 4.0 program. Following the first locus genotype frequency calculation, 724 markers, all SNPs, showed the highest level of segregation distortion (≥*** i.e., $p = 0.01$ to 0.0001) and were excluded. Thus, 2779 markers were used for the subsequent calculations. At a LOD of 15, seven groups were formed that contained more than 20 markers. Rub284a mapped on the LG3 of 'Glen Ample'. Two other SSRs mapped on LG1, whilst the other two mapped on LG2 and LG4. Eight hundred and seventy-seven markers were successfully mapped to the 'Glen Ample' linkage map (Table 2). This map spanned 660.53 cM, with LG3 as the longest with a genetic length of 112.91 cM and LG6 as the shortest with a genetic length of 47.80 cM (File S2b).

The order of the genetic positions (cM) of most of the markers corresponded to the order of their physical positions for both maps; nevertheless, several markers did not correspond. In addition, in some cases the SNP markers already assigned a *Rubus* chromosome number due to the uniquely aligned results, mapped on different linkage groups. Figure 3 shows the SNP markers of the 'Black Jewel' and 'Glen Ample' maps and their locations in the *Rubus* V3.0 chromosomes. The graphical illustration shows the physical gaps of both maps where SNPs could not be identified and/or mapped, and suggests a similar pattern of gaps for both species in the chromosomes, except in chromosomes one and five, for which there were too few markers in 'Black Jewel' for comparison (Figure 3).

Table 2. Summary of the characteristics of the genetic maps of 'Black Jewel' and 'Glen Ample'.

'Black Jewel'							'Glen Ample'						
LG	**No. of Markers LOD15**	**No. of Markers Mapped**	**Length of Map cM**	**Maximum Gap cM**	**Minimum Gap cM**	**Average Gap cM**	**LG**	**No. of Markers LOD15**	**No. of Markers Mapped**	**Length of Map cM**	**Maximum Gap cM**	**Minimum Gap cM**	**Average Gap cM**
1	30	23	58.80	7.74	0.34	2.67	1	312	123	103.75	4.78	0.25	0.85
2	81	68	59.23	5.61	0.07	0.88	2	384	120	103.76	2.79	0.08	0.87
3	123	96	97.69	3.72	0.15	1.03	3	586	200	112.91	1.59	0.06	0.57
4	74	47	47.72	3.92	0.35	1.04	4	320	140	96.03	3.82	0.01	0.69
5	25	25	66.09	4.59	0.47	2.87	5	295	106	98.87	3.54	0.08	0.94
6	113	96	101.96	6.25	0.04	1.07	6	58	42	47.80	4.74	0.02	1.17
7	120	88	48.27	4.32	0.01	0.55	7	364	146	97.41	3.57	0.02	0.67
Total	566	443	479.76				Total	2319	877	660.53			

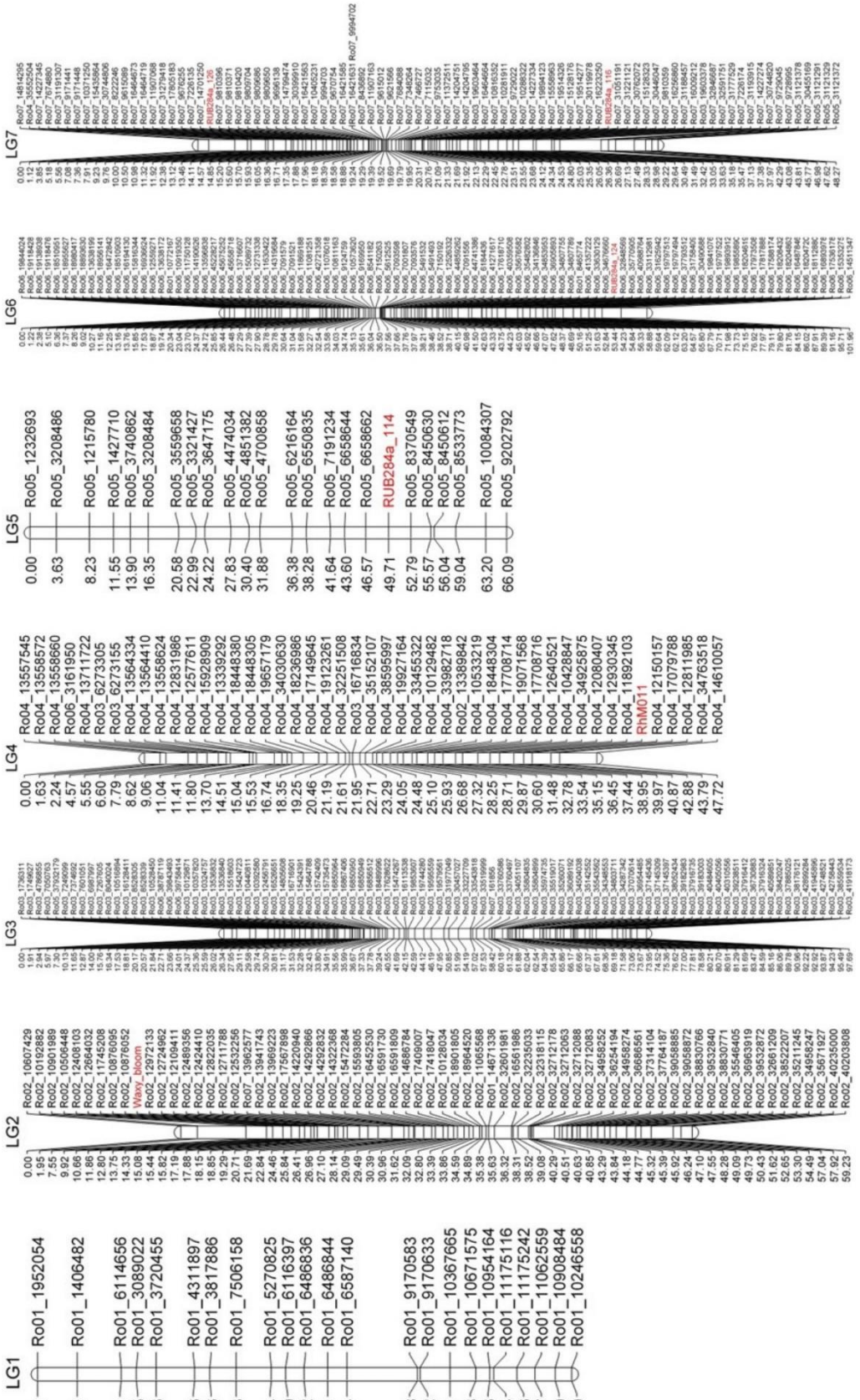

Figure 2. Genetic map of 'Black Jewel' showing seven linkage groups (LGs) that represent the seven chromosomes of *Rubus*. Waxy bloom maps on LG2 and is highlighted with the SSRs in red. Genetic distance is given in cM.

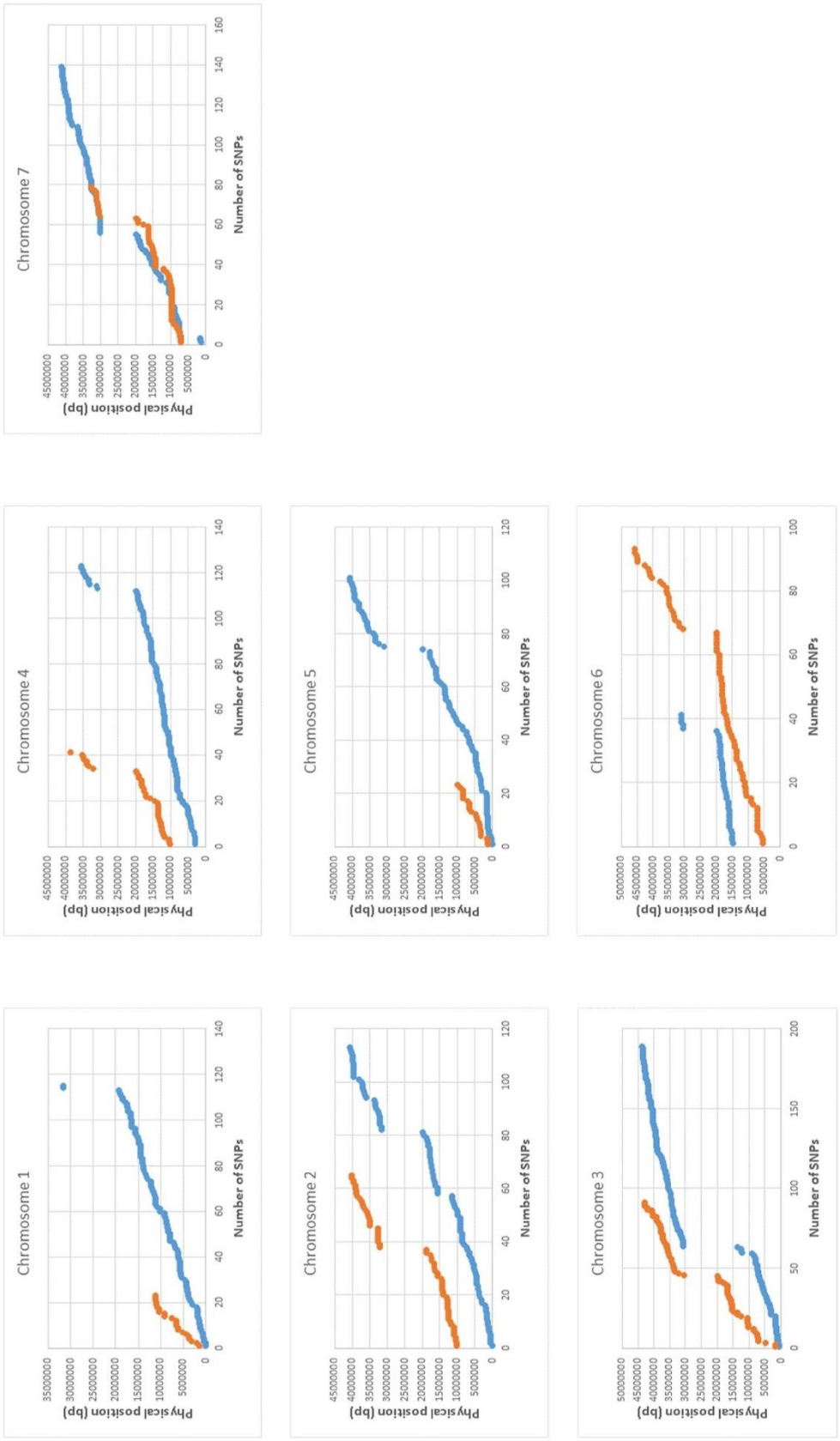

Figure 3. Comparisons of SNP markers in 'Black Jewel' (orange dots) and 'Glen Ample' (blue dots) maps, relative to their positions in the *Rubus* V3.0 chromosomes. Graph also shows regions (gaps) where SNPs were not identified/mapped for both species. A similar pattern of gaps is seen in both species.

3.5. Mapping of the Waxy Bloom Gene

The waxy bloom trait was mapped on LG2 of 'Black Jewel' at 15.08 cM in a 1.11 cM interval between the SNP markers Ro02_10876052 (14.33 cM) and Ro02_12972133 (15.44 cM). This represents a distance of approximately 2 Mb in the *Rubus* V3.0 genome [29]. Of all nine LG2 SSRs tested, only one, Rub284a, was polymorphic in 'Black Jewel', and none of the Rub284a loci mapped on LG2. All three SSRs in the linkage map of 'Black Jewel' are located on linkage groups different from what the literature suggests (Table 3). In fact, only one SSR in this study—Rub285a—maps on the same linkage group as was previously reported (Table 3)—i.e., on LG1 of 'Glen Ample'. Thus, it was impossible to ascertain the position of the waxy bloom gene relative to gene *H*. To ascertain the actual chromosome positions of the SSRs mapped in this study, their forward and reverse primers were blasted against versions one and three of the *Rubus* genome [29,33,34]. The positions of the SSRs in both genomes in comparison to our maps and already published *Rubus* maps is presented in Table 3 and shows that the alleles of Rub284a mapped on LGs 5, 6, and 7 in 'Black Jewel' and on LG3 in 'Glen Ample'. The chromosome locations of the sequences of most of these markers in the *Rubus* genome are in agreement with the linkage groups assigned in the maps presented in this study.

3.6. Annotated Genes in the Rubus V3.0 Reference Genome within the Physical Interval of the Waxy Bloom Gene

The two Mb physical interval containing the waxy bloom trait was scanned for annotated genes in the *Rubus* V3.0 genome [29]. Within this interval, 298 mRNAs were found (File S3a), with 136 of these having putative gene ontology annotations (File S3b). Noteworthy are genes bearing similar roles as those found in the sequences obtained from BAC clones near and spanning the gene *H* region [16]. Briefly, this includes carbohydrate metabolism, transcription factor activity, defense response, and oxidation-reduction process.

Table 3. Map positions of *Rubus* SSR markers in comparison to the *Rubus* genomes and other mapping studies.

	***R. occidentalis* Genomes**		**Map Locations in This Study in Comparison to Previous Studies**				
	Chromosomes Located		**This Study**		**Graham et al. [7]**	**Castillo et al. [24]**	
Rubus SSRs	*Rubus* V3	*Rubus* V1	'Black Jewel'	'Glen Ample'	'Glen Moy' × 'Latham'	'Glen Moy' × 'Latham'	'Autumn Bliss' × 'Malling'
Rub284a	– (F/R)	chr7 (F)	LG7	LG3	LG2	N/A	N/A
		chr6 (F)	LG6				
		chr5 (F)	LG5				
		chr4 (F)					
		– (R)					
RiM017	chr4 (F/R)	chr4 (F/R)	LG4 ‡	NP	N/A	LG7	LG7
RhM011	chr4 (R)	chr4 (R)	LG4	LG4	N/A	LG7	LG7
	chr3 (R)	chr3 (R)					
	– (F)	– (F)					
Rub123a	chr1 (F)	chr1 (F)	NP	LG1	LG6	N/A	N/A
	– (R)	– (R)					
Rub285a	– (F/R)	chr1 (F)	NP	LG1	LG1	N/A	N/A
		– (R)					
RhM043	– (F/R)	chr7 (F)	NP	LG2	N/A	NP	NP
		chr5 (F)					LG4 (secondary locus)
		– (R)					

F and R = forward and reverse sequence; ‡ Mapped at LOD of 14; – = no hits were found; NP = not polymorphic in the population; N/A = not applicable—i.e., not reported by the references.

4. Discussion

In the present study, we identified single nucleotide polymorphisms (SNPs) for black (*R. occidentalis*) and red (*R. idaeus*) raspberry cultivars using tunable genotyping-by-sequencing method and used informative SNPs to construct the respective genetic maps. Interestingly, we have also mapped, for the first time, the waxy bloom trait (gene *B*) on LG2 corresponding to chromosome two of the *Rubus* genome [29,33,34]. Genotyping-by-sequencing, a next-generation sequencing technique, is a high-throughput method that facilitates rapid, broad coverage targeted sequencing based on reducing genome complexity with restriction enzymes [35,36]. Widely applied to several species since it was first reported, there are only a few reports of the use of genotyping-by-sequencing for the identification of thousands of SNPs in *Rubus* [10,11,13]. Whilst two studies [10,13] were based on populations derived from *R. idaeus* cultivar crosses, one [11] was based on a population purely from a black raspberry cross. Therefore, this is the first report of the use of genotyping-by-sequencing to generate and map SNPs in progeny derived from black ('Black Jewel') and red ('Glen Ample') raspberries, although a progeny derived from black and red raspberries have previously been used to develop genetic maps [12].

