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SELF-FERTILIZATION AND MARKER-TRAIT ASSOCIATIONS IN SAINFOIN (*ONOBRYCHIS VICIIFOLIA*)

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List of abbreviations

2n	= zygotic number
BC	= backcross
bp	= base pairs
°C	= celsius
CH_4	= methane
cm	= centimeter
cM	= centimorgan
CP	= cross pollinated
CT	= condensed tannins
DNA	= deoxyribonucleic acid
e.g.	= for example
F_1	= filial generation from crossings of individual plants
g	= gram
GC rich	= guanine - cytosine rich
GCA	= general combining ability
H	= hydrogen
h^2	= heritability
i.e.	= that is
MAS	= marker assisted selection
$MgCl_2$	= magnesium chloride
min	= minute
mm	= millimeter
mM	= millimolar
N	= nitrogen
ng	= nanogram
NGS	= next generation sequencing
OH	= hydroxide
%	= percent
PC	= procyanidin
PCA	= principal component analysis
PCO	= principal coordinate analysis

PIC	= polymorphism information content
PD	= prodelphinidin
QTL	= quantitative trait loci
RF	= recombination frequency
RIL	= recombinant inbred line
RNA	= ribonucleic acid
s	= second
SCL	= single cross link
SIM	= single interval mapping
SPAD	= soil plant analysis development
TBE buffer	= Tris/Borate/EDTA buffer
UPLC-MS/MS	= ultra-performance liquid chromatography tandem mass-spectrometry
U	= unit
µL	= mycroliter
µM	= mycromol
UK	= United Kingdom
U.S.	= United States
UV	= ultraviolet

Summary

An extension of ruminant husbandry to satisfy the growing demand of milk- and meat products has negative consequences for the environment like e.g. increased methane gas emissions and wastewater. Additionally, higher production levels in animals are difficult to obtain because increased protein and energy levels must be provided within the fodder ration. There is also a continuous risk of diseases and parasite burdens in livestock due to resistance formation against conventional anthelmintic drugs. These problems could be tackled by the use of forage legumes with anthelmintic properties like sainfoin (*Onobrychis viciifolia*), which has health promoting characteristic mainly caused by the presence of polyphenols therein, especially condensed tannins. Condensed tannins are also known to reduce bloat and methane gas emissions in ruminants and improve protein absorption by protein-tannin-interactions in the rumen. Furthermore, the ability of sainfoin to fix atmospheric nitrogen by rhizobia bacteria association allows a reduction of nitrogen fertilizer input. However, sainfoin cultivation decreased during the last century, presumably due to cheap prices for inorganic fertilizer and competition from higher yielding forage crops. Consequently, sainfoin breeding attempts were reduced to a minimum. Existing varieties show poor and unstable biomass yields, reduced persistence and low seed yields. Breeding new, high performing varieties is indispensable to make sainfoin cultivation attractive to farmers.

Tetraploidy and, hence, complex inheritance patterns for traits are one important aspect breeders have to deal with in breeding sainfoin. Moreover, contradictory statements exist about the reproduction system in sainfoin, namely if sainfoin is strongly cross-fertilized or also able to self-fertilize. Here, molecular markers could help to unravel the breeding system in sainfoin and to clarify inheritance patterns on a molecular level. Detection of associations between molecular markers and traits would allow selection of individuals for desired traits on the basis of marker assisted selection (MAS). Thereby, localization of loci explaining variation in a quantitative trait (quantitative trait loci, QTL) within the genome could be improved with the establishment of a genetic linkage map which does not yet exist for sainfoin.

Due to the given necessity to assist sainfoin breeding, our study aimed to elucidate the breeding system of sainfoin as well as its consequences on plant performance (chapter 2), to test newly developed molecular markers which could be, later on, applied in genetic studies (chapter 3), to apply these markers to F_1 offspring of one biparental population to detect marker trait associations (chapter 4) and to establish a genetic linkage map based on this marker data (chapter 5). The present study was part of the European Marie Curie Initial Training Network 'LegumePlus' (PITN-GA-2011-289377) which aimed to conduct a holistic research of sainfoin.

Our first aim was to detect possible self-fertilization in sainfoin. Generally, sainfoin is described as mainly cross-fertilized, but few studies report high self-fertilization rates based on results from manual self-pollination by hand or using a recessive flower color marker as detection system. So far, information about self-fertilization under natural conditions is missing, as well as possible factors favoring self-fertilization. To address these questions, three biparental populations were established with bumble bee (*Bombus terrestris*) pollination in the greenhouse from five clones of two parental genotypes in each population. Using this approach, self-fertilization as well as cross-fertilization was possible, but the pollination was affected by the number of potential crossing partners and bumble bee movements. In addition to this approach, self-fertilization was assessed in three natural populations in the open field. For the first time, self-fertilization was assessed on the basis of dominant sequence related amplified polymorphism (SRAP) and co-dominant simple sequence repeat (SSR) molecular markers. In the greenhouse populations, high self-fertilization rates of 48.5, 61.5 and 64.8% were detected. Contrastingly, 0.0, 1.8 and 3.9% self-fertilization was detected in the three natural populations. The greenhouse populations were phenotyped over a period of three years to quantify possible inbreeding depression. It could be shown that seed yield per plant was highly affected (up to 79% reduction) by inbreeding depression, whereas plant height and vigor were less affected with a reduction of 23.8 and 17.2%, respectively.

Another emphasis of the study was to test sainfoin specific co-dominant SSR markers (chapter 3). Such markers are essential to realize genetic studies in sainfoin or, further, could be used for marker assisted selection. For this reason,

newly developed SSR markers were tested on a set of 32 sainfoin individuals. Thereby, 101 amplified markers showed allelic polymorphisms between the individuals. The number of alleles per marker lay between two and 24 alleles, whereby only five of total alleles were present in all individuals. Summarizing marker data using multivariate statistics revealed two main clusters, the first one containing individuals from Western Europe, whereas the second one contained individuals from Southern- and Eastern Europe and the U.S. The discovery of genetic differences by SSR markers indicates that those markers are useful for further genetic studies in sainfoin.

A set of tested SSR, together with SRAP markers, was used to search for marker trait associations (chapter 4) in 122 F_1 offspring of one greenhouse population developed within the framework of chapter 2. Finding such markers would allow selecting promising individuals already in early stages of the breeding processes. For this reason, all marker alleles were scored as present (1) / absent (0) in the individuals and tested for trait associations via simple regression analysis. Trait associated markers could be found for plant height, seed yield and vigor, whereby a correlation analysis among associated markers indicates that these are linked to at least two different QTL. Furthermore, one associated marker was found for prodelphinidin-share of condensed tannins.

At last, a set of SSR and SRAP markers was used to create a genetic linkage map of sainfoin based on 122 F_1 offspring from the above mentioned populations (chapter 5). JoinMap (for diploid and allopolyploid species) and TetraploidMap (for autotetraploid species) software packages were used accounting for the unknown origin of polyploidy in sainfoin. It could be shown that none of the software produced a satisfactory linkage map, because most of the markers grouped to only one linkage group. Partially, certain linkages between markers could be found by both software, but, based on the used population, no definite map could be developed and no preference for one of the two software packages could be given.

The results of the present study showed high self-fertilization rates in sainfoin with accompanied inbreeding depression. Consequently, a self-incompatibility system can be excluded. These results are directly useable to optimize breeding methods. Furthermore, the usability of newly developed markers for further

genetic studies was proven for sainfoin. The detected marker trait associations for agronomic and compositional traits could be applied to select superior individuals in plant breeding programs to bred new sainfoin varieties.

Zusammenfassung

Ein Ausbau der Wiederkäuerhaltung, aufgrund der wachsenden Nachfrage nach Milch- und Fleischprodukten, belastet die Umwelt durch steigende Methangaseinträge und anfallende Abwässer. Bezüglich der Tiergesundheit ist es eine Herausforderung genug Energie und Protein über die Fütterung zuzuführen um die Produktionsleistung zu steigern. In den Tierbeständen treten zudem Krankheiten und Parasiten auf, welche mit Medikamenten chemischen Ursprungs immer schwerer einzudämmen sind da z.B. Parasiten Resistenzen gegen gebräuchliche Anthelminthika entwickelt haben. Eine Alternative ist die Nutzung von Futterpflanzen mit anthelmintischen Eigenschaften wie die ausdauernde Leguminose Esparsette (*Onobrychis viciifolia*). Die Esparsette besitzt Polyphenole, darunter kondensierte Tannine, welche anthelminitisch wirken und zudem Blähungen und den Methangasausstoss reduzieren. Zusätzlich wird das in der Futterration enthaltene Protein durch Interaktion mit diesen Tanninen besser vom Tier absorbiert.

Obwohl die Esparsette wertvoll für die Tierernährung ist und ihr Anbau Ressourcen spart (aufgrund atmosphärischer Stickstofffixierung durch assoziierte Knöllchenbakterien), ging der Anbau im letzten Jahrhundert stetig zurück. Gründe hierfür sind günstige Preise für anorganischen Dünger und Konkurrenz durch andere, ertragreichere Kulturarten. Die Esparsette wird in Folge dessen kaum noch gezüchtet. Vorhandene Sorten haben oft geringe und schwankende Biomasseerträge über die Anbaujahre, eine reduzierte Persistenz und wenig Saatgutertrag. Die Zucht neuer, leistungsstarker Sorten ist unabdingbar, um den Esparsettenanbau für Landwirte attraktiv zu machen. Die züchterische Bearbeitung der Esparsette stellt nicht nur aufgrund der Tetraploidie und daraus resultierender komplexer Vererbungsgänge von Merkmalen eine Herausforderung dar. Des Weiteren existieren widersprüchliche Aussagen darüber, ob die Esparsette ausschliesslich fremdbefruchtet wird. Die Unterstützung der Züchtung mit Hilfe molekularer Marker würde es ermöglichen, sowohl die Frage der Befruchtungsart in der Esparsette zu klären, als auch Vererbungsgänge auf molekularer Ebene zu beschreiben. Durch Nutzung dieser Marker für das Auffinden von Marker-Merkmalsassoziationen

könnte gezielt auf gewünschte Merkmale selektiert werden. Um die Effektivität der Selektion zu verbessern ist die Erstellung einer genetischen Kopplungskarte von Bedeutung, da es so möglich ist die Chromosomenregion welche für das quantitative Merkmal (QTL) verantwortlich ist genauer zu lokalisieren.

Aufgrund der Notwendigkeit die Esparsetten Züchtung zu unterstützen, ergaben sich folgende Ziele für unserer Studie: die Aufklärung des Befruchtungssystems der Esparsette und dessen Auswirkung auf die Leistung der Pflanze (Kapitel 2), molekulare Marker für genetische Studien zu testen (Kapitel 3), diese molekularen Marker zum Auffinden von Merkmalsassoziationen in einer Kreuzungspopulation anzuwenden (Kapitel 4) und eine genetische Kopplungskarte der Esparsette zu erstellen (Kapitel 5). Die Studie wurde dabei im Rahmen des europäischen Marie Curie Initial Training Network, 'LegumePlus' (PITN-GA-2011-289377) durchgeführt, welches sich mit der ganzheitlich Erforschung der Esparsette befasst.

Die erste Fragestellung der Studie bezüglich des Befruchtungssystems (Kapitel 2) gründet sich auf widersprüchliche Angaben der Literatur zu diesem Thema. Wird die Esparsette zumeist als überwiegend fremdbefruchtet beschrieben, so finden sich wenige Studien die von geringen bis teilweise hohen Selbstbefruchtungsraten sprechen. Diese Ergebnisse stützen sich dabei auf morphologische Marker (Blütenfarbe) und Samenansatz durch gezielten Handbestäubungen mit eigenem Pollen. Eine Erfassung der Selbstbestäubungsrate unter natürlichen Bedingungen und unter Berücksichtigung von Aspekten welche Fremd- oder Selbstbefruchtung fördern wurde noch nicht erbracht. Um dieser Frage nachzugehen, wurden drei Kreuzungen mit je zwei fünffach verklonten Elternpflanzen im Gewächshaus mittels Hummelbestäubung (*Bombus terrestris*) durchgeführt. Hierbei war es möglich, dass die Pflanzen mit dem eigenen oder fremden Pollen bestäubt wurden. Der Bestäubungsvorgang war durch die Anzahl an vorhandenen Bestäubungspartnern und Hummeln eingeschränkt. Zusätzlich wurde die Bestäubungsrate auf drei natürlichen Flächen im Freiland ohne Einschränkungen erfasst. Die Selbstbefruchtungsraten wurden in unserer Studie erstmals anhand dominanter sequence related amplified polymorphism (SRAP) und zwei co-dominanter simple sequence repeat (SSR) molekularer Marker

bestimmt. In den Gewächshauspopulationen konnten hohe Selbstbefruchtungsraten von 48.5, 61.5 und 64.8% erzielt werden. Im Gegensatz dazu waren die Selbstbefruchtungsraten auf den natürlichen Flächen vernachlässigbar gering mit 0.0, 1.8 und 3.9%. Um eine mögliche Inzuchtdepression in der Esparsette zu quantifizieren, wurden die drei Gewächshauspopulationen über drei Jahre phenotypisiert. Es konnte dabei gezeigt werden, dass die Saatguterträge pro Einzelpflanze in den Selbstungsnachkommen stark zurückgehen (bis zu 79%), wobei die Pflanzenhöhe und die allgemeine Wüchsigkeit von einer etwas geringeren Inzuchtdepression betroffen waren (23.8% und 17.2%).

Der zweite Schwerpunkt der vorliegenden Studie befasste sich mit dem Testen von spezifischen co-dominanten molekularen SSR Markern für die Esparsette (Kapitel 3). Solche molekularen Marker sind für die Durchführung von genetischen Studien in der Esparsette unerlässlich. Zudem können sie, falls sie mit einem phänotypischen Merkmal assoziiert sind in der praktischen Züchtung verwendet werden. Zu diesem Zweck wurden neue SSR Marker an einem Pool aus 32 diversen Esparsetten Pflanzen verschiedener Herkunft angewendet. Dabei konnten 101 amplifizierte Marker Polymorphismen zwischen den Individuen aufzeigen. Die Anzahl von Allelen pro Marker reichte dabei von zwei bis 24 über alle Individuen. Zudem fanden sich nur fünf Allele die in allen Individuen vorkamen. Die genetische Charakterisierung der Individuen zeigte, dass diese sich in zwei Gruppen aufspalten. Gruppe 1 beinhaltet Individuen aus Westeuropa, wohingegen Gruppe 2 Individuen aus Süd- und Osteuropa und den USA aufweist. Die durch die SSR Marker aufgezeigten genetischen Unterschiede und Gemeinsamkeiten in den Individuen zeigen, dass sich diese Marker auch für weitere genetische Studien in der Esparsette nutzen lassen

Die Nutzung eines Sets dieser charakterisierten SSR Marker und dominanter SRAP Marker zur Erfassung von Marker-Merkmalassoziationen war ein weiteres Teilziel unserer Studie (Kapitel 4). Das Auffinden solcher Marker würde es in Zukunft erlauben, Individuen gezielt, schon in frühen Züchtungsstadien, nach erwünschten Merkmalen zu selektieren. Für die Suche nach solchen Assoziationen wurden 122 F1 Kreuzungsnachkommen aus einer der drei Gewächshauspopulationen (Kapitel 2) untersucht. Zur Detektion dieser

Assoziationen wurden alle Marker Allele als anwesend (1) / abwesend (0) in den Individuen erfasst und mittels einfacher Regressionsanalyse auf vorhandene Assoziationen zu phenotypisierten Merkmalen getestet. Merkmalassoziierte Marker konnten dabei für Pflanzenhöhe, Saatgutertrag und Wüchsigkeit gefunden werden. Des Weiteren wurde ein assoziierter Marker für den Prodelphinidin-Anteil in kondensierten Tanninen gefunden. Die Korrelation unter den Markern deutet zudem darauf hin, dass mindestens zwei QTL mit diesen Markern gekoppelt sind und sich für eine gezielte Merkmalsselektion eignen.

In einem letzten Schritt sollte aus einem Set von SSR und SRAP Markern eine genetische Kopplungskarte für 122 Kreuzungsnachkommen aus der oben genannten Kreuzungspopulation erstellt werden (Kapitel 5). Aufgrund des ungeklärten Ursprungs der Polyploidie in der Esparsette wurden die Software JoinMap (für diploide und allopolyploide Arten) und TetraploidMap (für autotetraploide Arten) angewandt. Es zeigte sich, dass mit keiner der beiden Softwares eine umfassende Kopplungskarte für die Esparsette erstellt werden konnte, da die meisten Marker Allele in eine Kopplungsgruppe gruppieren. Die aufgezeigten Marker Kopplungen waren dabei nur teilweise in beiden Softwares zu finden. Letztendlich liess sich auf Basis der verwendeten Population keine definitive Kopplungskarte erstellen und keine Aussage konnte darüber getroffen werden, welche Software die geeignete ist, um eine Kopplungskarte für die Esparsette zu erstellen.

Die Resultate unserer Studie zeigen dass hohe Selbstbefruchtungsraten in der Esparsette möglich sind und diese von einer Inzuchtdepression begleitet werden. Somit lässt sich ein Selbstinkompatibilitätssystem ausschliessen. Diese Erkenntnisse sind direkt nutzbar um praktische Züchtungsmethoden zu optimieren. Des Weiteren konnte die Nutzbarkeit neu bereitgestellter Marker für die Esparsette bewiesen werden, was zukünftige genetische Studien in der Esparsette fördern könnte. Die Assoziation einzelner SSR Marker mit agronomischen Merkmalen und Inhaltsstoffen kann zur Selektion von Individuen für Pflanzenzuchtprogramme genutzt werden und somit die Züchtung neuer Esparsettensorten unterstützen.

Chapter 1 General Introduction

„Ich habe niemals einen angenehmeren Anblick gesehen, als die Hügel um Milden herum, die überall von dem Purpur der blühenden Esparsette glänzen“.

„I have never seen a more pleasant view, as the hills around Milden, which glow all over from the purple of flowering sainfoin”

(von Haller 1772)

Livestock production is increasing world-wide, raising the demand for animal forage of high nutritional quality (Thornton 2010). To allow for a high level of animal productivity, species-appropriate rations should combine adequate fiber contents with high energy and protein concentrations. Grassland, which cover 33% of the agricultural area in Europe, presents a valuable source for locally produced ruminant forage that is by far not completely exploited yet (Peeters 2009). The production of high quality roughage from grassland is cost-efficient and could help to decrease the input of concentrated feed (Ivemeyer et al. 2014). In addition, roughage rations composed of diverse grassland species positively influence concentrations of valuable fatty acids and antioxidants, contributing to improved quality of meat and milk products (Stypinski 2011; Girard et al. 2016). By choosing plant species with specific adaptive capacities, grassland allow for extending forage production to less favorable areas or to mitigate challenges such as increasing temperatures or drought periods. Thereby, legume species are a valuable option to complement grassland mixtures: with their ability to fix atmospheric nitrogen via association with rhizobia, higher yields can be realized with less input of nitrogen fertilizers (Lüscher et al. 2014). Besides the typical, widely used grassland legumes such as red clover (*Trifolium pratense*) and alfalfa (*Medicago sativa*), many other, partially neglected species exist, which could offer some advantages over those traditional species. Sainfoin (*Onobrychis viciifolia*) is an outstanding candidate, as it combines the possibility to produce protein with equal quality as from alfalfa with secondary compounds that support animal health in different ways (Kaldy et al. 1979; Aufrere et al. 2008). Such beneficial effects for animal nutrition and husbandry were demonstrated in different feeding trials, ranging from reduced bloat over increased live weight and reduced worm burdens to decreased methane emissions. Contrastingly, from an agronomical point of view, sainfoin exhibits several disadvantages which make it less attractive for a wider distribution.

Despite its potential benefits, sainfoin is yet an underutilized crop and breeding activities are at a very low level when compared to other forage species. However, the development of new, superior varieties is essential for a wider distribution of sainfoin. To advance breeding programs, more detailed knowledge on the genetic basis of sainfoin, including the predominant type of fertilization (self- vs. cross-

fertilization), has to be provided. Furthermore, genetic markers need to be developed to describe the genetic makeup (genotype) of different sainfoin plants and to find possible marker-trait associations, that could be used to enhance selection progress in breeding programs. Hence, the aims of the present thesis were to (i) analyze the mode of reproduction in sainfoin with regard to cross- and self-fertilization (chapter 2), (ii) to investigate the usability of newly developed co-dominant molecular markers to assess genetic diversity (chapter 3), (iii) to test if these co-dominant and other dominant markers are associated with different agronomic and compositional traits (chapter 4), and (iv) to use these markers to establish a first linkage maps for sainfoin (chapter 5).

1.1 Grassland production systems

1.1.1 Importance of grassland

Grasslands are a major class of ecosystems consisting of diverse species. They can be divided into three groups: natural grassland formed by undisturbed ecological succession, semi-natural grassland involving naturally occurring herbs and grasses used for forage production, as well as intensive grassland consisting of sown and highly productive grass and legume species (reviewed in Hejcman et al. 2013). The benefits from grasslands are manifold: they provide animal forage, are habitat for several species, maintain biodiversity, contribute to the preservation of clean water, prevent floods, and sequestrate and store carbon in the soil (Scurlock and Hall 1998; O'Mara 2012). The total global value of such so-called ecosystem services provided by grass- and rangeland reached US$ 232 ha^{-1} $year^{-1}$ (reviewed in Gaujour et al. 2012). Grassland is also of high economic importance for many farmers, for example in Switzerland, where the majority of the gross income is based on milk production (Jeangros and Thomet 2004).

Species composition of grassland can be quiet diverse dependent on region, climate and usage. Furthermore, management intensity is a major factor defining grassland communities. For example, intensive production systems with high fertilization favor grasses tolerable to frequent cutting such as perennial ryegrass (*Lolium perenne*) or timothy (*Phleum pratense*), whereas legumes loose

their comparative advantage of symbiotic N-fixation and are generally reduced (reviewed in Gaujour et al. 2012). In Switzerland, grasses are the dominant element of intensive grassland, often accompanied by white clover (*Trifolium repens*) as the legume component (Jeangros and Thomet 2004). The demands to grassland communities are high: as forage source they should improve milk and meat quality and stay productive with changing environmental conditions e.g. due to climate change (Lüscher et al. 2014). A higher diversity of grassland species is seen as favorable in order to stabilize and enhance productivity of the system and improve the forage value (Kölliker and Boller 2010). The introduction of minor species as well as wild relatives of cultivated plants is, therefore, desirable.

1.1.2 Use of legumes

Legumes (Fabaceae) are a family which comprises 18'000 to 19'000 different species within the order Fabales, including annual and perennial herbaceous plants, shrubs and trees (reviewed in Graham and Vance 2003). For human welfare, legumes have a unique position within the plant kingdom, as they are the most important protein source for animal as well as human nutrition. For example, soybean (*Glycine max*) is a major crop of worldwide economic importance. Although soybean is associated with intensive agriculture and monocultures, legumes are also candidates for sustainable agriculture due to their ability to fix atmospheric nitrogen, which is driven via the association with root nodule forming rhizobia bacteria (Drevon et al. 2015). Fixed atmospheric nitrogen can also be furnished to non-leguminous companion species, reducing the need for inorganic N fertilizers (Travis 1993; Peoples et al. 1995). In grassland, legumes are, therefore, valued as partners for mixtures because nitrogen is the nutrient limiting productivity the most (Frame et al. 1998). Furthermore, legumes have been shown to restore soil nutrient levels in crop rotations after intensive cultivation (Peoples et al. 1995).

Legumes are also an ideal source of proteins in roughage, i.e., forage rich in crude fiber that usually includes fresh, ensiled or dried herbage, maize or beets. Because the forage value of roughage is seldom meeting the protein demands of highly productive farm animals, livestock farmers often complement rations with

concentrated feed on basis of soybean. However, concentrated feed is seldom produced on farm and must be imported from regions where monocultures dominate the landscapes, with severe impacts on the environment such as soil degradation, pollution of ground water via intensive fertilizer and pesticide application, as well as destruction of primary forests for provision of arable land. The use of homegrown protein from legume species produced within grassland communities or as part of rotation farming could drastically decrease the use of concentrated feed and, hence, is economically and environmentally sustainable.

1.2. Genetic improvement of plants: background information

1.2.1 Mating systems in plants

In the plant kingdom, three types for reproduction via seeds are known: self-fertilization (autogamy), cross-fertilization (allogamy) and rather seldom occurring parthenogenesis (apomixis). The type of reproduction has a major influence on the genetic composition of plants overall referred to as homozygosity or heterozygosity. For given loci (position in the genome), heterozygosity describes the presence of different alleles (possible state of a locus), whereas homozygosity describes the presence of identical alleles. In self-fertilizing individuals (self-pollinated; selfings) homozygosity is increased, whereas heterozygosity is increased in cross-fertilizing individuals (cross pollinated; crossings). The preference of plants for self- or cross-fertilization is not always exclusive under the presence of both sexes in one plant (monoecious plants), but mechanisms exist in some species to enhance one or the other. Self-fertilization often occurs due to enclosed pistil and anthers, preventing foreign pollen from entering the flower (cleistogamy). In contrast, cross-fertilization could be guaranteed by physical or genetic barriers preventing self-fertilization with own pollen or pollen from close relatives. Physical barriers consist of flower architectural traits, like e.g. a stigma overtowering the anthers (heterostyly). Genetic barriers include a shifted maturity of stigma and anthers (dichogamy; protandry and protogyny) or plants with separated sexes (dioecious plants). Furthermore, genetic self-incompatibility systems exist which prevent pollination

due to recognition of own or related pollen (sporophytic and gametophytic self-incompatibility, Lloyd and Schoen 1992 a).

There are several reasons why cross-fertilization and, therefore, an increased heterozygosity, is favored, like the plasticity of populations to withstand different environmental conditions and the avoidance of inbreeding depression. Inbreeding depression describes the decreased performance of plants for traits such as vigor or fecundity with increased homozygosity after self-fertilization. Oppositely, heterosis describes the supererogation of plants produced by cross-fertilization in comparison to their inbred parents (Becker 1993; Birchler 2013). Species which are predominantly cross-fertilizing tend to suffer severe from inbreeding depression when subjected to self-fertilization. This is mainly due to the accumulation of detrimental recessive alleles, which are initially unnoticed due to presence of non-detrimental alleles in heterozygous plants. Inbreeding depression occurs if plants get homozygous for detrimental recessive alleles, but is also affected by the disturbance of other mechanisms like the loss of overdominance at loci with heterozygote advantage and the loss of additive gene actions (Lande et al. 1994; Charlesworth and Willis 2009). In species with naturally higher proportion of self-fertilization, the effects of inbreeding are often alleviated due to selection against deleterious alleles in earlier generations.

1.2.2 Polyploidy in plants

Reproduction is the key element for the sustainment of species, favoring those individuals which are able to adapt to changing conditions. Polyploidy, the genome wide multiplication of the chromosome number is one mechanism enabling individuals to gain advantages over others. Several domesticated crops are polyploids like wheat (*Triticum* aestivum, hexaploid), cotton (*Gossypium hirsutum*, tetraploid) and potato (*Solanum tuberosum*, tetraploid). Polyploid plants often have an advantage over diploids via increased plant growth and vigor, larger flowers and larger seeds (Bretagnolle and Lumaret 1995). Furthermore they may have altered physiology which allows adaption to unfavorable climates as drought (reviewed in te Beest et al. 2012). Two different types of polyploidy can be distinguished: autopolyploidy and allopolyploidy. Autopolyploid plants own multiple copies of chromosomes from one and the same

diploid ancestor genome or of themselves as is the case for induced polyploids. Allopolyploids are derived from the hybridization of different diploid ancestor genomes. Auto- and allopolyploid plants vary in chromosome pairing behavior during cell division in sexual reproduction (meiosis). Autopolyploids only possess homologous chromosomes, i.e. sets of chromosomes owning the same genes and originating from the same ancestral genome. In contrast, allopolyploids possess homologous chromosomes as well as homeologous chromosomes, i.e. chromosomes owning similar genes, but originating from different ancestor genomes. Homologous chromosomes pair during prophase of meiosis, whereas homeologous chromosomes will not pair. In autopolyploids, homologous chromosomes are forming groups during metaphase, with pairs of two (bivalent), three (trivalent), four (quadrivalent) or more chromosomes (multivalent formations), resulting in a so-called polysomic inheritance. Contrastingly, in allopolyploids, only homologous chromosomes are pairing together, resulting in exclusively bivalent formations and a so-called disomic inheritance in case of an allotetraploid species (Ramsey and Schemske 2002). Bivalent formation is similar to the behavior in diploids with the two homeologous chromosomes being independent of each other. These differences in chromosome pairing behavior have influence on chromosome formation and, therefore gene inheritance and exchange (crossing over, Fig. 1.1). The knowledge of gene inheritance patterns is essential for breeding species with desired gene combinations, like e.g. combining resistance genes.