Millions of reads resulting from tGBS and the subsequent identification of thousands of SNPs in this study is consistent with other studies in *Rubus* [10,11,13]. For example, Hackett et al. [13] reported an average number of reads of 2,042,242, similar to the 2,163,421 found in this study. The stringency of the filtering criteria of SNP sets vary from study to study and usually results in a lesser number of markers. The total number of 47,794 SNPs initially identified here was first reduced to 18,700 following the determination of SNPs which could be called in at least 50% of the samples, termed MCR50 (minimum call rate 50), and further reduced due to the parental genotype information—'Black Jewel': 1059; 'Glen Ample': 3498. Other studies reported final reduced SNP numbers—for example, 9143 [10] and 8019 [13], due to different filtering criteria. However, the number of SNPs heterozygous for the black raspberry cultivar is much less than for the red raspberry cultivar. This is presumably due to the low heterozygosity levels of *R. occidentalis*. Bushakra et al. [11] reported a similar occurrence in their study, where only 1545 SNPs were suitable for black raspberry, of which only 399 could finally be mapped. In the current study, of 1059 SNPs, only 436 (excluding SSRs) were mapped. The high levels of heterozygosity of *R. idaeus* in our study is consistent with other studies [10,13].

The genetic maps of 'Black Jewel' and 'Glen Ample' span lengths of 479.76 and 660.53 cM, respectively, and are comparable to similar studies that used GBS technology [10,11,13]. However, there were some inconsistencies in the genetic order of the markers and their physical order. This lack of collinearity could be attributed to two possibilities: inaccuracies in the recombination frequencies and/or the poor assembly of the genome sequence. A major problem of GBS maps is that double recombinations could be found within a small genetic window, which in principle is impossible [37]. This has the potential to overestimate the overall size of linkage groups, and a possible consequence of this is the wrong order of some markers. On the other hand, Jibran et al. [34] resolved multiple inconsistencies between the genetic and physical orders of GBS markers in the *R. occidentalis* genomes [29,33], although 25% of the discrepancies remained unsolved. That a better collinearity would have been achieved had the tGBS reads in the current study been aligned to the *Rubus* V1.1 genome [34] is speculative. In addition, a few SNPs assigned to a certain chromosome from physical alignment mapped on a different linkage group, thus suggesting the plausibility of duplications in the genome. There is no information on the collinearity of GBS SNP genetic and physical orders in previous mapping studies [10,11,13]. Hackett et al. [13], whose map was based on the 'Glen Moy' × 'Latham' progeny, developed and used a 'Glen Moy' draft genome as a reference and opined that the scaffolds aligned comparably to the genome of black raspberry. Up to 97% of scaffolds [11] and approximately 83% of the reads in the current study aligned to single positions in the *Rubus* genome. Nevertheless, the correct alignment of scaffolds to the genome does not necessarily translate to the unique alignment of SNP sequences. Another surprising outcome in the current study was the contrasting map locations of *Rubus* SSRs [6,7,24]. Only Rub285a mapped on a linkage group it was previously reported to map on—i.e., LG1 [7]. Furthermore, the positions of the forward and

reverse sequences of SSR primers on the *Rubus* genomes [29,33,34] appeared to be in contrast with their previously mapped location, and in some instances no hits were found (Table 3). Many of the *Rubus* SSRs were developed from 'Glen Moy' (*R. idaeus*) genomic DNA, and this 'Glen Moy' × 'Latham' population [5–7] has been widely used in *Rubus* [13,38]. The other sets of SSRs applied in this study were developed from 'Meeker' (*R. idaeus*) and 'Marion' blackberry (hybrid) [24]. It is unclear whether these discrepancies suggest diversity in the *Rubus* genus reflecting the different cultivars. Nevertheless, the discrepancies deserve attention. The public availability of red raspberry draft genomes [13,39] will throw light on the locations of several of these *Rubus* SSR fragments. The development and mapping of *de novo* SSR markers from the *R. occidentalis* genome will improve molecular mapping studies in this vastly untapped species.

Further, we report the mapping of the waxy bloom trait (gene *B*) on LG2 of 'Black Jewel' (Figure 2) in a population derived from *R. occidentalis* (mother) and *R. idaeus* (father). Differences in gene *B* determine the presence (*B*) and absence (*b*) of waxy bloom [17]. Canes of *R. occidentalis* are heavily glaucous [15]. Several *R. occidentalis* cultivars are assumed to be heterozygous for waxy bloom, since they have previously produced bloomless progeny in certain crossings [21,40]. 'Black Jewel' could be the European equivalent of one of the 'Jewel' cultivars, as there are no known cultivars in the US under the name 'Black Jewel' and vice versa. A discrepancy in naming is not the only uncertainty with working with both *R. idaeus* and *R. occidentalis* cultivars. Even the genotype authenticity is not to be assumed as certain, as cultivars have been repeatedly found to not be true-to-type, resulting from mix-ups or mutations [41–43]. As such, care must be taken with comparisons with other studies, even if they used the same cultivars. 'Glen Ample', the father cultivar in this study, has non-glaucous canes and is therefore homozygous recessive to gene *B*. The trait segregated in a 1:1 ratio in the population, thus confirming the heterozygosity of the trait in the donor parent. Gene *B*, responsible for waxy bloom, is on the same chromosome as gene *T*, which determines the type of cane pigment [15]. It was postulated based on segregation that gene *B* must be between gene *T* and gene *H*, responsible for cane hairiness, and distal to gene *S*, which determines the presence or absence of spines [22]. Graham et al. [7] mapped gene *H* on LG2 of 'Glen Moy' in a population derived from 'Glen Moy' and 'Latham', both red raspberry cultivars. It was also reported that the phenotypic marker of gene *H* was the most significant marker correlating with the degree of spininess. Whereas gene *H* mapped at 48 cM [7], gene *B* maps at 15.08 cM of 'Black Jewel' (Figure 2). Based on Jennings [15,22] and Graham et al. [7], it makes sense that gene *B* maps on LG2 of 'Black Jewel'. Unfortunately, however, it was impossible to ascertain the putative genetic positions of gene *H* relative to gene *B* by analyses of LG2 SSR markers, since only one SSR, Rub284a, was polymorphic in 'Black Jewel' and did not map on LG2. The poor polymorphism of *R. idaeus* SSRs in 'Black Jewel' (*R. occidentalis*) observed in our study is a microcosm of the results in Bushakra et al. [11], although we have focused on only LG2 SSRs following the preliminary mapping results, which indicated that gene *B* maps at LG2 of 'Black Jewel'. MacKenzie et al. [16] characterized the region, which contained gene *H*, and proposed PDF2/GL2, a homeobox gene, amongst many others, as responsible for cane pubescence. Functions of other putative genes found on the BAC clone that spanned the gene *H* region as well as the adjoining BAC clones included transcription factors, disease resistance, carbohydrate transport metabolism, and oxidative reduction, amongst others [16]. The physical region-containing gene *B* in the *Rubus* genome [29] contains several genes, but genes were found with similar functions such as carbohydrate metabolism, transcription factor activity, defense response, and oxidation-reduction process. This is some evidence of similarity in the regions, and suggests that locus *B* and *H* may not be too distal.

5. Conclusions

An interspecific progeny derived from *R. occidentalis* and *R. idaeus* that segregates 1:1 for waxy bloom was the basis of this study. The genetic maps constructed with SNPs derived from tunable genotyping-by-sequencing and a few *Rubus* SSR markers allowed for the mapping of gene *B*, controlling waxy bloom, on chromosome 2 of *R. occidentalis*. However, poor polymorphism of *R. idaeus* SSR markers

was observed and led to an inability to characterize chromosome 2 relative to *R. idaeus* chromosome 2, where gene *H* was previously mapped. In addition, the maps we present show discrepancies in the map locations of previously mapped SSRs. Nevertheless, the genetic maps created in this study add to the handful of maps in the *Rubus* reservoir, and would contribute to our understanding, particularly as it pertains to the respective genomes of *R. occidentalis* and *R. idaeus*, in terms of sequence assembly and marker anchorage. In conclusion, to our knowledge, this is the first report of the mapping of gene *B* in *Rubus*. However, we do not propose any genes from the *R. occidentalis* genome as gene *B* due to the relatively large physical region.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/10/1579/s1: File S1: sequence of all SNPs identified in this study and the *Rubus* chromosome they align to; File S2: (a) excel map file of 'Black Jewel' and (b) excel map file of 'Glen Ample'; File S3: (a) Annotated genes in the physical interval of waxy bloom (b) available functional annotation within the waxy bloom physical interval.

Author Contributions: D.P. performed the phenotyping and genotyping with SSRs and wrote parts of the manuscript. M.v.R. supervised part of the research and performed some analyses of the population. J.K. and T.B. extracted putative genes in the waxy bloom physical interval on the *Rubus* V3.0 genome. A.P. performed SSR and marker analyses. H.F. conceived the research, got funding, and supervised D.P. O.F.E. performed the SSR genotyping, analyses, and mapping and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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3. Additional results

In addition to the results described in the manuscripts in chapter 3, more results were obtained with relevance to the thesis, but not published as a finished manuscript yet. These results will be presented throughout this chapter.

3.1. A naturally occurring flower mutation in large fruited raspberry is caused by a transposon insertion into the *LEAFY* gene

A sepaloid flower mutation was found on several plants belonging to a raspberry breeding population at the Julius Kühn Institute (JKI) in Dresden (Germany). The petals and stamens of these plants are mostly converted to sepals and carpels. This phenotype is interesting for breeding as it has more carpels than wild-type flowers and it develops into large fruit. The same sepaloid flower phenotype was already described in raspberry by Lewis (1939) and Keep (1964) in the cultivar 'Burnetholm'. However, nothing is known about the genetic mechanisms explaining the abnormal flower type. Finding the molecular cause and heredity of the development of excessive carpels would be valuable information for breeders.

To identify the gene responsible for the flower mutation, MADS-box genes of *Rubus occidentalis* were identified through an HMMER search and amino acid sequence comparison with *Arabidopsis thaliana* MADS-box genes. Using these *R. occidentalis* genes as a template, primers were developed for floral development genes to use in *R. idaeus*. As its expression in mutant phenotype flowers was disturbed, *PISTILLATA* (*PI*) was chosen as a candidate gene responsible for the mutation. Although two *PI* homologues were found in genomic and cDNA sequences, they had shown no allelic differences in wild-type and sepaloid plants. Consequently, upstream regulators of *PI* were chosen as new candidate genes. From these candidates, *LFY* amplified a different length fragment on genomic DNA of both types of plants. After cloning and sequencing these *LFY* fragments, a transposon insert was found to be the source of the length difference. Corresponding to the two *LFY* fragments, two primer pairs were developed to differentiate between the alleles. The transposon insertion was shown to be associated with the sepaloid phenotype in the segregating raspberry population. Furthermore, the raspberry population was phenotyped for flower phenotypes and fruit properties to study if affected plants do indeed develop larger fruit.

3.1.1. Material and Methods

Plant material

A large fruited chance seedling of raspberry (*Rubus idaeus* L.), derived from seed of commercially sold fruit, was open pollinated and the resulting fruit was harvested. Seeds were extracted by a household hand blender, scarified with HCl as described by Jennings and Tulloch (1965) and stratified for six weeks at 4 °C in a 1:1 mix of moist peat and sand. The seed soil mixture was poured onto a germination tray filled with soil and covered with a layer of sand. The germinated seedlings were transported to the field in the same year, resulting in a half-sib population of 131 plants. The population is named RiSEP_Him15K7 (RiSEP).

Phenotyping

Flowers of the RiSEP population plants were phenotyped and grouped into three phenotypes (type 1-3). Type 1 flowers contain five sepals and five petals, as well as numerous stamens and carpels, which conforms with the structure/morphology of wild-type flowers of raspberry. Type 2 flowers contain six sepals and six petals, as well as numerous stamens and carpels. Type 3 flowers contain over 15 sepals and carpels, but no petals and stamens. Typical flowers of each phenotypes were collected in the three phenological stages: small bud, large bud and open flower. Subsequently, they were photographed as full structure. Open flowers of each phenotype were dissected to their separate floral organs and photographed as well. Plants were phenotyped as type 1 if they only had type 1 flowers, as type 2 if they had type 2 flowers in addition to type 1 flowers, and they were phenotyped as type 3 if they only had type 3 flowers. The phenotyping data was collected over four years. The phenotype summary was determined by using the highest phenotype value recorded through the four years for the plant.

Open blooms of type 2 and type 3 phenotypes were collected from four individuals and from a control plant with wild-type flowers of the cultivar ‘Goldmarie’. The floral organs sepals, stamens and carpels were counted on 40 type 2 and type 3 flowers each and 20 wild-type flowers.

Fruits of the RiSEP population individuals were harvested and weighed on a Sartorius BP 110S scale (Göttingen, DE), measured longitudinally and their drupelets were counted.

Statistical analysis

Carpel and fruit phenotyping figures were created with the software R (R Core Team, 2017; Wickham, 2009). Statistical significance was determined with the same software by using the Kruskal-Wallis test, with a Wilcoxon test for pairwise comparisons for the carpel and fruit phenotyping figures, and using chi-squared test for the phenotype distribution.

Floral homeotic genes

A. thaliana floral homeotic amino acid sequences were downloaded from The Arabidopsis Information Resource (TAIR) (Tab. ES1a). A Hidden Markov Model search (pHMMER) was used on the *R. occidentalis* genome v1.0.a1 and v3.0 protein datasets (VanBuren et al., 2016, 2018) with the SRF-TF domain (Serum Response Factor-type transcription factor, PF00319) from the Pfam database (http://pfam.xfam.org/). These predicted *R. occidentalis* floral homeotic amino acid sequences were aligned in Mega-X 10.0.5 (Kumar et al., 2018) with the *A. thaliana* sequences mentioned above through a ClustalW algorithm. PI and AP3 sequences of other Rosaceae species were added in the same alignment. These sequences from apple, woodland strawberry, rose and peach PIs and rose AP3s were used in addition to the RidPI1 and RidPI2 sequences obtained through cloning. Following alignment, an UPGMA sequence similarity tree was created with 125 bootstrap replicates. The PI amino acid sequences of *Malus domestica* Borkh. (Q9AVU7_MALDO), *Prunus persica* Batsch. (A7LCX0_PRUPE), *Rosa rugosa* Thunb. (Q9FZN1_ROSRU) and the two AP3 amino acid sequences of *Rosa rugosa* (Q93X10_ROSRU, Q7X9I8_ROSRU) were downloaded from UniProt (https://www.uniprot.org). The second protein with high sequence similarity to *Rosa rugosa* PI, (A0A2P6PXI5_ROSCH) was found by BLASTing Q9FZN1_ROSRU against the *Rosa chinensis* database in Uniprot. The two *Fragaria vesca* genes (FvH4_2g27860 and FvH4_2g27870) were downloaded from the Genome Database for Rosaceae (GDR) (https://www.rosaceae.org) after BLASTing with the *A. thaliana* PI sequence. Functional annotation for the *R. occidentalis* sequences used in the alignment were downloaded from the GDR as well, as the GO (gene ontology) assignments from InterProScan of the *R. occidentalis* v3.0 genome (VanBuren et al., 2018).