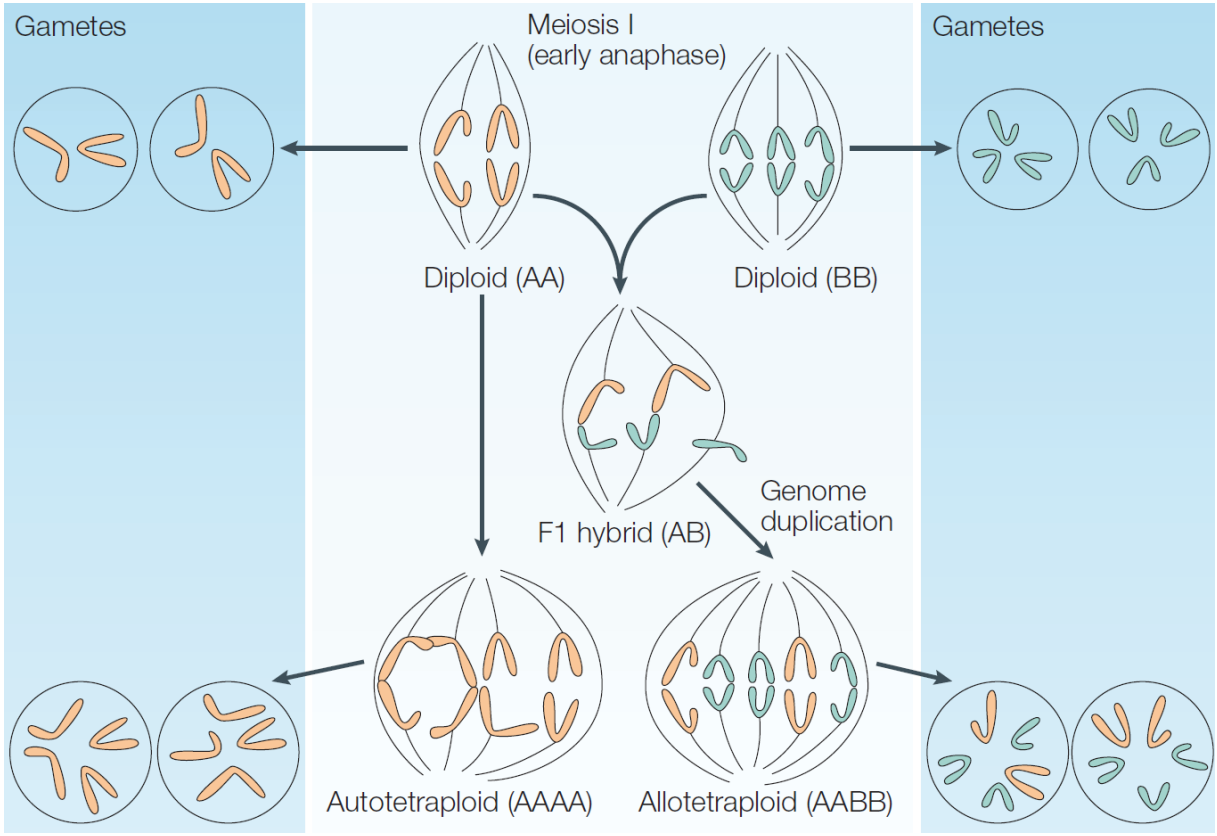

Fig. 1.1 Chromosome formation in autotetraploids and allotetraploids in metaphase during meiosis. Taken from Comai (2005)

1.2.3 Breeding methods in forage plants

The major aims for breeding forage plants are improvement of the annual dry matter yield, adaption to local environments, resistance against pests and diseases, stress tolerance and increased digestibility (Humphreys 1997; Humphreys 2005; Christoph Grieder, personal communication, 2016). Thereby, the breeding method is determined by the mating system of the particular species (Posselt 2010). Most forage species are cross-fertilized and need foreign pollen for successful reproduction within genetically diverse populations (Rea and Nasrallah 2008). For example, the most important grass species Italian ryegrass (*Lolium multiflourm*) and perennial ryegrass are wind pollinated, whereas the most important legumes red clover white clover and alfalfa are pollinated via insect vectors, cross pollination in both groups being secured by self-incompatibility (Cornish et al. 1979; Campbell 2000; Casey et al. 2010; Riday and Krohn 2010a). Hence, open pollinated or synthetic variety breeding, aiming at

the preservation of heterozygosity within a breeding population, are the main breeding methods employed for forage crops. In the past, forage varieties were bred as open pollinated varieties, whereby a number of superior plants are selected from a base population via mass selection, based on their phenotype determined on spaced plants in the breeding nursery. The process could be repeated several times (recurrent selection) and could also involve the introduction of new plant material and genetic resources. The resulting varieties represent a population of different genotypes, sufficiently large to avoid inbreeding depression. In polycross schemes, which are often employed today, selected individuals (each representing a different genotype) are vegetatively propagated (cloned) and interplanted together for random mating among the different genotypes. After separate harvest of seeds, progeny of each genotype can be grown to determine the general combining ability (GCA), i.e. the relative performance of the offspring of a parental genotype in relation to all other offspring of the polycross. Based on GCA, the best parental plants can be evaluated and clones or remaining seeds from these plants can be used to create the new, so-called synthetic variety. Topcross schemes are similar to polycross schemes, but involve crossing every plant genotype to a common pollinator (tester) instead of random pollination among all genotypes.

Increasing the performance of forage plants from grassland species will remain an ever ongoing process for forage plant breeders. A method frequently used in breeding forage plants is chromosome doubling, mostly from a diploid to a tetraploid set of chromosomes (induced autotetraploidy). This polyploidization is achieved by treating plant seedlings with colchicine (Blakeslee and Avery 1937), a metabolite of the autumn crocus (*Colchicum autumnale*) disturbing the cell cycle during metaphase of mitosis. Thereby, the reduction division is aborted and resulting cells exhibit a doubled chromosome set. In comparison to their diploid origin, induced polyploid plants often show increased vigor and biomass yield, larger seeds and higher digestibility, which is a consequence of higher cell-content to cell-wall ratios due to larger cell size (reviewed in te Beest et al. 2012). Another possibility to increase plant performance or to create new variations is the induction of mutations via chemical treatment or ionizing radiation (Posselt 2010). However, this method is not always successful. Future research will also

focus on the development of hybrid varieties in forage grasses and legumes, as synthetics do not strongly exploit heterosis (Aguirre et al. 2012). Hybrids, as usually used in maize (*Zea mays*) breeding, are the crosses between inbred lines of two different heterotic pools and express strong heterosis. Here, a prerequisite is the possibility to develop inbred lines, what can be usually achieved via self-fertilization. In this perspective, self-fertilization is a desired trait and will be possible in forage plants after finding or creating plant genotypes with broken self-incompatibility system (Riday and Krohn 2010b).

1.2.4. Genetic markers to assist plant breeding

Traditional plant breeding is a time consuming process with several years of phenotyping, testing of breeding candidates and selection. Most forage species require vernalization and only flower after the year of establishment. Therefore, they have a long generation interval, which, in turn, results in a low breeding progress. As part of long lasting meadows, forage species should exhibit a good persistence, a trait of which the assessment is a process consuming years. The knowledge of the plants' pedigree as well as the heritability (h^2, the share of genetic variation to the phenotypic variation) of a trait can be used to predict the performance of the offspring of selected plants. Here, traditional breeding is often assisted by molecular markers. A direct use of molecular markers in plant science is the designation of parentage (Kölliker et al. 2005). A more labor-intensive approach is the identification of molecular markers which are associated with quantitative trait, so-called quantitative trait loci (QTL, Kearsey and Farquhar 1998). QTL are sections on the plant chromosome harboring a gene regulating a quantitative trait. Genetic markers linked to a QTL are, therefore, very likely being inherited together with the regulating gene and indirect selection for the trait is possible via selection for the marker phenotype. Thereby, molecular markers have the advantage that they are not influenced by environmental factors, can be assessed on young plants, and have a theoretical heritability of $h^2 = 1$. Indirect selection of genotypes superior for a given trait via selection for markers linked to a QTL is referred to as marker assisted selection (MAS). The use of MAS was successfully established and used in several plant species such as in potato for the presence of resistance genes against potato late blight

(*Phytophtora infestans*, Sliwka et al. 2010), in maize for earliness and grain yield (Bouchez et al. 2002), and in rice (*Oryza sativa*) for resistance against bacterial blight (*Xanthomonas oryzae pv. oryzae*, Singh et al. 2001).

In forage plants, most traits have a quantitative and polygenic (controlled by many genes) character and their improvement is laborious and expensive. Therefore, MAS could be a valuable tool, e.g. as proposed for the improvement of nitrogen-use efficiency or crown rust (*Puccinia coronate*) tolerance in perennial ryegrass (Dolstra et al. 2003; Pauly et al. 2012). For species like sainfoin that are underutilized due to weaknesses in agronomic performance, MAS offers prospects to improve such species and make them economically more attractive (chapter 4).

For detection and application of QTL in plant breeding, availability of numerous and cheap genetic markers is a precondition. Several marker systems, broadly divided in dominant and co-dominant markers, are available today: Restricted Length Fragment Polymorphism (RFLP, co-dominant), Amplified Length Polymorphisms (AFLP, dominant), Random Amplification of Polymorphic DNAs (RAPDs, dominant), Sequence Tagged Sites (STS, co-dominant), Single Nucleotide Polymorphisms (SNP, co-dominant) and Simple Sequence Repeats (SSR, co-dominant). Dominant markers are widely distributed due to their easy application in non-model organisms (Meudt and Clarke 2007), but bearing the disadvantage of only showing the dominant allele and not being suitable to measure heterozygosity. Co-dominant markers, in contrast, also show recessive alleles and, therefore, the heterozygous state at a marker locus. The identification of recessive alleles is important, as they could carry advantageous or deleterious, rare properties. The development of co-dominant markers for non-model species was elaborate and expensive, but the new next generation genotyping-by-sequencing technologies (NGS) enabled the development of vast amounts of co-dominant SSR and SNP markers by high-throughput sequencing (Elshire et al. 2011; Zalapa et al. 2012). Different NGS platforms are available, e.g. Illumina MiSeq and HiSeq, Roche 454 and Ion Torrent PGM. Parallel handling of large sample amounts is possible, yielding billions of DNA bases. Those NGS technologies are also attractive possibilities to create sainfoin specific co-dominant markers.

1.3 Sainfoin

1.3.1 Taxonomy

Sainfoin is a perennial legume from the tribe Hedysarae of the subfamiliy Fabaoidae within the family Fabaceae. The taxonomy of the tribe Hedysarae remains quite controversial with different ordering of sections (Yildiz et al. 1999; Lewis et al. 2005; Kar et al. 2014). Summarizing available information, the most likely taxonomic affiliation is that within the tribe Hedysarae, sainfoin belongs to the genus *Onobrychis* Mill., therein to the subgenus *Onobrychis* and therein to the section *Onobrychis* (Fig. 1.2, Woodgate et al. 1999). The confusion in the taxonomy is also reflected by the number of synonyms listed for sainfoin which are *Hedysarum onobrychis* L., *Onobrychis onobrychis* (L.) Karsten, *Onobrychis sativa* Lam., *Onobrychis viciifolia* Scop. subsp. sativa (Lam.) Thell. and *Onobrychis vulgaris* Güldenst. (Porcher 2004). This lack of clarity might also lead to problems to differentiate between different species in the same section.

Within the species of sainfoin (*Onobrychis viciifolia)*, two botanical types are distinguished: common types (*Onobrychis viciifolia* Scop.var. communis Ahlef.) and giant types (*Onobrychis viciifolia* Scop. var. bifera Hort.). Common types have their origin in central Europe and are also referred to as single cut types, due to the slow vegetative regrowth after the first cut (Badoux 1964). They show a good persistence of up to eight years. The growth type remains more prostrate at the beginning of cultivation and flowering starts in the year after establishment. Giant types (*Onobrychis viciifolia* Scop. var. *bifera* Hort.) have their origin in the Middle East (Badoux 1964). They are designated as double-cut types due to their fast establishment and regrowth as well as a strong vigor. Giant types flower up to three times per year, but are less persistent than the common types (Bell 1948; Piano and Pecetti 2010). Modern sainfoin varieties are often based on common and giant forms to combine benefits of the two groups.

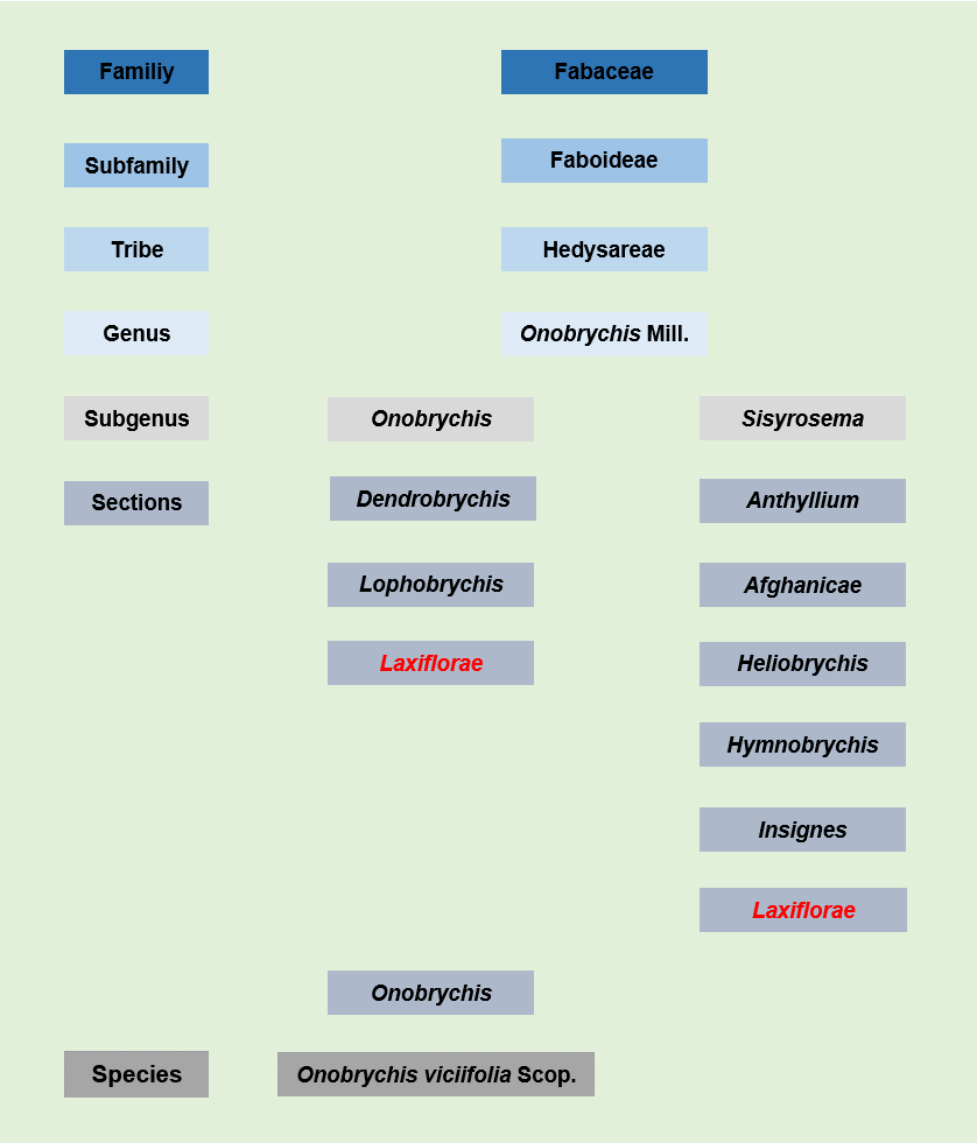

Fig. 1.2 Taxonomic position of sainfoin (*Onobrychis viciifolia*, adapted from information reviewed in Woodgate et al. 1999). The affiliation of section *Laxiflorae* (red) to either subgenus *Onobrychis* or subgenus *Sisyrosema* has not yet been clarified

1.3.2. Origin and historical distribution

Sainfoin is already being cultivated for several decades in temperate Asia and Europe (Goplen et al. 1991; Frame et al. 1998; Hybner 2013). Yet, its origin has not been fully clarified, but the majority of the literature refers to the Middle East and Asia as a center of origin (Smith 2007; Azuhnwi et al. 2012; Hayot Carbonero et al. 2012; Toluei et al. 2013). In Armenia, cultivation started as early as in the 10th century (Akopian 2009). It seems likely that sainfoin thence spread to neighboring countries such as Russia and Turkey, as an introduction for

cultivation in Russia took place in the mid-14th century (Dzyubenko and Dzyubenko 2009). Its first appearance in western Europe was in southern France during the 15th century (Piper 1914). Later, sainfoin gained high importance in France until the beginning of the 18th century, also reaching its peak distribution in whole Europe (Chorley 1981; Hanelt 2001). Around the 17th century, sainfoin cultivation activities started in the UK and Germany (Piper 1914; Doyle et al. 1984; Liu 2006) and around the 18th century in Italy (Piper 1914). The Swiss botanist Albrecht von Haller reported cultivation of sainfoin in Germany in one of his writings (von Haller 1772), and as he was open-minded to new agricultural plants, he possibly introduced this species to Switzerland: in 1759, he received larger amounts of seeds from the French Charles Bonnet, who sent them over the lake of Geneva to Vevey (Switzerland). Beyond Europe, the introduction of sainfoin to the United States of America is controversially discussed, with Goplen (1991) dating the beginning of its cultivation to the 1900s, whereas Roseberg (1993) specified it to 1786. However, sainfoin has never became a major forage crop in the USA, neither with the introduction of the "Cooper Mix" in 1970, a pasture mix with sainfoin as the seed base.

1.4. Basic characteristics of sainfoin

1.4.1 Morphology

Sainfoin is an herbaceous plant growing in a prostrate or erect manner (Fig. 1.3a and b). The biomass comprises stems and multifoliate leafs with ten to 28 pairs of pinnately leaflets and one single terminal leaflet arising from the middle of the vegetation disk. The leaf to stem ratio can be highly variable, with smaller plants tending to have a higher leaf to stem ratio (Malisch et al. 2015). The appearance of the stem varies with genotype, ranging from fine and soft to thick and stiff and from green to completely red (Fig. 1.3c). Also, the shape and color of the leaflets vary between genotypes.

The root system of sainfoin comprises a deep tap root, which reaches depths of 110 to 200 centimeters in the soil, allowing the acquisition of substantial amounts of subsoil water and nutrients and mediating a good tolerance to

drought spells with drying upper soil layers (Fleischmann 1932; Agrarökonomie 2006).

Fig. 1.3 Sainfoin plants in the year of establishment showing **a)** prostrate and **b)** erect growth, as well as **c)** red stems (Photos: Katharina Kempf).

The pink flowers stands, consisting of about 80 small florets, are an axillary raceme (grape-like) in conical shape (Fig. 1.4a-c). White flowers are also possible, but occur very seldom (Hayot Carbonero 2011). Flowering starts at the base of the flower stand, reaching its top after some days. The time of flowering can be highly variable among different genotypes. After pollination by insects, green oval to kidney shaped seeds develop, containing one true seed (0.5 x 0.3 cm) that is much smaller than the coated seed (0.8 cm x 0.5 cm, Fig 1.4d). Seed ripening starts from the base to the top of the flower stand with browning of the seeds (Langer and Hill 1991).

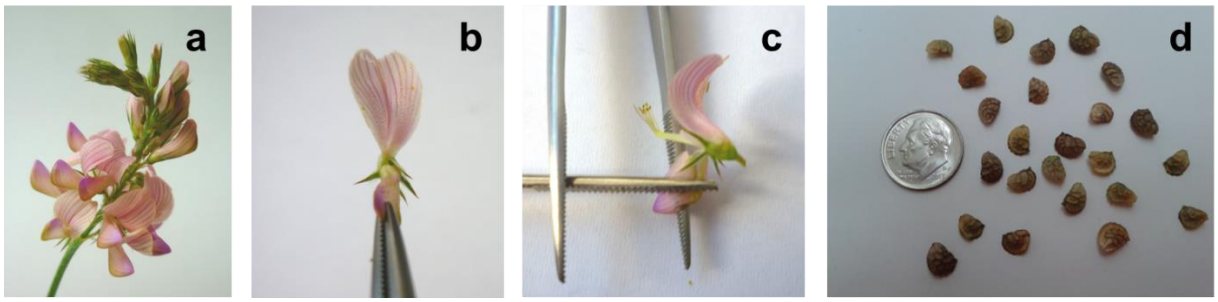

Fig. 1.4 a) partially mature sainfoin flower, **b)** single floret closed, **c)** open floret with anthers, pollen and stigma visible, **d)** mature coated sainfoin seeds (Photos: Katharina Kempf).

1.4.2. Chemical properties of sainfoin

Sainfoin is known to be rich in condensed tannins (synonym: proanthocyanidins), which belong to the group of secondary plant metabolites. In contrast to primary plant metabolites that are responsible for growth promotion, secondary plant metabolites are often involved in plant defense (Heil et al. 2002; Min et al. 2003; Volf et al. 2015), while they are mostly water soluble and bind to proteins and other macromolecules in aqueous solutions (Bate-Smith 1962, Haslam 1989 cited in Serrano et al. 2009). Tannins in general are polyhydroxyflavan oligomers or polymers. Four groups are defined based on their chemical structures: phlorotannins (occur mainly in marine organisms), condensed tannins (CTs), hydrolysable tannins (HTs) and complex tannins (Serrano et al. 2009). CTs are the most diverse group of tannins and are built of procyanidin (PC) and prodelphinidin (PD) subunits. PCs consists of two or more monomeric (+)-catechin or (-)-epicatechin units, whereas the PDs consist of (+)-gallocatechin or (-)-epigallocatechin units (Fig. 1.5a). The HTs (gallic acid derivates, gallotannins and ellagitannins) are, in view of their structure, the most complex tannins (Salminen and Karonen 2011). Salminen and Karonen (2011) suspected that ellagitannin (Fig. 1.5b, c, a class of hydrolysable tannins) oxidation might form a major chemical defense against herbivores, what is against the previous opinion that the protein binding activity in CTs alone is responsible for plant defense mechanisms.

CTs, as present in sainfoin, are associated with several benefits if fed to animals. The protein utilization in ruminants fed with diets enriched with CTs is improved, leading to faster growth rates, increased milk production and quality (Girard et al. 2016). The better utilization is caused by a reduced protein digestion by microorganisms in the rumen when proteins are bound to tannins. Complexation of proteins is assumed to happen at pH of 6 – 7, as present in the rumen. With changing pH in the abomasum (pH < 3.5) and the small intestine (pH > 7), proteins unbind from the complex again, leading to increased protein availability for gastric or pancreatic digestion (reviewed in Mueller-Harvey 2006). From these so-called ruminal escape proteins, more dietary amino acids can be absorbed and utilized by the animal.

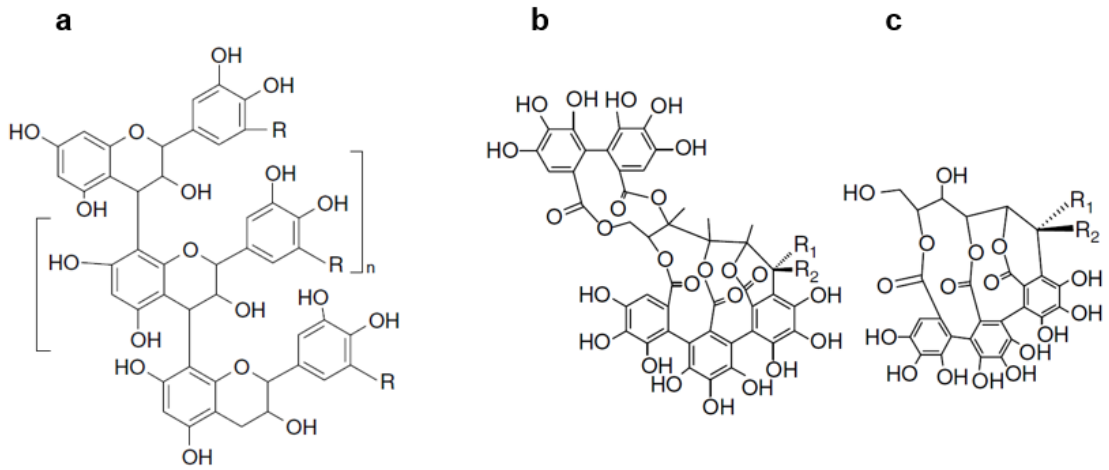

Fig. 1.5 a) condensed tannin (CT, as present in forage plants) R = H procyanidins; R=OH: prodelphinidins; **b)** ellagitannin (as present in chestnut) R_1 = OH, R_2 = H: castalagin; R_1 = H, R_2 = OH: vescalagin; **c)** ellagitannin (as present in chestnut), R_1 = OH, R_2 = H: castallin; R_1 = H, R_2 = OH: vescalin. Taken from Mueller-Harvey (2006)

The presence of CTs has also direct impact on animal health by preventing bloat. Bloat is a severe problem in ruminants causing the impairment of the digestive and respiratory function and, not rarely, the death of the animal due to cardiac or respiratory arrest. Bloats are caused by gas accumulated in the rumen and reticulum after the ingestion of readily digestible feed with intensive fermentation, e.g. from alfalfa and white clover (Sottie 2014). Frequently, stable foams generated by bacterial slime and plant leaf protein hinder the release of fermentation gas via eructation, leading to froathy-bloat (Wang et al. 2012). Condensed tannins are able to inhibit rumen microorganisms and foam formation via binding and precipitation of proteins (Waghorn and Jones 1989). Another positive aspect of the presence of CTs is the reduction of greenhouse gas emissions by reducing ruminal degradability of fiber (McMahon et al. 1999; Bueno et al. 2015). Thereby, the chemical structure of CTs seems to affect the total CH_4 reductions (Hatew et al. 2015; Saminathan et al. 2015).

CTs also play a role in the control of gastrointestinal parasites in animal production systems. Because increasing formation of resistance in parasite populations to synthetic anthelminthic drugs, CTs are a desired alternative nutraceutical, impairing the development of nematodes. CTs decrease the development of infectious larval stages and egg excretions, also reducing

contamination of pastures. Different hypotheses on the anthelmintic mode of action of CTs exist. One hypothesis is that CT directly influences the worm biology by reducing fecundity, whereas another hypothesis supposes that the host immune response is improved after ingestion of CT rich forage (Tzamaloukas et al. 2006; Hoste et al. 2012). Though the mode of action is not completely known yet, the susceptibility of parasites varies depending on structural variations in CTs (Quijada et al. 2015).

Despite their many advantages, the use of tannins in animal science is a tightrope. The binding of proteins, carbohydrates and lipids bears also the risk that the availability of these nutrients might get too low (Butler 1992). The source of tannins as well as an adequate dose is, therefore, fundamental to enhance the positive effects. The differentiation of beneficial and non-beneficial tannins seems dependent on several aspects and is yet not fully examined. The group of tannins surely has an impact, whereby PCs, PDs and ellagitannins are considered as predominantly animal health promoting. The ratio of PCs to PDs in CTs might also play a role, as well as presence of other plant compounds, binding strength of CTs and their molecular weight (Mueller-Harvey 2006).

1.5. Cultivation and use

1.5.1 Nowadays distribution

Sainfoin is nowadays commonly cultivated in Turkey with an area of about 94,000 ha in 2001, whereby the plant is mainly produced in middle and eastern Anatolia (Eken et al. 2004; Tufenkci et al. 2006). There, ecotypes, i.e., populations locally evolved in the respective regions and adapted to the environmental conditions, are of high importance (Tan and Dumlu 2009). In Russia, sainfoin is mainly cultivated in forests, steppe and mountainous areas, where it is under use for light grazing, but moreover for hay production (Dzyubenko and Dzyubenko 2009). In Switzerland, sainfoin is recommended as a minor component for dry to fresh fertile meadows, yellow oat grassland, bromegrass meadows and on ecological compensation areas which are useful to connect isolated habitats (Agroscope 2009). Wild grown sainfoin plants were found all over Switzerland

with more frequent findings in areas bordering Germany and France (info-flora 2015). In Germany, the abundance is highest in Baden-Württemberg and Franconia, with the majority of plants growing naturally rather than being under cultivation (BfN 2013; Meyer 2015). In France, sainfoin covered 24% of the total cultivated legume area during the last centuries (Huyghe 2007), but has been largely replaced by alfalfa nowadays. In general, the situation for France and Germany with decreasing importance of sainfoin since the middle of the 20th century seems to be reflective for the whole of Europe, including the UK, where the crop almost disappeared (Doyle et al. 1984). In the U.S., sainfoin is gaining more and more interest as a forage plant in Montana and New Mexico, what could also be achieved thanks to the development of site specific adapted varieties. There, sainfoin stands are used for direct grazing and cutting for hay production and they are also appreciated as parts of crop rotations (Smith 2007; Roesler 2015). The cultivation of sainfoin is nowadays also considered as a valuable option for Australia and New Zealand, because of its health promoting benefits by preventing bloat in ruminant production and resistance to aphids which cause severe losses in alfalfa (Fortune 1985; Rumball and Claydon 2005).

1.5.2 Cultivation

The weak persistence and, hence, performance of this perennial plant is probably the main reason why farmers have not widely adopted sainfoin. Here, the poor competitive ability of sainfoin against weeds and other plants, as well as the missing adaption to diverse conditions are the driving force for the loss of plants (Burke 1840). Pure sainfoin stands usually persist three to four years (Tan and Dumlu 2009). Conditions favoring the competitive ability of sainfoin and, therefore, its cultivation, are dry and warm climates with fair irrigation. Contrastingly, high salt contents, waterlogging and frost are disadvantageous for its persistence and cultivation (Doyle et al. 1984; Goplen et al. 1991; Neuhoff 2009). Sainfoin grows preferentially on calcareous loam, marly chalk and sandy or oolitic soils with pH >6.5 (Burke 1840). Under warm and dry climate and chalky soil conditions, sainfoin could even outperform red clover and alfalfa due to its higher drought resistance when compared to red clover and lower demands for deep soils when compared to alfalfa (Doyle et al. 1984; Neuhoff 2009).

Sainfoin as a legume forms symbiosis with rhizobia (Scott Hill 1980). Artificial inoculation is sometimes recommended, but not necessary for regions with abundant legume cultivation and wide distribution of different related strains of rhizobia bacteria in the soils (Carsten Malisch, personal communication, 2015). Only under insufficient N status, either due to missing rhizobia association in some regions or poor soil conditions, application of nitrogen fertilizers is needed, as the low N status would otherwise result in stunted growth (Goplen et al. 1991).

Fig. 1.6 Weed competition in sainfoin stands in the third growing season (2014) without plant protection (Photo: Katharina Kempf).

Recommended sowing rates for sainfoin range between 120 to 200 kg per hectare with 15 cm distance between single seeds (Agrarökonomie 2006; Neuhoff and Bücking 2006; ufasamen 2015). Severe weed pressure during the establishment phase can reduce the density of sainfoin stands. Under organic farming with restricted plant protection, weed occurrence (Fig. 1.6) in established stands reached up to 42.7% (Neuhoff and Bücking 2006). As alternative to pure stands, the cultivation of sainfoin in mixtures bears the advantage that weeds could be suppressed by companion plants, which are not too aggressive against sainfoin itself. Sainfoin mixtures are cheaper in production and the dry matter yield could rise by 15% compared to pure stands (Doyle et al. 1984). The climatic conditions and the site play an important role in finding the optimal mixture partner for

sainfoin. In Switzerland, optimal partners were found to be perennial ryegrass and meadow fescue (*Festuca pratensis*), both suppressing weeds but not decreasing sainfoin yield, whereas chicory (*Cichorium intybus*) and white clover tended to be too suppressive (Carsten Malisch, personal communication, 2015). Contrary, chicory seemed to be the optimal partner in the U. K. (Mora Ortiz and Smith 2016).

Pests and diseases and their influence on sainfoin persistence cannot be generalized for all regions of cultivation. In the U.S. and Canada, root- and crown rot diseases are important (Goplen et al. 1991). For Western Europe, clover rot (*Sclerotinia trifoliorum*) and brown spots (*Stemphylium sarciniforme*) are considered being relevant diseases of sainfoin (Schubiger 2015).

1.5.3. Usage

Sainfoin is used as forage source for ruminants, mostly served fresh, as hay or pellets, whereas preservation as silage presents difficulties due to its low carbohydrate content (Agridea 2012). Direct grazing is restricted to dry areas with moderate irrigation (Goplen et al. 1991; Agridea 2012). Dry matter yield varies dependent on variety, environmental conditions and cultivation conditions (Wilman and Asiedu 1983). Generally, production levels are highest for the first cut in the year after establishment and decreases in the following years. For Switzerland 60 to 100 dt/ha dry matter yield were reported for the first cut under conventional conditions (Agridea 2012). Similar yield levels were also reported under organic farming conditions for Germany with 75 dt/ha (Neuhoff and Bücking 2006), whereas yields in the U.S. seem to be somewhat lower with 40 dt/ha (Killen 2012).

Besides its usage as animal forage, sainfoin is a valuable plant in rotation farming as catch crop to restore soil nutrient resources via acquisition of atmospheric nitrogen for subsequent cultures (Agrarökonomie 2006). Furthermore, humus development and soil aeration are improved via the deep soil penetration of the extensive root system, reducing the need for ploughing (KÖL 2012). Sainfoin is also mentioned as promising species for use in wildlife habitat restoration or mine land reclamation (Gray and Koch 2008). Its characteristic as good insect forage source supporting development of different,

even non-specialized, bee species, make it a valuable species to support natural insect communities (Haider et al. 2014).

1.6. Genetic improvement of sainfoin

1.6.1. Reproduction system

Sainfoin is considered being mainly cross-fertilized, with self-fertilization being supposed to occur seldom. The existence of a self-incompatibility system was suspected, but no conclusive evidence was provided (Tasei 1984). The architecture of the flower hampers spontaneous self-fertilization because the stigma overtowers the anthers (Knuth 1906; Demdoum 2012). So far, only few studies were conducted to estimate the rate of self-fertilization in sainfoin (Thomson 1938; Knipe and Carleton 1972; Knipe 1972; Demdoum 2012). Self-fertilization in general could be achieved by human interference via hand pollination in emasculated plants and by wind or insect pollination. Using hand pollination in different studies, only low rates of seed set of 5.11, 5.0 and 15.5% could be reached (Thomson 1938; Knipe 1972; Demdoum 2012). In a trial of Knipe and Carlton (1972), white flowering sainfoin plants were planted in a foil tent together with pink flowering plants and bumble bees ensuring pollination. From the offspring of these white flowering plants, 72 to 92% showed white flowers, verifying a high degree of self-fertilization, as the white flower color shows a recessive inheritance. Thus, the visit of insects such as honey or bumble bees seems to be the driving force for successful pollination, either with own or foreign pollen. This result showed that insect pollination could overcome difficulties in producing selfed sainfoin plants. To verify these results and possibly use insect pollination for production of inbred lines in breeding programs, an efficient detection system other than the flower color marker use by Knipe (1972) is needed. Here, molecular markers would be an excellent choice to distinguish offspring originating from self- and cross-fertilization.

1.6.2. Polyploidy

Besides the mode of reproduction, information on the origin of tetraploidy in sainfoin is also scarce. This is a relevant issue for breeding sainfoin because the likelihood of trait inheritance to offspring is different for alleles or genes under autotetraploid or allotetraploid gene segregation. This likelihood influences success and time needed for breeding steps. For example, an autotetraploid genotype AaAa can produce three different types of gametes (Aa, AA and aa), whereas an allotetraploid genotype AaAa can produce four types of gametes (AA, Aa, aA and aa, Comai 2005). The possibly oldest study on polyploidy in sainfoin proved the tetraploid chromosome number 2n = 28 and considered sainfoin being of the *Primula kewensis* type without evidence for allo- or autotetraploidy (Fyfe 1946). The *Primula kewensis* type describes the observation of the first fertile hybrid derived from crosses of two diploid ancestor plants, in which restoration of fertility occurs after spontaneous chromosome doubling, resulting in offspring which is able to reproduce genetically stable offspring (Upcott 1939). However, more recent studies found indications for autotetraploidy, based on an autotetraploid-like arrangement of chromosomes during metaphase 1 of meiosis as observed by microscopy, and a tetraploid inheritance pattern of isoenzymes (Sacristan 1965; De Vicente and Arus 1996). Besides gene segregation studies, the inheritance of phenotypic traits represents another way to unravel the origin of polyploidy. Selfing heterozygous sainfoin individuals with stigma mutations by hand pollination resulted in gene segregation ratios suggesting allotetraploidy (Knipe 1972).

Problems to clearly identify the type of tetraploidy might occur due to the possible common occurrence of polysomic (expected for autotetraploids) and disomic (expected for allotetraploids) segregation and inheritance patterns of chromosomes and genes in plants. At the one hand, quadrivalent chromosome pairing as occurring in autotetraploids could also arise in allotetraploid species due to a higher numbers of homologous segments in closely related ancestors or mutations in genetic mechanisms preventing non-homologous chromosome pairing (Stebbins 1950; Sybenga 1996). At the other hand, an autotetraploid species could direct to diploid-like bivalent chromosome pairing to stabilize the

polyploidy or due to genotypes with preferential bivalent formation (Kuckuck et al. 1972; Comai 2005).

1.6.3 Breeding activities

Nowadays, sainfoin breeding activities are marginal and only few varieties are available worldwide. The European Commission listed 22 sainfoin varieties registered in the section "Agricultural plant species" in the "plant variety catalogue and databases" of 2015 (European Commission 2015). The origin of those varieties spans only a few countries, with the majority coming from Italy, Switzerland and Eastern and Southern Europe. The admission dates lead back to the 1940s and 1960s, followed by a gap of twenty years in which no variety was registered, and starts again in the 1990s. This gap might reflect political reforms supporting more intensive agriculture during the 1980s (Hayot Carbonero 2011). New varieties are often re-admissions from older varieties. For example, the varieties Vinovsk, Visnovsky, Višňovský might all lead back to the same original cultivar Visnovsky. The Swiss variety Perdix, released in 2011, originates directly from the older Swiss variety Perly (Beat Boller, personal communication, 2012). The amount of sainfoin accessions available as genetic resources in gene banks is also limited, with only 79 accessions listed until 2012 (Feuerstein 2012). In Germany, no seed multiplication was performed in 2010, and the amount of imported seeds accounted only for 120 tons (Feuerstein 2012).

Contrastingly to the situation in Europe, research and variety development in Western U.S. and Canada was most progressive during the 1970s, when sainfoin was promoted as an alternative to alfalfa (Cash et al. 2010). In the U.S., the variety "Shoshone", released 2005, was developed from sainfoin plants surviving infestations with the northern root-knot nematode (*Meloidogyne hapla*, Gray and Koch 2008). Three years later in 2008, the variety "Delaney" was released in the U.S. as multi-cut variety with improved regrowth and yield (Hybner 2013). In Canada, the new variety "ACC Mountainview" was promoted which matures earlier than the standard varieties and performing well in mixed stands with alfalfa (Acharya 2015).

1.6.4. Breeding goals and genetic resources

The overall goal in breeding sainfoin is the creation of new varieties showing high biomass yields and improved persistence. Persistence itself is related to the susceptibility to different pests and diseases, as well as the competitive strength against weeds and adaption to regional climatic conditions.

So far, the demand for genotypes adapted to dry and warm areas was served by genotypes native to the Soviet Union and Turkey, which performed well in dry regions of the U.S. compared to Western European varieties (Goplen et al. 1991; Gray and Koch 2008). For western Canada, adapted varieties exist showing winter hardiness and exhibiting a strong spring vigor (Goplen et al. 1991). Furthermore, Canadian sainfoin varieties should perform well under intercropping with alfalfa in order to reduce bloat potential of the forage, wherefore improved sainfoin populations are also selected under mixed cropping with alfalfa (Sottie 2014). Contrary, cultivation trials in New Zealand showed that varieties from other countries do not perform well and less genetic variation for adaption to cool season conditions could be found (Rumball 1982). Furthermore, the slow autumn growth resulted in poor annual yields, a problem New Zealand sainfoin breeding programs have to deal with besides improved winter growth (Percival and Cranshaw 1986). These genetic limitations might be another reason why sainfoin has difficulties to gain agronomic importance in New Zealand (Rumball and Claydon 2005). In Europe, the problems are equivalent to New Zealand, with genotypes lacking adaption to wet and cold climates, whereas the development for drought regions was satisfactory. For example, Spanish sainfoin cultivars showed poorer performance for morphological and productive parameters grown in rainfed than under irrigated conditions (Delgado et al. 2008). A lack of genetic variability for adaption to moderate temperatures and abundant humidity could also be demonstrated in trials of Liu (2006) in the U.K., analyzing biomass yield in eight sainfoin varieties.

Besides the climatic adaption, resistance to different diseases and pests is desired dependent on area of cultivation. The existing variation in worldwide genetic pools of sainfoin for resistance traits seemed low or barely tapped. In the U.S., resistance to the alfalfa weevil, nematodes and root-rot knot nematode

Meloidogyne hapla is desired (Wofford and Gray 1987; Ruckman 2008). The available resistance in common varieties against the northern root-knot nematode showed low levels of resistance (Wofford and Gray 1987). Testing different accessions for resistance against *Fusarium solani*, a pathogen causing root and crown rot, control varieties showed higher resistance than non-cultivated individuals, indicating a co-occurrence of resistance and better agronomic performance (Auld et al. 1977) and highlighting the need for constant improvement of disease resistances.

Furthermore, given the suitability of sainfoin for animal nutrition, the improvement of tannin content and composition will gain importance in the future. Breeding for a generally high CT concentration might result in a drawback, as it is, so far, not clarified which composition of tannins are the most effective. For example, species with higher total tannins compared to sainfoin showed adverse effects for protein utilization in animals (reviewed in Mueller-Harvey 2006). Nevertheless, it seems reasonable to breed for high leaf to stem proportions, as leaves make up the biggest share of tannins in sainfoin and are the palatable part in the forage ration (Haering et al. 2007; Malisch et al. 2015). The possibility of influencing tannin content and composition via breeding is feasible, as significant variation could be found in 27 distinct sainfoin accessions grown under equal conditions (Malisch et al. 2015).

Summarizing available information, there is a lack of varieties for adaption to cold and wet climates, as well as resistance to pests. The existing sainfoin resources have to be screened worldwide for plants that could close these gaps. Furthermore, it will be useful to describe such individuals genetically in order to identify genes corresponding to the better adaption. Using the knowledge and genetic marker resources created during this process will be crucial to further advance breeding programs.

1.7 Objectives of the thesis within the LegumePlus project

The present thesis was embedded in the European project "LegumePlus" (PITN-GA-2011-289377). The project was funded by the Marie Curie Initial Training Network within EU's the 7th framework program. The focus of this project was on interdisciplinary research on the forage legume sainfoin. Within the project, the disciplines of chemistry, animal nutrition, parasitology, agronomy and plant breeding were covered. The objectives of the present thesis were to provide basic knowledge for breeding sainfoin on the basis of molecular markers.

Chapter 2 deals with a fundamental information gap about the ability of self-fertilization in sainfoin. Sainfoin is considered to be an outbreeding species. However, the rare data on self-fertilization and different speculative hypotheses still impose a problem. For developing new breeding strategies, it is essential to know the possible amount of self-fertilization in sainfoin. The aim of this study was the analysis of self-fertilization in sainfoin under different pollination set-ups:

1. Insect pollination by bumble bees (*Bombus terrestris*) in the greenhouse with the amount of plants, and thus pollen, restricted to two pairs of five clones.
2. Open-pollination in three fields grown with populations of sainfoin without restrictions in the number of plant.

The amount of self-fertilization was then measured using dominant sequence related amplified polymorphisms (SRAP) and two new, co-dominant simple sequence repeat (SSR) genetic markers

Chapter 3 is about the characterization of sainfoin specific SSR markers. The SSR markers were newly discovered by NIAB Cambridge (United Kingdom) via RNA sequencing. The SSR markers were characterized in collaboration with NIAB and Aberystwyth University (United Kingdom) for amplification and polymorphisms in a panel of 32 diverse individuals, to assess their potential to measure genetic diversity in sainfoin.

Chapter 4 builds on the work performed in the previous chapter. A selection of 53 co-dominant SSR markers and 16 dominant SRAP marker combinations were used for the molecular characterization of a biparental F_1 population originating from cross-fertilization of the pollination trials performed within the framework of chapter 2. The genetic marker data were combined with information on the phenotypic performance of the population from a field trial to find marker trait associations for several agronomic and compositional traits.

Chapter 5 describes a first approach to establish a genetic linkage map for sainfoin in one biparental F_1 population (see chapter 2) on the basis of 48 co-dominant SSR markers and dominant 16 SRAP marker combinations.

Chapter 6 gives a discussion and overview of the results. Furthermore, possible future approaches and application of our findings for sainfoin breeding will be highlighted.

Chapter 2 Evidence and consequences of self-fertilization in the predominantly outbreeding forage legume *Onobrychis viciifolia*

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2.1 Abstract

Background: Sainfoin (*Onobrychis viciifolia*) is a promising alternative forage plant of good quality, moderate nutrient demand and a high content of polyphenolic compounds. Its poor adoption is caused by the limited availability of well performing varieties. Sainfoin is characterized as tetraploid and mainly outcrossing, but the extent of self-fertilization and its consequences was not investigated so far. This study aimed at assessing the rate of self-fertilization in sainfoin under different pollination regimes and at analyzing the consequences on plant performance in order to assist future breeding efforts.

Results: The self-fertilization rate was assessed in three sainfoin populations with artificially directed pollination (ADP) and in three populations with non-directed pollination (NDP). Dominant SRAP (sequence-related amplified polymorphism) and co-dominant SSR (simple sequence repeats) markers were used to detect self-fertilization in sainfoin for the first time based on molecular marker data. High rates of self-fertilization of up to 64.8% were observed for ADP populations in contrast to only up to 3.9% for NDP populations. Self-fertilization in ADP populations led to a reduction in plant height, plant vigor and, most severely, for seed yield.

Conclusions: Although sainfoin is predominantly outcrossing, self-fertilization can occur to a high degree under conditions of limited pollen availability. These results will influence future breeding efforts because precautions have to be taken when crossing breeding material. The resulting inbreeding depression can lead to reduced performance in self-fertilized offspring. Nevertheless the possibility of self-fertilization also offers new ways for hybrid breeding based on the development of homogenous inbred lines.

2.2 Background

Legumes are particularly valuable component of permanent and temporary grasslands, as they increase forage yield and quality and simultaneously decrease the need for nitrogen fertilization through symbiotic N_2 fixation (Lüscher et al. 2014). The perennial legume sainfoin (*Onobrychis viciifolia*) combines a multitude of positive characteristics of grassland legumes. It is adapted to drought prone areas and few important pests and pathogens are reported for this species (Hayot Carbonero 2011). The name sainfoin is derived from the French words "sain" and "foin" which means "healthy hay" and implies the health- promoting features of this species. Sainfoin is characterized by high contents of condensed tannins which, at a moderate level, support protein digestion and help to reduce bloat in sheep (Theodoridou et al. 2012; Dentinho et al. 2014) or cattle (Mezzomo et al. 2011). Tannins are also valued for their anti-parasitological effects against gut parasites (Paolini et al. 2003). Feeding sainfoin may help to reduce the use of medications in animal husbandry.

The use of sainfoin in ruminant nutrition is focused on roughage production in pure or mixed stands. For this, predominantly tetraploid varieties (2n=4x=28) are used, but diploid populations (2n=2x=14) also exist in natural grasslands. Based on comparative cytological studies, an autopolyploid inheritance was suggested for sainfoin (Sacristan 1965). This was verified by a preponderance of tetrasomic gene segregation, which is characteristic for autotetraploid species, as shown in a study based on isozyme variation (De Vicente and Arus 1996). However, the latter study also found some evidence for disomic segregation and some authors have suggested an allopolyploid condition of sainfoin, although no direct evidence was given (Zarrabian et al. 2013). Sainfoin is insect-pollinated with six insect species acting as pollinators (Richards and Edwards 1988), i.e. bumble bees (*Bombus huntii*, *Bombus occidentalis*, *Bombus rufocinctus* and *Bombus fervidus*), honey bees (*Apis mellifera*) and alfalfa leafcutter bees (*Megachile rotundata*). Sainfoin was described to be mainly cross-fertilizing (Tasei 1984; Bennett et al. 2001), but a gametophytic or sporophytic self-incompatibility has not been described. Cross-pollination may be mediated by the architecture of the flower, where the position of pistil and anthers could prevent

self-pollination (Knuth 1906; Özbek 2011; Demdoum 2012). However, self-fertilization has been observed to a certain extent (Thomson 1938; Knipe and Carleton 1972; Negri 1984; Demdoum 2012). Demdoum (2012) verified that pollen tube growth occurred after self-pollination, but directed self-pollination by hand resulted in only small numbers of seeds.

Sainfoin was traditionally widely used in monoculture for hay production and is nowadays mostly used as a component of mixed meadows in extensive agriculture. Although forage yield and quality as well as animal health supporting properties make sainfoin an ideal choice for ruminant forage production, sainfoin is not widely adopted in today's agriculture. The poor adoption is mainly due to lower forage yield compared to other legumes (Sheehy and Popple 1981), a low persistency mostly due to a poor adaptation to wet areas and cold winters (Simonnet and Carlen 2011), and a weak competitive ability against other species (Liu et al. 2010). These disadvantages lowered the interest in sainfoin and breeding efforts have been reduced in the last 30 years to a minimum. Consequently, there is a general lack of well adapted varieties. In the plant variety catalogues & databases of the EU (European Commission 2015) only 22 sainfoin varieties are listed, compared to 218 and 385 varieties for red clover (*Trifolium pratense*) and alfalfa (*Medicago sativa*), respectively. In addition, seed of sainfoin varieties is often scarce due to low seed yield, which impairs seed multiplication. Another reason for the low breeding progress in sainfoin might be the still limited knowledge on the genetics of this species (Hayot Carbonero 2011). The majority of sainfoin varieties are developed as population or synthetic varieties and hence comprise a wide range of different heterozygous genotypes. The amount of gene heterogeneity in such populations allows on the one hand adaptation to diverse environmental conditions. On the other hand, deleterious alleles are hidden in such populations and could emerge after some generations. In other species such as maize (*Zea mays*), sugar beet (*Beta vulgaris*) and rye (*Secale cereale*), breeding success was accelerated by the development of hybrid varieties, which exploit heterosis (Moll et al. 1962; Helmerick et al. 1963; Geiger and Wahle 1978). Hybrid varieties are based on a pair-cross between two homozygous plants with different genetic background. The combination of favorable alleles in the offspring leads to increased

performance known as hybrid vigor. Hybrid breeding is so far not considered for sainfoin due to the outbreeding fertilization system and the resulting difficulties of producing homozygous parental plants. Assessing the rate of self-fertilization and its consequences in sainfoin will indicate, whether the development of inbred lines for hybrid breeding is feasible.

The rate of self-fertilization may be directly influenced by pollen availability through crossing partners, mainly depending on the amount of simultaneously flowering individuals of the same species (Jain 1976). Pollen availability may be markedly different in natural conditions in the field than under controlled conditions such as in breeding nurseries or pollination cages. However, detailed information on self-pollination rates under different conditions is not available for sainfoin, partially due to the lack of large scale availability of sequence specific molecular markers. Marker systems not relying on *a priori* sequence information are applicable to a wide range of species and, therefore, may offer a means to study self-fertilization in sainfoin. The sequence-related amplified polymorphism (SRAP) marker technique relies on the amplification of GC rich regions of the genome and produces dominant markers that can be distinguished based on different amplicon lengths (Li and Quiros 2001).

A major limitation associated with self-pollination in predominantly outbreeding species is the decrease in plant performance and fitness associated with inbreeding depression, i.e., the accumulation of deleterious alleles in the progeny. Knowledge on the extent of inbreeding depression following self-fertilization in a species is important for breeding decisions such as the selection of parental plants for bi- or multiparental crosses or the development of homozygous lines for hybrid breeding.

The main objective of this study was to increase the knowledge on the extent of self-fertilization in sainfoin and its consequences on plant performance and fitness in order to provide the basis to optimize breeding strategies for the development of better varieties and to promote a wider adoption of sainfoin cultivation. In particular, we aimed at developing a method to assess self-fertilization in sainfoin with dominant SRAP (sequence-related amplified polymorphism) and co-dominant SSR (simple sequence repeats) markers. This method will be used to compare the extent of self-fertilization under two

pollination regimes, i.e., artificial directed and natural non-directed pollination. Based on the knowledge of self-fertilized offspring and non-self-fertilized offspring the effect of inbreeding on agronomic traits such as plant height, plant vigor, flowering time and seed development will be analyzed.

2.3 Methods

2.3.1 Plant material and field trial

Three populations of sainfoin (*Onobrchis viciifolia*) generated through artificially directed pollination in the greenhouse (ADP) and three populations generated under non-directed pollination (NDP) in the field were examined for rates of self-fertilization. For generation of ADP populations, plants from the four varieties *Onobrychis viciifolia* 'Visnovsky', 'Brunner', 'Perly' and 'Perdix', which differ for origin, flowering time, growth habit and mean vigor, were selected (Boller and Günter 2009; Azuhnwi et al. 2011). All varieties were of the multiple flowering type *bifera*, which shows a fast development with flower emergence in the year of sowing and restart of flowering after cutting (Badoux 1964). Five clones were established from each of six sainfoin plants via stem cuttings, which were placed in wet soil without adding growth promoting substances. Cuttings were covered with plastic foil for two weeks to preserve humidity and established plants were grouped pairwise in separate greenhouse chambers for seed production (Table 2.1).

Table 2.1 Overview of populations derived from artificially directed pollination (ADP) and non-directed pollination (NDP).

ADP population	Plants (Total)	Parent 1	Parent 2	NDP population	Plants (Total)	Maternal parent
ADP 1	145	Visnovsky_1[a]	Perly_1[c]	NDP 1	103	Perly[c]
ADP 2	218	Visnovsky_2[a]	Perly_2[c]	NDP 2	109	Perdix[c]
ADP 3	237	Brunner_1[b]	Perdix_1[c]	NDP 3	110	Perly[c]

[a] Agrogen, spol. s.r.o., Troubsko, Czech Republic
[b] Agroscope, Zurich, Switzerland
[c] Agroscope, Nyon, Switzerland

Artificially directed pollination were conducted in January 2012 by placing bumble bee (*Bombus terrestri*) hives ("*Bombus Maxi Hummeln",* Andermatt Biocontrol, Switzerland) into each chamber for three weeks. Seeds from successful pollinations were germinated in May 2012 on moistened filter paper in petri dishes at 20°C under normal daylight (ISTA 2009). The final number of offspring per ADP population varied from 145 to 237 (Table 2.1). The seedlings were transferred to turf pots and nursed in the greenhouse for two months. In July 2012, the plants were planted at the field site in Delley (Delley, Fribourg, Switzerland) with a distance of 50 cm between plants. Plants were arranged in two rectangular blocks, both with an equal proportion of plants originating from each cross to balance potential environmental effects. Within blocks, plants were randomly arranged in rows each consisting of ten offspring of the same maternal plant.

Populations based on naturally non-directed pollination (NDP) were selected from three different field sites of rectangular shape. The site of NDP 1 was a mixed meadow containing the sainfoin variety Perly located in an urban area in Zurich (Zurich, Switzerland). Sites of NDP 2 and NDP 3 were seed multiplication trials for the *Onobrychis viciifolia* varieties Perdix and Perly, both located in a rural area in Delley (Delley, Fribourg, Switzerland). Maternal plants were identified at eight positions in each field, which were chosen at the corners and in the middle of the field for NDP 2 and NDP 3. Plant material was sampled from these plants for DNA extraction and seeds were harvested and germinated in the greenhouse to build up the three NDP populations (Table 2.1).

Sites for the field trial and for sampling plant material were provided by DSP Delley seeds and plants Ltd (Delley, Fribourg, Switzerland; ADP1-3, NDP2-3) and Agroscope, ISS (Zurich, Switzerland; NDP1).

2.3.2 Phenotyping of ADP populations

Traits associated with agronomic performance were assessed in the first main season in 2013 on a single plant basis. Plant height was measured in summer 2013 (length of stretched plants from base to the last leaflet). The Plant vigor, was visually scored on a scale from 1 (weak) to 9 (strong) in summer 2013. Flowering time was determined in days after first of May 2013 when a plant

showed at least three open flowers (Azuhnwi et al. 2011). In the first main season, seed number and weight were assessed by destructive harvest. As sainfoin seeds ripen time-delayed from the base to the top of the inflorescence, the risk to loose seeds before full maturity of all seeds is high (Curtis 1829). To reduce possible loss of seeds, tillers carrying seeds were cut 10 cm above ground in July 2013 and directly put into cotton bags. After drying at 30 °C for two days, plants were threshed manually to avoid seed damage that might interfere with seed counting. Seeds were separated from the plant material by rough sieving (5 mm grid size), followed by cleaning with an air separator (Kurt Pelz Maschinenbau, Bonn, Germany) and fine sieving (1.6 mm grid size). Cleaned seeds were then counted and weighed on a single plant basis.

2.3.3 DNA extraction and marker genotyping

Fresh leaf material from ADP was sampled in October 2012 and from NDP in July 2013. Afterwards the plant material was freeze dried over a period of 48 h. The dried plant material was then ground with a ball mill (Cell tissue Analyzer 2, Quiagen, Hilden, Germany) for subsequent DNA extraction using the illustra™ DNA Extraction Kit PHYTOPURE (GE Healthcare, Little Chalfont Buckinghamshire, United Kingdom) following the manufacturer's instructions. The DNA concentration was determined by gel electrophoresis with a mass standard (High DNA Mass Ladder, Invitrogen™, Life Technologies, Carlsbad, USA). Marker genotyping was performed using dominant sequence-related amplified polymorphism (SRAP) markers (Li and Quiros 2001) and two co-dominant SSR markers (Marina Mora Ortiz, personal communication, 2014). Four fluorescently labelled forward and reverse primers (me1 to me4 and em1 to em4; Table 2.2; Li and Quiros 2001) were used in 16 combinations in the parental plants and offspring of the ADP populations and in eight combinations in the maternal plant and offspring of the NDP populations (Table S2.1). The PCR reactions were performed using an iCyler (Biorad, Hercules, USA) with a sample volume of 20 µL, each containing 10 ng DNA template, 1 x Go Taqflexi buffer (Promega, Madison, USA), 1 mM $MgCl_2$ (Promega), 0.2 mM dNTPs (Promega), 0.6 mg/ml BSA, 0.2 µM fluorescently labelled forward primer, 0.2 µM reverse primer and 0.5 U polymerase G2 (Promega). The PCR conditions consisted of 5

min at 94° C, followed by 5 cycles of 94 °C for 1 min, 35 °C for 1 min and 72 °C for 1 min, followed by 35 cycles at 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min. The reaction ended with 7 min at 72 °C (Li and Quiros 2001). For fragment analysis, 1 µL of the undiluted PCR product was mixed with 0.5 µL LIZ 600 (GeneScan™-600LIZ® Size Standard; AB applied biosystems, Forster City, USA) and 10 µL Formamide (Hi-Di™ Formamide; AB, applied biosystems) in a 384 well plate and heated for 5 min at 94 °C. After cooling down, samples were analyzed with an Applied Biosystems 3500/3500XL Genetic Analyzer. Resulting SRAP fragments were scored for presence or absence of marker alleles using GeneMarker (Softgenetics, V2.4.0 Inc., State College, USA). To allow for the distinction between cross- and self-fertilization in ADP populations, only marker alleles present in one parent and absent in the other parent (nulliplex alleles) were recorded. For NDP populations, only fragments which were absent in the maternal plant and present in at least one of the offspring were scored. In addition to the SRAP markers, two previously developed unpublished co-dominant SSR markers were used with the same DNA samples (Table 2.2). PCR reactions were conducted in a volume of 20 µL, containing 10 ng DNA, 1 x Go Taqflexi buffer (Promega, Madison, USA), 2.5 mM $MgCl_2$ (Promega), 0.2 mM dNTPs (Promega), 0.2 µM fluorescently labelled forward primer, 0.2 µM reverse primer and 0.5 U Polymerase G2 (Promega), using conditions as for a touchdown PCR with 4 min at 94° C, 12 cycles of 30 s at 66° C with - 1°C decrease at each cycle plus 30 s at 72° C, and 30 cycles of 30 s at 94° C, 30 s at 54°C plus 30 s at 72°C, followed by 7 min at 72°C. Fragment analysis was performed as described for SRAP markers.

Table 2.2 SRAP and SSR primers used to determine the rate of self-fertilization

Marker	Forward primer (5`-3`)		Reverse primer (3`- 5`)		Reference
SRAP	me1	TGAGTCCAAACCGGATA	em1	GACTGCGTACGAATTAAT	Li and Quiros, 2001
SRAP	me2	TGAGTCCAAACCGGAGC	em2	GACTGCGTACGAATTTGC	Li and Quiros, 2001
SRAP	me3	TGAGTCCAAACCGGAAT	em3	GACTGCGTACGAATTGAC	Li and Quiros, 2001
SRAP	me4	TGAGTCCAAACCGGACC	em4	GACTGCGTACGAATTTGA	Li and Quiros, 2001
SSR	OVLegPl17_F	GGGTGTTAGTTATCCATTTCC	OVLegPl17_R	ACATACTAGCCTTCTGGGGTA	Mora Ortiz, "pers. comm"
SSR	OVLegPl27_F	AATGGAATCTCGGAGACAG	OVLegPl27_R	GGAAGAAGACGAAGTAGTAGGA	Mora Ortiz, "pers. comm"

2.3.4 Detection of self-fertilization

In ADP populations, an offspring was considered the result of a self-fertilization (selfing) when all marker alleles scored as absent in one parent (nulliplex) were also absent in the offspring. All remaining offspring were classified as the product of a cross-fertilization (crossings). The SRAP marker data were additionally used for a principal component analysis (PCA) to visualize a clustering dependent on origin of cross- or self-fertilization. For comparison, PCA was also performed on simulated data representing 200 dominant marker scores for two heterozygous parents and 50 self-fertilized and 50 cross-fertilized progeny per parental plant (Sheet S2.1). For SSR markers, all offspring containing marker alleles that were unique to the pollen donor plant (i.e. not present in the maternal plant) were classified as crossings. SSR data was used to complement the results from the SRAP analysis. In NDP populations, offspring with SRAP and SSR marker alleles not present in the maternal plant were classified as crossings, whereas the remaining offspring were considered as putative selfings.

2.3.5 Statistical analysis

Phenotypic data of ADP populations were analyzed on a single plant basis using general linear models to assess the effect of population, parental plant and breeding type of offspring (selfing vs. crossing) on plant height, seed yield, plant vigor and flowering time:

$$y_{ikj} = \mu + p_i + pg_{ik} + pb_{ij} + pgb_{ikj} + e_{ikj},$$

where μ is the general intercept, p_i is the effect of the ith ADP population, pg_{ij} the effect of the kth parent and pb_{ij} the effect of the jth breeding type, both nested within the ith ADP population, pgb_{ijk} the effect of the jth breeding type nested within the kth parent and ith population, and e_{ikj} is the residual error.

Effects of NDP population and sampling position on the self-fertilization rate were analyzed with generalized linear models using the following logistic regression model

$$\text{logit}[P(\text{Self-Fert})] = \mu + p_i + s_{ij},$$

where logit[P(Self-Fert)] is the logit of the self-fertilization rate, μ is the general intercept, p_i is the effect of the i^{th} population and s_{ij} is the effect of the j^{th} sampling position within the i^{th} population. Because sampling positions "corner" and "middle" were only applied for NDP 2 and NDP 3, NDP 1 was excluded from this analysis and a further model, reduced by the sampling position term (p_i), was applied on total numbers per population only to test for differences among the three populations.

All statistical analyses and calculations were performed within the R-environment (R Core Team 2014), using functions prcomp() for principal components analysis of SRAP marker data, lm() for general linear models for analysis of phenotypic data and glm() for generalized linear models.