Sequence analysis and visualization

Nucleotide and amino acid alignment was performed with CLC Main Workbench 8.1.3 (Qiagen, Hilden, DE) and BioEdit 7.2.5 (Hall, 1999). Local BLASTs were done in BioEdit 7.2.5 (Hall, 1999). The transposable element found in *RidLFY-2* was analyzed with TEclass (Abrusán et al., 2009). Images were assembled and created with Photoshop CS4 11.0 (Adobe Inc., 2017) and Inkscape 0.92 (Inkscape Project, 2017).

Nucleotide and amino acid sequence information

The sequence information of *RidPI1*, *RidPI2*, *RidLFY-1* and *RidLFY-2* can be found in the electronical appendix directory ES3.

DNA and RNA isolation

Young leaf tissue (0.05 g) was processed with the DNeasy Plant Mini Kit (Qiagen, Hilden, DE) following the manufacturer's protocol to extract genomic DNA. The DNA pellet was resuspended in 50 µl of the elution buffer included in the kit.

For RNA isolation, bud and flower samples of each phenotype were collected from flowers of the three different phenological stages. Complete and separated flower organ samples were prepared and stored at -80 °C. For separating the flower organs of type 3 flowers, sepals were defined as the first five sepaloid structures, petals as the second five sepaloid structures, carpels as the carpeloid structures and stamens as the remaining structures. RNA isolation was conducted with the Invitrap Spin Plant RNA Mini Kit (Stratec, Birkenfeld, DE) following the manufacturer's protocol with minor changes to extract RNA from the samples, using 0.05 g plant material. No DTT or b-Mercaptoethanol was used and Lysis Solution RP was incubated for 15 min at room temperature. All samples were subsequently treated with the DNA-free DNA Removal Kit (Thermo Scientific, Waltham, MA, USA) according to manual with specifications below to remove genomic DNA before cDNA synthesis. A 30 minute incubation at 37 °C and 2 µl inactivation agent was used. 1 µg RNA per sample was used for cDNA

synthesis, which was performed with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) with Oligo $(dT)_{18}$ primer.

Primer development

The black raspberry genome sequence versions 1.1 (Jibran et al., 2018) and 3.0 (VanBuren et al., 2018) were used to develop primers. Genes of interest from *A. thaliana* were BLASTed against the black raspberry genome. Primers were created with the Primer3 tool (Koressaar and Remm, 2007; Untergasser et al., 2012) and ordered at Eurofins Genomics (Ebersberg, DE) (Tab. 3.1).

PCR and cloning of amplicons

***PI* genomic DNA cloning**

PI genomic DNA fragments were amplified with Long PCR Enzyme Mix (Thermo Scientific, Waltham, MA, USA) using 80 ng template from type 1 and type 3 plants. Genomic DNA was purified with the DNeasy PowerClean Pro Cleanup Kit (Qiagen, Hilden, DE) following the manufacturer's protocol. The PCR program was the following: initial denaturation at 94 °C for 2 min, 10 cycles of 94 °C for 20 s, 58 °C for 30 s and 68 °C for 6 min, 20 cycles of 94 °C for 20 s, 58 °C for 30 s and 68 °C for 6 min plus 5 s increments with a final extension step of 68 °C for 10 min.

A 1:100 dilution of genomic DNA fragments were reamplified with Phusion High-Fidelity DNA Polymerase (Thermo Scientific, Waltham, MA, USA), with the following PCR program: initial denaturation at 98 °C for 30 s, 33 cycles of 98 °C for 10 s, 63 °C for 30 s and 72 °C for 1 min with a final extension step of 72 °C for 10 min. These now blunt ended fragments were cloned with the TOPO XL-2 Complete PCR Cloning Kit with One Shot OmniMAX 2 $T1^R$ chemically competent *Escherichia coli* cells (Thermo Scientific, Waltham, MA, USA) following the manufacturer's protocol with a 30 min ligation.

Table 3.1. Primers used in this study. Names, sequences and annealing temperatures of the primers used in particular experiments.

Experiment	Name	Sequence	AT (°C)
Cloning *PI*	RiPI CDS2 F	ATG GGG AGG GGT AAG ATT GAG ATT	58
	RubPI CDS R	TTA CAT TCT GTC GTG GAG ATT AGG	
Cloning *LFY*	T InFU Insert F	AAT TCG AGC TCG GTA CTC CAC AAA ATA GGG CTA GGA C	55
	T InFU Insert R	AAC AGC TAT GAC CAT ACA GTT ACA CAT AAA ACG AAC GG	
	T InFU pUC19-V F	ATG GTC ATA GCT GTT TCC TGT GTG	55
	T InFU pUC19-V R	TAC CGA GCT CGA ATT CAC TGG C	
RT-PCR RidPI1	RiPI WT M13 F	TGT AAA ACG ACG GCC AGT ACA TGG AAG ATG CGT ATC ACC A	52
	RubPI CDS R	TTA CAT TCT GTC GTG GAG ATT AGG	
RT-PCR RidPI2	RiPI MT2 M13 F	TGT AAA ACG ACG GCC AGT CAC AAG CAG ACG ATA AAA	52
	RoPI mt e7R	TTT CAT TCT CTA TCG TGG TCG TCG	
RT-PCR LFY	RoLFYe3 1 F	AGG AGC GAA CTA CAT TAA CAA GC	52
	RoLFYe3 1 R	CGC TGA AAA TGG CAT CAA TGT C	
RT-PCR ef	EF F	ATT GTG GTC ATT GGY CAY GT	56
	EF R	CCA ATC TTG TAV ACA TCC TG	
***RidLFY-1* marker**	RiLFY TP F	GGA GAG GTA CGG CAT CAA GG	49
	RiLFY TP w R	GTT CCA CCC AAA TGT AAT AAA TGT	
***RidLFY-2* marker**	RiLFY TP F	GGA GAG GTA CGG CAT CAA GG	51
	RiLFY TP mt 2 R	GGT GCT GAC AGA AGT AGA GAA CA	

PI cDNA cloning

Type 1 and type 3 plant cDNA was amplified with Phusion High-Fidelity DNA Polymerase (Thermo Scientific, Waltham, MA, USA) using 3 µl template of 1:50 dilution. The PCR program was the following: initial denaturation at 98 °C for 30 s, 30 cycles of 98 °C for 10 s, 66 °C for 30 s and 72 °C for 30 s with a final extension step of 72 °C for 10 min.

The cDNA fragments were treated with DreamTaq enzyme at 72 °C for 20 minutes to create TA-ends. They were subsequently cloned with the TOPO TA Cloning Kit for Subcloning, with One Shot TOP10 chemically competent *E. coli* cells (Thermo Scientific, Waltham, MA, USA) following the manufacturer's protocol with a 10 min ligation.

Resulting positive colonies were purified with the GeneJET Plasmid Miniprep Kit (Thermo Scientific, Waltham, MA, USA) and sequenced at Eurofins Genomics (Ebersberg, DE).

LFY genomic DNA cloning

LFY genomic DNA fragments were amplified with CloneAmp HiFi PCR Premix (Takara Bio Europe SAS, Saint-Germain-en-Laye, FR) using 20 ng template from type 1 and type 3 plants. The PCR program was the following: 30 cycles of 98 °C for 10 s, 55 °C for 10 s and 72 °C for 15 s for type 1, 2 min for type 3 template, with a final extension step of 72 °C for 2 min. The vector pUC19 was linearized with the same protocol as the type 1 plant DNA, with 2 ng template. The fragments were cloned with the In-Fusion HD Cloning Kit (Takara Bio Europe SAS, Saint-Germain-en-Laye, FR) according to the manufacturer's protocol for spin-column purified fragments.

RT-PCR flower organs

Type 1 and type 3 whole flower and separated flower organ (sepal, petal, stamen and carpel) cDNA was amplified with Type-it Kit (Qiagen, Hilden, DE) using 1 µl template of 1:50 dilution. The PCR program was the following: 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. Annealing temperatures are listed in Table 3.1.

Allele specific PCR for the *RidLFY* gene

Type 1 and type 3 genomic DNA was amplified with the Type-it Kit (Qiagen, Hilden, DE) using 30 ng DNA as template. The PCR program was the following: initial denaturation at 95 °C for 5 min, 30 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. Annealing temperatures are listed in Table 3.1.

3.1.2. Results

Floral phenotyping

There were three floral phenotypes found in the RiSEP raspberry population during the four years of phenotyping (Fig.3.1). Out of 131 plants, ten plants were categorized as type 1, 65 plants type 2 and 19 plants type 3 as their summary phenotype. There were 37 plants that were not phenotyped in any of the years due to not flowering or the death of the plant. Not all plants showed consistency in their floral phenotype; 21 plants had at least in one year a different phenotype than the other years.

A random sampling of open blooms showed that when cut apart to their floral organs, type 2 flowers had more carpels than the other two types (Fig.3.1.E). In a small floral organ phenotyping evaluation, type 2 flowers had significantly more carpels than type 3 or the wild-type control (Fig.3.2).

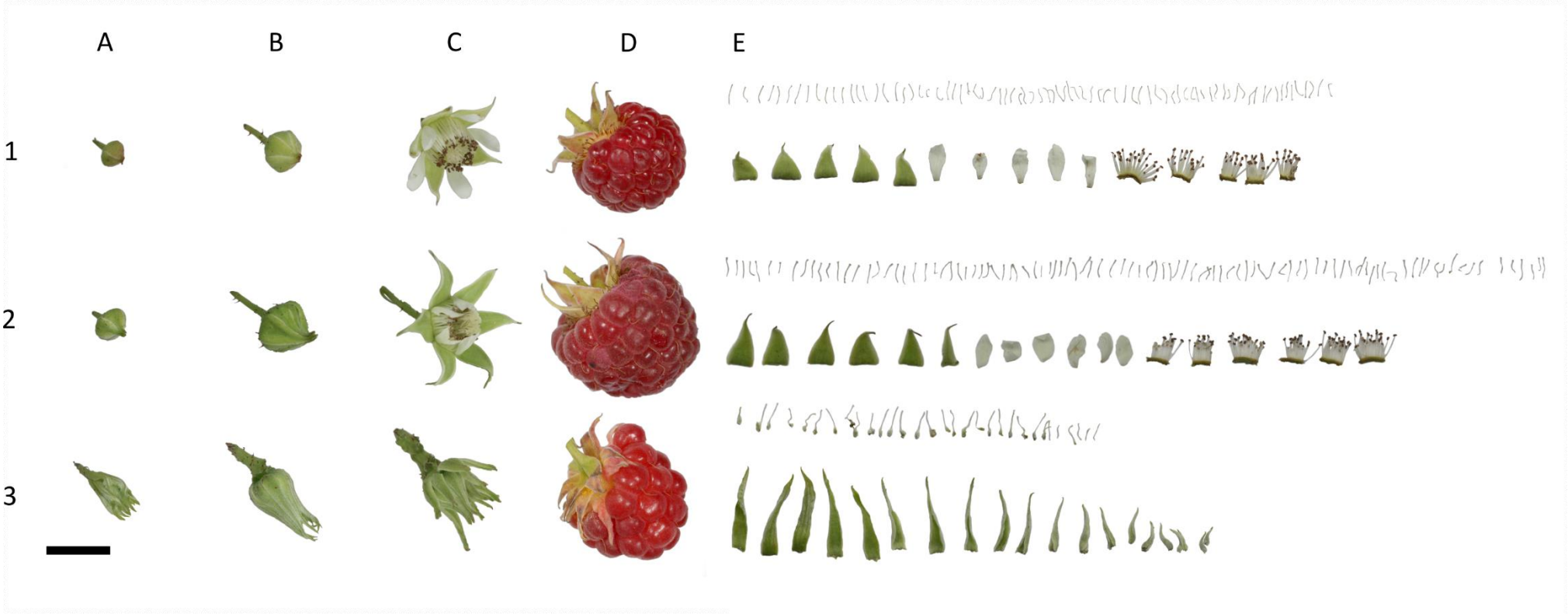

Figure 3.1. Floral stages in three flower and fruit phenotypes found in the RiSEP raspberry population. (1) Type 1 with five sepals and petals, stamens and carpels present. (2) Type 2 with six sepals and petals, stamens and carpels present. (3) Type 3 with sepals and carpels, no petals and stamens. (A) Small bud, (B) large bud, (C) bloom, (D) fruit and (E) flowers cut apart to their floral parts. Bar = 10 mm.

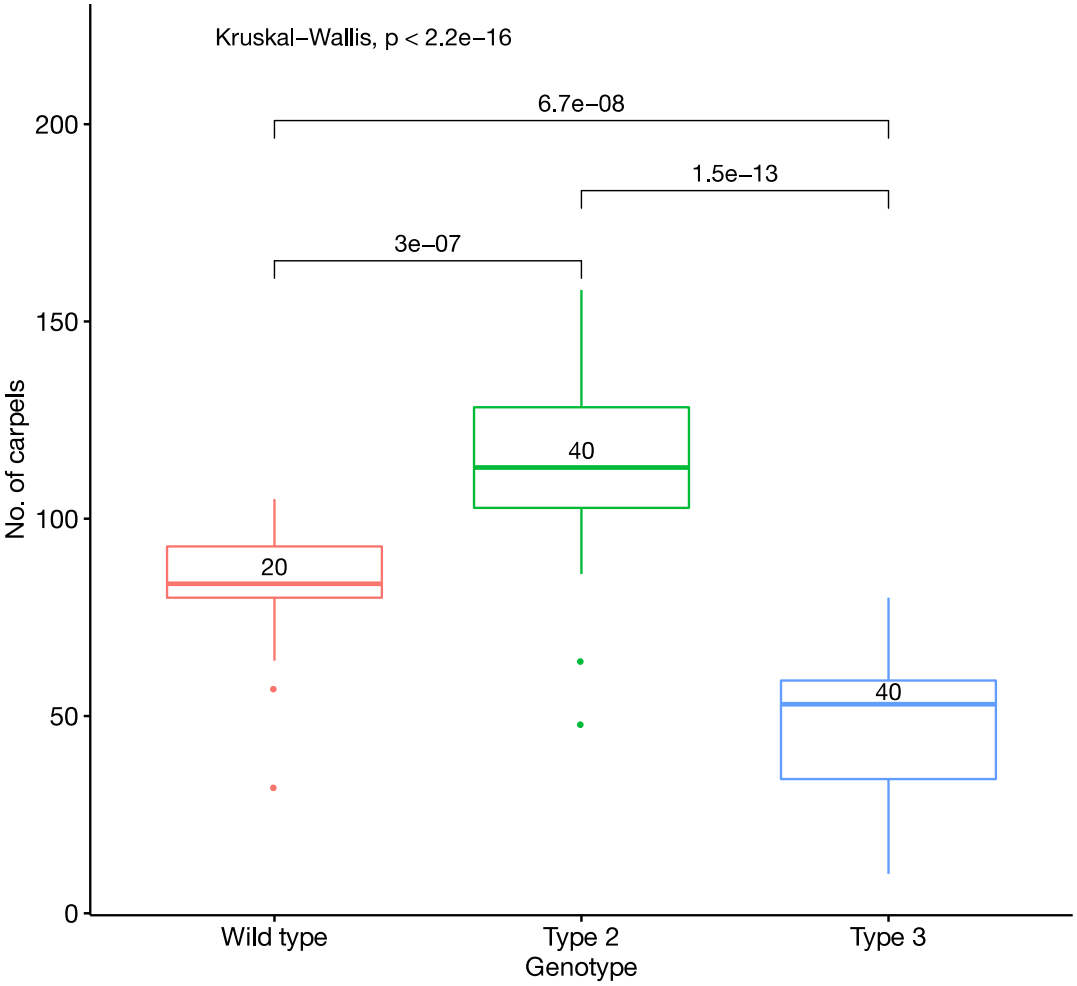

Figure 3.2. Boxplots of number of carpels per flower for two phenotypes found in the RiSEP raspberry population compared to a wild-type control 'Goldmarie'. Variance analysis calculated via Kruskal-Wallis, pairwise comparison via Wilcoxon test in R, significance between types is noted on horizontal lines, with n on its corresponding boxplot. Boxplots show the median value as thick line, interquartile range as the hinges, minimum and maximum values (if not exceeding 1.5 times the interquartile distance) as whiskers and outliers exceeding the 1.5 interquartile distance as single data points.