2.4 Results

2.4.1 Self-fertilization in ADP populations

For the three ADP populations (Table 2.1), the number of markers obtained from SRAP analysis ranged from 80 to 195 (Table 2.3). Using these markers, high self-fertilization rates could be identified for all three ADP populations (51.0 to 66.2%), which were largely verified by SSR analysis (Table 2.3). Combined analysis using both marker systems revealed slightly lower self-fertilization rates (48.5 to 64.8%), because some offspring identified as selfings by SRAP markers were clearly identified as crossings based on SSR markers. Self-fertilization rates varied within populations dependent on the maternal parent (Table 2.3).

Table 2.3 Self- and cross-fertilizations in populations from artificially directed pollination (ADP) determined by SRAP and SSR markers.

ADP populations	Maternal subpopulations[a]	No. SRAP marker	Number of plants/ selfings (selfings %)	
			SRAP	SRAP/SSR
ADP 1		195	145/96 (66.2 %)	145/94 (64.8 %)
	Visnovsky_1	104	141/95 (67.4 %)	141/93 (66.0 %)
	Perly_1	91	4/1 (25.0 %)	4/1 (25.0 %)
ADP 2		188	218/134 (61.5 %)	218/134 (61.5 %)
	Visnovsky_2	81	110/49 (44.5 %)	110/49 (44.5 %)
	Perly_2	107	108/85 (78.7 %)	108/85 (78.7 %)
ADP 3		166	237/121 (51.0 %)	237/115 (48.5 %)
	Brunner_1	86	126/34 (27.0 %)	126/30 (23.8 %)
	Perdix_1	80	111/87 (78.4 %)	111/85 (76.6 %)

[a] Maternal subpopulations originated from five clones of one single maternal plant

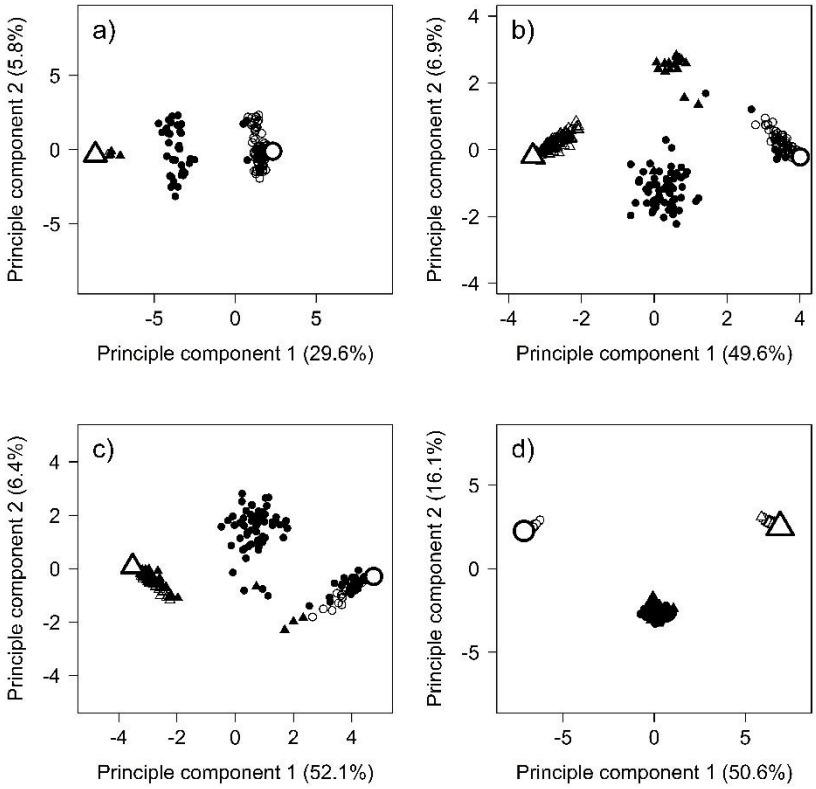

Fig. 2.1 Principal component analysis of offspring from ADP populations and simulated data by SRAP marker data. Letters in brackets denote the populations: **a)** ADP 1, **b)** ADP 2, **c)** ADP 3 and **d)** simulated data. Large circles and triangles represent the two parents, small grey circles/triangles the offspring from self-fertilization of the respective parents and small black circles/triangles the offspring from cross-fertilization between the two parents.

Principal component analysis (PCA) based on SRAP data revealed distinct grouping of offspring depending on breeding type (Fig. 2.1, a-c). The first principal component (29.6 to 52.1% explained variance) mainly differentiated between crossings (black symbols) and selfings (grey symbols) with the latter clustering mostly around the respective parent (Fig. 2.1). The second principal component (5.8 to 6.9% explained variance) mainly separated crossings based on their parent. For all ADP populations, some of the identified crossings clustered close to the respective selfings. This may be due to the one sided nulliplex-marker evaluation for each maternal plant. We checked for non-maternal alleles at positions where the maternal plant carries the nulliplex allele, which characterize the offspring individual as crossing. Offspring with only few non-maternal alleles would be also considered as crossing, if these individuals additionally show high similarity in the non-nulliplex alleles they group closely to selfings of the respective maternal parent. However, the grouping observed was largely congruent with the one based on simulated data with 200 individuals and 200 marker loci (Fig. 2.1, d).

2.4.2 Self-fertilization in NDP populations

For the three NDP populations (Table 2.1), the number of SRAP markers ranged from 40 to 122 (Table 2.4). In these populations, generally low rates of self-fertilization (i.e. 5.8, 0.9 and 4.5%) were observed. After excluding possible false classifications using SSR markers, the rate of self-fertilization decreased to 3.9% for NDP 1, to 0.0% for NDP 2 and to 1.8% for NDP 3 (Table 2.4).

NDP 1 showed the highest self-fertilization rate characterized by SRAP markers only and combined with SSR. The self-fertilization rate was not significantly different among NDP populations. Furthermore, no significant effect of the sampling site (corner vs. center of the field) could be detected within NDP 2 or NDP 3.

Table 2.4 Self- and cross-fertilizations in populations from non-directed pollination (NDP) determined by SRAP and SSR markers.

NDP populations	Maternal subpopulations[a]	No. SRAP markers	Number of plants/ selfings (selfings %)	
			SRAP	SRAP/SSR
NDP 1		635	103/6 (5.8 %)	103/4 (3.9 %)
	NDP 1_1	86	12/0 (0.0 %)	12/0 (0.0 %)
	NDP 1_2	83	11/0 (0.0 %)	11/0 (0.0 %)
	NDP 1_3	93	13/0 (0.0 %)	13/0 (0.0 %)
	NDP 1_4	84	12/1 (8.3 %)	12/1 (8.3 %)
	NDP 1_5	62	13/1 (7.7 %)	13/0 (0.0 %)
	NDP 1_6	76	13/3 (23.1 %)	13/3 (23.1 %)
	NDP 1_7	106	15/1 (6.7 %)	15/0 (0.0 %)
	NDP 1_8	45	14/0 (0.0 %)	14/0 (0.0 %)
NDP 2		688	109/1 (0.9 %)	109/0 (0.0 %)
	NDP 2_C1	100	15/0 (0.0 %)	15/0 (0.0 %)
	NDP 2_C2	65	13/0 (0.0 %)	13/0 (0.0 %)
	NDP 2_C3	89	13/0 (0.0 %)	13/0 (0.0 %)
	NDP 2_C4	79	13/0 (0.0 %)	13/0 (0.0 %)
	NDP 2_M1	122	14/0 (0.0 %)	14/0 (0.0 %)
	NDP 2_M2	77	13/0 (0.0 %)	13/0 (0.0 %)
	NDP 2_M3	116	15/1 (6.7 %)	15/0 (0.0 %)
	NDP 2_M4	40	13/0 (0.0 %)	13/0 (0.0 %)
NDP 3		676	110/5 (4.5 %)	110/2 (1.8 %)
	NDP 3_C1	116	13/0 (0.0 %)	13/0 (0.0 %)
	NDP 3_C2	51	10/1 (10.0 %)	10/0 (0.0 %)
	NDP 3_C3	64	14/1 (7.1 %)	14/1 (7.1 %)
	NDP 3_C4	100	13/1 (7.7 %)	13/0 (0.0 %)
	NDP 3_M1	78	15/1 (6.7 %)	15/0 (0.0 %)
	NDP 3_M2	116	15/0 (0.0 %)	15/0 (0.0 %)
	NDP 3_M3	79	15/0 (0.0 %)	15/0 (0.0 %)
	NDP 3_M4	72	15/1 (6.7 %)	15/1 (6.7 %)

[a]Maternal subpopulations originated from one single maternal plant.
NDP _C sampled at the corners and _M sampled in the middle of the field site.

2.4.3 Phenotypic characterization of ADP populations

The number of days until flowering for individual plants ranged from 17 days to 65 days (Fig. 2.2). On average, selfings of Perly_1 (28 days), Perly_2 (34 days) and Perdix_1 (33 days) showed earlier flowering than selfings from Visnovsky_1 (47 days), Visnovsky_2 (48 days) and Brunner_1 (50 days). The average flowering time for crossings ranged from 35 days to 44 days and showed less variation when compared to selfings. Overall, significant differences for flowering time were only observed between crossings from Brunner_1 and Perdix_1 and for selfings from Visnovsky_2 and Perly_2 as well as from Brunner_1 and Perdix_1. Significant differences between crossings and corresponding selfings were found for Visnovsky_1, Visnovsky_2 and Brunner_1 (Fig. 2.2).

Seed yield ranged from 0.0 g (plants without seed set) to 121.5 g and was mostly lower for selfings compared to crossings. For ADP 1, mean seed yield in crossings of Visnovsky_1 was significantly reduced by 67% in corresponding selfings. In ADP 2, selfings showed seed yields reduced by 69.4 and 70.3% compared to crossings for Visnovsky_2 and Perly_2, respectively, the latter difference not being significant. In ADP 3, selfings showed seed yields reduced by 79.1 and 37.6% compared to crossings for Brunner_1 and Perdix_1, the latter difference not being significant.

Table 2.5 Analysis of variance (ANOVA) for traits in populations from artificially directed pollination (ADP)

Phenotypic trait	Model[a]	Df	MS	F value	Pr (>F)
Flowering time	Population	2	1096.6	19.2	8.3e -9***
	Population : breeding type	3	1639.2	28.7	< 2.2e -16***
	Population : parent	3	3158.2	53.4	< 2.2e -16***
	Population : breeding type : parent	3	1079.9	18.9	1.0e -11***
	Residual	567	57.0		
Seed yield	Population	2	20072.0	67.7	< 2.2e -16***
	Population : breeding type	3	40508.0	136.7	< 2.2e -16***
	Population : parent	3	8457.0	28.5	< 2.2e -16***
	Population : breeding type : parent	3	3273.0	11.0	4.7e -7***
	Residual	561	296.0		
Plant height	Population	2	3814.8	20.7	2.1e -9***
	Population : breeding type	3	10760.5	58.5	< 2.2e -16***
	Population : parent	3	4119.3	22.4	1.0e -13***
	Population : breeding type : parent	3	3940.9	21.4	3.7e -13***
	Residual	569	184.0		
Plant vigor	Population	2	60.6	31.0	1.6e -14***
	Population : breeding type	3	61.7	31.6	< 2.2e -16***
	Population : parent	3	11.7	5.9	5.2e -16***
	Population : breeding type : parent	3	6.1	3.1	0.03*
	Residual	567	1.9		

[a] Complete model = Population + Population: Breeding Type + Population: Breeding Type: Parent
Breeding type = crossing or selfing.
MS = Mean squares. * = P < 0.05; ** = P < 0.01; *** = P < 0.001

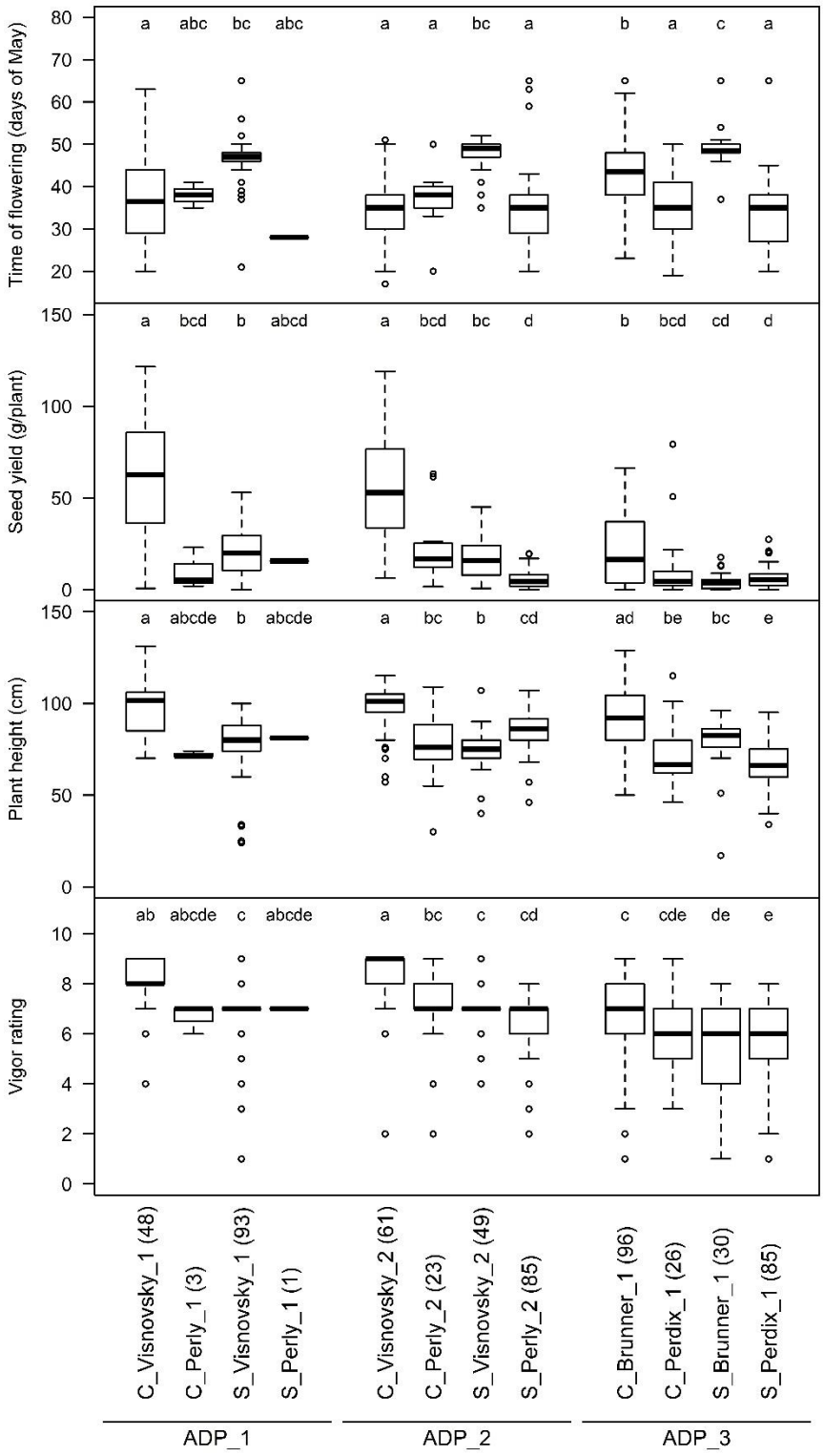

Fig. 2.2 Differences of traits in populations from artificially directed pollination (ADP) dependent on cross- and self-fertilization. C_ = offspring from cross-fertilization; S_ = offspring from self-fertilization. Numbers in brackets refer to the total number of plants in this group. Different letters state significant differences.

Overall plant height ranged from 17 to 131 cm. Average plant height was significantly lower for selfings of Visnovsky_1, Visnovsky_2 and Brunner_1 when compared to corresponding crossings (Fig. 2.2). In ADP 1, plant height of selfings was significantly reduced by 20.4% for Visnovsky_1. In ADP 2, plant height of selfings was significantly reduced by 23.8% for Visnovsky_2, but (non-significantly) increased by 10.1% for Perly_2. In ADP 3, reductions in plant height of selfings compared to crossings ranged from 7% to 12.8% for Perdix_1 and Brunner_1, respectively. Vigor scores, reflecting the overall performance of plants, were also affected by self-fertilization. The scores were significantly higher in crossings with 8.1 compared to 6.7 for selfings of Visnovsky_1 and with 8.3 compared to 7 of Visnovsky_2. Across all ADP populations, breeding type, population and maternal parent, as well as their interactions had a significant influence on flowering time, seed yield, plant vigor and plant height (Table 2.5).

2.5 Discussion

2.5.1 High rates of self-fertilization can be induced

To the best of our knowledge, this is the first report on self-fertilization rates in sainfoin under different pollination regimes and based on molecular genetic marker data. The high rates of self-fertilization detected under artificially directed pollination (ADP) of up to 64.8% allow for the conclusion that a strict self-incompatibility system is not functional in this species. Previous reports (Thomson 1938; Demdoum 2012) on self-fertilization in sainfoin reported clearly lower self-fertilization rates than those observed with ADP in this study, which might be mainly attributed to the different experimental conditions, such as plant isolation, manual or insect pollination and plant material. Under plant isolation, self-fertilization rates of 0.98% (Thomson 1938) and 1.1% (Demdoum 2012) were observed, which was clearly lower than the rates observed for ADP populations in this study, but comparable to rates found in non-directed pollination (NDP) populations. Following strict manual self-pollination, seed set rates of only 5.1% (Demdoum 2012) to 15.5% (Thomson 1938) were observed, reflecting the low rates of successful self-fertilizations. In these studies, manual

pollination may have been hindered by morphological barriers to self-pollination in sainfoin flowers. Open-pollination by insects in a tent with two clones of two genotypes resulted in self-fertilization rates from 72 to 92%, as detected using flower color as marker (Knipe 1972). This is more comparable to our findings in ADP populations. In our study, rates of self-fertilization showed strong dependency on the maternal genotype. This could be due to a potential difference in flowering time between the two genotypes, which may have favored self-fertilization in earlier flowering genotypes. This is in congruence with the observation that genotypes with higher selfing rates such as Perly_2 or Perdix_1 showed earlier flowering in the field. In contrast to the ADP populations, we found very low rates of self-fertilization in NDP populations (0 to 3.9%), which might be caused by the large number of mature flowers on the three field sites and the ample availability of pollen from neighboring plants. In addition, the presence of different pollinator species and their diverse activity patterns lead to a more constant pollen supply over the day, potentially decreasing the rate of self-fertilization (Richards and Edwards 1988). In order to assess whether the number of neighboring plants influences the self-fertilization rate, we tested for differences among sampling positions located at corners or in the middle of the fields for NDP 2 and 3, but no significant effect of the field position was found. However, self-fertilization rates were higher in NDP 1, which was a mixed meadow with a sainfoin proportion of approximately 20% when compared to NDP 2 and 3, which were pure stands.

2.5.2 Power to detect selfings

Up to now, few sequence specific markers have been developed for sainfoin and the transferability from other species is limited (Demdoum et al. 2012). Therefore, we used sequence-related amplified polymorphism (SRAP) analysis allowing to generate a large number of anonymous, dominant markers (Li and Quiros 2001). Dominant markers have been successfully used to detect self-fertilization if marker alleles were unique in each parent (Brazeau et al. 1998; Clegg et al. 1999). Unique parental alleles can be tracked in the offspring and used for the detection of cross- or self-fertilization. The disadvantage of dominant markers is the loss of information about the genotype of an individual shown by

the higher variance of estimates obtained from dominant loci compared with co-dominant loci (Milligan and McMurry 1993). Consequently, the dominant nature of SRAP markers makes a characterization of self-fertilization or cross-fertilization ambiguous, since nulliplex genotypes can also arise from a cross of two tetraploids that are not both nulliplex, e.g. 0 0 0 0 and 1 0 0 0 (0 = allele absent, 1 = allele present) leading to false positives in the classification of self-fertilization. However the probability of a nulliplex state after crossing two markers (0 0 0 0 x 1 0 0 0) with a probability of 0.5 for each marker locus decreases rapidly with increasing marker numbers and converges to zero with marker numbers larger than 50. In comparable studies with Caribbean corals (*Favia fragum* and *Porites astreoides*) it has been shown that 30 dominant marker were sufficient to detect all crossings (Brazeau et al. 1998). Those marker numbers were lower than the marker numbers used in our study. Supplementary analysis with co-dominant SSR markers largely supported the accuracy of SRAP marker results (Table 2.3). A general limitation of marker fragment analysis could arise from miss-scoring fragments. However, repeated independent scoring and a large number of markers help to minimize this problem (Milligan and McMurry 1993). Therefore, SRAP markers demonstrated highly efficient for distinguishing offspring resulting from self- or cross fertilizations.

2.5.3 Inbreeding depression dependent on trait

Plants which mainly rely on cross-fertilization often suffer from strong decline in performance after self-fertilization. This inbreeding depression is particularly pronounced in grassland species such as ryegrass (*Lolium perenne*, Bean and Yok-Hwa 1972; Pauly et al. 2012) or red clover (Taylor and Anderson 1980) with a strong self-incompatibility system. Existence and extent of inbreeding depression for sainfoin could crucially influence breeding decisions since care would have to be taken to select for genetically diverse crossing partners. Alternatively, a low inbreeding depression would allow for the development of inbred lines as a basis for hybrid breeding. Nevertheless, until now, no detailed data on inbreeding depression on plant performance was available for sainfoin. In our study, plant height and plant vigor were affected by self-fertilization in all three ADP populations (Fig. 2.2). One generation of inbreeding had lowered

height and vigor of selfings when compared to crossings. On the other hand, the better performance of crossings may also have been due to heterosis (Shull 1914). In our study, the decrease in performance was surprisingly strong for a potentially heterozygous tetraploid plant. In autotetraploids, recessive homozygous genotypes will be less frequent than in diploids, and inbreeding depression is expected to be lower (Posselt 2010). For example, with two alleles at a frequency of 0.5 each, homozygous recessive genotypes will be present at a rate of 0.25 in diploids, but only of 0.0625 in tetraploids. Severe inbreeding depression in autotetraploids was explained by a loss of complementary gene interactions in the first few generations of inbreeding (Bingham et al. 1994). Sainfoin is a natural tetraploid, for which tri- and tetraallelic interactions are of higher importance than for artificially induced tetraploids, where diallelic interactions are predominant (Gallais 2003). Such higher order interactions will be quickly lost through inbreeding, partly explaining the observed inbreeding depression for traits in the ADP populations. In addition, the high copy number in polyploids and the large genome size allow mild deleterious mutations to accumulate which can also lead to increased inbreeding depression (Ozimec and Husband 2011). In our study, we found that not only the breeding type significantly determined the plant height, seed yield, flowering time and vigor in all populations, but also the maternal plant influenced the plant performance (Fig. 2.2, Table 2.5), what might be attributed to different levels of heterozygosity in the maternal genotypes.

The difference in flowering time observed among plants is unlikely to influence the total seed yield, because earlier flowering does not extend the generative phase (Galloway and Etterson 2007). For ADP populations, a reduction in seed yield of up to 79.1% (Fig. 2.2) was observed for selfings. This is remarkably high when compared to species such as alfalfa, where seed yield reductions of 55% after one generation of inbreeding were observed (Gallais 1984). Two factors could play a major role for inbreeding depression of seed yield in sainfoin. On the one hand, the fitness of the maternal plant plays an important role as seeds acts as sinks for nutrients and assimilates (Alonso-Blanco et al. 1999) and a good overall fitness of the maternal plant is indispensable for high seed yield. On the other hand, the possibly changed genetic composition after selfing, e.g. loss of genes or interactions and the accumulation of deleterious alleles, might have

contributed to inbreeding depression. Environmental conditions may also play an important role for total seed production (Dechaine et al. 2014). Our experimental setup did not allow for assessment of genotype x environment interactions, but selfings and crossings were randomly distributed across the experimental field. For flowering time, no significant difference between crossings and selfings, but a significant influence of the maternal genotype was observed (Fig. 2.2). Selfings from the maternal genotype Visnonsky showed the tendency of later flowering than the corresponding crossings and selfings of Perly. Selfings of Perdix showed also the same trend to earlier flowering which could be attributed to the fact that the variety Perdix originated from the variety Perly (Beat Boller, personal communication, 2012). Crossings showed an intermediate time of flowering reflecting the combination of genes from early and late flowering parents. This pattern of flowering time indicates additive inheritance of this trait and is in accordance with earlier studies in maize or chickpea (Buckler et al. 2009; Kumar et al. 2009).

2.6 Conclusions

This study clearly showed that a high degree of self-fertilization could be achieved in sainfoin under controlled conditions and using insect pollination. The selfings showed significant inbreeding depression for plant height, plant vigor and seed yield. Although the dominant reproduction mechanism seems to be outbreeding, a higher rate of inbreeding can be observed under selective conditions, as they are also often present in pair- or polycross breeding schemes, i.e., open pollination within a limited set of selected elite parents. Hence, creating polycrosses composed of a sufficiently large number of parents that are strictly homogenous in flowering time is of highest importance to avoid inbreeding of the earliest genotypes. For maintenance breeding of varieties, large numbers of genotypes may help to reduce the risk of inbreeding. For targeted pair-crosses, it might become necessary to emasculate the plants which were selected as maternal parents to avoid self-fertilization or at least to carefully check the progeny for potential selfings using genetic markers.

On the other hand, if self-fertilization is easily accomplished, superior sainfoin varieties may be developed through hybrid breeding. For this, homogenous inbred lines from well performing and good combining genotypes have to be established and will be crossed to create a superior hybrid offspring. Therefore, our results provide a valuable basis to define strategies for the implementation of hybrid breeding in sainfoin.

The assessment of self-fertilization in sainfoin fills a gap in knowledge of this species and the results could be applied for developing novel breeding schemes. Finally, improving underestimated species like sainfoin and integrating those plants in practical cultivation may help to enhance biodiversity in future agriculture.

2.7 Authors' contributions

KK established the field trial for this study, carried out the molecular analysis using SRAP and SSR marker, performed the genetic data analysis and drafted the manuscript. CG carried out the statistical data analysis and participated in writing the manuscript. AW and FW discussed the results and participated in writing the manuscript. SR participated in the molecular analysis and contributed to the data interpretation. RK supervised the project, assisted in the data analysis, discussed the results and contributed to draft the manuscript. All authors read and approved the final manuscript.

2.8 Acknowledgments

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We thank Delley seeds and plants Ltd. for providing the field site and assisting in the field trial. We would also like to thank Beat Boller (Agroscope), for providing the plant material and Marina Mora Ortiz (NIAB, Cambridge, UK) for providing the SSR markers used in this study. Finally, we thank the group of

Molecular Ecology at Agroscope, Stefan Oberlin and Robert Spiess for their support in the lab and in the field.

2.9 Supplementary Information

Table S2.1 SRAP primer combinations in populations of artificially directed pollination (ADP) and in non-directed pollination (NDP)

SRAP combinations	Forward primer (5`-3`)	Reverse primer (3`- 5`)	ADP populations	NDP populations	Reference
me1em1	TGAGTCCAAACCGGATA	GACTGCGTACGAATTAAT	✓	✓	
me2em2	TGAGTCCAAACCGGAGC	GACTGCGTACGAATTTGC	✓	✓	
me3em3	TGAGTCCAAACCGGAAT	GACTGCGTACGAATTGAC	✓	✓	
me4em4	TGAGTCCAAACCGGACC	GACTGCGTACGAATTTGA	✓	✓	
me1em2	TGAGTCCAAACCGGATA	GACTGCGTACGAATTTGC	✓	✓	
me2em3	TGAGTCCAAACCGGAGC	GACTGCGTACGAATTGAC	✓	✓	
me3em4	TGAGTCCAAACCGGAAT	GACTGCGTACGAATTTGA	✓	✓	
me4em1	TGAGTCCAAACCGGACC	GACTGCGTACGAATTAAT	✓	✓	Li and Quiros, 2001
me1em4	TGAGTCCAAACCGGATA	GACTGCGTACGAATTTGA	✓	-	
me2em1	TGAGTCCAAACCGGAGC	GACTGCGTACGAATTAAT	✓	-	
me3em2	TGAGTCCAAACCGGAAT	GACTGCGTACGAATTTGC	✓	-	
me4em3	TGAGTCCAAACCGGACC	GACTGCGTACGAATTGAC	✓	-	
me1em3	TGAGTCCAAACCGGATA	GACTGCGTACGAATTGAC	✓	-	
me2em4	TGAGTCCAAACCGGAGC	GACTGCGTACGAATTTGA	✓	-	
me3em1	TGAGTCCAAACCGGAAT	GACTGCGTACGAATTAAT	✓	-	
me4em2	TGAGTCCAAACCGGACC	GACTGCGTACGAATTTGC	✓	-	

Sheet S2.1

R-code for simulation

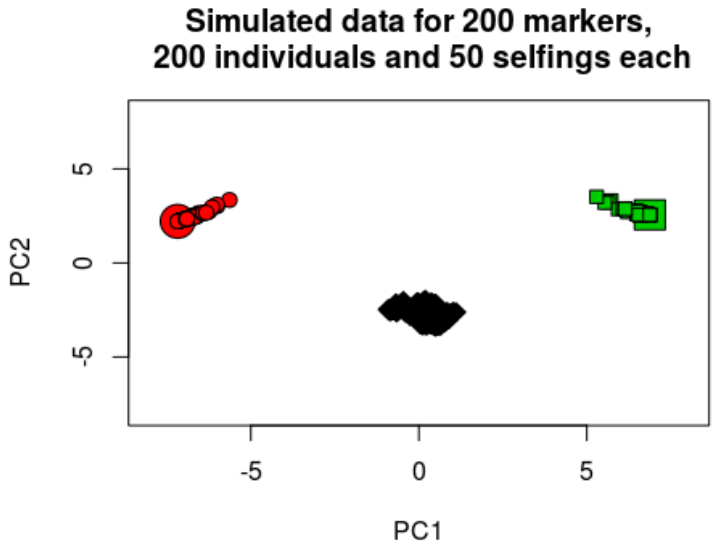
Roland Kölliker

June 4, 2015

```
##Simulation of dominant marker data for offspring of two tetraploid heter
eozygous
##plants under cross- and self-fertilization
##Written by Roland Kölliker (roland.koelliker@agroscope.admin.ch), 4.6.15
##Arguments:
##nl=number of loci
##t.bal=proportion of zeros in parental genotype
##ncross=number of crossings
##nself1=number of selfings for parent1
##nself2=number of selfings for parent2
##nco=number of recombinations,
##maxco=max number of markers per co
ffselfsim <- function(nl=200, t.bal=0.5, ncross=100, nself1=50, nself2=50,
nco=1, maxco=nl/28, ...){
```

```
  #Parental marker-phenotypes
  p1 <- sample(c(0,1), nl, replace=T, prob=c(t.bal, 1-t.bal))
  p2 <- 1-p1

  #parental genotypes
  pi1 <- NULL
  pi2 <- NULL

  #table with possible allele combinations (1,1,1,1 is not an option since
this does
  #not segregate in the progeny)
  all <- as.matrix(expand.grid (rep(list(c(0,1)), 4)))

  #create index vector
  for (i in 1:nl){
    pi1[i] <- ifelse(p1[i]==0,1, sample(2:16,1))
    pi2[i] <- ifelse(p2[i]==0,1, sample(2:16,1))
  }
  #create parental genotypes
  pa1 <- all[pi1,]
  pa2 <- all[pi2,]

  cbind(p1,pa1)
  cbind(p2,pa2)



  #function to create gametes with recombination

  #(t.x <- matrix(rep(c("A","B","C","D"), each=nl), ncol=4)) #test to see
whether this works

  ffgam <- function(x) {
    t.g1 <- x[, sample(1:4,4)]
    tt.g1 <- t.g1
    for (i in 1:nco){
      anco1 <- sample(maxco,1) #number of markers concerned
      loco1 <- sample(1:(nl-anco1),1) #locatin of co
      anco2 <- sample(maxco,1) #number of markers concerned
      loco2 <- sample(1:(nl-anco2),1) #locatin of co

      tt.g1[loco1:(loco1+anco1),1] <- t.g1[loco1:(loco1+anco1),2]
      tt.g1[loco1:(loco1+anco1),2] <- t.g1[loco1:(loco1+anco1),1]
      tt.g1[loco2:(loco2+anco2),3] <- t.g1[loco2:(loco2+anco2),4]
      tt.g1[loco2:(loco2+anco2),4] <- t.g1[loco2:(loco2+anco2),3]
      ga1 <- cbind(tt.g1[,sample(1:2,1)], tt.g1[,sample(3:4,1)])
    }
    return(ga1)
  }

  ##create genotypes of crossings for autopolyploids
  ntot <- ncross+nself1+nself2

  d.prog <- matrix(nrow=ntot, ncol=nl)
  for (i in 1:ncross){
```

```
    c.auto <- cbind(ffgam(pa1), ffgam(pa2))
    d.prog[i,] <- apply(c.auto,1, function(x) ifelse(any(x==1),1,0))
  }
  #selfings parent 1
  for (i in (ncross+1):(ncross+nself1)){
    c.auto <- cbind(ffgam(pa1), ffgam(pa1))
    d.prog[i,] <- apply(c.auto,1, function(x) ifelse(any(x==1),1,0))
  }
  #selfings parent2
  for (i in (ncross+nself1+1):ntot){
    c.auto <- cbind(ffgam(pa2), ffgam(pa2))
    d.prog[i,] <- apply(c.auto,1, function(x) ifelse(any(x==1),1,0))
  }
  d.prog <- data.frame(d.prog)
  rownames(d.prog) <- c(paste0("C",1:ncross), paste0("SA",(ncross+1):(ncro
ss+nself1)), paste0("SB",(ncross+nself1+1):ntot))
  head(d.prog)
  d.final <- rbind(p1, p2,d.prog)
  head(d.final)

  r.pca <- prcomp(d.final)
  plot(r.pca$x, pch=c(21,22,rep(23,ncross),rep(21,nself1), rep(22,nself2))
, bg=c(2,3,rep(1,ncross), rep(2,nself1), rep(3,nself2)), cex=c(3,3,rep(1.3
,ntot)), ...)
}

set.seed(1895)
ffselfsim(main="Simulated data for 200 markers,\n 200 individuals and 50 s
elfings each", xlim=c(-8,8), ylim=c(-8,8))
```



Chapter 3 Characterization of novel SSR markers in diverse sainfoin (*Onobrychis viciifolia*) germplasm

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3.1 Abstract

Background: Sainfoin is a perennial forage legume with beneficial properties for animal husbandry due to the presence of secondary metabolites. However, worldwide cultivation of sainfoin is marginal due to the lack of varieties with good agronomic performance, adapted to a broad range of environmental conditions. Little is known about the genetics of sainfoin and only few genetic markers are available to assist breeding and genetic investigations. The objective of this study was to develop a set of SSR markers useful for genetic studies in sainfoin and their characterization in diverse germplasm.

Methods: A set of 400 SSR primer combinations were tested for amplification and their ability to detect polymorphisms in a set of 32 sainfoin individuals, representing distinct varieties or landraces. Alleles were scored for presence or absence and polymorphism information content of each SSR locus was calculated with an adapted formula taking into account the tetraploid character of sainfoin. Relationships among individuals were visualized using cluster and principal components analysis

Results: Of the 400 primer combinations tested, 101 reliably detected polymorphisms among the 32 sainfoin individuals. Among the 1154 alleles amplified, 250 private alleles were observed. The number of alleles per locus ranged from two to 24 with an average of 11.4 alleles. The average polymorphism information content reached values of 0.14 to 0.36. The clustering of the 32 individuals suggested a separation into two groups depending on the origin of the accessions.

Conclusions: The SSR markers characterized and tested in this study provide a valuable tool to detect polymorphisms in sainfoin for future genetic studies and breeding programs. As a proof of concept, we showed that these markers can be used to separate sainfoin individuals based on their origin.

3.2 Introduction

Onobrychis viciifolia, commonly known as sainfoin, belongs to the tribe *Hedysareae* and the family Fabaceae. It is a tetraploid (2n = 4x = 28) perennial forage legume, rich in proteins and secondary plant metabolites. Its center of origin is attributed to the Middle East and Central Asia. It was introduced into Europe in the 15th century and was rapidly adopted by farmers due to its high fodder value, especially for working horses (Koivisto and Lane 2001). Nowadays, sainfoin is cultivated only in small areas for fodder production and on ecological compensation areas. Its cultivation steadily declined since the 1950's, due to the expanding availability of inorganic fertilizers and the preference for higher yielding legume crops such as alfalfa (*Medicago sativa*) or red clover (*Trifolium pratense*, Burton and Curley 1970; Sheehy and Popple 1981; Frame et al. 1998). In the last few years, however, sainfoin has gained renewed interest due to its animal health promoting properties associated with the presence of condensed tannins (CT) and other complex phytochemicals in the foliage. Benefits include anthelminthic properties and prevention of potentially lethal bloat associated with most other forage legumes (Marais et al. 2000; Min et al. 2003; Ramirez-Restrepo 2005; Hoste et al. 2006; Mueller-Harvey 2006). In addition, sainfoin shows a range of beneficial agronomic features. As a legume, it is able to fix atmospheric nitrogen in root nodules by rhizobia, reducing the need for nitrogen fertilizers (Goplen et al. 1991; Koivisto and Lane 2001). Furthermore, soil fertility is improved by increased humus development through its deep rooting capability and low input requirements once established (KÖL 2012). Used as a component of permanent grassland, sainfoin is a valuable alternative for areas suffering from intensification, as it increases soil fertility and has become a popular addition to non-cropped environmental planting; sainfoin provides good resources for native insects and high quality fodder for livestock (KÖL 2012).

Despite its advantages, a wide distribution of sainfoin is hampered by the often poor agronomic performance and the lack of sainfoin varieties adapted to different environmental conditions. The main weaknesses of sainfoin lie in its low tolerance to waterlogging and frost as well as in its poor competitive ability in the early stages of development. Therefore, targeted breeding activities are needed to

select for sainfoin individuals better adapted to a broad range of environmental conditions. Breeding activities have also been impaired by the lack of knowledge of the genetic diversity of the species and its mode of inheritance. Further investigation and development of tools for marker assisted breeding has been hampered by the limited availability of species-specific molecular markers. So far, most studies have focused on the use of cross-amplifiable EST-SSRs, mainly from *Medicago* and *Glycine* species; ITS markers based on nuclear internal transcribed spacer regions and dominant SRAP markers (Demdoum et al. 2012; Hayot Carbonero et al. 2012; Lewke Bandara et al. 2013; Kempf et al. 2015). The use of co-dominant SSR markers developed in other species yielded only a low number of alleles per locus in sainfoin (from five to seven in bulks of ten individual plants, Demdoum et al. 2012). The development of highly informative, specific markers for sainfoin is indispensable to create a genetic knowledge base and assist breeding by marker assisted selection (MAS, Sorrells and Wilson 1997).

Simple Sequence Repeats (SSRs) or Microsatellites (Litt and Luty 1989) are composed of tandemly repeated sections of DNA (Powell et al. 1996). SSR markers show co-dominance of alleles and are randomly distributed along the genome, particularly in low-copy regions (Morgante et al. 2002; Kumar et al. 2009). Considering the complex tetraploid sainfoin genome and the lack of knowledge about its genetics, SSRs are the markers of choice. SSRs are multi-allelic in contrast to next generation high-throughput sequencing (NGS) derived SNP marker which are bi-allelic. This makes SSR markers highly variable and useful for distinguishing even between closely related populations or varieties (Smith and Devey 1994). Furthermore, SSR are easily detected using standard PCR methods and are transferable to related taxa (Chen et al. 2015). The development of NGS has recently enabled the identification of a large set of set of SSR sequences from sainfoin (Mora Ortiz et al. 2016).

In this study, our aim was to develop and characterize a comprehensive set of markers based on recently identified SSR sequences in a panel of 32 sainfoin individuals of different origin (Mora Ortiz et al. 2016).

3.3 Material and Methods

3.3.1 Plant material

In order to include a large range of genetic diversity, we selected a set of 32 individual sainfoin plants from 29 different accessions (Table 3.1), originating from a range of geographical regions and showing differences for tannin content and composition (Demdoum et al. 2012; Hayot Carbonero et al. 2012; Lewke Bandara et al. 2013; Malisch et al. 2015). These accessions were grown in the glasshouse at NIAB (Cambridge, UK) and in the field at Agroscope (Zurich, Switzerland). Young leaf material was collected from each single plant, ground in liquid nitrogen and stored at -80°C until subsequent DNA extraction.

Table 3.1 *Onobrychis viciifolia* individuals used for marker characterization in this study.

Individual	Variety	Status	Origin	Source
ID_01	247	NA	Morocco	GRIN
ID_02	Buceanskij	NA	Romania	GRIN
ID_03	CPI 63750	NA	Turkey	GRIN
ID_04	CPI 63764	wild	Turkey	GRIN
ID_05	CPI 63820	NA	Spain	GRIN
ID_06	CPI 63826	NA	Spain	GRIN
ID_07	NA / RCAT028437	NA	Hungary	GRIN
ID_08	Ökotyp Wiedlisbach	ecotype	Switzerland	ISS
ID_09	Premier	landrace	Switzerland	ISS
ID_10	Rees A	cultivar	UK	GRIN
ID_11	TU86-43-03	cultivated	Turkey	GRIN
ID_12	Nova	cultivar	Canada	GRIN
ID_13	Visnovsky	cultivar	Czech Republik	ISS
ID_14	Perly	cultivar	Switzerland	ISS
ID_15	Brunner	landrace	Austria	ISS
ID_16	Perdix	cultivar	Switzerland	ISS
ID_17	Cotswold Common	cultivar	UK	RAU
ID_18	Perly	cultivar	Switzerland	RAU
ID_19	Somborne	cultivar	UK	RAU
ID_20	Ibaneti/ RCAT028292	NA	Romania	RCAH
ID_21	Bivolari/RCAT028294	cultivar	Romania	RCAH
ID_22	NA/170582	NA	Hungary	RCAH
ID_23	CPI 637554/ 192995	NA	Turkey	GRIN
ID_24	CPI 63767 / 212241	cultivar	USA	GRIN

ID_25	Na/228352	wild	Iran	GRIN
ID_26	CPI 63781/ 236486	NA	Turkey	GRIN
ID_27	Cholderton Hamshire Common	cultivar	UK	GRIN
ID_28	Visnovsky	cultivar	Czech Republic	GRIN
ID_29	Zeus	cultivar	Italy	Cotswold Seeds Ltd
ID_30	Zeus	cultivar	Italy	Cotswold Seeds Ltd
ID_31	Ambra	cultivar	Italy	private
ID_32	Esparcette	cultivar	UK	private

RAU: Royal Agricultural University, Gloucestershire, UK; **RCAH:** Research Centre for Agrobotany, Tápiószele; Hungary; **GRIN:** Germplasm Resources Information Network, Washington, USA; **ISS**: Agroscope Institute for sustainability science, Zurich, Switzerland

3.3.2 DNA extraction

DNA was extracted using the Nucleon Phytopure Genomic DNA extraction kit (GE Healthcare, Little Chalfont Buckinghamshire, U.K.) following the manufacturer's instructions. This method has been shown to be suitable for extraction of high quality DNA from *Onobrychis viciifolia*, in which high levels of polyphenol and condensed tannins have been reported to interfere with a successful DNA extraction using other approaches (Hayot Carbonero 2011). DNA quality and quantity was assessed using gel electrophoresis and spectrophotometry.

3.3.3 PCR and gel electrophoresis

A total of 400 SSR primers designed from *Onobrychis viciifolia* transcriptome data (Mora-Ortiz et al. 2016), were tested with unlabeled primers for amplification in the 32 plants using an iCyler (Biorad, Hercules, USA) in a volume of 10 µL, with 10 ng DNA, 1 x Go Taqflexi buffer (Promega, Madison, USA), 2.5 mM $MgCl_2$ (Promega), 0.2 mM dNTPs (Promega), 0.2 µM forward primer, 0.2 µM reverse primer and 0.5 U Polymerase G2 (Promega). The conditions followed a touchdown PCR approach with 4 min at 94 °C, 12 cycles of 30 s at 66 °C with -1 °C decrease at each cycle plus 30 s at 72 °C, and 30 cycles of 30 s at 94 °C, 30 s at 54 °C plus 30 s at 72 °C, followed by 7 min at 72 °C. PCR products were separated by gel electrophoresis. Amplicons were separated on 1%

agarose in 1x TBE buffer, stained with ethidium bromide and visualized under UV light.

3.3.4 M13 PCR and capillary electrophoresis

Those 101 primer pairs that successfully amplified fragments in the 32 individuals (Table 3.2) were further characterized for polymorphisms using the M13 (-21) tail primer genotyping protocol (Schuelke 2000). The PCR reactions were conducted in an iCycler (Biorad) in a sample volume of 10 µL, each containing 20 ng DNA template, 1x Go Taqflexi buffer (Promega), 1.5 mM $MgCl_2$, (Promega), 0.2 mM dNTPs (Promega), 0.16 µM forward primer carrying the M13-tail, 0.04 µM reverse primer and 0.16 µM fluorescently labelled M13-primer, 0.5 U polymerase GoTaq G2 (Promega).

PCR conditions were 2 min at 94 °C, 30 cycles of 30 s at 94 °C, 45 s at 56 °C and 45 s at 72 °C, followed by 8 cycles of 30 s at 94 °C, 45 s at 53 °C and 45 s at 72 °C. The final extension step was conducted at 72 °C for 10 minutes. An aliquot of 1 µl of the PCR product was diluted in 10 µl HiDi™ formamide (Applied Biosystems®, Thermo Fisher Scientific, Waltham, MA, USA) and 0.2 µl Rox 500™ oligonucleotide 'size ladder' (Applied Biosystems®) for capillary electrophoresis on the Genetic Analyzer 3730 (Life Technologies, Carlsbad, CA). Alleles were scored using the GeneMarker software (Softgenetics, V2.4.0 Inc., State College, USA).

Table 3.2 SSR primer sequences used for amplification in 32 *Onobrychis viciifolia* individuals and characteristics of SSR motifs

Marker	Motif	Repeats	Predicted size	Forward primer (5`-3`)	Reverse primer (5`- 3`)
OVK002	AG	9	164	CCCACCAGACAAAAAGAATA	GCTTTCCCCTTCATCAACTAT
OVK003	TA	8	122	GATAGAATTCGTTTGTTGGTG	ATCTTTGTAACTGTTCGCTCA
OVK017	AC	8	158	GGGTGTTAGTTATCCATTTCC	ACATACTAGCCTTCTGGGGTA
OVK027	CTCG	6	129	AATGGAATCTCGGAGACAG	GGAAGAAGACGAAGTAGTAGGA
OVK034	GCT	6	150	GTGAGATGAGCTTGGACATT	AGATAACTAACTGCAGGCAAG
OVK036	AGGT	6	150	GTGTTAAAGGGGTGAAAACAT	CATTTTGACAAACCAGTATCC
OVK038	ATT	6	166	CCACATACGAGACAGAATAGG	CTGAAAATTGATCGATACTGG
OVK042	GTT	6	144	GGAACGGTTAATTTCTGATTT	AGAATTCCGTACAAGTCGAG
OVK045	AGA	6	148	CCAAAAATCATCAATCAACAC	TTGAACAAGGGTTAGGGTTAT
OVK046	AGTG	6	151	TCAACCACATTATAAAACCTCA	CGCGAAATCATAGTTCACTT
OVK054	GAA	6	201	TTGCAGAGATAACACTCACCT	TCCTGAAAAACCTAATCACAA
OVK055	GAT	6	189	GAAGATATTTCAAAGCAGCAA	CATGCTACCACTAGCAGAAGT
OVK063	TTG	6	188	AATTGCAACTGAAACTGAAAC	ACTGCTACCCTCTCCATAAAT
OVK068	GGA	6	195	GACCACCCGCAGCTCAAC	GTCTTCTTCCCCCATATTTAG
OVK072	ACC	7	199	TTGCCTTAGTCAGTTACCTTG	GTGGAGAGAATGAGAGAACCT
OVK073	GAC	6	200	GTAGACAACCGTATCTGGACA	AAGATGGAAGGTTCTAGTTCG
OVK077	TTA	6	249	GTCCCTCTCTCTCAAATTGTT	AGGTTAATGGAGCTTAGTGGT
OVK089	CAT	6	257	CAAAGTCATACCAATCACCAT	TCTTGGAAGCACTTGTTACTC
OVK093	CCA	6	259	CCAAGTGTTTGAAAGTCTCAG	TGAGAGTTCGTTCAAGGTAGA
OVK094	TTGCG	5	255	ACCGATCTTAGGATAGATGGA	ACTTTTGGTTGCTTAGTCGAT
OVK096	TCA	6	249	GAGCGTTGCATTTACATTTAC	CATCCTCCTTTACACCCTAAT
OVK097	GTGA	6	252	TCTATAGAGATGAGGCGACAA	CGCCCCTAACTAACCTACTAC
OVK099	TGAG	6	247	AGAAAATGGAAGCAACAGAGT	ACAAATAGCAGCTCCCTTC
OVK101	CTAA	6	254	GTTGAGTTTCAGACACAGAGC	AATAGCTCCCACAATAACTCC
OVK102	TGT	6	249	CCAAAGGGTGTTTTATTTTCT	GGAAGAAATTAAGCAAATGGT
OVK107	AG	8	193	AAGTTAAAACTTTGCGTTGTG	GACGTTGTTCTGGATTTCTTC
OVK111	GGT	7	206	TATAGACCTTCTCCTCCCAGA	GTGAAAGTCACAAATCCAAAG
OVK119	CAG	6	199	ACCCTCCTTCTCTCCTTATTT	GACGAGAGAACTCGTTTATGA
OVK122	TC	9	211	GCAGATAGCACAGTTATCGAC	GAACCACACACACAGAATCA
OVK123	ACA	9	200	CACCCATTAACTATCATGGTC	CAAGCCCTTTGTGAGATACTA
OVK124	TGA	9	211	GCCTTTTCTGTGACTCGTAA	GCTCCATTCCCATTTATAGTT
OVK125	CATTT	5	193	AAATTTAAGCACCGGAATAAC	AAAGCAAAAGGGCTACTAAAG
OVK126	TC	8	197	CGACAAAACTATTTAGGCAAA	GGGAAGAGATCATAAACCCTA
OVK127	AT	8	200	GCCCAAAATGTATTATCCTTC	AGAACAGACAGATATGCAAGC
OVK131	TA	8	200	TCTATCTGGGTGTTGTTTTGT	CTGTTTGAATATCGATTACCAC
OVK133	TG	8	196	TGCTTCAGCATTATTGTAACAT	TGCACTTCTCCATACTTCCTA
OVK138	CTAA	6	250	TAATATGGTGCAAGTTCCAAT	TTCTACGCTTAGCTCAAACC
OVK141	CACG	6	239	GAGGAGGTACATACAGCACAG	CAACCTCCTCGTTATCTTTTT
OVK142	GT	8	243	AACATGACTACTGTGAACAAGG	CGAACATGTAATTGATCCAAG
OVK155	GTG	6	251	CAGGTTTGAAGTAGCAGAGAA	GTAGACCACGCATACTGAATC

OVK158	GACT	6	257	TCAGAGTGTGTTGTGTTGTGT	AGTGAAGCAAATGTGTGATTT
OVK159	TG	8	251	CATTATTGCCTAGCATTGTTC	ATTTCACCATCAAGTATGCAC
OVK161	TTCC	6	249	AAAGCTTTCTACACGTTGGTA	TGGGTTTTTACACTCTGTGAT
OVK165	ACA	6	267	TTTCAAACACTCACTCACTCC	TCGGATTTGTGACCTAACTC
OVK168	TGA	6	253	AATTATCACCCACTGCTATGA	GGTTTCCATCACTGTTTGTTA
OVK172	AGC	6	256	TTATTAAACCTGCGTCTTCTG	GTAGAGCTGTGGGCTTTATCT
OVK173	CT	8	253	TCGTTCTCGTGATTATTCTGT	CCTCTATTCAAATAGGGCAAT
OVK174	GGCCC	5	246	ACATGATCGTGAATATGAAGC	CAGCAGCAATCAATATATCATC
OVK175	CA	9	250	GTAAAATATCAAGCAGGAGCA	AAACTATGCAGACACCCTGTA
OVK177	CTG	7	257	TCTGTTGATTTAAGGAGACGA	CTCTTGCTCATATTTTCCTCA
OVK181	AAG	6	257	AGGAAGAAGAAGAAGAAGCAG	TTCTCCTTTAACCACAACCTT
OVK183	TGAT	6	256	GAGGGTAAGAGAGAGTGGAAG	CTTGCCTGATATCTTCTCAAA
OVK196	AGC	6	286	TTTTGAGAGTGTGGAAGGTTA	AGTATGAGCCTGATGATGATG
OVM003	TC	9	297	CCGTCTGTTTAATCATTCACT	GAAAGGAAAGGTTATTGGAGA
OVM004	ATTT	5	290	GGGAATTCTTAAATCTCATGG	ATGCATGGTACTGGGTCGT
OVM025	CAA	6	297	TTCTGAACAACAACAACAACA	GTCCAGGAGCTAAGTAACCAT
OVM031	TGA	6	306	ATTGGTTTCTAAGGAGGACTG	GCAATACTCCTCTGCCTAGTT
OVM033	CTC	6	300	CAAGGCTTATTTGGTTAACAG	ATACTATTTCCCATGCCTACC
OVM034	TTC	6	308	GCATTTCATCAAACACTTTTC	TTGGTTTGAATCTGTGAGACT
OVM035	TTC	7	303	TCATCAAACACTTTTCGTTCT	TTGGTTTGAATCTGTGAGACT
OVM038	GAAG	6	297	CACAGGACAAGAGTGAGAGAG	TCATGATACCACGAATTTTTC
OVM043	GAG	7	167	TAGTATGGCTGAAATCAAAGG	ATATCATAAGGGCAACAGTGA
OVM048	AT	9	157	GACATTGAAATCAAACAATCC	AACACTTGTCATGTTTCCAAG
OVM049	TGA	7	150	AACAAACAAGAGGAAAAGGAG	TATGTGCTTATCAGGCATTTT
OVM050	ATCC	6	161	ATGAGCATGAAGAGTTTCAGA	ACACATCTACGACTTCTTTCG
OVM053	GTGGA	5	149	CACCAAAAGCATAGCAATAGT	GCTTGAATTGAATGAGAAATG
OVM057	TTG	6	153	CCTTGAGGAGGAATAATAGGA	GACATCATCATCACCTTCACT
OVM058	AT	9	150	GTCAAGTCATACCCATACGAG	CAGTGTAACCATATGCACAAA
OVM059	AGA	6	149	ACTCCAACTCCAACTCAGAAC	AAGCGAAGAAGAGAGTGAGAT
OVM060	CT	8	159	ATGTAATCAAAAGGTGCAGAA	AGCTTCCAAAACAGTGTATGA
OVM061	GTA	6	150	TTAACACACGTACGTACCACA	TTTGTCGTTGATCGTTAAGTT
OVM062	AG	8	139	GGAAAAAGGTTTGGATAGATG	AAGTTTTCCCCACACTATTCT
OVM064	AT	8	353	GCATGCACAGAATTAAGTTTC	AGAAGGTCCTTTGAAAATCAG
OVM065	CT	8	352	AAGACAGCGAGTTACCAATCT	GATTGAAACTGAGTAGCGATG
OVM067	CTT	6	352	CAACCTTAATACCAACCTTCC	AAAAGTAGCCAGAGAGCAAAT
OVM068	CCT	6	333	CTACAACTCACCGAAACTCAC	CGATTTCTGCCTCTTTATTCT
OVM069	AATG	6	357	ATGTTGTACAGATGAGCTTCG	TAGTGAGCAAACCTATTTTGG
OVM072	GAA	6	350	TTGATGTGGTTGATCCTATTC	GATGTCAACATCTTGGTCATTA
OVM073	ACA	6	346	GTTCTCAAACGCACTATCAAC	AAAATCTTGTAGGGATTCGAT
OVM076	AAC	7	348	CCCATTCTTCATCTTTCTCTC	TGCTTCCATAATCAGTGAAAT
OVM081	GT	9	350	TCTAGCACAATGTTTTGGATT	TATTGAGTTGAAGCAGACCAT
OVM083	CT	8	347	CACACAAACACAAAACTCACA	GATCGGAGAAAAGAAGAAGAG
OVM086	GAA	6	350	TCATACAAAGTTCCTTCCGTA	ATTGCCAATAACAGTGAAGAG

OVM090	CCA	6	151	AATCAATGGAGGAGGATAAAC	GAAGGTTGAAAAGGGAATAAA
OVM091	ATC	6	188	AACCACCCTTAATTCCATAAG	AGATAAAAGCCGCAAAAGTAT
OVM092	CAC	6	157	GGACCAACAAAGAGGATTATT	CCCTTGCTTGAAGTGTTACTA
OVM094	GTTT	5	163	ATTCATGGGGACAATAAATTC	CAAGAGAATGAATGAATCAGC
OVM099	GA	9	149	TATGTATTGCAGAATCACAGC	TATTACCCTTTTCCATCTTCC
OVM100	AAG	6	151	GAACTAGATTTGCGGCATT	CCCACACCCTTATCCTTATTA
OVM110	AT	8	154	CTGGACGAAAACAACATATTC	GTTGGCTTTGGTACTGACATA
OVM116	GAT	7	151	AACTACACGCACGTAATGAAT	TGGTTTGATAAACACCTCAAG
OVM120	TTC	6	152	TTCAGTGTCACTTTCCTCATT	AGAAGTTGTCATGTCAAGGAA
OVM122	TGG	6	156	ATGAATCTTGTACGGAATCTG	GAAGAAAAAGCCATAAACACC
OVM125	AAATT	5	151	ATTCTTTCAACAAGCAAGTGA	CTGCAATTCCATCCTATTTTA
OVM126	TCC	6	188	ACTAAGAACCACCCAAAACAT	TGAGAAGATGGAGAAGATGTG
OVM128	TGTT	6	155	GAGAAGCATAACCAAAATCCT	TGGAAGAAAAGAAACTTCTGA
OVM129	TG	8	133	AATTGGATTCATGTGTTAGGA	GAAGTGGAGCCAAAACCT
OVM130	AG	9	154	GCAAATTATCACCATGCAC	CGTGAAGAAAATCGGTACTTA
OVM131	AGA	6	153	GAAATAACGCAGGCAGATAC	AATTAGAGGCTTCGACTTGTT
OVM132	GAC	6	142	ACGGTAATCAGTAGTGACAGC	GTGTGACAGAAAATGGGATTA
OVM133	TTTC	5	171	TAGCATCAAGGTTGGAAATAG	CTAGGCTACCTGAATCAAACA

3.3.5 Data analysis

All statistical analyses and calculations were performed using R statistical software (R Core Team 2014). The polymorphism information content (PIC) of SSR markers was calculated as the mean of the PIC of each allele, using the formula for dominant markers from Roldan-Ruiz et al. (2000) as;

$$PIC_i = 2f_i (1 - f_i),$$

where PIC_i is the polymorphism information content of allele i and f_i is the frequency of occurrence of allele i (fragment present) in the 32 individuals. From single alleles, average (PIC_{Av}), minimum (PIC_{Min}) and maximum (PIC_{Max}) PIC values were calculated for each SSR marker.

In order to calculate genetic distance measures, SSR alleles were coded as individual markers with 1 for presence and 0 for absence of the allele as binary data. Pairwise genetic distances between individuals were calculated as modified Rogers' distance D_w (Wright 1978) which shows the extent of genetic diversity

between two individuals (Lee et al. 1989) ranging from 0 (no diversity between individuals) and 1 (maximum diversity).

Genetic relationships were visualized using cluster analysis and the R-function pvclust() (Suzuki and Shimodaira 2006) based on Euclidean distance that was rescaled to D_w for plotting purposes (D_w and Euclidean distance show a linear relationship, Fig. S3.1). Probability values (p-values) were calculated for each cluster using multiscale bootstrap resampling (Shimodaira 2002, 2004) to calculate approximately unbiased (AU) p-values (Efron 1979). The k-means clustering algorithm (MacQueen 1967) was applied to the D_w values using a sequence of k = 2 clusters to 32 clusters. The Calinsky criterion (Calinski and Harabasz 1974) was then calculated for each number of k as implemented in the R function cascadeKM() and the optimum number of clusters was determined at the maximal value. Population structure was further investigated by principal component analysis performed on binary raw data of individual alleles.

3.4 Results

3.4.1 SSR analysis

SSR markers showed a high degree of polymorphism and overall, 1154 alleles were found with an average of 11.4 alleles per marker locus (Table 3.3). Among those 1154 alleles, only five alleles (from SSR OVK042, OVK172, OVM031, OVM072 and OVM100) were non-polymorphic, and hence, present in all individuals studied.

With only two alleles in the 32 individuals, SSR OVK042 had the lowest number of alleles, whereas OVK158 had the highest number with 24 amplified alleles. The minimum rate of allele occurrence was 0.03125, corresponding to occurrence in only one genotype (i.e. a private allele of an individual genotype). In total, 250 private alleles were detected and these were equally distributed across the examined set of individuals and markers. With regard to individuals, the highest number of private alleles over all markers was found for individual ID_08 (14 private alleles) and the lowest number was found for ID_17 (three private alleles). The origin of the individual did not appear to affect the occurrence of

private alleles. With regard to markers, the most private alleles were observed in OVM064 (eight private alleles), whereas 16 markers (15.8%) had no private alleles at all.

The average polymorphism information content (PIC_{Av}) ranged from 0.14 (OVK141) to 0.36 (OVK101, Table 3.3). A detailed look at the PIC values of individual alleles in the different markers exhibited minimum PIC values per SSR (PIC_{Min}) between 0, (OVK042, OVK172, OVM031, OVM072, OVM100) and 0.17 (OVK131) and maximum PIC values per SSR (PIC_{Max}) between 0.3 (OVK 172) and 0.5 (16 different markers, Fig. S3.2).