Isolation of flowering gene homologues

To identify PI in *R. idaeus*, amino acid sequences of putative MADS-box genes were identified in the *R. occidentalis* genome version v1.0.a1 protein dataset through a pHMMER search with the SRF-TF domain. The identified sequences were aligned with amino acid sequences of the known *A. thaliana* MADS-box genes. In addition to already annotated MADS-box genes, several MADS-box gene candidates were identified (e.g. Ro04 G02528, Ro03 G05331, Ro02 G34836, Ro01 G01137 and Ro02 G35014) in *R. occidentalis* that were previously not

annotated. One of these new MADS-box genes in *R. occidentalis* had high amino acid sequence similarity with the *A. thaliana* PI. This gene, Ro02 G35014, was used for primer design. Using the primers RiPI CDS2 F and RubPI CDS R a fragment of about 2800 bp was amplified on genomic DNA of *R. idaeus* type 1 and type 3 plants (Fig. S2).

Sequencing of the PCR products resulted in two distinct gene sequences (*RidPI1* and *RidPI2*). Both sequences were present in type 1 plants, but also in type 3 plants. They are not alleles but two distinct paralogues. The two genes have similar sequences near their start and stop codons which enabled their amplification in one reaction. *RidPI1* is around 2579 bp long, has seven exons and six introns. *RidPI2* is 2826 bp long and has seven exons and six introns as well. There are 82 nucleotide substitution differences between the exons of the two sequences. There are two gaps, six and 15 nucleotides long, in the seventh exon of *RidPI2* compared to *RidPI1*. *RidPI1* has two three-nucleotide gaps compared to *RidPI2* in their seventh exon.

After translating the new sequence information of *RidPI1* and *RidPI2* to amino acid sequences, a new pHMMER search was conducted as described above, but using the *R. occidentalis* protein database version v3.0 instead of v1.0.a1. There were 82 MADS-box predicted amino acids identified in *R. occidentalis* this way (Fig.3.3). An additional *R. occidentalis* sequence (Ro02 G35013) was found to also have a high sequence similarity to *A. thaliana* PI. A sequence alignment with the two PI amino acid sequences of *R. idaeus* and *R. occidentalis* and PI sequences of *M. domestica*, *P. persica*, *F. vesca*, *R. rugosa* and *R. chinensis* was performed (Fig. 3.3). Both RidPI1 and RidPI2 show similarity to *A. thaliana* PI. RidPI1 is similar to the PI of *R. occidentalis* (Ro02 G35014), *F. vesca, R. rugosa*, *M. domestica* and *P. persica*. RidPI2 is similar to the PI of *R. occidentalis* (Ro02 G35013), the *R. chinensis* predicted protein Rc A0A2P6PXI5 and the second PI of *F. vesca*. As for the remaining organ identity genes aside from PI, two AP3 homologues Ro02 G34836 and Ro01 G01137, one AP1 homologue Ro04 G02528, and one AG homologue Ro03 G05331 were found in *R. occidentalis*. The two *R. occidentalis* AP3 homologues showed high sequence similarity with one of the two AP3 homologues of *R. rugosa*, each. Out of the 82 *R. occidentalis* candidate genes, 76 in total show high sequence similarity to one or more MADS-box genes of *A. thaliana*.

Figure 3.3. Dendrogram of MADS-box proteins of *Arabidopsis thaliana* and *Rubus occidentalis* with a PI subtree of predicted PI proteins from *Malus domestica*, *Prunus persica*, *Fragaria vesca*, *Rosa rugosa*, *Rosa chinensis* and the two *R. idaeus* PI paralogues, RidPI1 and RidPI2. Sequence alignment and dendrogram construction were performed in MEGA-X using UPGMA method and 125 bootstrap replicates. The PI branch is highlighted in blue; the two *R. idaeus* PI paralogues RidPI1 and RidPI2 are highlighted in orange. The PI of *M. domestica* is labeled Md Q9AVU7 PI, of *P. persica* is labeled Pp A7LCX0 PI, of *F. vesca* are labeled FvH4 2g27860 1 PI and FvH4 2g27870 1 PI, of *R. rugosa* is labeled Rr Q9FZN1 PI and the second rose PI of *R. chinensis* is labeled Rc A0A2P6PXI5 PI. The two AP3 paralogues of *R. rugosa* are labeled Rr Q93X10 AP3 and Rr Q7X9I8 AP3.

There are several differences in the amino acid sequence between the two paralogues RidPI1 and RidPI2, most of them near the 3'end. There are in total 45 amino acid differences between the two sequences. There are eight amino acid differences in the MADS-box of RidPI2 compared to RidPI1 (Fig.S1). These eight differences are: T15S, N18K, K25Q, N33K, S51T, R53K, N55V and Q56E. The functional annotations of the *R. occidentalis* MADS-box gene candidates can be found in Table ES1d.

Spatial expression of *RidPI* genes in type 1 and type 3 flowers

The expression of the identified *PI* genes of *R. idaeus* was studied on cDNA of whole flower buds and separated flower organs of type 1 and type 3 flowers, respectively. An RT-PCR using gene specific primers for *RidPI1* (RiPI WT M13 F and RubPI CDS R) and *RidPI2* (RiPI MT2 M13 F and RoPI mt e7R) resulted in comparable expression pattern for both genes (Fig. 3.4). Both *RidPI* genes are clearly expressed in whorls 2 and 3 (petals and stamens) of type 1 flower samples, whereas only weak expression was found in whorls 1 and 4 (sepals and carpels). The expression of both genes was weak in all whorls of type 3 flower samples.

Identification and expression of possible upstream regulators

To find out the reason for this low *RidPI* gene expression in type 3 flowers, possible upstream regulator genes of *RidPI* were isolated. Using the genome sequence of *R. occidentalis*, homologues of the *ASK1*, *UFO* and *LFY* genes were identified. Gene specific primers for each of the genes were developed and tested on genomic DNA. PCR fragments of *ASK1* and *UFO* showed the same length between type 1 and type 3 gDNA samples (Fig. S3).

In contrast, the *LFY* homologue has shown a fragment size difference. Amplicons of different sizes were obtained with gDNA samples of type 1 and type 3 plants (Fig. S4). For type 1 plants, an amplicon of 2,478 bp was detected. This gene sequence was named *RidLFY-1*. *RidLFY*-1 was only found in type 1 samples, whereas a much longer fragment of 6,215 bp was detected using gDNA of type 3 samples. This sequence was designated as *RidLFY-2* and contained an insert of a 3,737 bp long transposon between exons 1 and 2 (Fig. 3.5). This transposon can be classified as a DNA transposon (Abrusán et al., 2009). It has a direct repeat segment of 10 n (GAG GCT

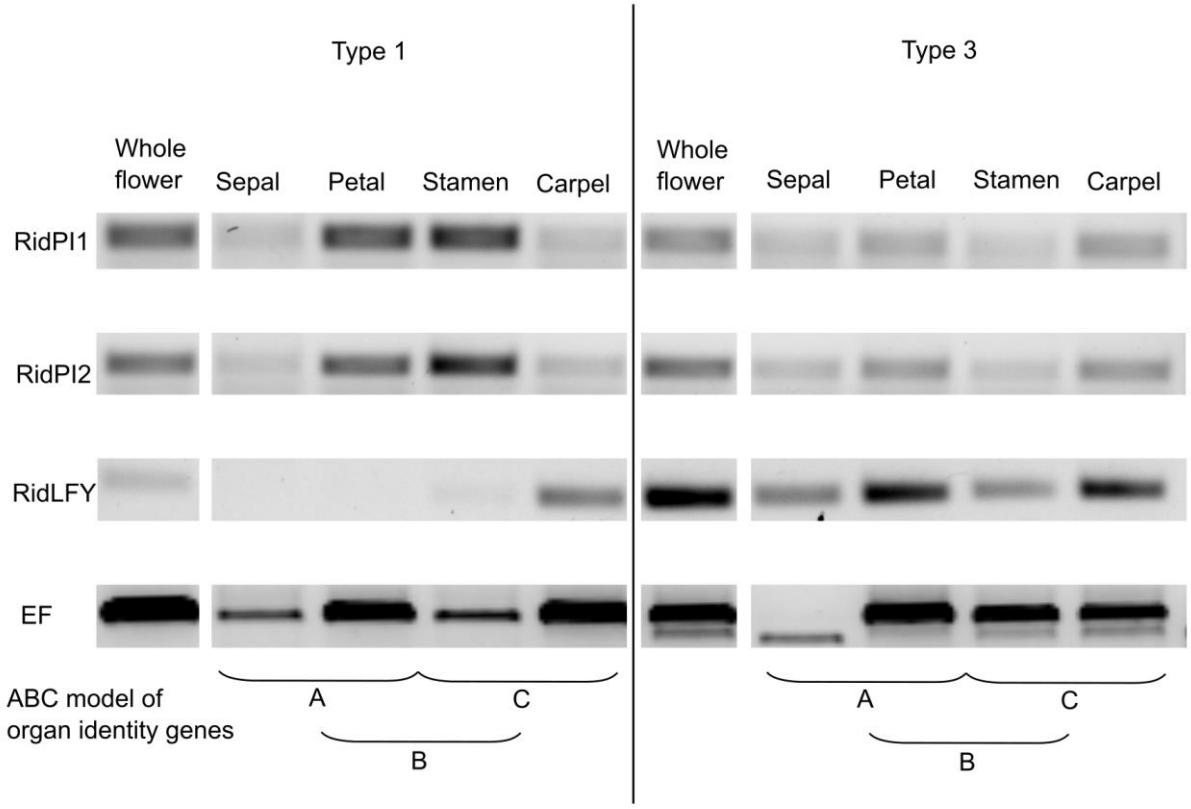

Figure 3.4. Agarose picture of whole flower and whorl cDNA RT-PCR, amplified with *PI* and *LFY* primers. Templates were taken from type 1 and type 3 open flowers, whole flowers and separated flower organs: sepals, petals, stamens and carpels. Fourth row depicts fragments amplified with the housekeeping gene *ELONGATION FACTOR 1 ALPHA*. The ABC model of organ identity genes is showing in which whorl the class genes are active.

AGA C) at its start and end, and an 11 n long inverted terminal repeat (TGG ACA GAA GA – TCT TCT GTC CA).

RidLFY-2 has a three-nucleotide insertion and three one-nucleotide substitutions in exon 2 and a one-nucleotide substitution in exon 3 compared to *RidLFY-1*. Only two of these result in a change in the amino acid sequence in RidLFY-2; one missense substitution, A219G, and the duplication of a glutamic acid (E) at 202, making this allele one amino acid longer than RidLFY-1 (Fig. S5).

In type 1 flowers, transcripts of *RidLFY* were only detectable in whorl 4, whereas in type 3 flowers the gene was expressed in all flower whorls (Fig.3.4).

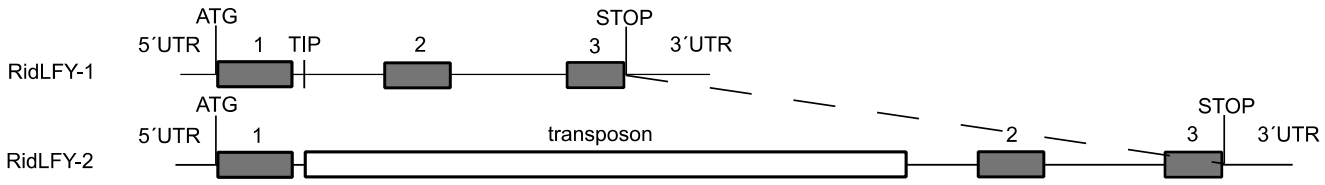

Figure 3.5. Schematic representation of the *RidLFY-1* and *RidLFY-2* gDNA sequences. Sequence schematic is to scale. The grey boxes signify exons, between exons 1 and 2 is the insertion point on *RidLFY-1* where the two sequences start to diverge because of the transposon in *RidLFY-2*.

Based on the sequence of the two *LFY* alleles, allele specific primers were designed. These primers were tested on gDNA samples of all plants of the RiSEP half-sib family. Seven out of 131 plants had only the *RidLFY-1* allele, 50 plants contained both alleles, whereas 37 plants contained only the *RidLFY-2* allele. The missing 37 plants were not tested due to perishing before harvesting DNA samples.

Association between the mutant phenotype and the occurrence of *RidLFY-2*

Phenotyping data and phenotype summaries for all plants in relation to their *RidLFY* allele status is shown in the electronical appendix (Tab. ES2). All plants phenotyped as type 1 had only the *RidLFY-1* allele. All plants phenotyped as type 3 had only the *RidLFY-2* allele, with nine plants phenotyped as type 3 one time out of the four years. Most plants phenotyped as type 2 were heterozygous to the alleles, except for 19 plants, which had only the *RidLFY-2* allele. The distribution of the phenotyping results of four years to the *RidLFY* allele status of plants assessed by the chi-squared test resulted in a p-value of 1.58e-15. Thus, phenotype is significantly linked to the *RidLFY* allele status in the RiSEP population.

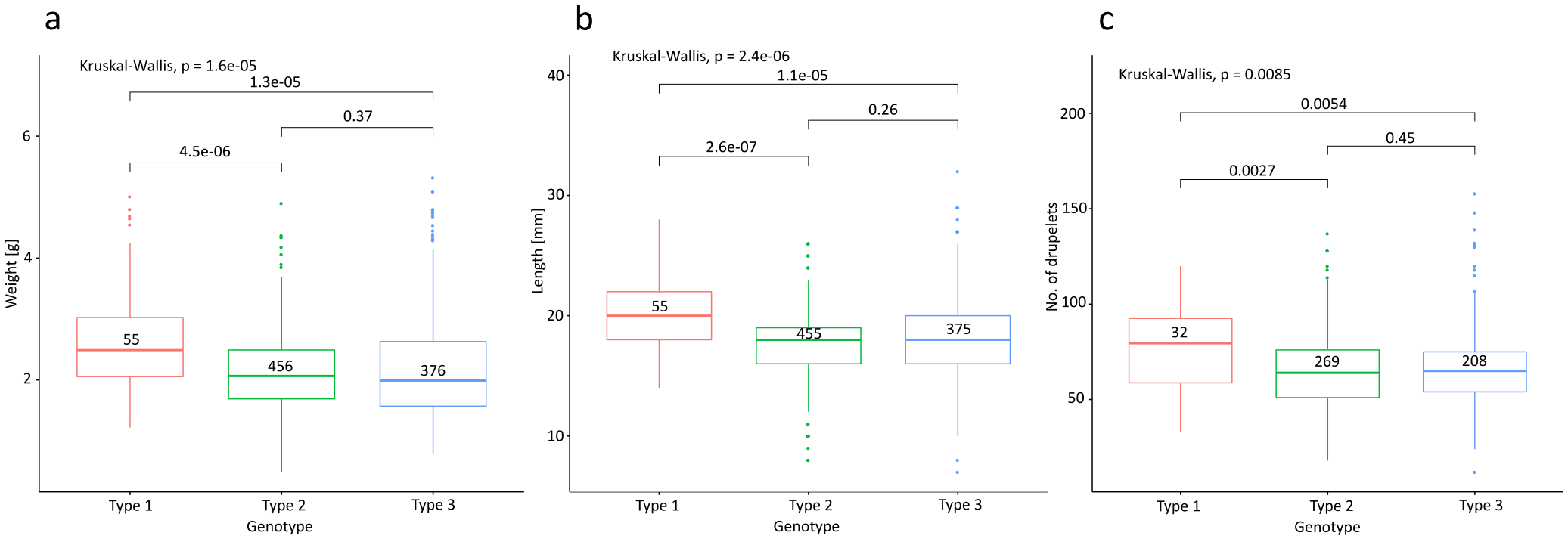

Figure 3.6. Boxplots of fruit weight (a), fruit length (b) and number of drupelets (c) in a raspberry aggregate fruit for the three genotypes found in the RiSEP raspberry population. Variance analysis calculated via Kruskal-Wallis, pairwise comparison via Wilcoxon test in R, significance between types is noted on horizontal lines, with n on its corresponding boxplot. Boxplots show the median value as thick line, interquartile range as the hinges, minimum and maximum values (if not exceeding 1.5 times the interquartile distance) as whiskers and outliers exceeding the 1.5 interquartile distance as single data points.

Fruit phenotyping

To visualize the fruit phenotyping values, the RiSEP population individuals were put into three genotype categories based on their *RidLFY* alleles. These genotypes were type 1 for plants with only the *RidLFY-1* allele, type 2 with both alleles and type 3 for plants with only the *RidLFY-2* allele. All three of the recorded qualities, weight, length and the number of drupelets of the fruit were significantly larger in type 1 fruit than both types 2 and 3 (Fig. 3.6). Types 2 and 3 fruit had no significant difference to each other in all three fruit phenotyping qualities. The genotype categories caused considerably unequal sample sizes, as there were only seven plants with type 1 genotype, more than five times less than the other two genotypes. This resulted in considerably smaller sample size for type 1 plants compared to types 2 and 3.

4. General discussion

The research presented in this thesis was conducted in order to develop molecular genetic tools to aid raspberry breeding. Since the main aim was concentrated around the important traits of adaptability to new growing techniques, resistance to diseases and pests, fruit quality and yield, the following experiments were undertaken to answer the four main objectives:

In manuscript 1, raspberry cultivars from multiple sources were tested with SSR markers for trueness-to-type to examine the extent of cultivar mismatches in Germany. Manuscript 2 analyzed SSR marker genotyping results of progenies from open pollinated cultivars, in addition to a hand-pollination and a pollen-restricted topcross experiment to identify self-incompatible cultivars. In manuscript 3, an interspecific hybrid population of *R. occidentalis* × *R. idaeus* was genotyped with SNP markers obtained through GBS, as well as SSR markers with the goal of mapping a resistance trait, waxy bloom. Finally, in the additional results, a raspberry population with a naturally occurring flower mutation was phenotyped for three disparate flower types. In order to find the gene responsible for the mutated flower phenotype, the SRF-TF domain was used to identify MADS-box genes in the draft genome of *R. occidentalis*, which were then used for primer development in *R. idaeus*. After our first candidate, *PI*, was demonstrated not to be responsible, its upstream regulator, *LFY* was investigated as a candidate gene.

The insights gained from these manuscripts and additional results present the following answers to the four main objectives stated in the general introduction:

1. Nine out of 33 cultivar samples were not true-to-type. Although it is only a small sampling without statistical significance, this result raises awareness to the issue of cultivar mismatches in the German raspberry trade.
2. Out of the 16 cultivars tested, ‘Rumla’ proved to be highly self-incompatible in all three experiments, while the other cultivars revealed more flexibility to tolerate self-fertilization. The pre-testing described in our study could be used to identify self-incompatible cultivars like ‘Rumla’ that would present an issue as a monoculture in protected growing.

3. Gene *B*, responsible for the resistance trait waxy bloom, was mapped to LG2, equivalent to chromosome 2, in *R. occidentalis*.
4. Several previously unannotated MADS-box genes in *R. occidentalis* were described, additional to two *PI* homologues in *R. idaeus*. We demonstrated that neither of these two paralogous genes, *RidPI1* and *RidPI2*, is responsible for the mutated flower phenotype, although their expression is downregulated in such flower tissue. We have also presented two alleles of a *LFY* homologue in *R. idaeus*, *RidLFY-1* and *RidLFY-2*, with *RidLFY-2* containing a transposon in comparison to *RidLFY-1*. The three flower types phenotyped in the population correlate with their *RidLFY* allele genotype. The findings of this study indicate that in genotypes containing *RidLFY-2*, *PI* expression is downregulated through the presence of *LFY* in later flower stages.

The results summarized above were presented in-depth in the three manuscripts and as additional results in the previous two chapters. Rather than repeating the content of the manuscripts by discussing their results in detail, the key ideas of the thesis will be addressed in the current chapter. The broader meaning of the thesis will be explored by the connections and relationships between the manuscripts. In addition, findings previously not discussed will be presented as well. Furthermore, an overarching conclusion and possible applications and indications for raspberry growing, breeding and research, the latter proposed as future work, will be presented in the following sections.

4.1. Trueness-to-type of raspberry cultivars

Trueness-to-type has always been important in the growing and breeding of clonally propagated plant species. DNA fingerprinting through molecular markers presents a convenient alternative for pomological cultivar testing. The use of molecular markers is especially important in a crop like raspberry, were phenotypical identification is difficult. In all three manuscripts, we found plants that were not true-to-type. In manuscript 1, nine out of 33 samples were not true-to-type. In manuscript 2, two out of the 16 mother plants were cultivar mix-ups and 7 out of 360 progenies showed single allele discrepancies. Finally, in

manuscript 3, one of the 146 individuals was found not to be the progeny of the 'Black Jewel' × 'Glen Ample' cross. Additionally, pre-testing the parent cultivars of the crossing population revealed one of the four 'Black Jewel' plants in our experimental fields not matching the population's SSR marker profile (unpublished data). Clearly, the issue is prevalent and it has consequences in multiple disciplines of raspberry growing, breeding, trade and research. As mentioned in manuscript 1, in light of the high prevalence of mix-ups, testing plant material with molecular markers is necessary for both research and commercial purposes. As mix-ups can occur during propagation or shipping of plant material, nursery staff must proceed with utmost care during making cuttings, and maintaining clear labelling for tissue culture and shipping of plants alike. In research, a high comparative value of studies is very important. For this reason, cultivar mix-ups can lead to issues in genetic mapping as well. Bushakra et al. (2012) were unable to compare their results with a previous study by Graham et al. (2004) because 'Latham', a parent in both mapping populations, was not the same genotype. As mapping is still an expensive experiment, using the right genotype is of the utmost importance for the longevity of results.

In a previous study at our Institute, Girichev et al. (2015) couldn't confirm the pedigree of 'Meeker' ('Willamette'× 'Cuthbert' (Jennings, 1988)) based on its SSR marker profile. However, the trueness-to-type of all five 'Meeker' samples we tested can be validated using the pedigree information of the cultivar, with the marker profiles of 'Willamette' (manuscript 2), and 'Cuthbert' from Bassil et al. (2012). In addition, our 'Meeker' samples also match the sample Bassil et al. (2012) received from the Hutton Institute. Although there is a 5-6 n difference between our results, these appear consistently for all four markers and three cultivars tested in both studies. Consequently, the discrepancies are most likely due to technical differences in capillary electrophoresis platforms and can be ignored.

Bassil et al. (2012) tested two 'Meeker' samples in their study above. The samples differed in one allele, for which the authors named a cultivar specific predisposition to somaclonal variation as a possible cause. We found similar, one allele, discrepancies between progenies and their mother cultivars in seven samples (manuscript 2). However, these plants were not created by tissue culture, but were products of sexual propagation. As there is a reportedly high mutation rate in raspberries (Janick, 2009), both discrepancies are more likely due to spontaneous mutation. Assessing the mother cultivars of manuscript 2 from multiple sources

for single allele discrepancies could determine whether the reason is spontaneous mutation. As for Bassil et al.'s (2012) hypothesis, DNA samples of cultivars and their tissue cultured clones could be compared to investigate the influence of somaclonal variation on SSR marker fragment size.

The probability of identity (PoI) analysis showed that the 15 markers used in manuscript 1 were more than enough for secure cultivar identification. Although SSR markers have certain weaknesses, like the possibility of size homoplasy shown in other species (Barkley et al., 2009; Samarina et al., 2021), their use in cultivar identification is considerably less prone to errors than in interspecies taxonomic or phylogeny studies. Indeed, all not true-to-type samples tested in manuscript 1 differ in 8-13 markers out of the 15. As the PoI is under 0.05 after four markers, a standard set of eight markers in two multiplex PCR reactions could be a convenient way of raspberry cultivar identification. A similarly sized eight-SSR fingerprinting set was published recently for blackberry parentage and identity testing (Zurn et al., 2018).

However, this is not the same for all *Rubus* species. Dossett et al. (2012a, 2012b) reported low allelic diversity and inability to distinguish between *R. occidentalis* cultivars with 18 and 21 SSR markers. Five of those markers were also tested in our, and in Girichev et al.'s (2015) studies (RhM003, Rubus275a, Rubus270a, Rubus123a, Rubus223a). Dossett et al. (2012a) have also found that although wild species accessions had high allelic diversity, they had lower than expected heterozygosity at every polymorphic locus and suggested a degree of inbreeding caused by bottlenecking and/or isolation of wild populations as reason. These results revealed how *R. occidentalis* wild accessions have lower heterozygosity than cultivars (Dossett et al., 2012a), but they don't show how they both compare to *R. idaeus*. Using the results for the five markers common in Girichev et al. (2015) and Dossett et al.'s (2012a) study reveals that *R. idaeus* cultivars have significantly higher allelic diversity and expected heterozygosity than *R. occidentalis* cultivars and a significantly higher observed heterozygosity than *R. occidentalis* wild accessions (Tab.4.1). Furthermore, *R. idaeus* cultivars' observed heterozygosity is almost twice as high as *R. occidentalis* cultivars' on average, with higher values at every marker, even though this difference is not statistically significant.

Table 4.1. Comparison of marker data analysis between *R. occidentalis* cultivars, wild accessions and *R. idaeus* cultivars by Dossett et al. (2012a) and Girichev et al. (2015). Allelic diversity, expected heterozygosity (He) and observed heterozygosity (Ho) for 5 SSR primer pairs used in both studies. Values of Girichev et al. (2015) were recalculated after excluding non-*R. idaeus* cultivars. Statistical significance was analyzed by one-way ANOVA followed by a Tukey HSD multiple pairwise-comparison in R.

Primer	Dossett et al., 2012a						Girichev et al., 2015		
	R. occidentalis cultivars n = 21			*R. occidentalis* wild accs. n = 125			*R. idaeus* cultivars n = 75		
	Alleles	He	Ho	Alleles	He	Ho	Alleles	He	Ho
RhM003	3	0.56	0.81	6	0.57	0.22	7	0.78	0.89
Rubus275a	5	0.66	0.76	20	0.91	0.35	19	0.87	0.92
Rubus270a	2	0.44	0.57	9	0.78	0.23	18	0.80	0.88
Rubus123a	2	0.09	0.1	4	0.51	0.21	25	0.92	0.85
Rubus223a	1	0.00	0.00	6	0.54	0.25	14	0.81	0.61
Mean	2.6**	0.35**	0.45	9	0.66	0.25**	16.6**	0.84**	0.83**

** Significant at $p < 0.01$

4.2. Differences in allelic heterozygosity between *R. occidentalis* and *R. idaeus*

Our results from manuscript 3 are consistent with the comparison of previous studies above (Tab.4.1, Dossett et al., 2012a; Girichev et al., 2015): we found that the *R. idaeus* cultivar 'Glen Ample' had three times as many heterozygous SNP markers than the *R. occidentalis* cultivar 'Black Jewel'. Due to the nature of linkage mapping based on polymorphic markers, this reflected in the number of markers mapped (877 vs. 443) and in the length of the linkage maps (660,53 vs. 479,76 cM) between the cultivars as well. The low heterozygosity of 'Black Jewel' (or 'Jewel' in the USA) can partly be explained by its pedigree, as it is a backcross of ('Bristol' × 'Dundee') × 'Dundee' (Jennings, 1988). However, as *R. occidentalis* wild accessions' observed heterozygosity is even lower than cultivars' (Dossett et al., 2012a), this is not only due to an artificial crossing. Furthermore, the two species have reportedly differing inbreeding coefficients as well. Bushakra et al. (2012) calculated an inbreeding coefficient of 0.35 for the *R. occidentalis* parent of their mapping population. In contrast, the mean inbreeding coefficient of 137 cultivars was 0.118 in *R. idaeus* (Dale et al., 1993). As mentioned in the general introduction, it has been suggested that self-compatibility in *R. idaeus* occurred more recently than in *R. occidentalis* (Keep, 1968a; Lewis and Crowe, 1958). The difference in

heterozygosity supports this hypothesis as well, as it would make sense that a species that has been longer self-compatible has more selfings in its pedigree and is thus less heterozygous than a species that became self-compatible recently. Moreover, loss of self-incompatibility occurs far more commonly than its gain (Igic et al., 2006, 2008). With these facts in mind, the most probable scenario is that *R. occidentalis* gained self-compatibility after the split from their common self-incompatible ancestor, and *R. idaeus* continued to be self-incompatible until its domestication.

Due to the low heterozygosity of *R. occidentalis* and thus poor polymorphism of its SSR markers, gene *B* could only be mapped to a 2 Mb physical interval, and its position relative to gene *H* couldn't be ascertained (manuscript 3). Future work should concentrate on enhancing the quality of the linkage map by fine mapping the gene *B* region. This could be achieved by using additional SSR markers derived from *R. occidentalis* as opposed to the ones we used from *R. idaeus* to genotype the population. *R. occidentalis* was found to have twice as high efficiency of polymorphic SSR recovery from its own species than from *R. idaeus*, while the latter had no difference between donor species (Dossett et al., 2015). Therefore, *R. occidentalis*-derived markers could be used without restrictions in the *R. idaeus* parent too, making it possible to confirm gene *B*'s position relative to gene *H*. Furthermore, a tighter mapping could deliver a marker for the trait as well.