Table 3.3 Characterization of the 101 polymorphic sainfoin markers. PIC_{Av}, PIC_{Min} and PIC_{Max} give the average, minimum and maximum allele-wise polymorphism information content values, NoA_{Tot} the total number of alleles, NoA_{Priv} the number of private alleles, Min_{AF} the minimum allele frequency and Max_{AF} the maximum allele frequency value

Marker	PIC_{Av}	PIC_{Min}	PIC_{Max}	NoA	NoA_{Priv}	Min_{AF}	Max_{AF}	Size
OVK002	0.22	0.06	0.47	9	3	0.03	0.63	154 - 175
OVK003	0.23	0.06	0.47	11	2	0.03	0.38	92 - 124
OVK017	0.22	0.06	0.50	19	5	0.03	0.47	148 - 184
OVK027	0.28	0.06	0.50	9	2	0.03	0.59	120 - 140
OVK034	0.27	0.06	0.49	12	1	0.03	0.56	138 - 154
OVK036	0.35	0.17	0.50	7	0	0.09	0.69	133 - 154
OVK038	0.19	0.06	0.40	14	4	0.03	0.28	155 - 186
OVK042	0.25	0.00	0.50	2	0	0.50	1.00	183 - 186
OVK045	0.29	0.12	0.43	6	0	0.09	0.94	138 - 148
OVK046	0.31	0.06	0.49	12	1	0.03	0.56	138 - 157
OVK054	0.29	0.12	0.49	15	0	0.06	0.44	274 - 290
OVK055	0.20	0.06	0.38	8	2	0.03	0.84	135 - 159
OVK063	0.24	0.06	0.50	13	2	0.03	0.72	179 - 200
OVK068	0.25	0.06	0.43	9	3	0.03	0.31	186 - 213
OVK072	0.32	0.12	0.50	4	0	0.06	0.81	193 - 198
OVK073	0.29	0.06	0.50	11	1	0.03	0.53	186 - 210
OVK077	0.23	0.06	0.45	9	2	0.03	0.78	233 - 264
OVK089	0.27	0.06	0.49	9	2	0.03	0.44	279 - 299
OVK093	0.23	0.06	0.50	14	6	0.03	0.56	234 - 271
OVK094	0.24	0.06	0.48	14	4	0.03	0.66	208 - 244
OVK096	0.21	0.06	0.48	20	6	0.03	0.41	215 - 294
OVK097	0.22	0.06	0.38	3	0	0.13	0.97	240 - 248
OVK099	0.25	0.06	0.49	13	2	0.03	0.75	232 - 270
OVK101	0.36	0.06	0.50	7	1	0.03	0.72	339 - 352

OVK102	0.23	0.06	0.34	4	1	0.03	0.22	239 - 251
OVK107	0.29	0.06	0.45	15	1	0.03	0.72	206 - 234
OVK111	0.26	0.06	0.48	7	2	0.03	0.75	213 - 232
OVK119	0.30	0.06	0.47	10	1	0.03	0.72	216 - 252
OVK122	0.24	0.06	0.45	8	1	0.03	0.66	330 - 341
OVK123	0.26	0.06	0.50	10	3	0.03	0.75	208 - 237
OVK124	0.26	0.06	0.49	15	1	0.03	0.44	218 - 267
OVK125	0.29	0.06	0.50	9	1	0.03	0.72	197 - 222
OVK126	0.25	0.06	0.49	15	3	0.03	0.56	198 - 233
OVK127	0.28	0.06	0.49	6	1	0.03	0.44	204 - 222
OVK131	0.17	0.06	0.48	15	3	0.03	0.59	183 - 228
OVK133	0.25	0.06	0.50	13	3	0.03	0.63	205 - 239
OVK138	0.21	0.06	0.49	13	6	0.03	0.56	232 - 267
OVK141	0.14	0.06	0.47	15	7	0.03	0.38	242 - 269
OVK142	0.25	0.06	0.50	12	3	0.03	0.47	256 - 285
OVK155	0.24	0.06	0.49	14	3	0.03	0.56	234 - 282
OVK158	0.19	0.06	0.40	24	6	0.03	0.28	273 - 375
OVK159	0.23	0.06	0.50	14	4	0.03	0.81	268 - 290
OVK161	0.25	0.06	0.40	12	1	0.03	0.28	220 - 276
OVK165	0.19	0.06	0.50	20	6	0.03	0.50	273 - 311
OVK168	0.24	0.06	0.50	11	3	0.03	0.81	258 - 284
OVK172	0.16	0.00	0.30	5	0	0.06	1.00	268 - 279
OVK173	0.23	0.06	0.48	18	5	0.03	0.59	268 - 316
OVK174	0.23	0.06	0.48	5	2	0.03	0.75	245 - 266
OVK175	0.19	0.06	0.38	10	2	0.03	0.88	252 - 267
OVK177	0.27	0.06	0.49	7	2	0.03	0.59	267 - 286
OVK181	0.19	0.06	0.38	19	4	0.03	0.25	343 - 381
OVK183	0.24	0.06	0.49	17	1	0.03	0.44	266 - 289
OVK196	0.21	0.06	0.50	8	2	0.03	0.53	297 - 314
OVM003	0.31	0.12	0.47	10	0	0.06	0.69	299 - 321
OVM004	0.21	0.06	0.45	18	6	0.03	0.34	380 - 426
OVM025	0.33	0.17	0.49	7	0	0.09	0.84	306 - 324
OVM031	0.26	0.00	0.50	13	1	0.03	1.00	292 - 353
OVM033	0.29	0.06	0.50	8	1	0.03	0.69	308 - 330
OVM034	0.22	0.06	0.50	17	5	0.03	0.53	307 - 355
OVM035	0.22	0.06	0.50	17	5	0.03	0.53	301 - 350
OVM038	0.19	0.06	0.43	14	4	0.03	0.31	311 - 351
OVM043	0.30	0.06	0.49	10	2	0.03	0.66	173 - 203
OVM048	0.29	0.12	0.43	6	0	0.06	0.72	174 - 186
OVM049	0.31	0.06	0.50	9	1	0.03	0.50	162 - 198
OVM050	0.20	0.06	0.49	13	6	0.03	0.72	168 - 198
OVM053	0.32	0.06	0.50	11	1	0.03	0.50	134 - 182
OVM057	0.35	0.17	0.49	5	0	0.09	0.66	165 - 180

OVM058	0.23	0.06	0.49	15	3	0.03	0.44	135 - 178
OVM059	0.24	0.06	0.48	7	2	0.03	0.59	156 - 174
OVM060	0.23	0.06	0.50	21	4	0.03	0.50	172 - 219
OVM061	0.19	0.06	0.50	10	4	0.03	0.84	143 - 175
OVM062	0.30	0.06	0.49	12	2	0.03	0.59	151 - 170
OVM064	0.16	0.06	0.47	16	8	0.03	0.38	380 - 444
OVM065	0.25	0.06	0.49	14	4	0.03	0.69	360 - 391
OVM067	0.33	0.17	0.49	6	0	0.09	0.66	366 - 380
OVM068	0.26	0.12	0.43	8	0	0.06	0.88	343 - 368
OVM069	0.26	0.06	0.48	13	2	0.03	0.59	454 - 479
OVM072	0.28	0.00	0.50	7	1	0.03	1.00	365 - 387
OVM073	0.20	0.06	0.45	21	5	0.03	0.34	446 - 511
OVM076	0.22	0.06	0.48	17	3	0.03	0.41	347 - 376
OVM081	0.18	0.06	0.40	17	5	0.03	0.28	353 - 396
OVM083	0.30	0.06	0.50	11	2	0.03	0.63	365 - 384
OVM086	0.32	0.06	0.50	10	1	0.03	0.63	371 - 391
OVM090	0.30	0.06	0.49	8	2	0.03	0.84	158 - 180
OVM091	0.34	0.06	0.50	8	1	0.03	0.47	184 - 217
OVM092	0.23	0.06	0.43	7	2	0.03	0.81	163 - 185
OVM094	0.33	0.12	0.50	7	0	0.06	0.81	190 - 207
OVM099	0.18	0.06	0.50	11	5	0.03	0.50	165 - 198
OVM100	0.24	0.00	0.48	5	0	0.09	1.00	163 - 179
OVM110	0.21	0.06	0.49	18	5	0.03	0.44	163 - 185
OVM116	0.28	0.06	0.49	15	2	0.03	0.56	138 - 204
OVM120	0.34	0.06	0.50	6	1	0.03	0.88	169 - 187
OVM122	0.31	0.06	0.50	4	1	0.03	0.91	164 - 180
OVM125	0.26	0.06	0.50	10	3	0.03	0.47	161 - 180
OVM126	0.22	0.06	0.50	18	4	0.03	0.53	191 - 229
OVM128	0.26	0.06	0.47	9	1	0.03	0.81	173 - 190
OVM129	0.25	0.06	0.49	14	4	0.03	0.56	146 - 173
OVM130	0.20	0.06	0.47	20	7	0.03	0.38	152 - 187
OVM131	0.34	0.06	0.50	8	2	0.03	0.56	159 - 198
OVM132	0.30	0.06	0.47	8	2	0.03	0.78	157 - 176
OVM133	0.20	0.06	0.47	14	3	0.03	0.63	177 - 212

The overall length of SSR fragments detected ranged from 91 to 511base pairs (bp). Markers with two base pair motifs had a slightly higher number of repeats (eight to nine) when compared to markers with three to five bp motifs (five to seven repetitions). The total fragment length observed did not differ between motif lengths (data not shown). Contrastingly, the number of alleles found for

SSRs with two bp motifs was higher (13.5 alleles on average), compared to SSRs with longer motifs (10.7 alleles). The average number of alleles per sainfoin genotype was 230.1 over all SSR markers, leading to an average of 2.3 alleles per SSR marker and genotype. The lowest number of alleles was found for genotype ID_25 with 191 alleles, the highest for ID_07 with 268 alleles.

3.4.2 Diversity of *Onobrychis viciifolia* individuals

The allocation of individuals to groups by overall similarity of alleles was assessed using k-means partition comparisons. Those k-means statistic (Fig. 3.1, left) give the grouping of individuals (assigned by different colors) dependent on number of groups chosen. Individuals were assigned into two to ten groups, with a more homogenous grouping for two and three groups. The Calinski criterion (Fig. 3.1, right), giving the most likely grouping by the highest value reached, indicated a grouping of individuals into two groups by a value >3.

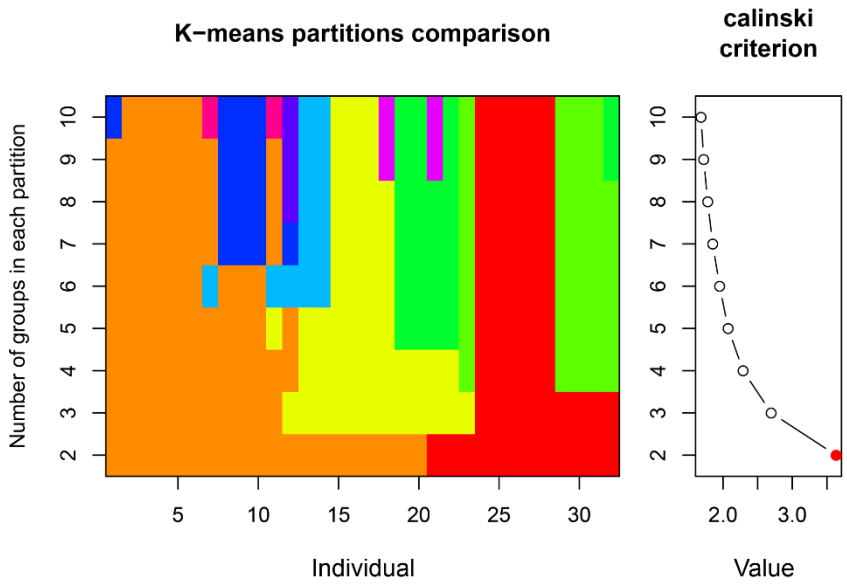

Fig. 3.1 Group separation of individuals as assessed by k-means partitioning for k = 2 to 10 with colors indicating different groups (left). The optimum number of groups (k) according to maximum Calinski criterion was determined to be two (right).

The cluster dendrogram based on the modified Roger's distance (Fig. 3.2) also displayed a partitioning of individuals in two main groups, which were separated by a modified Roger's distance value of 0.47. Individuals belonging to the same

variety located in the same main branch for the varieties Perly (ID_14, ID_18; 0.4), Visnovsky (ID_13, ID_28; 0.39) and Zeus (ID_29, ID_30; 0.48). The variety Perdix is an advanced variety originating from the variety Perly and the Perdix genotype (ID_16) clusters closely to one of the Perly individuals (ID_14).

The first, smaller branch of the cluster (Fig. 3.2, right hand side) consisted mainly of individuals originating from Switzerland and the U.K, whereas the majority of the second, larger branch was comprised of individuals from Southern and Eastern Europe as well as individuals from USA, Morocco and Canada. However, AU values showed no significance (values <95) for most branches.

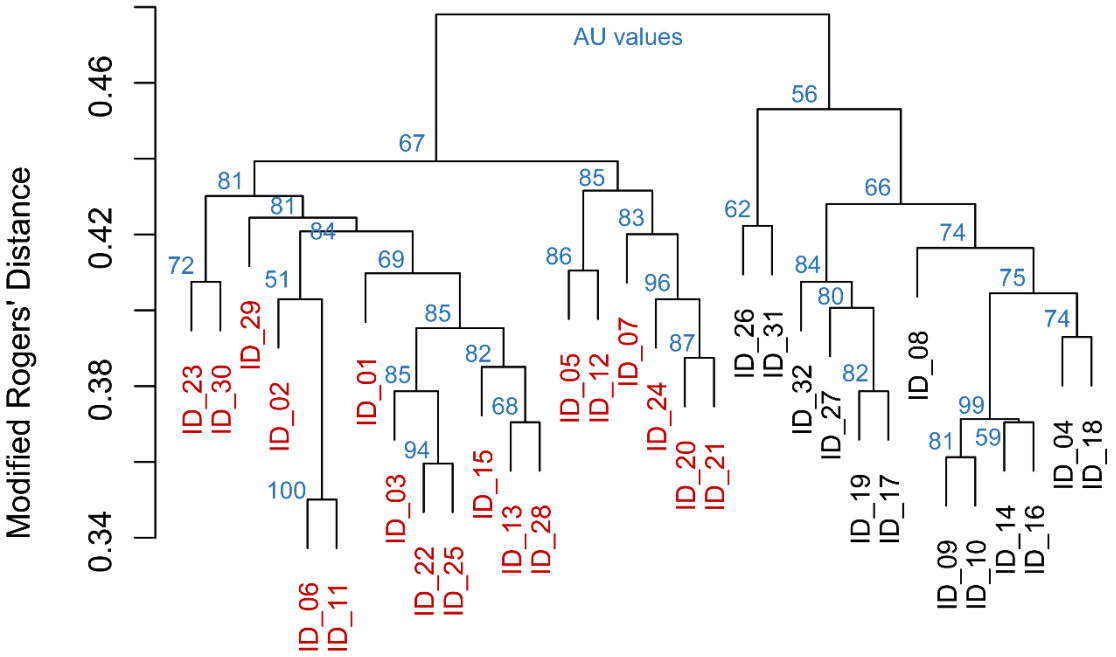

Fig. 3.2 Cluster Dendrogram of individuals based on the modified Rogers' distance. Values at branches are AU p-values (blue). Different colors of genotype labels give the affiliation to the two groups determined by k-means partitioning.

Principal component analysis (PCA; Fig. 3.3) showed a pattern comparable to that observed from cluster analysis with individuals of the two main clusters mainly being separated by the first principal component which explained 10.3% of the total marker variation. The second principal component illustrates the intragroup variation with 4.9%.

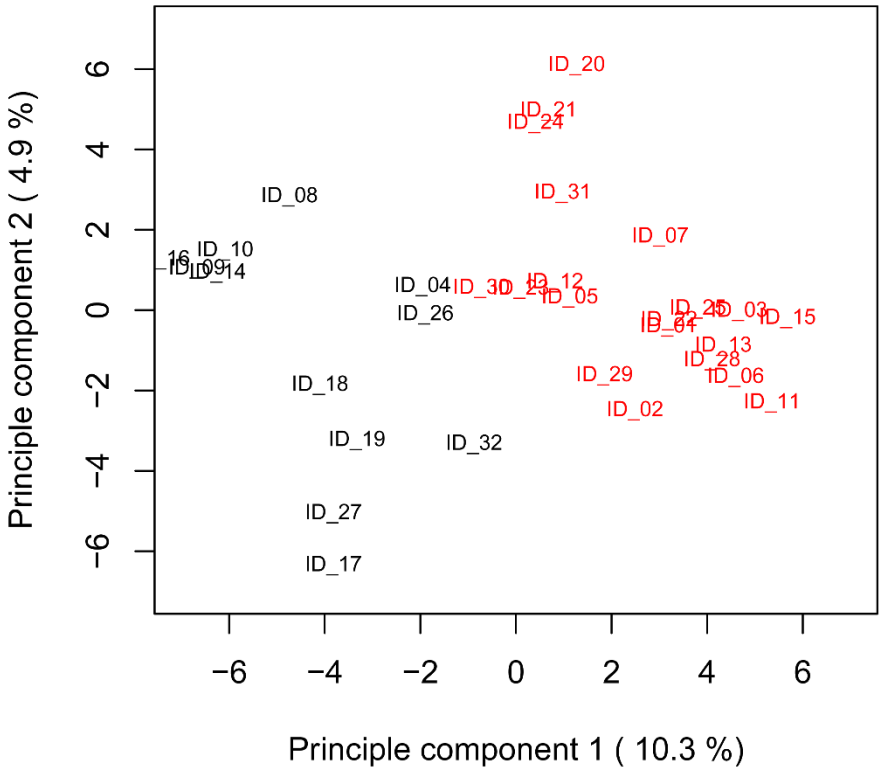

Fig. 3.3 Principal component analysis of 32 sainfoin (*Onobrychis viciifolia)* individuals based on 1054 alleles of 101 SSR markers. Different colors give the affiliation to the two groups as determined by k-means partitioning.

3.5 Discussion

The 101 SSR markers newly developed from sainfoin revealed a high degree of polymorphism. In addition to differences in multiples of the repeat motif, we also found alleles differing by fractions of the multiple motif length. Such variations could have arisen from insertions, deletions and translocations in the flanking region of the SSR (Robinson et al. 2013). Such mutations in the flanking region might also contribute to the high degree of polymorphism in our marker data set. The SSR sizes predicted through sequencing and the actual size distribution observed in the 32 individuals was consistent for most of the markers. Discrepancies can largely be explained by the fact that SSR motifs were developed from individuals not represented in the present study. In total, we found 1154 alleles at 101 loci resulting in 11.4 amplified alleles per SSR on average. This is twice the amount found by Demdoum et al. (2012), who found 5.83 alleles by transferring markers from barrel clover (*Medicago truncatula*) and

soybean (*Glycine max*) to sainfoin. Fragments were smaller for the specific marker set in this study (92 to 511 bp) compared to markers adopted from other species (79 to 865 bp, Demdoum et al. 2012). The larger sizes of alleles from cross-species amplification could be attributed to interspecific differences to the donor species due to repeat length variation within the SSR region and indels in the flanking region (Peakall et al. 1998). Avci (2014) amplified 725 alleles from 18 SSR markers in diverse *Onobrychis* subspecies using markers from pea and barrel clover. The higher number found by these authors could be explained by the larger diversity of germplasm used, which originated from different subspecies.

SSR marker studies with other tetraploid species using diverse panels of individuals showed lower numbers of alleles per marker compared to the present study, e.g., 7.2 alleles in sugar cane (*Saccharum officinarum*, Pinto et al. 2006), 6.7 alleles in switchgrass (*Panicum virgatum*, Narasimhamoorthy et al. 2008) and 6 alleles in peanut (*Arachis hypogaea*, Hopkins et al. 1999).

A few markers were observed with less than five alleles among the 32 individuals. These may still be useful in future studies, since this study represents an initial screening of single individuals and not an extensive population survey. Additionally, using only the most polymorphic markers would bias the overall genetic diversity e.g. in conservation studies (Vali et al. 2008).

The challenge in analyzing SSR alleles in tetraploids lies in determining the dosage of each allele, which is often impossible using capillary electrophoresis for individuals carrying less than four different alleles at a specific marker locus. The PIC content gives an estimation of the information content of a marker and is traditionally calculated by the formula of Botstein et al. (1980). This was developed for diploid species, for which the allele frequency is either known or can be inferred from the allele occurrence (presence/absence). For tetraploid species, the allele frequency is difficult to derive from the allele occurrence due to different allele doses (one to four alleles). Hence, the formula for diploids could not be used for tetraploid sainfoin. Thus, the PIC was calculated separately for each allele, on the basis of allele occurrence counts, using a formula adopted from Roldan-Ruiz (2000) and averaging the PIC across all loci of one locus (Tehrani et al. 2008). Here, the maximum value that can be reached is 0.5, which

corresponds to alleles found in 50% of the population. Small values, on the other hand, correspond to very abundant or to very rare alleles. Deciding whether a SSR marker is useful also depends upon the scientific issue. Taking into account different allele-based PIC values of an SSR locus (Fig. S3.2), therefore, gives the most holistic picture of the SSR marker. High PIC values of alleles (0.5 – 0.4) are useful for inside population studies, e.g., to trace marker trait associations, whereas low PIC values (0.0 – 0.1) of single alleles could be more useful for studies of evolution or genetic drift (Silvertown and Doust 1993). The average PIC values in this study indicated that most markers had alleles which could be found in a group of individuals and are suitable for several approaches in future studies. These PIC values were comparable to those found by Tehrani et al. (2008), which were between 0.16 and 0.44 in *Lolium persicum*. The large number of private alleles is a clear indication of genetic distinctness of the individuals, which was anticipated in view of their diverse origins.

Genetic diversity is a prerequisite for selection in variety development. So far, there is limited information on the genetic diversity of sainfoin available. Use of AFLPs and SSR markers from other species were not able to reveal genetic diversity in distinct Spanish sainfoin accessions (Sardaro et al. 2003; Demdoum et al. 2012). The values of that study, given by Nei`s similarity values, which represent the proportion of shared fragments on the basis of binary data and corrected by the marker number (Lamboy 1994), reached values of 0.73 to 0.8 (Sardaro et al. 2003; Demdoum et al. 2012). A conversion of those values to genetic distance values by the formula –ln (Nei`s similarity values, Frankham et al. 2010) resulted in Nei`s genetic distance values of 0.31 and 0.22. In a study of sainfoin genetic diversity using RAPD markers in ten landraces from East Azerbaijan and in 36 Iranian sainfoin populations, Nei`s genetic distance values of 0.32 and 0.25, respectively, were observed (Rasouli et al. 2013; Hejrankesh et al. 2014). In our study, highest modified Roger`s distance of 0.48 corresponds to alleles not shared between our two cluster groups, which is almost 50% (Fig. 3.2). The smallest Roger`s distance values with 0.35, corresponds to an approximate Nei`s distance value of 0.43 (Fig. S3.1), which is higher than the low values observed in other studies (Sardaro et al. 2003; Demdoum et al. 2012; Hejrankesh et al. 2014). The majority of among-genotype comparisons showed higher values.

The higher values of genetic diversity found in the present study may reflect the high variability of the markers developed and the selection of 32 individuals of contrasting origin. Despite the fact that individuals of the same cultivars in this predominantly outbreeding species can show considerable variability (Posselt 2010), the individuals from the same cultivar grouped clearly together in the present study (Fig. 3.2 and 3.3, Table 3.1).

The 32 individuals investigated separated into two clear groups based on different multivariate analyses. The first main group comprised mainly individuals from Switzerland and the U.K., whereas the second group contained individuals originating from South and East Europe as well as USA, Canada and Morocco. A similar grouping of accessions could be found in earlier studies between sainfoin accessions from Western Europe and those from Eastern Europe and Asia (Demdoum et al. 2012; Hayot Carbonero et al. 2012). This clear genetic distinction between the individuals from Western Europe and those from Eastern Europe and beyond could reflect adaptation to diverse climatic conditions either naturally or as a result of local selection by growers (Silvertown and Doust 1993). Under genetic isolation and limited gene exchange, differentiation in the sainfoin germplasm with accompanied morphological separation seems likely (Zarrabian et al. 2013).

3.6 Conclusions

This study reports the first characterization of specific co-dominant SSR markers for sainfoin. The 101 SSR markers characterized in this study showed a high degree of polymorphism and clearly demonstrated the differences between sainfoin individuals with diverse origin, on a molecular genetic level. The genetic differences found in our panel separated the individuals into two groups, with a clear correlation to the geographical origin of those individuals. SSR markers, such as those characterized here, will be very useful in future genetic analyses, such as paternity or pedigree analysis in breeding programs, as well as more detailed analysis of genetic diversity in this forage crop. Furthermore, the

development of new varieties could be crucially improved by choosing distinct genepools and minimizing inbreeding depression.

3.7 Acknowledgements

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3.8 Authors' contributions

KK and MMO contributed equally to the publication. KK and MMO contributed to the experimental design performed all analyses and lead the writing of the manuscript. LMJS assisted in data collection, variety choice, structuring the work program and helped to draft the manuscript. RK and LS conceived the study and assisted with data collection, data analysis and drafting the manuscript. All authors read and approved the final version of the manuscript.

3.9 Supplementary Information

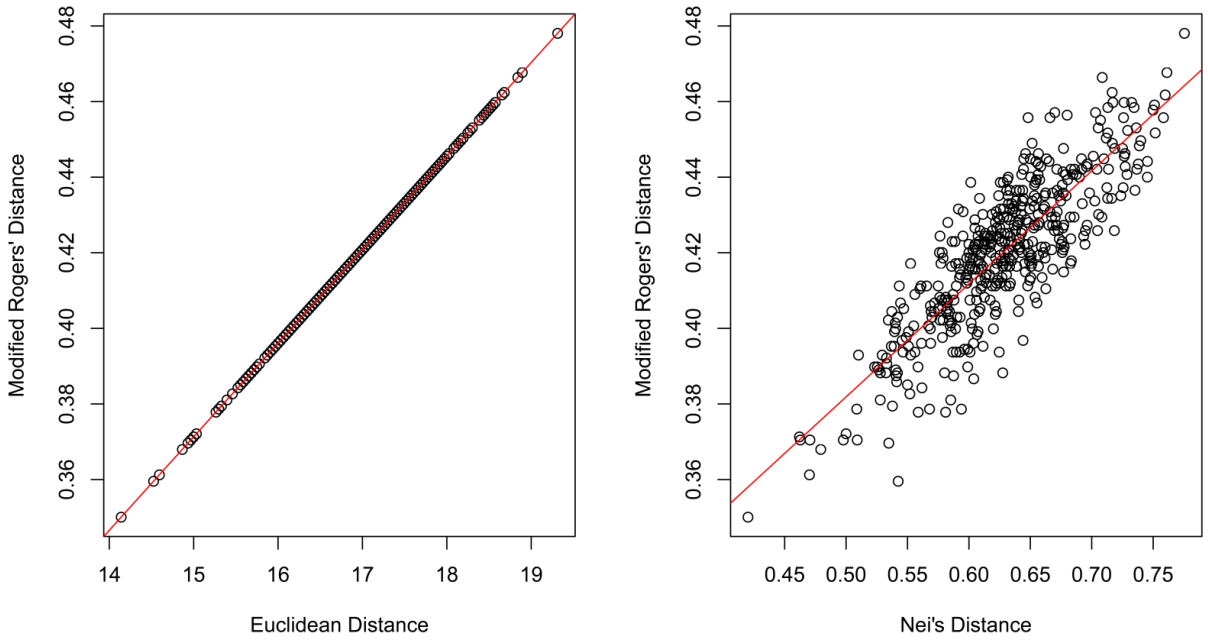

Figure S3.1 Relationship between modified Roger's Distance to Euclidian Distance and to Nei's Distance.

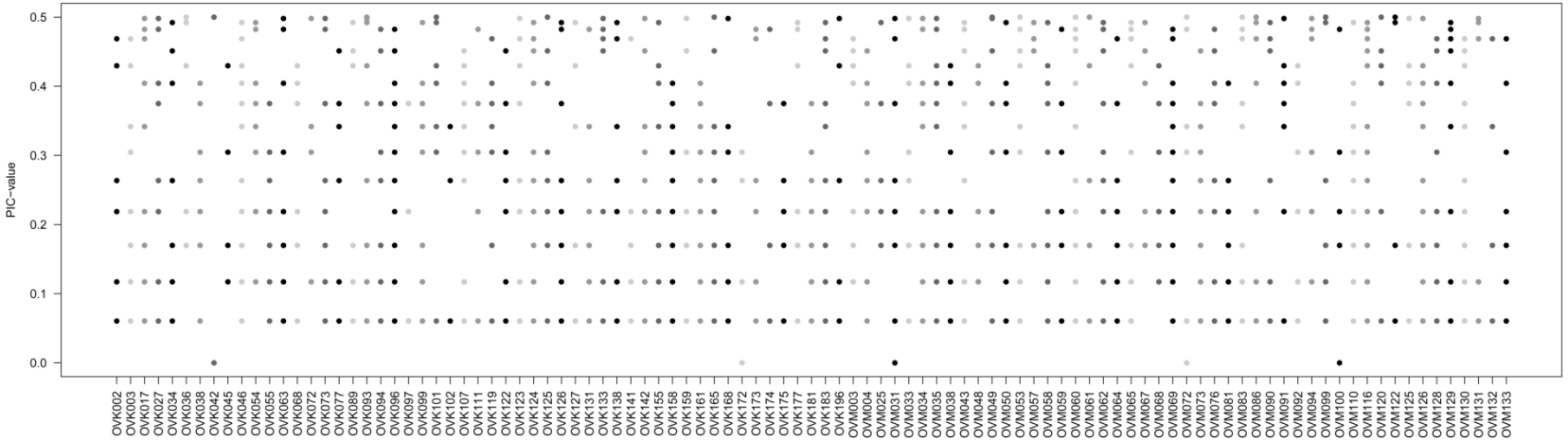

Figure S3.2 Polymorphism Information Content (PIC) values for individual alleles at SSR loci. Different grey levels are used for better visual differentiation among alleles of the different SSR markers.

Chapter 4 Marker- trait association analysis for agronomic and compositional traits in sainfoin (*Onobrychis viciifolia*)

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4.1 Abstract

Sainfoin (Onobrychis viciifolia) is a perennial forage legume with great potential for use in sustainable agriculture due to its low input requirements, good drought tolerance, and production of forage rich in polyphenolic compounds, which are beneficial for animal health. However, its distribution and cultivation are limited due to its moderate agronomic performance and a general lack of well adapted, highly yielding cultivars. Faster progress in breeding is imperative, but is often hampered by the complex inheritance of traits and limited knowledge on the genetic composition of this tetraploid, outbreeding species. Molecular genetic tools might aid phenotypic selection; however, to date no information on marker-trait associations is available for sainfoin. Hence, the goal of the present study was to detect marker-trait associations in a biparental F1 population. Single plants were screened for recently developed genetic markers and phenotyped for important agronomic traits and concentrations of different polyphenolic compounds. Significant trait-associated markers (TAM) were detected for plant height (11), plant vigor (1), and seed yield (7). These three traits were positively correlated with each other and shared some TAMs. Correlations among markers suggested that two independent loci control these three vigor-related traits. One additional, independent TAM was detected for the share of prodelphinidins in total condensed tannins. Our results provide insight into the genetic control of important traits of sainfoin, and the TAMs reported here could assist selection in combination with phenotypic assessment.