While mapping in the interspecific population, we found discrepancies in the map positions of markers with previous mappings (Castillo et al., 2010; Graham et al., 2006), as well as between *R. occidentalis* and *R. idaeus*. As for the former, aside from cultivar mix-ups discussed in section 4.1 (Bushakra et al., 2012), or actual differences due to high heterozygosity between cultivars (i.e. varietal polymorphisms), the quality of the genome assemblies and mappings could also present a reason. The current linkage group nomenclature first introduced by Bushakra et al. (2012) was obtained by aligning the 'Latham' genetic map to *F. vesca*, *M. domestica* and *P. persica* draft genomes with common orthologous markers. The draft genomes of *R. occidentalis* used *F. vesca* for anchoring as well (Jibran et al., 2018; VanBuren et al., 2016, 2018). Furthermore, the first draft genome of *R. idaeus*, which was published recently as a preprint by Wight et al. (2019), was anchored by linkage maps from its own species (Ward et al., 2013). All mappings and draft genomes above were made by computational gene prediction and anchoring with Rosaceae genomes. However, even the

small genome of the model organism *A. thaliana* is still being improved, with a recent re-annotation correcting 10% of gene models (Cheng et al., 2017). In a recent publication about *Actinidia chinensis var. chinensis*, kiwifruit, multiple genetic mapping and sequence resources combined with EST sequencing and RNA-Seq data were used to manually annotate a new genotype (Pilkington et al., 2018). This approach resulted in 33,123 protein isoforms, of which only 9,4% were found in the previous genome assembly, and revealed evidence of local and global gene duplications (Pilkington et al., 2018). During our linkage mapping in the interspecific population, a few SNPs were mapped to a different linkage group from the chromosomes of their physical alignment, which points to the likelihood of gene duplications in the *R. occidentalis* and *R. idaeus* genomes as well (manuscript 3). A community annotation project like Pilkington et al.'s (2018), using the already available mapping and genome data of *R. idaeus* and *R. occidentalis,* could be used to improve the draft genomes of both species and shed light to the marker location discrepancies found in manuscript 3. The discrepancies between the map positions of markers between *R. idaeus* and *R. occidentalis* mentioned above raise a question of the transferability of SSR markers between the species, and the suitability of *R. occidentalis* as the sole basis for *R. idaeus* marker development. In the additional results, we used the *R. occidentalis* draft genome to identify floral development genes and used these as a basis for primer development to find the gene responsible for the mutated phenotype in the RiSEP population.

4.3. *R. occidentalis* as basis for developing *R. idaeus* molecular genetic tools

In an initial phenotyping of the population, three flower phenotypes were found with different amount of carpels. One of the three phenotypes, type 2, had significantly higher number of carpels than type 3 and wild-type control flowers. As the number of drupelets is directly related to the number of pollinated carpels, this trait could be used to develop a marker for fruit size. Our hypothesis was that the variance of three phenotypes were caused by the loss of function mutation of one single gene. This gene being homozygous to the non-mutated allele in type 1 plants, heterozygous in type 2 plants and homozygous to the mutated allele in type 3 plants. A recessive inheritance for sepaloidy was suggested by Lewis (1939) and Keep (1964). *PISTILLATA* was chosen as the candidate gene for this mutation since it is responsible

for the development of the affected whorls, and is proven to be the gene in question in a similar phenotype in apple (Yao et al., 2001). Although *APETALA3* is also an organ identity gene active in whorls 2 and 3, it has multiple homologues in *M. domestica*, *R. rugosa* and *R. occidentalis* and thus more robust against mutations affecting phenotype (Hibino et al., 2006; Kitahara et al., 2004). Hence we found it less likely to be a source of the mutation in *R. idaeus*.

The two *PI* homologues, *RidPI1* and *RidPI2*, were both found in type 1 and type 3 genomic DNA samples. This makes our hypothesis of the loss-of-function mutation of *PI* causing the mutated flower phenotype not possible. The presence of *RidPI1* and *RidPI2* in type 1 and type 3 plants could mean that they are either two different alleles of the same gene, or they are two paralogous genes in both of these genotypes. After repeating the pHMMER search in the *R. occidentalis* v3.0 protein database (VanBuren et al., 2018), each of the two *R. idaeus* PIs aligned to a separate amino acid sequence. These two proteins are encoded by two sequential genes, Ro02 G35013 and Ro02 G35014, in *R. occidentalis*. This supports the assumption that *RidPI1* and *RidPI2* are sequential paralogues in *R. idaeus* as well.

The amino acid sequences of the two paralogous genes *RidPI1* and *RidPI2* showed high sequence similarity to the PI of *A. thaliana*. As mentioned above, they also showed high sequence similarity each to two PIs encoded by adjacent genes in *R. occidentalis,* where *PI* is as of yet not described. This sequential paralogue pattern can also be found in *F. vesca* and *R. chinensis,* where two adjacent genes code for predicted proteins with high sequence similarity to the two *R. occidentalis* and *R. idaeus* PIs each. Davis et al. (2010) described the two *F. vesca PI*s as tandemly duplicated. Although only one PI was published in *R. rugosa*, MASAKO BP, by Hibino et al. (2006), Liu et al. (2018) described five predicted PI proteins in *R. chinensis*. Out of these five predicted proteins, RcPI4 is identical to MASAKO BP and RcPI3 differing in only one amino acid from the *R. chinensis* PI homologue A0A2P6PXI5.

Thus, *R*. *idaeus* and *R*. *occidentalis* join *F. vesca* and *R. chinensis* in having at least two *PI* homologues each. In contrast, in *M. domestica* and *P. persica* only one *PI* is known (Yao et al., 2018; Zhang et al., 2008). This points to a gene duplication event after the separation of *Amygdaloideae* and *Rosoideae* (Xiang et al., 2016). Gene duplication occurs often in plants but the duplicates also tend to be eliminated or silenced, even with positive changes, like increased expression or heterozygosity. A more secure way of being fixed in the genome is through divergence in function, as in the timing or the pattern of expression (Otto and Yong,

2002). It is not unprecedented for gene doubling to occur with the MADS-box genes, as these flower development genes did get created by gene doubling of an ancestral MADS-box gene (Floyd and Bowman, 2007). In contrast, only one *AP1* and *AG* analogue was found in *R. occidentalis* and thus do not seem to be doubled. Although *AP2* belongs to the class A flower organ development gene group, it is not a MADS-box gene, and therefore no homologue was found in *R. occidentalis*.

Study of the expression in open flower samples of the two *PI* paralogues showed that they both function in the right whorls (petals and stamens) in type 1 samples, but their expression is low in all type 3 samples. This suggests a similar functionality during flower development for the paralogues. Future studies could use gene inactivation experiments, for example in stably transformed plants, RNAi-based gene silencing, VIGS (virus induced gene silencing) or genome editing; by comparing phenotypes and expression patterns after inactivation of one or both *PI*s it could be clarified if they can be substituted for class B function or have a different function from each other. The same could be done for the two newly found *AP3* paralogues found in *R. occidentalis*.

Both *PIs* have lower expression in all type 3 flower whorls than in that of type 1 (Fig. 3.4). The transcription of *PI* is therefore affected in type 3 flowers, even if there is no difference on the genomic DNA level. A disturbance of a gene regulating class B genes could lower *PI* expression. Gene expression of class B genes is regulated by *ASK1*, *LFY* and *UFO* (Levin and Meyerowitz, 1995). Out of these three candidate genes only *LFY* showed a noticeable fragment length difference between type 1 and 3 plant gDNA (Fig. S3). With this information, we chose *LFY* as our new candidate gene for the flower mutation.

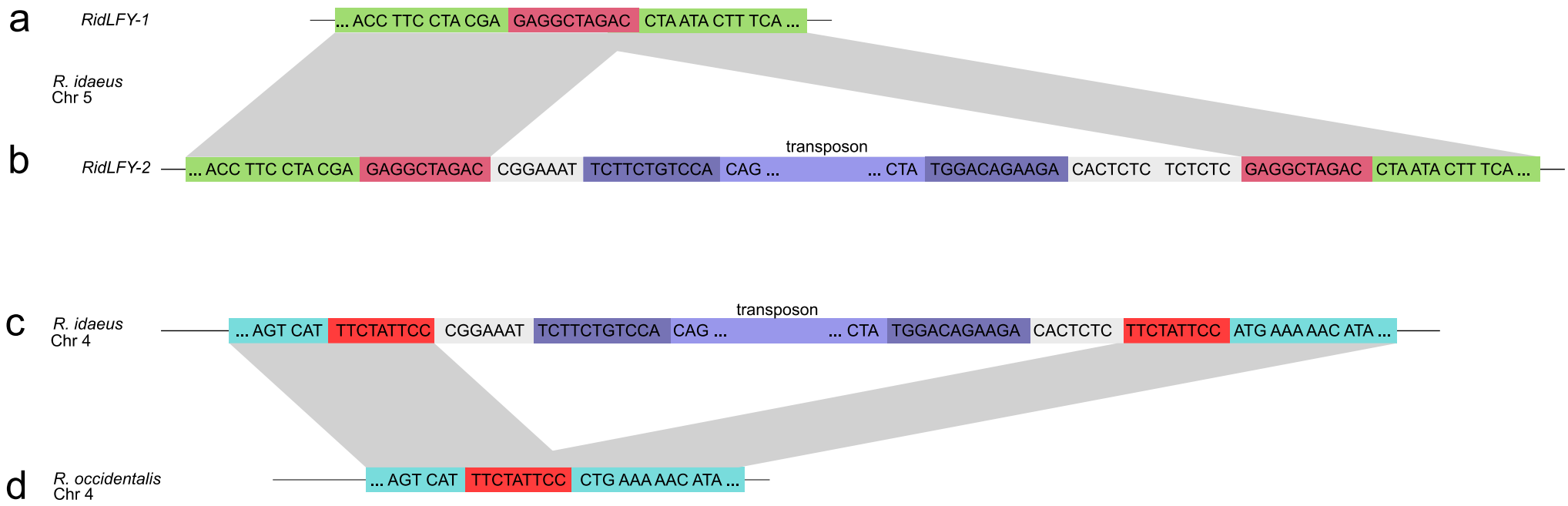

Figure 4.1. Comparison of the transposon and insertion site found in *Rubus idaeus* and *Rubus occidentalis*. The insertion site is in intron 1 of *RidLFY* on chromosome 5 in the RiSEP population, (a) in RidLFY-1 with no transposon insertion and (b) in RidLFY-2 with a transposon. (c) In the *R. idaeus* genome assembly (Wight et al., 2019), the transposon is inserted in a noncoding sequence between the genes *Rr0342* and *Rr0343*, on chromosome 4. (d) In the *R. occidentalis* genome assembly (VanBuren et al., 2018), no transposon is inserted in the homologous region on chromosome 4, between the genes *Ro04_G23784* and *Ro04_G23785*. Sequence schematic is not to scale, ellipses mark sequence cut-offs. The transposon sequence is shown in purple, with the inverted terminal repeats dark purple. Flanking direct inserts are shown in pink for RiSEP and in red for *R. idaeus* and *R. occidentalis* draft genomes. The sequence originally found in the insertion site is shown in green for RiSEP and in blue for *R. idaeus* and *R. occidentalis* draft genomes. The sequences not found in the insertion site are shown in white. The grey bars indicate the shifting of the insertion site sequences compared to the sequence with the transposon.

The allele *RidLFY-2*, which is only present in type 2 and 3 plants, has a 3,737 nucleotide long transposable element between its first and second exons. The transposon can be further characterized by sequence comparison with the *R. idaeus* and *R. occidentalis* draft genome assemblies (VanBuren et al., 2018; Wight et al., 2019) via BLAST (Fig. 4.1). The transposon can be found on scaffold SC0000015 in *R. idaeus* as a continuous sequence with 100% sequence identity. It is surrounded by a direct insert differing from the RiSEP sequence and is located between the genes *Rr0342* and *Rr0343*. These genes are homologues of *Ro04_G23784* and *Ro04_G23785*, respectively, in *R. occidentalis*, found on chromosome 4. However, the transposon sequence can not be found in *R. occidentalis.*

The *LFY* homologue *Rr028331* can be found on scaffold L1SC0000008 in *R. idaeus*, and on chromosome 5 in *R. occidentalis* (as *Ro05_G01825*). There are short sequences surrounding the transposon in both *R. idaeus* sources that did not originally belong to the insertion site. These sequences are the same in both sources beside a dinucleotide repeat in *RidLFY-2* (Fig. 4.1). Such tandem repeats often arise from replication errors like slippage during meiosis (Walsh, 1987). Thus, it seems that the transposon is the same in both sources, on chromosome 5 in RiSEP plants and on chromosome 4 in 'Joan J', the cultivar used in the *R. idaeus* draft genome assembly. It is likely that the transposon is present in other *R. idaeus* cultivars as well, moving to different parts of the genome.

Although this transposon is not in the coding sequence and should not change the *LFY* mRNA sequence, it could disturb its expression via induction of alternative splicing or by changing its intron regulated expression (Gallegos and Rose, 2019; Varagona et al., 1992). A similar occurrence was described by Yao et al. (2001) in sepaloid apple cultivars. In their study, the researchers identified a transposon inserted in the intron of *MdPI* to be responsible for the flower mutation. However, the transposon is not the only sequence difference between *RidLFY-1* and *RidLFY-2*. There are two missense mutations in *RidLFY-2*, not in the two functional domains of LFY (Fig. S5). These domains, the Sterile Alpha Motif oligomerization domain (SAM) and the C-terminal DNA-binding domain (C-terminal DBD), are important for genomic binding of LFY (Sayou et al., 2016). Since the missense mutations are not in either of the functional domains they might not disturb normal LFY function.

The expression pattern of *LFY* in type 3 full bloom matches that of *A. thaliana lfy* mutants; in wild-type flowers *LFY* is only expressed before floral stage 10 (Weigel et al., 1992). This can be

observed with type 1 raspberry plants where *LFY* expression is low in open flowers. However, type 3 plants continue to express *LFY*, which in turn blocks class B gene expression and thus causes the mutated phenotype (Weigel et al., 1992). This means that *RidLFY-2* is still active in type 3 flower organs in open blooms. The slightly disturbed phenotype of type 2 plants could be explained by this as well, as they have both *LFY* alleles. The intact *RidLFY-1* allele supports normal flower development while the expression of *RidLFY-2* in open blooms is disturbing *PI* function. However, further research should be done to confirm that the sepaloid phenotype is indeed caused by the *LFY* mutation. For this purpose, genome editing or stable transformations with *RidLFY-2* could be used in other *R. idaeus* cultivars to induce changes in phenotype and gene expression. Moreover, the *RidLFY* alleles could be further functionally characterized with a yeast one-hybrid assay in order to analyze their protein-DNA interaction with *PI*. For this purpose, the promoter region of *PI* would be cloned in a yeast one-hybrid-compatible plasmid. The RidLFY allele capable of binding to the *PI* promoter could be identified by activating the bait reporter gene with its prey activation domain in close proximity.

The flower phenotyping data is linked significantly with the *RidLFY* allele genotyping categories. However, there are some deviations in phenotyping values. The foremost cause of the discrepancy in phenotyping is due to temperature change; we observed that the phenotypes of the individual plants were not constant from year to year, or even during the same flowering season. The extent of the mutation was more pronounced with increasing temperature. This means that for example in colder weather or earlier in the year plants would be phenotyped as type 1, but with increasing temperature they developed type 2 flowers and were thusly classified as type 2. This was also seen in plants placed in a warmer environment, in the greenhouse, compared to cultivation outdoors. This reinforces that a *LFY* mutant is the deciding factor for the development of the mutated flower phenotype, as *LFY* expression is shown to be higher with increasing temperature in several plant species, like *Narcissus tazetta*, *Populus tramula x P. tremuloides* and *Citrus sinensis* (Noy-Porat et al., 2013; Rinne et al., 2018; Tang and Lovatt, 2019). Further studies that consider this temperature dependency will need to be undertaken. Clones of the three genotypes could be placed in a controlled temperature environment with different temperature treatments to investigate this. A remaining small number of deviation in phenotyping values cannot be explained by temperature differences. These cases are most likely due to misreading plant labels or mixing

up the phenotyping scores of neighboring plants. The nature of phenotyping a large number of plants in the field does carry the risk of human error.

Although we could not show significantly larger fruit size in type 2 plants, the potential of larger fruit is still present with the development of a larger number of carpels. A study with an equal sample size of the three genotypes could deliver a more robust fruit phenotyping dataset. This could be arranged by propagation of clones of the desired genotypes or by crossing type 1 and type 3 plants with a tester cultivar. In the latter case, there would be no type 3 plants in the resulting F1 progeny, so crossings between two F1 type 2 plants could deliver a new population with higher number of individuals. Additionally, a population developed like this would not be affected by inbreeding depression, which does affect raspberry plants negatively in growth and mortality. A new population like that could be utilized in breeding as well. Using the *LFY* mutation to create new cultivars heterozygous to the *RidLFY* gene can present an advantage for raspberry growers, even without proven larger fruit size. Firstly, type 2 plants develop more carpels than wild-type flowers, which carries the potential for larger fruit. Secondly, they have more petals than the wild type and are thus more visible for pollinators. A higher visitation of pollinators can result in higher fruit weight in raspberry because of the higher number of pollinated carpels alone.

Finally, the floral development genes we found in *R. occidentalis* could serve as a base for future studies to identify and sequence genes in *R. idaeus*, adding to the *PI* and *LFY homologues* found in this study. However, great care must be taken when developing primers, as the differences between the two species have to be considered. Rather than taking long time optimizing unsuccessful PCR reactions, new primers should be designed and used instead. This is especially true for primer design in non-coding regions, where a higher mutation rate can be generally observed due to low selective pressure. As an example for the sequence difference between the two species, the two *PI* genes we found had 8 and 47 respective amino acid differences compared to their homologues in *R. occidentalis*. For Ro02_G35014 and RidPI1, the substitutions throughout the 212 amino acids are: N15T, S16N, M33N, H55N, E56Q, V92I and R147L (Fig. S1). As for Ro02_G35013 and RidPI2, in 131 amino acids, R25Q and S51T were substitutions, with a gap from 65 to 103 in RidPI2 (Tab. ES1b-c).

The use of the *R. occidentalis* draft genome for the development of molecular genetic tools in *R. idaeus* is justified by the close relation of the two species, as well as the high collinearity of

their genomes in the draft genome preprint of Wight et al. (2019). Furthermore, aligning their SNP markers relative to the *R. occidentalis* v3.0 draft genome results in a similar pattern in five out of the seven chromosomes (manuscript 3). However, based on our results of marker location discrepancies, and differences in heterozygosity and sequence information from manuscript 3 and the additional results, the two species do exhibit some differences.

The difference in heterozygosity points to a differing tolerance of inbreeding between the two species. As mentioned in the general introduction, although *R. idaeus* flowers can self-fertilize, the selfed progenies exhibit inbreeding depression. In contrast, *R. occidentalis* is known not to be affected by inbreeding depression (Ourecky, 1975a). However, Dossett et al. (2012a) used inbreeding depression as an explanation for the higher heterozygosity of cultivars compared to wild accessions in *R. occidentalis*. They postulated that by selecting for best performers, breeders inadvertently selected for heterozygosity as well by eliminating lower yield caused by inbreeding depression (Dossett et al., 2012a).

4.4. Inbreeding depression and self-incompatibility of raspberry cultivars

Inbreeding depression is one aspect of the SI system typical for Rosaceae crops. Its genetic control in *Rubus*, similarly to that of SI, still remains unclear. The SI system of Rosaceae, an S-RNase-based GSI, originated about 120 million years ago, based on phylogenetic analyses of the T2-RNase gene family (Igic and Kohn, 2001; Steinbachs and Holsinger, 2002; Vieira et al., 2008). The common ancestor of Asterid and Rosid has a presumed GSI, suggesting SI occurrence before these clades separated (Igic and Kohn, 2001; Sassa, 2016). As mentioned in the introduction, the GSI S-locus encodes for pollen and pistil specific genes. However, there is some diversity in the workings of the regulatory models of GSI among rosaceous species.

Maleae species have a non-self-recognition system with multiple pollen S proteins, SFBBs (S LOCUS F-BOX BROTHERs) (Sassa, 2016). In this system, pollen expressed SFBBs recognize non-self S-RNases, leaving the self S-RNase untargeted and free to inhibit pollen tube growth (Claessen et al., 2019). According to the current preferred model of action, protein degradation, after interaction with one or multiple SFBB proteins, S-RNases are ubiquinated and subsequently degraded by the 26S proteasome (Claessen et al., 2019). Protein

degradation is mediated by an SCF complex, consisting of F-box proteins, Skp1, Cullin1 and Rbx1 (Xu et al., 2013). In a non-recognition system, competitive interaction (CI) disrupts SI in tetraploids with heteroallelic diploid pollen because the SFBBs recognize both S-RNases (De Franceschi et al., 2012).

In contrast to Maleae, species in the *Prunus* genus have a self-recognition system, and a single polymorphic F-box gene, *SFB* (*S HAPLOTYPE-SPECIFIC F-BOX*) (Sassa, 2016; Sassa et al., 2010; Yamane et al., 2003). Additionally, three *SLFL* (*S-LOCUS F-BOX-LIKE*) genes are part of the S-locus and express in pollen, but in contrast to *SBF* have low or no allelic polymorphism (Matsumoto et al., 2008). In a self-recognition system, SFB interacts with Skp1 and Cullin to form SCF complexes (Matsumoto et al., 2012). S-RNases, when not recognized by SFB, undergo ubiquitination by *SLFL*, and are finally degraded via the 26S proteasome pathway (Li et al., 2020; Matsumoto and Tao, 2019). In a non-compatible pollination, SFB blocks this detoxification, and S-RNases degrade the pollen tube unhindered. In contrast to Maleae, CI has not been observed in *Prunus* (Tao and Iezzoni, 2010).

The S-locus of *P. avium* was recently characterized via whole-genome sequencing, revealing divergent genomic structures between haplotypes (Shirasawa et al., 2017). Nevertheless, *S-RNase* and *SFB* genes were found close on the same contig, between 1.5 and 7.4 kb apart (Shirasawa et al., 2017). Similarly, the sequence of the S-haplotypes in *Pyrus communis* have shown significant variation in the orientation and position of the pollen S-gene(s) relative to the S-RNase gene (Okada et al., 2011).

The plant family phylogenetically closest to *Rubus* with an identified S-locus is *Rosa* (Vieira et al., 2021). The SI of *Rosa* is a non-self-recognition system, with one S-RNase gene and multiple F-box genes on its S-locus, similarly to Maleae (Vieira et al., 2021). *Rubus*, belonging to Rosoideae, separated from Roseae between 80 and 70 Mio years ago, based on their phylogeny and fossil calibrations (Xiang et al., 2016). Still, there is not enough evidence to declare this SI system as the same for *Rubus*, or when the latter lost its functionality. However, it has been already suggested that the loss of functionality in raspberry cultivars affects only the F-box gene(s), but not the one(s) controlling S-RNase (Keep, 1968a). This is supported by the fact that said incompatibility can be overcome with heat treatment (Hellman et al., 1982). A similar heat treatment is used in cherry to overcome stylar SI; Tsuruta et al. (2020) reported

lower S-RNase activity and longer pollen tube length in heat treated selfings compared to no heat treatment.

There is still further research to be carried out to characterize the genetic background of SI in raspberries. Using the S-RNase genes of *P. avium* (AJ298312), *M. domestica* (AB032247) and *F. nipponica* (gi561674690, gi561985884, gi561957436) as BLAST template against the draft genome assembly of Wight et al. (2019), as well as a pHMMER approach with the ribonuclease T2 family motif (PF00445.20), several candidate genes can be identified. Two of them, *Rr023867* and *Rr028956*, encode a protein construct with an isoelectric point (pI) above 8, which is characteristic of S-RNases (Igic and Kohn, 2001). Keep (1985) suggested that the S-locus is located on the same linkage group as gene H, based on segregations in progeny. *Rr023867* has a high sequence similarity to *Ro02_G19269*, which is located on chromosome 2 in *R. occidentalis*. In the flanking region of *Ro02_G19269* are multiple putative F-box protein coding genes, like *Ro02_G19268*, *Ro02_G35280* and *Ro02_G35281*. Thus, the region surrounding *Rr023867* is a good starting point for molecular characterization of the S-locus in *R. idaeus*. The cultivar 'Rumla', which we found to be self-incompatible, could be used in the development of a marker for this trait. Furthermore, a hand pollination experiment with 'Rumla', 'Lucana' and 'Preussen' should be performed to test a possible cultivar specific incompatibility.

Inbreeding depression symptoms are lower yield, smaller plant sizes and lower germination rate of seedlings (Charlesworth and Willis, 2009). In the RiSEP population, which was sown from an open pollination, several plants had a smaller habitus. As mentioned above in section 4.3, it is possible that some of the individuals in the population suffer from inbreeding depression, because of the commonly occurring self-fertilization of raspberry. This could be an explanation for why the fruit phenotyping results do not correlate with the *LFY* allele status, although they do with the flower phenotype. Fertilization problems often show up as crumbly fruit phenotype, which is similar in appearance to type 3 fruit. Jennings (1967) selfed 'Latham', a cultivar with crumbly fruit occurrence, and observed three fruit phenotypes in the resulting population: normal, crumbly and sterile. This crumbly phenotype was later mapped by Graham et al. (2015) to LG1 and LG3 with an association with ripening time. Alleles associated with longer ripening time on LG2, LG3 and LG5 are also associated with smaller root measurements (Graham et al., 2011). These root growth genes were proposed to be regarded

as general vigor genes by Graham et al. (2015), and were offered as a possible factor in crumbly fruit formation. As general vigor is lower in plants affected by inbreeding depression, this could also point to inbreeding depression as a cause for non-correlation of the fruit size affecting several plants in the RiSEP population. However, until the population is checked with SSR markers for selfing status, this can only remain as speculation.

Another explanation for the lack of correlation with fruit size could be the sampling method of the fruit phenotyping. In studies about berry fruit, often there is a difference made between primary and nonprimary fruit during sample collection, and only nonprimary fruit is evaluated (Dossett and Finn, 2005). We did not discern between nonprimary and primary fruit in our sample collection. In a study similar to ours, Willman et al. (2020) evaluated drupelet variance in *R. occidentalis*, although their populations exhibited a natural variance, as opposed to one caused by a floral mutation. In their study, nonprimary fruit was used to map drupelet count to a 2 cM/1.02 Mb long QTL on LG1 (Willman et al., 2020). Interestingly, the marker Ri18886 (Dossett et al., 2015), which is 10,1 cM from the QTL peak position (Willman et al., 2020), is 1,79 Mb close to Ro01_G01137, an *AP3* homologue we found in our additional results. As *AP3*, a class B floral development gene, is responsible for petal and stamen development, exploring the region around it and the QTL could deliver further genes of interest for flowering and fruit traits. Future work should target the region around Ri18886 for sequencing so that the QTL and the *AP3* homologue could be explored in both *Rubus* species.

4.5. Conclusions

The main purpose of this thesis was to improve raspberry breeding through the development of molecular genetic tools. This was performed by following the four main aims of the project:

The first aim was to explore the extent of cultivar mismatches present in the German raspberry trade. For this, we found that 9 out of the 33 samples was not true to type, therefore the trueness-to-type is not guaranteed in raspberry cultivars purchased from both online retailers and nurseries.

The second aim pertained to the investigation of the self-incompatibility of raspberry cultivars, here a self-incompatible cultivar, 'Rumla', was found, with consequences for the use in protected growing and breeding of this cultivar.

For the third aim, gene *B*, which is responsible for the breeding relevant trait waxy bloom, was mapped to chromosome 2 in *R. occidentalis*. Interestingly, discrepancies were found in marker map positions between *R. idaeus* and *R. occidentalis*, as well as with previous mappings.

The fourth and last aim was concerned with floral development genes and their role in a flower mutation. Here, a transposon inserted into *LFY* was found to have caused the mutation in *R. idaeus*, and 82 MADS-box candidates were identified in *R. occidentalis*.

These results can be used in raspberry breeding research to further our understanding in the genetic background of several key traits. Additionally, the issue of cultivar mismatches was revealed, thus creating awareness to this subject. Furthermore, research areas of floral development, self-incompatibility and disease resistance can all benefit from the findings of this study.

5. References

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6. Appendix

6.1. Supplementary materials

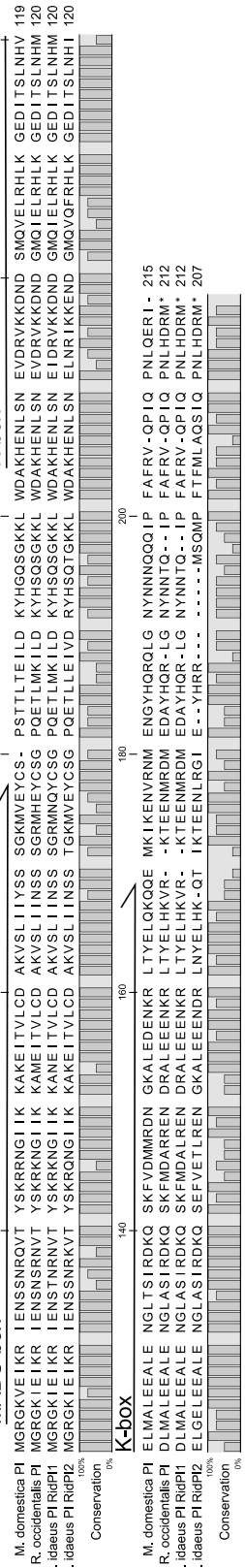

Figure S1. Amino acid alignment of the PISTILLATA sequence of *Malus domestica, Rubus occidentalis* and the two paralogues found in one *R. idaeus* population. Arrows span the functional domains MADS-box and K-box.

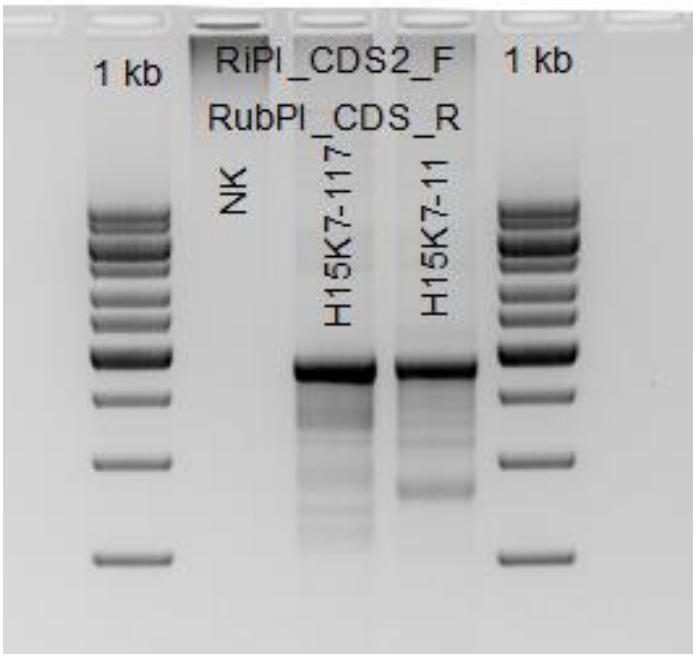

Figure S2. Agarose gel visualization of PCR fragments of *PI* of gDNA of *R. idaeus* type 1 and type 3 plants from the RiSEP population.