4.2 Introduction

The perennial, tetraploid legume sainfoin (*Onobrychis viciifolia*) has a long tradition as a valued forage source for working horses and other farm animals in Europe. With the cultivation of more productive species, application of inorganic fertilizers and the use of automated harvest systems, its cultivation decreased during the 20th century. Consequently, sainfoin has almost disappeared from current agricultural landscapes. However, the recently increased interest in more sustainable agricultural production makes this crop a valuable alternative for various reasons. First, sainfoin shows high tolerance to drought and low nutrient availability (Doyle et al., 1984), making it a valuable option for less favorable sites, which are expected to increase due to larger climatic variability accompanying climate change. Second, it is capable of fixing atmospheric nitrogen via association with rhizobia, reducing the need for nitrogen fertilization and improving soil quality (Hill, 1980). Third, as its most prominent feature, sainfoin contains a high concentration of beneficial secondary metabolites such as condensed tannins (CT, proanthocyanidins) and other polyphenolic compounds, which have anthelmintic properties against gut parasites, increase protein utilization, prevent bloating, and reduce methane emissions (McMahon et al., 2000; Hoste et al., 2012). Hence, sainfoin has the potential to be a valuable ruminant feed.

However, despite its advantages, the wide cultivation of sainfoin is hampered by its often poor agronomic performance and the limited availability of varieties adapted to temperate climates (Goplen et al., 1991). Major agronomic weaknesses also include poor competitive ability against weeds or companion species, slow establishment, low seed set, and weak persistence. Persistence is a complex trait that depends on several factors, of which plant vigor (strong and healthy growth) is a major determinant. In addition to plant vigor, plant height, leaf area, disease resistance, and seed production are also indicators for strength and capacity to survive and, consequently, to persist. Breeding improved varieties with regard to these agronomic traits is indispensable to increase farmers' acceptance of sainfoin and to promote its cultivation. Furthermore, targeted breeding for desired composition and concentrations of CT and other polyphenolic compounds

in sainfoin is also desirable, because nutraceutical properties are consistent with the demands for future plant breeding to assist animal welfare and health (Humphreys, 2005).

Sainfoin breeders have to contend with an allogamous (outbreeding) reproduction system, as is common in most forage species, and is often secured through self-incompatibility mechanisms (Humphreys, 2000; Posselt, 2010). Hence, under the absence of efficient methods to induce self-fertilization or perform controlled crosses between inbred individuals, varieties are classically bred as open pollinated varieties or synthetics. Starting from selection of superior plants within a base population, this process of developing a new variety takes at least ten years (Posselt, 2010). The resultant varieties exhibit high levels of genetic diversity, evoking a generally broad environmental adaptability. For sainfoin breeding, this large diversity presents an opportunity to select for superior genotypes improved for important traits such as persistence, seed yield and concentrations of CT, and other polyphenolic compounds (flavonols and their glycosides).

To date, information on the response of sainfoin to phenotypic selection and inheritance patterns of traits is scarce. Additionally, measuring complex traits, such as CT concentration and composition, is both complex and labor intensive (Engström et al., 2014). In addition, determination of seed yield and plant vigor is labor intensive and usually requires field experiments over several years. Indirect selection can accelerate breeding, and can be performed using traits that are easily determined and correlated with the target trait, or via genetic markers linked to the trait of interest (marker assisted selection [MAS]). The basic prerequisite for MAS is the availability of markers that are tightly linked to genes or quantitative trait loci (QTL). Such markers could be used to select for traits that are difficult to measure or dependent on the developmental stage, to maintain recessive alleles, to pyramid monogenic traits, and to speed up the breeding progress in general. In the case of polyploid species and traits subject to polygenic inheritance, both factors impede the efficient selection of genotypes based on their phenotype, and application of MAS would be especially valuable (Barrett et al., 2009). Detection of potential QTLs in sainfoin would, therefore, be particularly promising to improve breeding progress.

In other forage plant species, several QTLs suitable for MAS have been identified, such as those for vigor and seed yield in white clover (*Trifolium repens*; Faville et al., 2003), seed yield in red clover (*T. pratense;* Herrmann et al., 2006), or height and regrowth in alfalfa (*Medicago sativa;* Robins et al., 2007). In sainfoin, adequate numbers of markers are not yet available and, consequently, no studies have been performed to detect marker-trait associations or QTLs. Marker systems that do not rely on prior DNA sequence information offer a valuable approach for the initial characterization of minor species that generally lack available sequence information. Sequence-related amplified polymorphism (SRAP), which preferentially amplify in gene rich regions (Li and Quiros, 2001), have been successfully used for the molecular investigation of allogamy and autogamy in sainfoin (Kempf et al., 2015). Recently, RNA transcriptome sequencing, using next generation sequencing, has been successfully used to develop comprehensive simple sequence repeats (SSR) marker resources that can be applied to assess genetic diversity in sainfoin (Kempf et al., 2016; Mora-Ortiz et al., 2016). In order to use these newly developed marker resources to identify possible marker trait associations in sainfoin, the objectives of the present study were to (i) phenotype a biparental F_1 population of sainfoin for a wide range of traits under field conditions, (ii) investigate correlations among agronomic and compositional traits, and (iii) identify dominant SRAP and co-dominant SSR markers associated with phenotypic traits. Hence, the present study provides a first step towards MAS in sainfoin.

4.3 Material and Methods

4.3.1 Plant material

The plant material used in the present study consisted of a F_1 population (n=122) from a biparental cross between two plants of the varieties Brunner (parent 1; Agroscope, Zurich, Switzerland) and Perdix (parent 2, Agroscope, Nyon, Switzerland), and the first generation of plants resulting from self-fertilization (S_1 progenies) of the two parents (n=85 for parent 1, n=30 for parent 2). Crossings (F_1 progenies) and selfings (S_1 progenies) have previously been identified using SSR and SRAP markers (Kempf et al. 2015). The plant material was established in January 2012 by placing two sets of five clones of each parent in a greenhouse chamber for bumble bee pollinations (*Bombus terrestris*, "Bombus Maxi Hummeln", Andermatt Biocontrol, Switzerland). Seeds were harvested separately for each parent. Plants from pre-germinated seeds were then kept in the greenhouse for two months before being transplant as juvenile plants to the field site (Delley, Fribourg, Switzerland) in July 2012. All plants were randomly planted into rows of 10 plants, arranged in two rectangular blocks.

4.3.2 Phenotyping

Plants were grown in the field from 2012 to 2014, chemical composition was determined in 2012, and phenotyping of agronomic traits was performed in 2013. Due to a large loss of plants during the winter following the 2013 season, no phenotyping could be performed in 2014. Plant height was determined as the distance from the soil to the last leaflets of stretched plants in spring (HSp) and summer (HSu). Plant vigor (Vigor) was scored visually on a scale from 1 (very weak) to 9 (very strong). Susceptibility to rust disease (caused by *Uromyces onobrychidis*) was also visually scored on a scale from 1 (non-susceptible, no disease occurrence) to 9 (highly susceptible, high disease occurrence). Flowering time (FT) was recorded as the number of days after May 1st, when a plant showed three or more open flowers. Seed yield was measured as the total number of seeds per plant in 2013 (SN), as described by Kempf et al. (2015).

In spring 2013, the chlorophyll content of leaves was measured non-destructively using a SPAD chlorophyll meter (Konica Minolta SPAD 502Plus, Tokyo, Japan). This device measures the transmittance of the leaf in the red and infrared regions reporting a unit-less index, which correlates with the relative quantity of chlorophyll in the leaf tissue (Castelli et al., 1996). SPAD values (SPAD) were recorded on the uppermost fully developed leaf of each single plant, calculating the average of three measurements taken on three different leaflets outside the middle lamella. After taking SPAD measurements, the respective leaves were cut and their area (LA, cm^2) and length (LL, cm) were determined photographically: leaves were laid planar on a white paper and photographed with a full-frame (21 megapixel) digital single-lens reflex (DSLR) camera (EOS 5D MkII, Canon Inc., Tokyo, Japan), which was converted to a three-band vegetative stress camera (LDP LLD, Carlstadt, NJ, USA, www.maxmax.com), reporting normalized difference vegetation index (NDVI) values. On the basis of NDVI values, the image was separated into plant (high NDVI) and background (low NDVI) pixels, using a custom script within the R-environment (R Development Core Team, 2014).

For chemical analysis, plant material (10 fully developed leafs per plant) was sampled in 2012 and immediately freeze dried. The concentrations of prodelphinidins and procyanidins, the flavonols quercetin, kaempferol, and myricetin (including their glycosides), as well as the polyphenolic acids quinic acid and gallic acid, were analyzed using ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). The analysis was performed on an Acquity UPLC system (Waters Corp., Milford, MA, USA) coupled with a Xevo TQ triple-quadrupole mass spectrometer (Waters Corp., Milford, MA, USA) according to the methods described by Engström et al. (2014). The concentration of total CTs was calculated as the sum of the concentrations of its sub constituents, prodelphinidins and procyanidins. The share of prodelphinidins in the total CT (PD-share) was calculated by dividing the concentration of prodelphinidins by the concentration of total CT.

4.3.3 Genotyping

A subsample of the plant material used for chemical analysis was taken for DNA analysis. The dried leaves were ground using a ball mill (Cell tissue Analyzer 2, Qiagen, Hilden, Germany). DNA was extracted with the illustra™ DNA Extraction Kit PHYTOPURE (GE Healthcare, Little Chalfont Buckinghamshire, United Kingdom) according to the manufacturer instructions. DNA concentration was measured by gel electrophoresis with a mass standard (High DNA Mass Ladder, Invitrogen™, Life Technologies, Carlsbad, USA). For genotyping, SRAP markers and SSR markers were employed. For the SRAP marker system, four fluorescently labeled forward [me1 to me4] and reverse [em1 to em4] primers were applied in 16 combinations in all plants, while sainfoin specific primers (Mora-Ortiz et al., 2016) were used for SSR analysis (Table S4.1). PCR and fragment analysis were performed as described in Kempf et al. (2015) using an iCycler (Biorad, Hercules, USA) and an ABI 3500 Genetic Analyzer (Applied Biosystems, Forster City, USA). Allele counts for the SRAP markers were those used previously to analyze self-fertilization (Kempf et al., 2015), and the approach of counting alleles present in one parent and absent in the other was followed. SSR marker data was analyzed *de novo*.

4.3.4 Statistical analysis

Trait-associated markers (TAM) were detected by marker regression within crossings and selfings of parent 2 (due to the low number of plants [N = 30], selfings of parent 1 were omitted from the analysis). For the analysis, all SRAP and SSR alleles were coded based on their presence (1) or absence (0). P values of marker-trait correlations were obtained with one degree of freedom score test as implemented in the mmscore function of R-package GenABEL (Aulchenko et al., 2007; Chen and Abecasis, 2007). To correct for multiple testing effects, Benjamini-Hochberg q-values were calculated based on the P values obtained using the function qvaluebh95 to obtain significance values for markers at a false discovery rate of 5%. Additionally, population structure within crossings and selfings was investigated. For this, pairwise genetic distances between individuals were calculated based on all marker alleles using the modified Rogers' distance D_w (Wright, 1978), which shows the extent of genetic diversity

between two plants (Lee et al., 1989), ranging from 0 to 1. Principal coordinate (PCO) analysis was then performed on D_w values using the R function cmdscale (R Development Core Team, 2014). PCO analysis indicated the presence of certain unexpected population structures in crossings (Figure 4.1a), which demanded a correction during marker regression. For this, the kinship matrix was calculated (only for crosses) as the proportion of shared alleles among plants (Eding and Meuwissen, 2001) using the R function ibs and was considered as a variance-covariance matrix to correct for random genotypic effects, using the maximum likelihood implementation in the polygenic function of the GenABEL package. No population structure was visible in neither selfing group (Figure 4.1b, c). Therefore, kinship was not corrected for during marker regression in selfings of parent 2.

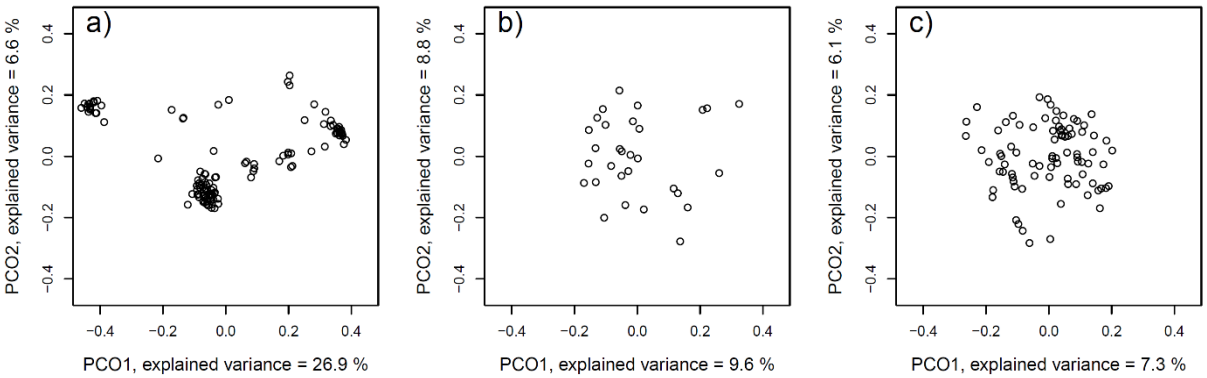

Fig. 4.1 Principal coordinate analysis for a) 122 F_1 progenies derived from a cross between parent 1 and parent 2, b) 30 S_1 progenies of parent 1, and c) 85 S_1 progenies of parent 2, based on 170 SRAP and 208 SSR marker alleles

For traits with significant TAM, a one-way ANOVA was performed to determine the proportion of explained variance and the effect of the TAM (difference between plants showing the presence and absence of the respective TAM). Correlations among phenotypic traits were calculated as Pearson's correlation coefficients. All statistical analyses and calculations were performed within the R-environment (R Development Core Team, 2014).

4.4 Results

4.4.1 Large variation in agronomic traits

All agronomical traits showed clear segregation among the crossings (F_1 progenies), with observed values following a nearly normal distribution except for number of seeds (SN), which was positively skewed due to a large number of plants having less than 500 seeds per plant (Figure 4.2). Therefore, a logarithmic transformation was applied to SN (ln[SN]) when testing for TAM. To a lesser extent, rust susceptibility also deviated from normal distribution, caused by many plants showing a low rust susceptibility (score = 2). SN per plant ranged from 0 to 4608 and large variation was also observed for vigor-related traits such as Vigor, HSu, LA, and LL (Figure 4.2, Table 4.1). The difference between the earliest and latest flowering plant, as assessed by FT, was 46 days. For agronomic traits, the observed ranges of values were comparable between crosses and selfings except for SN, where selfings (mean/max value of 316/1114 in selfings of parent 1 and 447/1483 in selfings of parent 2) showed largely reduced seed numbers per plant compared to crosses (mean/max values of 1265/4608; Figure S4.1, S4.2).

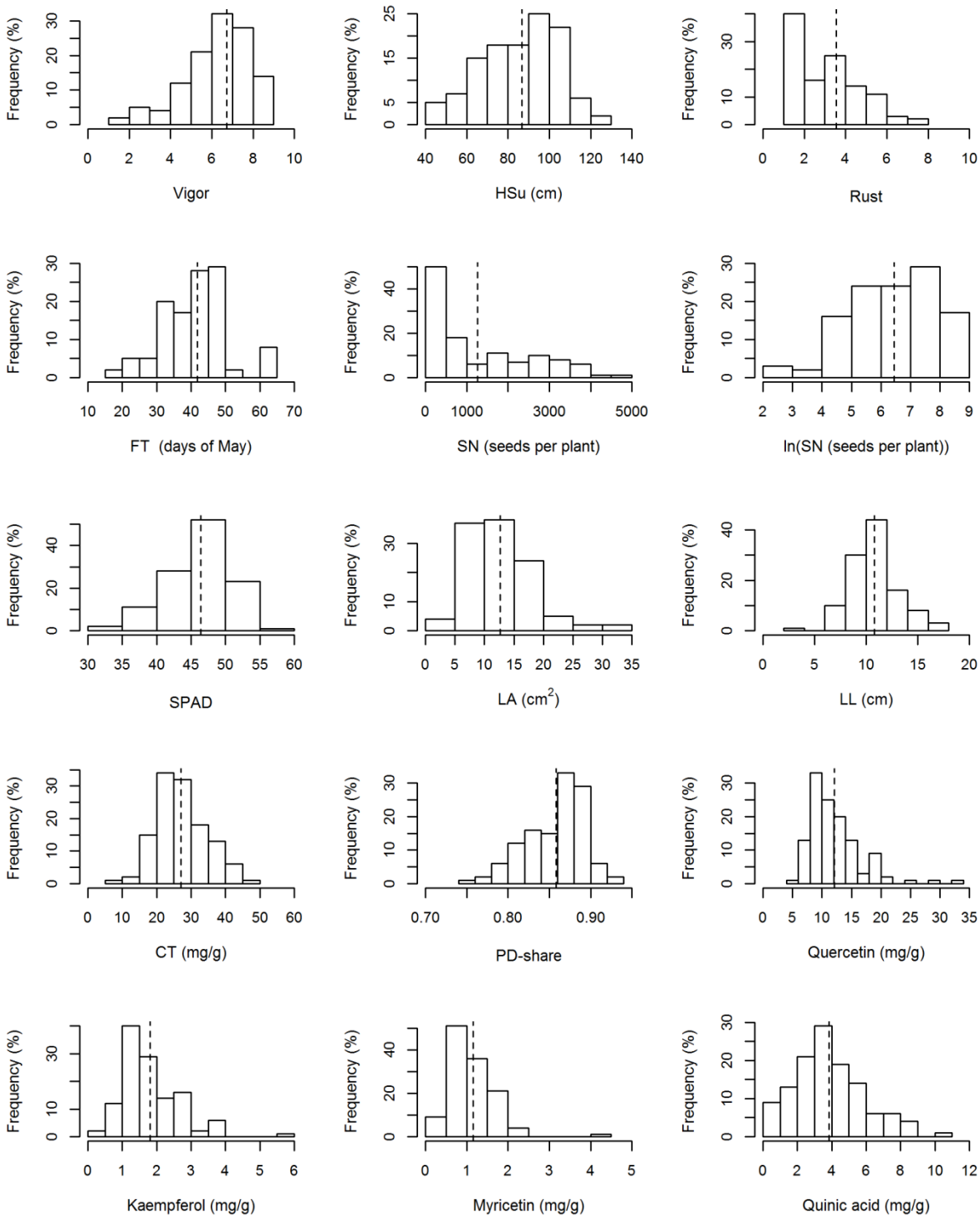

Fig. 4.2 Frequency distribution of agronomic traits and concentration of phenolic compounds in 122 F_1 progenies derived from a cross between parent 1 and parent 2. For abbreviations of traits see Table 4.1.

Table 4.1 Minimum, mean, and maximum observed value as well as standard deviation (SD) and number of plants with observations (# Ind) of agronomical and compositional traits examined in F_1 progenies derived from of a cross between parent 1 and parent 2. The number of SRAP and SSR markers showing significant association to the traits are indicated. Agronomical traits are time of flowering (FT), plant height in spring and summer (HSp, HSu), plant vigor (Vigor), seed number per plant (SN), leaf area and length (LA, LL), SPAD and susceptibility to rust diseases (Rust), all the traits being assessed in 2013 season. Among compositional traits, CT gives the total concentration of condensed tannins and PD-share the share of prodelphinidines on total CT.

	Range of observed values					# sig Marker	
Trait	Min	Mean	Max	SD	# Ind	SRAP	SSR
FT (days of May)	19	41.9	65	9.5	116	0	0
HSp (cm)	8.0	14.9	24.5	3.8	118	0	0
HSu (cm)	46	86.6	129	18.4	118	5	6
Vigor	1.0	6.7	9.0	1.7	118	0	1
SN	0	1265	4608	1254	118	1	6
LA (cm^2)	3.6	12.7	31.8	5.5	112	0	0
LL (cm)	3.8	10.8	16.8	2.3	112	0	0
SPAD	33	46.4	57.1	4.7	117	0	0
Rust	0.0	2.6	7.0	1.7	111	0	0
CT (mg/g)	9.7	27.0	47.2	7.0	122	0	0
PD-share	0.76	0.86	0.93	0.04	122	1	0
Quercetin (mg/g)	5.8	12.1	32.1	4.4	122	0	0
Kaempferol (mg/g)	0.25	1.8	5.59	0.86	122	0	0
Myricetin (mg/g)	0.3	1.15	4.3	0.54	122	0	0
Quinic acid (mg/g)	0.1	3.8	10.2	2.0	122	0	0
Gallic acid (mg/g)	0.09	0.16	0.33	0.05	122	0	0

4.4.2 Quantity and quality of condensed tannin varies among plants

Concentrations of polyphenolic compounds observed for crossings also showed a clear segregation, and were close to the normal distribution (Figure 4.2). For CT, concentrations ranged from 9.7 to 47.2 mg/g plant material, with a mean value of 27.0 mg/g, while the PD-share ranged from 0.76 to 0.93. (Table 4.1). Concentrations of polyphenolic compounds were also assessed in the parental plants, whereby parent 1 (Brunner) showed a CT concentration of 22.3 mg/g with PD-share of 0.88, while the corresponding values for parent 2 (Perdix) were 17.2 mg/g and 0.83, respectively (data not shown). The observed differences in the concentrations of polyphenolic compounds between crossings of parent 1 and parent 2 were also reflected by their respective selfings (Figure S4.1 and S4.2).

For all compounds except for quercetin, where the difference in the concentration between parents was low, selfings of the parent with the higher concentration also showed increased values compared to selfings of the parent with the lower concentration (data not shown). Apart from that, the range of the compositional traits was comparable between selfings and crosses.

4.4.3 Strong correlations among vigor related traits

Among agronomical traits, significant, positive correlations were observed among Vigor, HSp, HSu, and SN, with the correlation being strongest between HSp and HSu, (r = 0.76) and weakest between Vigor and HSp (r = 0.59; Table 4.2). These vigor-related traits were also positively correlated to LA and LL, but to a lower extent. FT was most strongly correlation with ln(SN) (r =-0.34), with later flowering plants exhibiting a lower seed yield.

Table 4.2 Phenotypic correlations among agronomical traits in 122 crossings (F_1 progenies) derived from a cross between parent 1 and parent 2. For abbreviation of traits see Table 4.1.

	Correlated trait							
Trait	HSp	HSu	FT	ln(SN)	Rust	SPAD	LA	LL
Vigor	0.59**	0.62**	-0.23*	0.75**	0.18	0.26**	0.26**	0.18
HSp		0.76**	-0.15	0.47**	0.09	0.36**	0.28**	0.22*
Hsu			-0.02	0.61**	0.10	0.47**	0.25**	0.20*
FT				-0.34**	0.08	-0.09	0.20*	0.29**
ln(SN)					0.30**	0.22*	0.20*	0.14
Rust						0.14	0.23*	0.31**
SPAD							0.14	0.11
LA								0.78**

* = P < 0.05; ** = P <0.01

4.4.4 Weak correlations between agronomic and compositional traits

Among polyphenolic compounds, CT concentration was positively correlated with PD-share (r = 0.60), indicating that prodelphinidins were most important for total CT concentration levels (Table 4.3). CT also showed weak, positive correlations to the other compounds, with the exception of kaempferol, which, together with myricetin, was negatively correlated to quinic acid. Correlations between agronomic and compositional traits were only significant in a few

instances (Table 4.3). Flowering time showed weak-to-moderate correlations with chemical composition, whereby later flowering was associated with higher concentrations of CT, quinic acid, and gallic acid, and lower concentrations of kaempferol and myricetin. Furthermore, Vigor, HSp, and SPAD showed weak, negative correlations to gallic acid concentrations. Correlations between selfings of parent 1 and parent 2 generally had lower significance levels due to the lower number of plants used (Table S4.2 and S4.3). However, the patterns of correlations observed were generally similar to those observed in crossings.

Table 4.3 Phenotypic correlations among compositional traits as well as between compositional and agronomic traits in 122 crossings (F_1 progenies) derived from a cross between parent 1 and parent 2. For abbreviations of traits see Table 4.1.

	Correlated trait						
Trait	CT	PD-share	Quercetin	Kaempferol	Myricetin	Quinic acid	Gallic acid
Vigor	0.12	0.12	0.01	0.09	0.02	0.03	-0.25**
HSp	0.21*	0.21*	-0.10	0.07	-0.05	0.15	-0.33**
Hsu	0.17	0.00	-0.20*	-0.06	-0.16	0.18	-0.17
FT	0.30**	0.04	-0.15	-0.34**	-0.33**	0.27**	0.26**
ln(SN)	0.19*	0.13	0.04	0.13	0.18	-0.01	-0.05
Rust	0.20*	0.23*	-0.06	0.05	-0.08	0.13	0.03
SPAD	-0.04	-0.06	-0.17	0.04	-0.23*	-0.07	-0.24*
LA	0.1	0.11	-0.10	-0.21*	-0.02	0.11	0.05
LL	0.18	0.13	-0.21*	-0.22*	-0.07	0.17	0.09
CT		0.60**	0.33**	0.04	0.22*	0.26**	0.26**
PD-share			0.29**	0.32**	0.28**	0.18	0.21*
Quercetin				0.32**	0.52**	-0.12	0.03
Kaempferol					0.32**	-0.28**	-0.15
Myricetin						-0.35**	0.07
Quinic acid							0.03

* = P < 0.05; ** = P <0.01

4.4.5 TAM were detected for three agronomic and one compositional traits

Across all individuals, 173 and 189 alleles were detected for the 16 SRAP and 48 SSR markers, respectively. In crossings, TAMs were detected for SN (7 TAM), HSu (11 TAM), Vigor (1 TAM,) and PD-share (1 TAM, Table 4.1, 4.4), whereas no TAMs were found within selfings of parent 2 (data not shown). Analyses of logarithmic- and non-transformed values of SN revealed significant values for the same markers (data not shown), thus untransformed SN values are reported for simplicity. Among the observed TAMs, SSR markers were more frequently found

to be linked to phenotypic traits (13 TAM) when compared to SRAP markers (7 TAM). TAMs associated to SN explained between 1.1 and 28.1% of the variation, where the presence of the allele had a positive effect on all SSR marker alleles +991 to 1448 seeds per plant) and a negative effect on the SRAP marker allele (-261 seeds per plant; Table 4.4). For HSu, values of explained variance for the 11 TAMs ranged from 4.4 to 28.6%, with the presence of the allele always having a positive effect and resulting in plants that were 7.9 to 25.6 cm taller. For compositional traits, only one TAM could be found for PD-share, with the SRAP marker allele explaining 12.3% of the observed phenotypic variance, and resulting in a slightly higher share of prodelphinidins.

Table 4.4 P-value, proportion of explained phenotypic variance (% expl. Var.) and effect size (Effect) of trait associated marker (TAM) alleles identified in F_1 progenies derived from of a cross between parent 1 and parent 2. For abbreviations of traits see Table 4.1.

Trait	Marker	Allele size	p value	% expl. Var.	Effect
SN (seeds/plant)					
	OVK_183	274	1.0E-06***	28.1	1447.93
	OVK_141	251	3.0E-04***	24.9	1352.43
	OVK_073	204	4.4E-06***	24.4	1333.25
	OVK_133	205	1.0E-04***	9.9	1025.61
	OVK_155	255	1.0E-04***	9.9	1025.61
	OVK_111	223	6.2E-06***	9.3	991.47
	me4em3	356	1.0E-03***	1.1	-260.66
HSu (cm)					
	OVK_111	223	4.7E-06***	28.6	25.62
	me1em1	299	1.0E-03***	27.3	25.46
	me1em1	175	6.0E-04***	25.4	23.66
	OVK_133	205	1.1E-03***	24.0	23.43
	OVK_155	255	1.1E-03***	24.0	23.43
	me4em1	104	4.0E-04***	22.8	19.12
	OVK_063	191	1.4E-03***	21.3	20.99
	me4em3	127	1.1E-03***	20.0	19.81
	OVK_183	274	2.0E-04***	8.2	11.45
	me1em1	217	1.0E-04***	7.6	10.12
	OVK_073	189	8.0E-04***	4.4	7.87
Vigor	OVK_183	274	4.5E-05***	23.9	1.77
PD-share	me4em4	525	1.0E-04***	12.3	0.02

*** = P < 0.001

For the majority of TAMs detected in crossings, the marker allele was transferred from one parental plant only (data not shown). This is also reflected by the occurrence of the marker alleles in respective selfings, whereby almost all plants

derived from one parent generally possessed the allele and plants derived from the other parent generally did not (Figures 4.3–5).

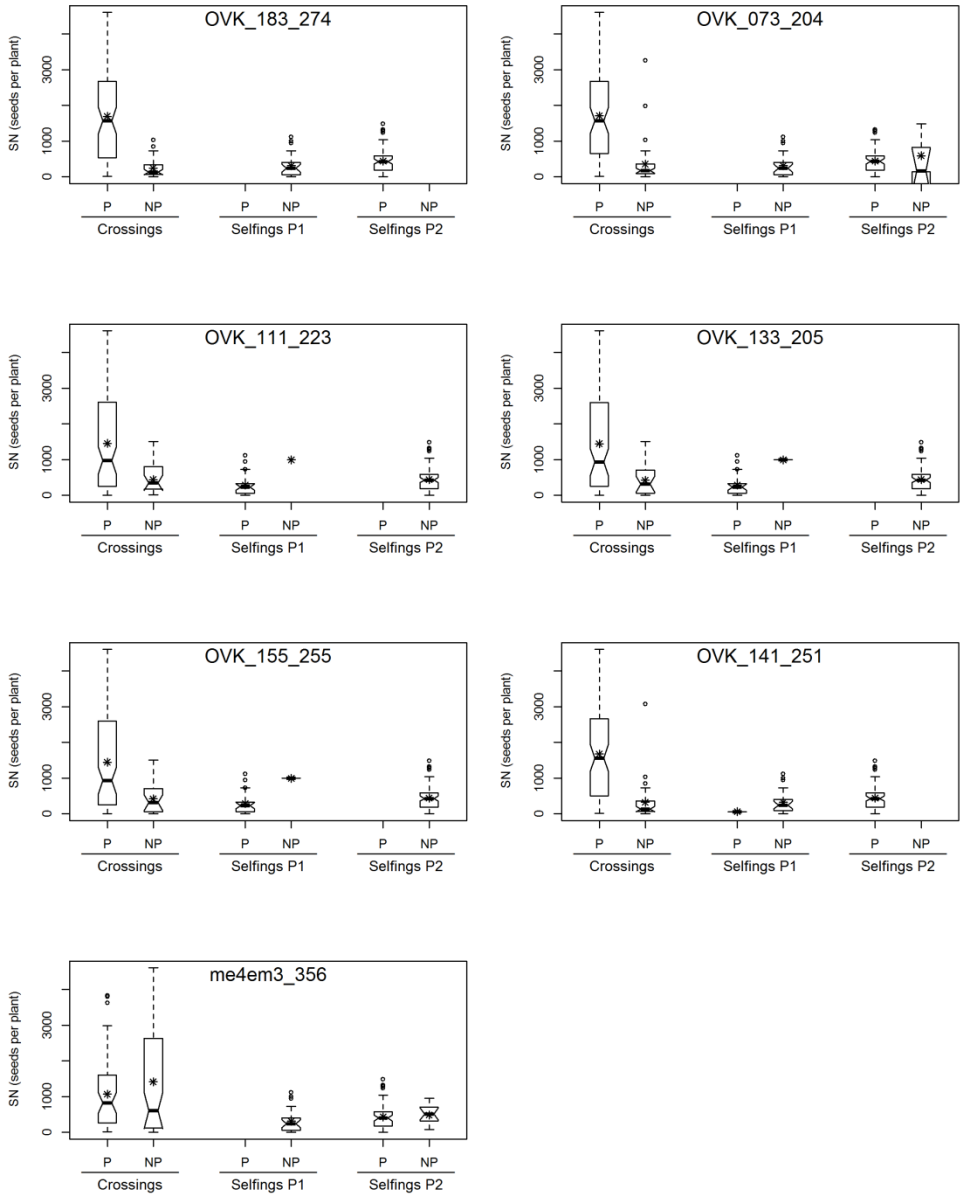

Fig. 4.3 Seed number per plant (SN) dependent on presence (P) and absence (NP) of the marker allele of TAM significantly associated with the trait for 122 F_1 progenies (Crossings) derived from a cross between parent 1 and parent 2, 30 S_1 progenies of parent 1 (Selfings P1) and 85 S_1 progenies of parent 2 (Selfings P2). Stars represent mean values per group.