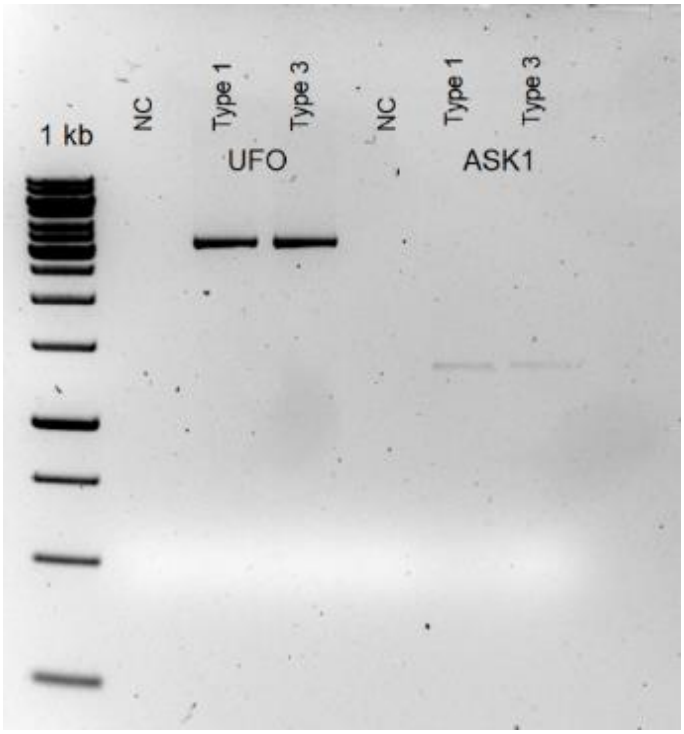

Figure S3. Agarose gel visualization of PCR fragments of UFO and ASK1 on gDNA of *R. idaeus* type 1 and type 3 plants from the RiSEP population. Primer development based on *A. thaliana* sequences: UFO (Ro03 G05828), ASK1 (Ro02 G02211).

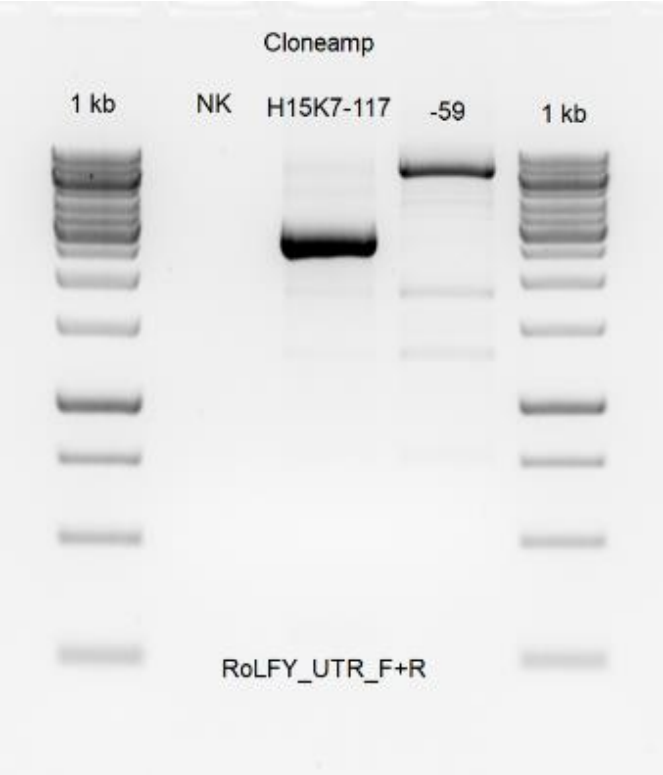

Figure S4. Agarose gel visualization of PCR fragments of LFY on gDNA of *R. idaeus* type 1 and type 3 plants from the RiSEP population.

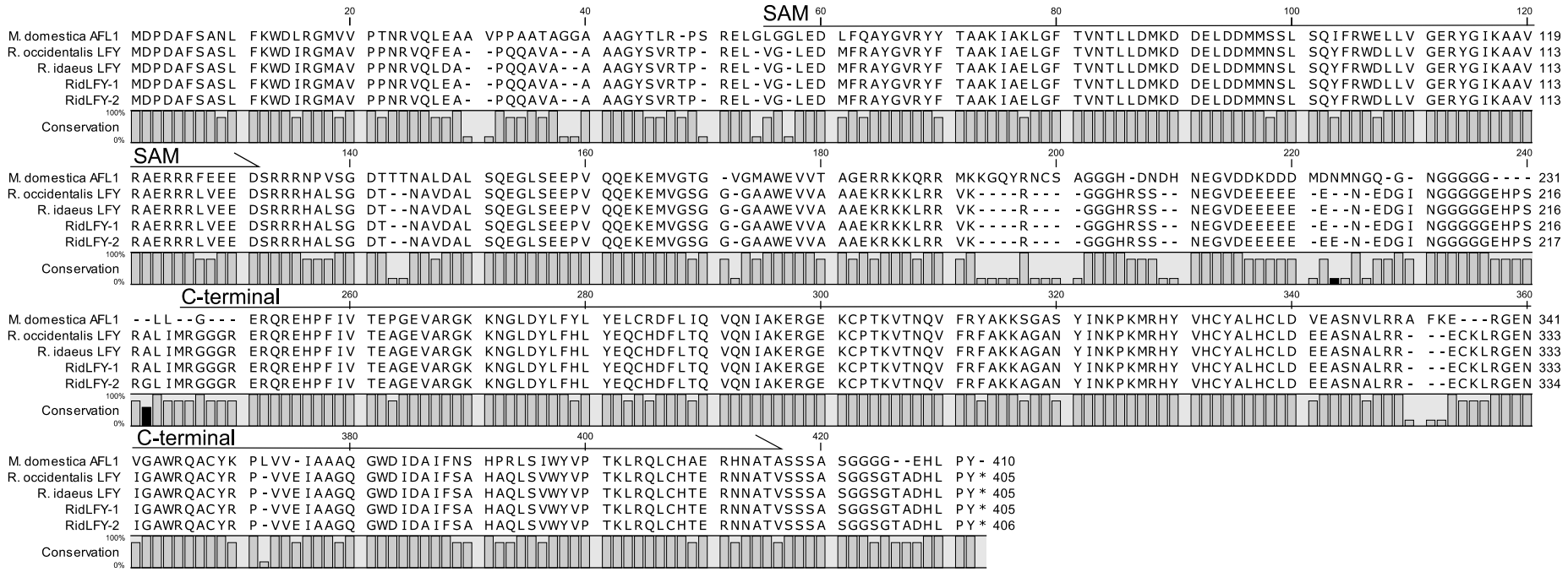

Figure S5. Amino acid alignment of the LEAFY sequence of *Malus domestica, Rubus occidentalis*, *R. idaeus* and the two paralogues found in one *R. idaeus* population. Arrows span the functional domains SAM oligomerization domain and C-terminal DNA-binding domain. The black colored conservation squares depict the two missense changes in RidLFY-2 compared to RidLFY-1.

6.2. Electronical appendix

Electronic supplementary material Table ES1a: *A. thaliana* MADS-box genes downloaded from TAIR.

Electronic supplementary material Table ES1b: *R. occidentalis* MADS-box candidate amino acid sequences.

Electronic supplementary material Table ES1c: Rosaceae MADS-box amino acid sequences.

Electronic supplementary material Table ES1d: Available functional annotation of the *R. occidentalis* MADS-box candidates.

Electronic supplementary material Table ES2: Flower phenotyping and *RidLFY* genotyping results of the RiSEP population.

Electronic supplementary material ES3: Sequence information of *RidPI1*, *RidPI2, RidLFY-1* and *RidLFY-2*.

6.3. Curriculum vitae

Dora Pinczinger

Curriculum vitae

Date of birth: 18. March 1985
Place of birth: Budapest, Hungary
Nationality: German, Hungarian

Experience

04/2021– **Scientific associate**, Julius Kühn-Institut, Breeding Research on Fruit Crops. Pre-breeding strategies for obtaining new resilient and added value berries (BreedingValue).

11/2016–01/2020 **Scientific associate**, Julius Kühn-Institut, Breeding Research on Fruit Crops. Development of new raspberry breeding clones with improved resistance towards cane diseases for German fruit production (HimRes).

06/2016–10/2016 **Scientific associate**, Julius Kühn-Institut, Breeding Research on Fruit Crops. Genetic research on apple via molecular biology tools and field phenotyping (AlternApp).

09/2014–12/2014 **Student research assistant**, Institute of Plant Genetics, Department of Molecular Plant Breeding, Leibniz Universität Hannover.

07/2013–10/2013 **Student research assistant**, Institute of Plant Genetics, Department of Molecular Plant Breeding, Leibniz Universität Hannover.

08/2010–10/2010 **Industry intern**, DLR Rheinpfalz, Germany.

09/2007–10/2008 **Forwarding agent**, logMASter Kft, Hungary.

Education

11/2018– **PhD student**, *Leibniz Universität Hannover*, Institute of Plant Genetics, Department of Molecular Plant Breeding, Germany. Supervision: Prof. Dr. Thomas Debener. Thesis: The development of molecular genetic tools to improve efficiency in raspberry breeding.

10/2012–04/2015 **Master of Science in Horticultural Sciences**, *Leibniz Universität Hannover*. Thesis: The function of glutathione S-transferase in *Euphorbia pulcherrima*.

10/2009–10/2012 **Bachelor of Science in Horticultural Sciences**, *Leibniz Universität Hannover*. Thesis: Analysis of microsatellite markers in the rose genome.

09/2006–06/2007 Industrial product design, *Budapest Műszaki Egyetem*, Hungary.

10/2003–03/2006 Environmental and bioengineering, *Universität Bayreuth*, Germany.

09/1999–06/2003 Secondary school, *Ungarndeutsches Wirtschaftsgymnasium Werischwar*, Hungary.

09/1991–06/1999 Primary school, *Nagymarosi Kittenberger Kálmán Általános Iskola*, Hungary.

Awards

2017 10th Young Scientists Meeting, Oral Presentations - Second Prize

Publications

Peer-reviewed journals

Pinczinger, D.; von Reth, M.; Hanke, M.-V.; Flachowsky, H. (2021), Self-incompatibility of raspberry cultivars assessed by SSR markers, Scientia Horticulturae 288, 110384.

Wöhner, Th.; Pinggera, J.; Fritzsche, E.; Pinczinger, D.; Hanke, M.-V. (2021), Insights into the susceptibility of raspberries to *Drosophila suzukii* oviposition, Journal of Applied Entomology 145(3), 182-190.

Pinczinger, D.; von Reth, M.; Keilwagen, J.; Berner, T.; Peil, A.; Flachowsky, H.; Emeriewen, O. F. (2020), Mapping of the waxy bloom gene in 'Black Jewel' in a parental linkage map of 'Black Jewel' x 'Glen Ample' (*Rubus*) interspecific population, Agronomy 10, 1579.

Pinczinger, D.; von Reth, M.; Hanke, M.-V.; Flachowsky, H. (2020), SSR fingerprinting of raspberry cultivars traded in Germany clearly showed that certainty about the genotype authenticity is a prerequisite for any horticultural experiment, European Journal of Horticultural Science 85(2), 79-85.

Conference contributions

Oral presentations

Pinczinger, D.; von Reth, M.; Keilwagen, J.; Berner, T.; Peil, A.; Flachowsky, H.; Emeriewen, O. F. (2021), Mapping of the waxy bloom gene in 'Black Jewel' in a parental linkage map of 'Black Jewel' x 'Glen Ample' (*Rubus*) interspecific population., 13th Young Scientists Meeting 2021 : 11th – 13th October - Abstracts (Berichte aus dem Julius Kühn-Institut 213), 34.

Pinczinger, D.; von Reth, M.; Hanke, M.-V.; Flachowsky, H. (2019), Differences in the self- and cross-fertilization ratio of raspberry cultivars., 12th Young Scientists Meeting 2019: 6th – 8th November in Kleinmachnow - Abstracts (Berichte aus dem Julius Kühn-Institut 206), 23.

Pinczinger, D.; Hanke, M.-V.; von Reth, M.; Flachowsky, H. (2019), Naturally occurring flower mutation in offspring of a large fruited raspberry chance seedling, 5th Plants and People Conference - (M)PIMP Future Foods - Challenges for Global Food Security, 3rd - 4th September in Potsdam.

Pinczinger, D.; Hanke, M.-V.; von Reth, M.; Flachowsky, H. (2018), Naturally occurring flower mutation in offspring of a large fruited raspberry chance seedling, 52. Gartenbauwissenschaftliche Jahrestagung Klimafolgen und Herausforderungen für den Gartenbau - Kurzfassung der Vorträge und Poster (BHGL Schriftenreihe 33), 67.

Pinczinger, D.; Hanke, M.-V.; von Reth, M.; Flachowsky, H. (2017), Naturally occurring flower mutation in offspring of a large fruited raspberry chance seedling, 10th Young Scientists Meeting 2017, 8th - 10th November in Siebeldingen - Abstracts (Berichte aus dem Julius Kühn-Institut 192), 36.

Posters

Pinczinger, D.; Hanke, M.-V.; von Reth, M.; Flachowsky, H. (2019), Naturally occurring flower mutation in offspring of a large fruited raspberry chance seedling, 53. Gartenbauwissenschaftliche Jahrestagung Future Food Production: Kurzfassung der Vorträge und Poster (BHGL-Schriftenreihe 34), 134.

Pinczinger, D.; Hanke, M.-V.; von Reth, M.; Flachowsky, H. (2019), SSR fingerprinting of raspberry cultivars traded in Germany to test trueness-to-type, 53. Gartenbauwissenschaftliche Jahrestagung Future Food Production: Kurzfassung der Vorträge und Poster (BHGL-Schriftenreihe 34), 135.

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Fritzsche, E.; Pinggera, J.; Pinczinger, D.; von Reth, M.; Wöhner, T.; Hanke, M.-V.; Flachowsky, H. (2018), Evaluation of raspberry and strawberry genetic resources for resistance to spotted wing drosophila (*Drosophila suzukii*), 11th Young Scientists Meeting 2018 : 14th - 16th November in Braunschweig - Abstracts - (Berichte aus dem Julius-Kühn-Institut 200), Braunschweig, 42.

Pinczinger, D.; Hanke, M.-V.; von Reth, M.; Girichev, V.; Schulte, E.; Flachowsky, H. (2017), Development of new raspberry breeding clones with improved resistance to cane diseases for German fruit production, 51. Gartenbauwissenschaftliche Jahrestagung Der Beitrag gartenbaulicher Produkte zur Ernährung und Gesundheit - Kurzfassung der Vorträge und Poster (BHGL-Schriftenreihe 32), 82.

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