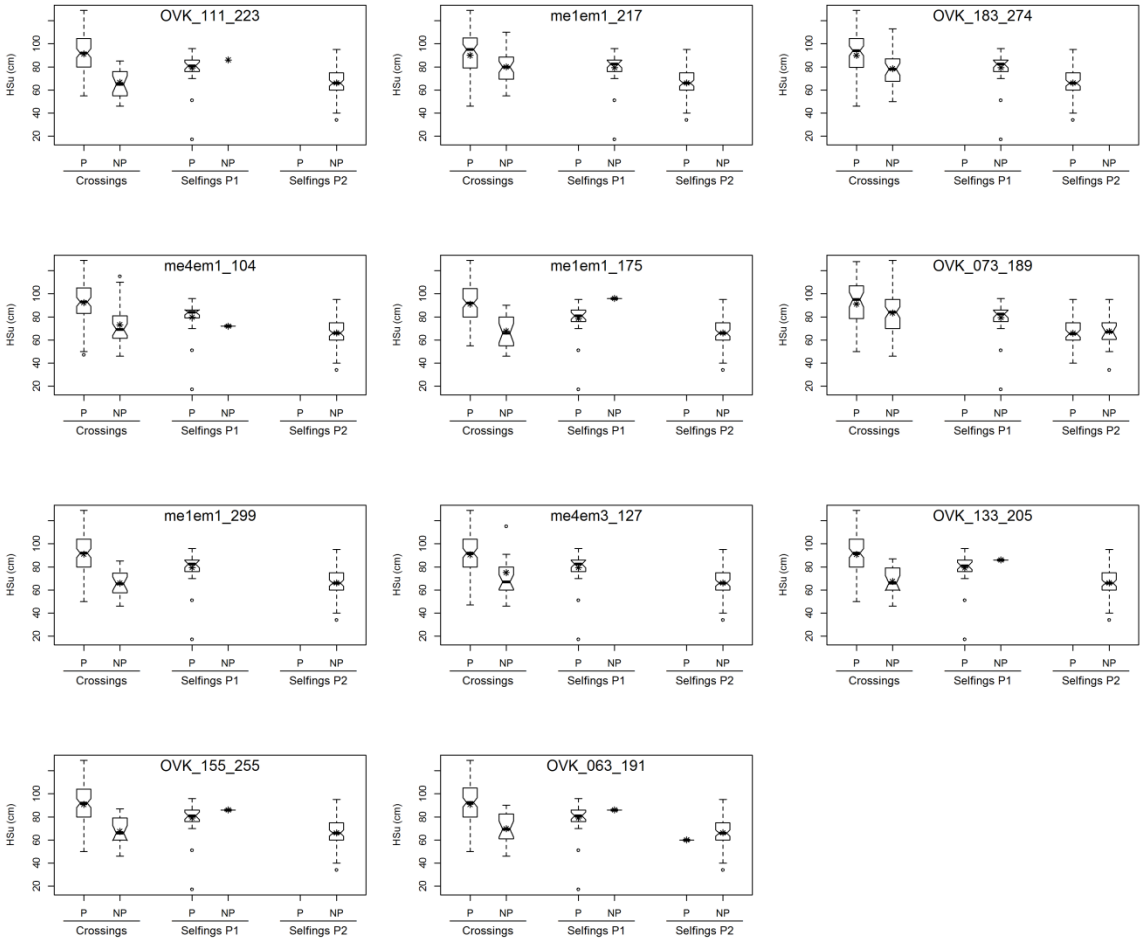

Fig. 4.4 Plant height during summer (HSu) in cm dependent on presence (P) and absence (NP) of the marker allele of TAM significantly associated with the trait for 122 F_1 progenies (Crossings) derived from a cross between parent 1 and parent 2, 30 S_1 progenies of parent 1 (Selfings P1) and 85 S_1 progenies of parent 2 (Selfings P2). Stars represent mean values per group.

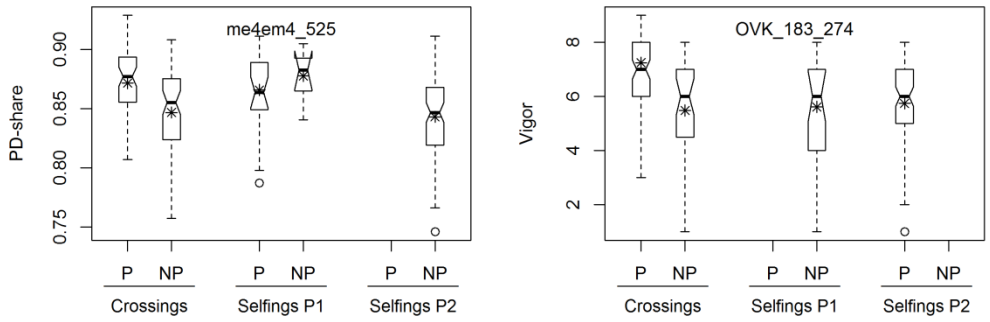

Fig. 4.5. Plant vigor ($Vigor_{13}$) and share of prodelphinidin on total condensed tannins (PD-share) dependent on presence (P) and absence (NP) of the marker allele of TAM significantly associated with the trait for 122 F_1 progenies (Crossings) derived from a cross between parent 1 and parent 2, 30 S_1 progenies of parent 1 (Selfings P1) and 85 S_1 progenies of parent 2 (Selfings P2). Stars represent mean values per group.

In crossings, the presence of TAM marker alleles mostly had a positive effect on plant performance, with only one SRAP marker-allele having a negative effect on seed yield. Due to the fact that plants of one selfing group generally possessed or lacked the respective marker-allele, the effects of alleles from TAM detected in crossings could not be confirmed within selfings of the same parent.

Several TAMs showed associations with multiple, mainly vigor-related traits. For example, OVK_183_274 was associated with SN, Vigor, and HSu, while OVK_133_205, OVK_111_223 and OVK 155_255 were associated with HSu and SN. Correlations among TAMs associated with vigor related traits (SN, Vigor, and HSu) showed two main clusters of marker alleles with strong correlations within and weak correlations outside the clusters (Figure 4.6). The TAMs me4em3_356 (associated to SN) and me4em4_127 (associated to PD-share) showed no clear affiliation to either of the two clusters.

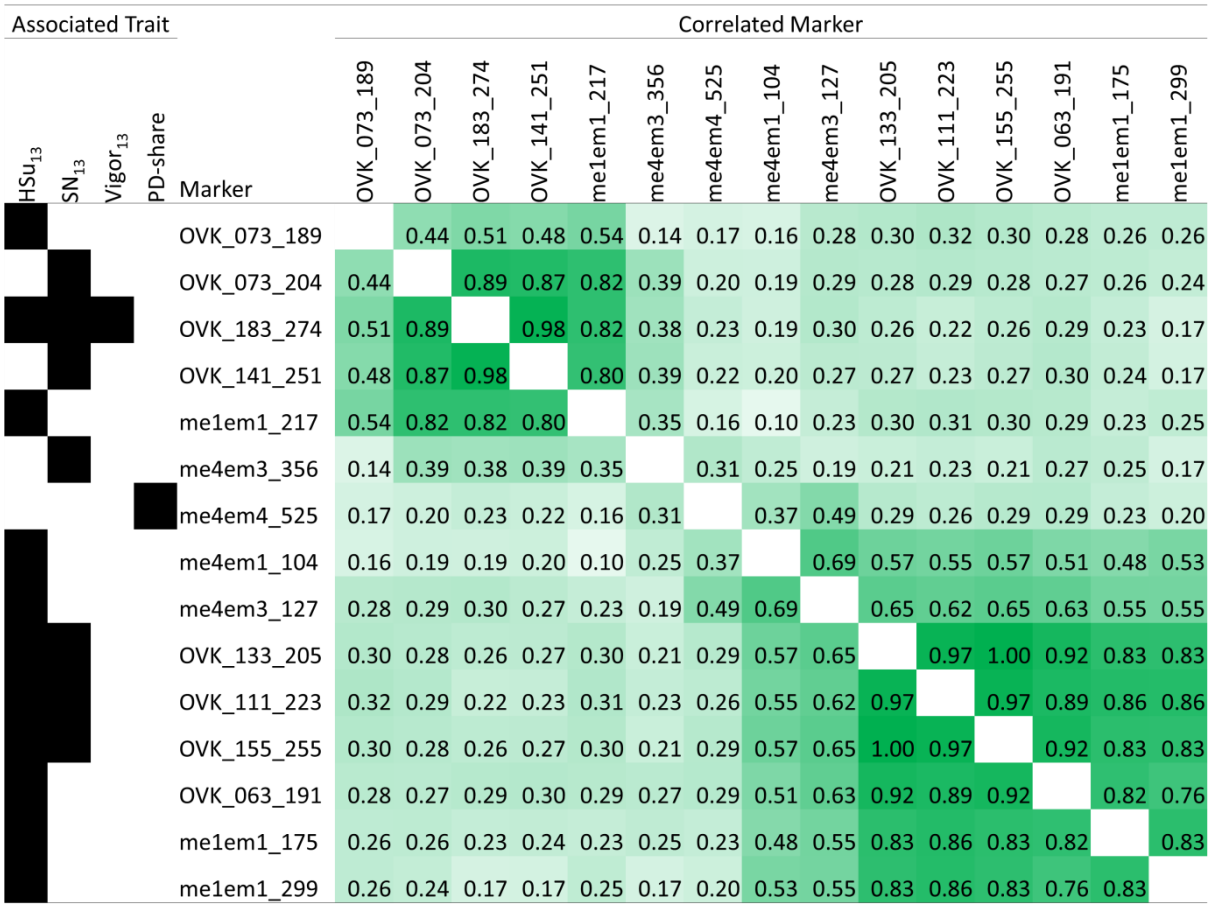

Fig. 4.6 Absolute values of correlation coefficients among trait associated marker (TAM) alleles showing significant association with at least one of the phenotypic traits plant height during summer (HSu), seed number (SN), plant vigor (Vigor) and share of prodelphinidins on total condensed tannins (PD-share). Black squares indicate significant associations of the TAM with one of the respective traits, intensity of green color reflects the size of the correlation coefficient.

4.5 Discussion

The present study reports, for the first time, associations between genetic markers and phenotypic traits in sainfoin, representing a preliminary, but essential step towards MAS for this species. The information gained on TAMs, and on correlations among phenotypic traits, could help to increase efficiency in sainfoin breeding. For example, favorable marker alleles of TAM can be traced in potential parental plants from new varieties to increase their performance. Furthermore, the work effort required in the selection for complex traits such as seed yield might be reduced by selection for correlated traits such as plant vigor or height that can be more easily determined.

4.5.1 Detected trait variation as basis for selection

The presence of sufficient variation in heritable traits is indispensable for the selection of superior individuals. In crossings, for flowering time, the difference between the earliest and latest flowering plant was more than 6 weeks (Table 4.1). This variation is larger than in other forage legumes like red clover, where the difference between the earliest and latest materials of a breeding program ranges between 2 and 3 weeks (Boller, unpublished results). Hence, this variation indicates the potential to create early and late flowering varieties, adapted to different climate regions. Differences in Vigor, Hsu (83 cm), LA (28.2 cm^2), and LL (13 cm) were also substantial, indicating the potential to breed generally stronger plants with a higher chance of survival.

For concentrations of polyphenolic compounds, including total CT, large variation could be found among crossings (Table 4.1). This within-population variation for CT concentration (range = 37.5 mg/g) was higher compared to that observed in earlier studies that analyzed variation among different sainfoin varieties, where ranges of 24.5, 21.8, and 18.5 mg/g were observed (Azuhnwi et al., 2011, 2012; Malisch et al., 2015). The benefit of most of the polyphenolic compounds analyzed in the present study is their antioxidant capacity and anthelmintic effectiveness (Thill et al., 2012). Some individual compounds from these groups are considered more abundant and effective in their anthelmintic properties than others. For example, breeding for higher flavonol composition, i.e., quercetin and its glycoside rutin, might improve the anthelmintic capacity of the forage (Barrau et al., 2005). The range of quercetin concentrations found in our study was between 5.8 and 32.1 mg/g (Table 4.1), showing a very strong correlation ($r = 0.99$) to rutin concentration (data not shown), which is comparable to the range found by Malisch (unpublished results) in a panel of 27 diverse accessions (1.9 to 21.3 mg/g). This indicates the potential to increase the concentration of this active compound via selection in existing breeding pools or by introgression of more exotic material.

4.5.2 TAM detection and validation

Principle coordinate analysis (Figure 4.1a) revealed the existence of population structure, which would not be expected in traditional bi-parental mapping populations, as was the case for our F1 progenies. This could be due to the fact that samples were coded for the presence and absence of SRAP and SSR marker alleles, resulting in two classes of marker phenotypes. Hence, the actual genotype of plants could be biased by the negligence of the allele dose (simplex, 1000; duplex, 1100; triplex, 1110; and quadruplex, 1111). Another possible explanation could be preferential chromosome pairing, a phenomenon observed in allohexaploid wheat and other species, which is caused by the presence of pairing between control loci (Sears, 1976). In the present study, using the kinship matrix as a variance-covariance matrix was a powerful tool to correct for such population structure effects when performing marker regression.

4.5.3 Detected TAMs and potential benefits

The three agronomical traits for which significant TAMs were found (SN, HSu, Vigor) are all related to general plant vigor, are moderately-to-strongly correlated among each other, and share the same TAM (Table 4.2, Figure 4.6), indicating a pleiotropic mode of action for the potential underlying gene loci. Increased seed yield is crucial for sainfoin, and its selection via TAM would be highly effective. This is because, to date, seed yield can only be assessed after the year of establishment in the field, and is highly labor intensive. Furthermore, seed yield is strongly influenced by the environment (Nowosad et al., 2016), reducing trait heritability and, therefore, the response to selection. An efficient strategy to increase seed yield might be to combine MAS using identified TAMs with indirect selection via positively correlated traits that are simple to determine. In our study, plant height ($r = 0.47, 0.61$) and vigor ($r = 0.75$) showed moderate-to-strong correlations with seed yield, i.e., more vigorous plants had a higher seed production potential, and could be used for the indirect selection of plants with increased seed yield. To a lower extent, indirect selection for higher seed yield is also possible by selecting genotypes with earlier flowering ($r = -0.34$).

For compositional traits, one significant TAM was identified for PD-share (Table 4.4). Our results show that it would not be possible to indirectly select for

chemical composition via simple agronomic traits due to the weak correlations between these two groups of traits (Table 4.3). The single TAM found in our study that was associated with higher PD-share could, therefore, be useful to efficiently select plants with a higher PD concentration. This could be desirable, because PDs are thought to be the most beneficial compound of total CTs with the strongest antiparasitic activity (Mechineni et al., 2014). Due to the positive correlation between the two traits (r = 0.60), MAS for PD-share would also increase the total concentration of CT.

4.5.4 Accuracy of TAM to infer the presence of QTLs in sainfoin populations

TAMs detected by simple marker regression are good indicators of the co-occurrence of a marker with QTL (Collard et al., 2005). However, the exact location of the marker within the genome, and the assignment of several TAMs to the same or a different QTL remains unknown without verification in a linkage map; to date, this is not available for sainfoin. However, patterns of correlations among markers or marker alleles might provide an indication of the assignment of TAMs to potential QTL. For example, in perennial ryegrass (*Lolium perenne*), highly correlated markers (r = 0.95) linked to seed yield were found to be associated with the same QTL (Studer et al., 2008). In our study, the two clusters of correlated TAMs suggested the existence of two different potential QTL regions for vigor-related traits, with five and eight associated TAMs, respectively (Figure 4.6). One TAM (me4em3_356) associated with SN could not be assigned to either one of the two clusters and might, therefore, describe a third potential QTL region affecting seed yield (Figure 4.6). This was also the case for the TAM associated with PD-share (me4em4_525), which likely belongs to a different, independent QTL.

The number and effect size of TAMs or potential QTLs, respectively, detected in our study was comparable to results reported for other forage species. For seed yield, three QTLs were detected in red clover and two in perennial ryegrass, explaining a maximum 15.3 and 32.8% phenotypic trait variation, respectively (Herrmann et al., 2006; Studer et al., 2008). This is comparable to 28.1% of the explained variance as observed in our study. For plant height, six QTLs were

reported for perennial ryegrass, and five QTLs were reported for switchgrass (*Panicum virgatum*), explaining 27.9 and 17.4% of phenotypic variation, respectively, which is consistent with the 28.6% found in the present study (Studer et al., 2008; Serba et al., 2015). For plant vigor in alfalfa, four QTLs were detected, explaining up to 23.3% of the phenotypic variance, which is close to the 23.9% found in the present study (McCord et al., 2014).

The precision with which a QTL can be localized by a genetic marker locus is proportional to the number of sampled meiosis (each one providing the opportunity for recombination between the marker and the trait locus) and, therefore, depends on the type of population and its size (Mackay, 2001). TAMs detected in our study are based on 122 F_1 progenies derived from crossing two non-inbred parental plants (parent 1, parent 2). Therefore, the number of meiotic divisions was limited to one per parental plant and progeny. A larger population size would have been desirable; however, due to the large number of self-fertilizations (Kempf et al., 2015), and the generally low seed set, this could not be accomplished. Hence, due to the limited number of crossing-over events, larger parts of chromosome might be linked (and thus, always inherited together). Therefore, distances of observed TAM to the true QTL might be large, and it is likely that the association might be lost in the next generation of plants, when crossing-over occurs between the QTL and observed TAM (Humphreys, 2005). For this reason and the allelic marker diversity between populations, the application of detected TAMs in other sainfoin populations might be challenging. However, they may still be useful in populations derived from crosses of one of the parents used in this study with other materials, e.g. for introgression of a given trait.

4.5.5 Future trends for sainfoin breeding and conclusions

Generally, some of the disadvantages associated with sainfoin could be compensated for by cultivation practices, e.g. by reducing competition from weeds and choosing appropriate companion plants to increase persistence. However, optimised cultivation practices cannot fully compensate the missing adaptation capacity of available sainfoin cultivars to diverse environmental conditions. Genetic improvement may, therefore, not be bypassed in the long term.

In this study, traits relevant for plant survival and reproduction were found to be positively correlated, showing potential for their simultaneous improvement via breeding. Furthermore, a subset of polymorphic SSR and SRAP markers showed its usability for screening populations consisting of self- and cross-fertilized progenies. Thereby, the identification of TAMs based on linear regression methods was feasible for vigor traits, seed yield, and one chemical compound. Those TAMs could be incorporated into sainfoin breeding programs.

In future studies on sainfoin breeding, more information on trait inheritance must be provided. It will be necessary to apply our marker systems to several, larger sainfoin populations with different genetic backgrounds. A further important step will be the establishment of linkage maps to gain information about the location of QTLs on the chromosome. The possibility of inbreeding, which was recently confirmed (Kempf et al., 2015), could, thereby, enable the development of experimental F_2 mapping populations or recombinant inbred lines. With a larger number of sampled meiotic divisions, linkage mapping in such populations, in combination with an increased number of markers and individuals, might allow a more precise determination of QTL positions than would be currently possible with our F_1 population.

4.6 Acknowledgements

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4.7 Supplementary Material

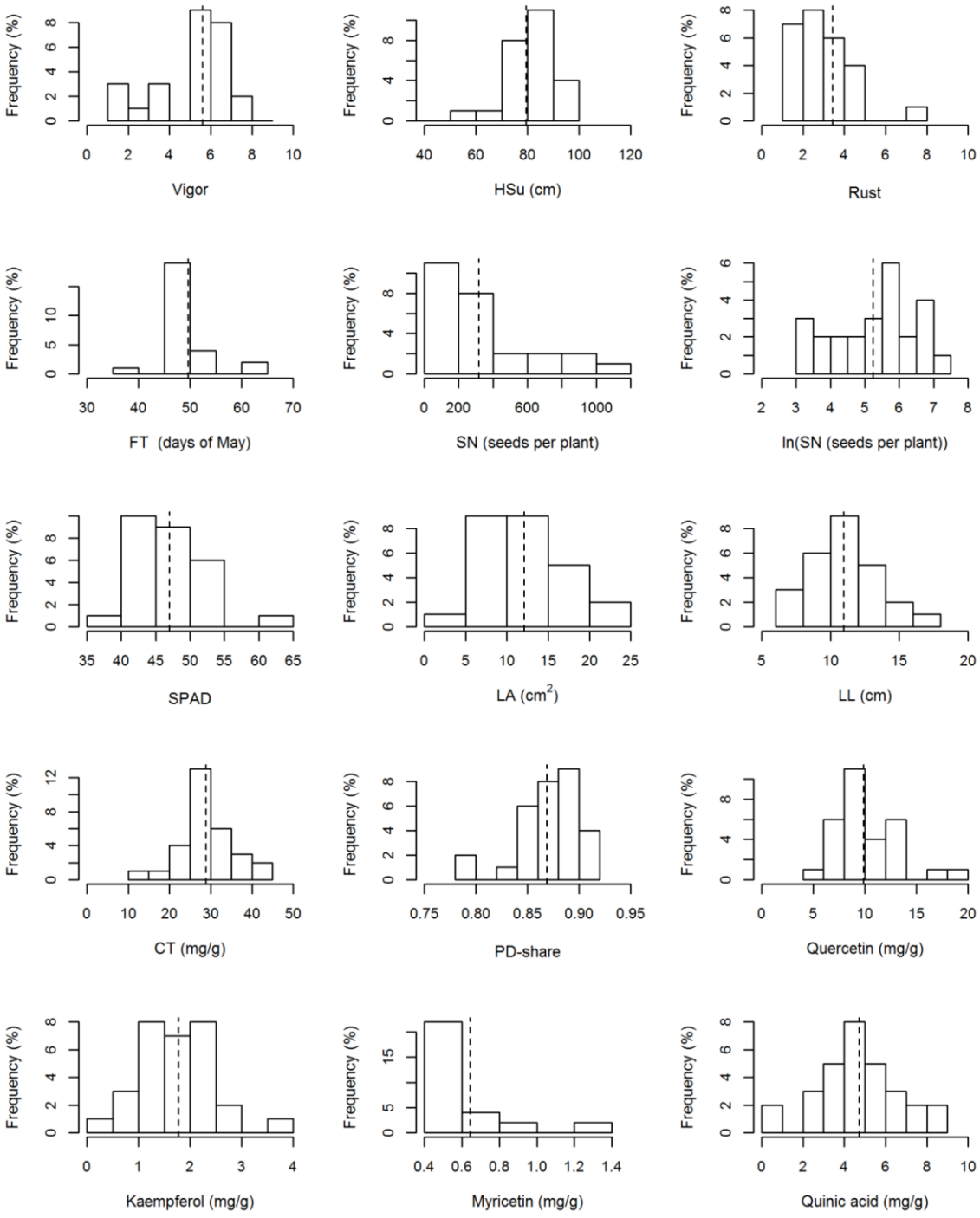

Fig. S4.1 Frequency distribution of performance in agronomic traits and concentrations of phenolic compounds in 30 S_1 progenies derived from parent 1

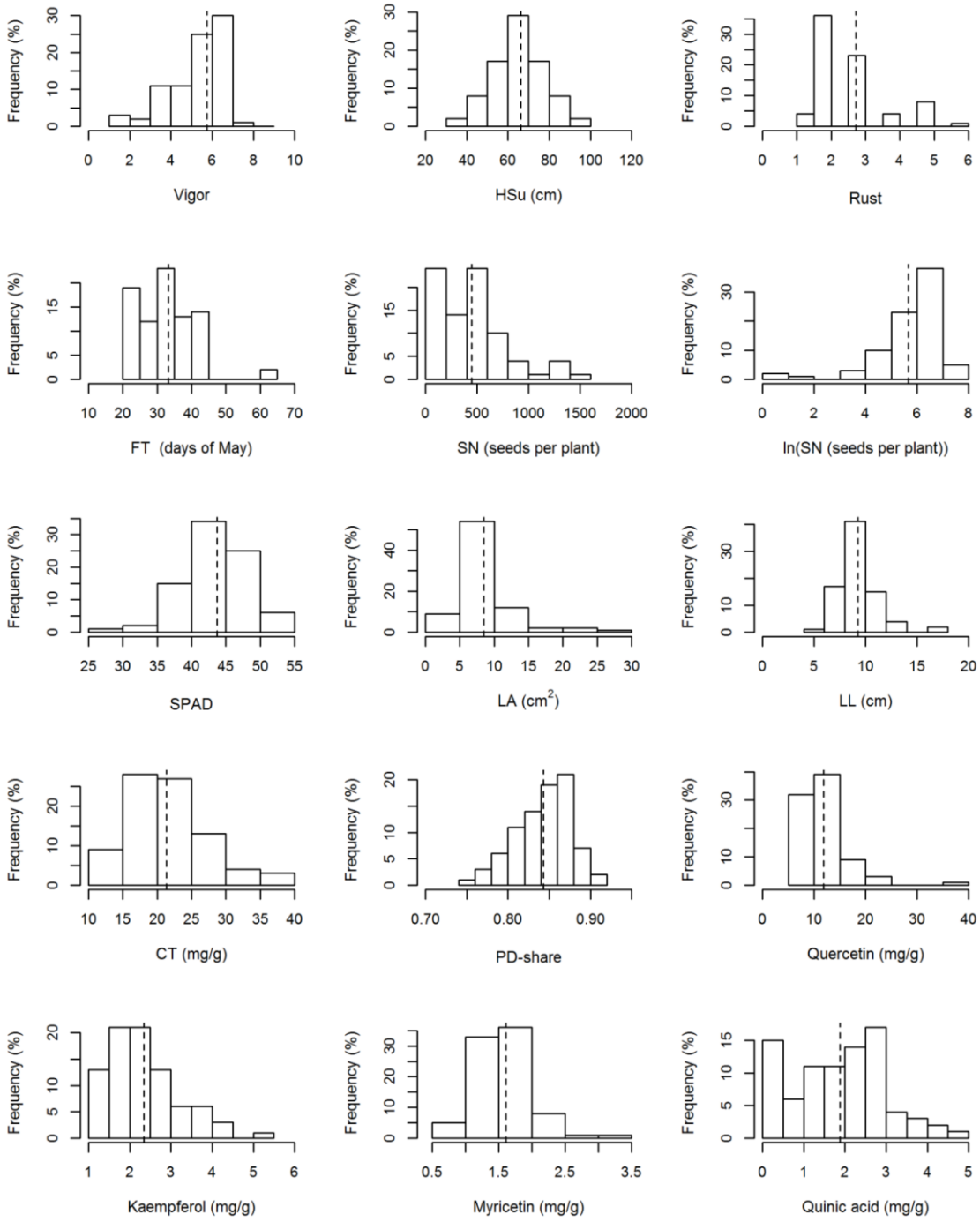

Fig. S4.2 Frequency distribution of performance in agronomic traits and concentrations of phenolic compounds in 85 S_1 progenies derived from parent 2

Table S4.1. Forward and reverse primers for SSR (Kempf et al. 2016) and SRAP (Li and Quiros 2001) markers used to test for marker trait associations

Marker	Forward primer (5‘ - 3‘)		Reverse primer (3‘ – 5‘)	
SSR	OVK002	CCCACCAGACAAAAAGAATA	OVK002	GCTTTCCCCTTCATCAACTAT
SSR	OVK003	GATAGAATTCGTTTGTTGGTG	OVK003	ATCTTTGTAACTGTTCGCTCA
SSR	OVK017	GGGTGTTAGTTATCCATTTCC	OVK017	ACATACTAGCCTTCTGGGGTA
SSR	OVK027	AATGGAATCTCGGAGACAG	OVK027	GGAAGAAGACGAAGTAGTAGGA
SSR	OVK034	GTGAGATGAGCTTGGACATT	OVK034	AGATAACTAACTGCAGGCAAG
SSR	OVK036	GTGTTAAAGGGGTGAAAACAT	OVK036	CATTTTGACAAACCAGTATCC
SSR	OVK038	CCACATACGAGACAGAATAGG	OVK038	CTGAAAATTGATCGATACTGG
SSR	OVK042	GGAACGGTTAATTTCTGATTT	OVK042	AGAATTCCGTACAAGTCGAG
SSR	OVK045	CCAAAAATCATCAATCAACAC	OVK045	TTGAACAAGGGTTAGGGTTAT
SSR	OVK046	TCAACCACATTATAAAACCTCA	OVK046	CGCGAAATCATAGTTCACTT
SSR	OVK054	TTGCAGAGATAACACTCACCT	OVK054	TCCTGAAAAACCTAATCACAA
SSR	OVK055	GAAGATATTTCAAAGCAGCAA	OVK055	CATGCTACCACTAGCAGAAGT
SSR	OVK063	AATTGCAACTGAAACTGAAAC	OVK063	ACTGCTACCCTCTCCATAAAT
SSR	OVK068	GACCACCCGCAGCTCAAC	OVK068	GTCTTCTTCCCCCATATTTAG
SSR	OVK072	TTGCCTTAGTCAGTTACCTTG	OVK072	GTGGAGAGAATGAGAGAACCT
SSR	OVK073	GTAGACAACCGTATCTGGACA	OVK073	AAGATGGAAGGTTCTAGTTCG
SSR	OVK077	GTCCCTCTCTCTCAAATTGTT	OVK077	AGGTTAATGGAGCTTAGTGGT
SSR	OVK089	CAAAGTCATACCAATCACCAT	OVK089	TCTTGGAAGCACTTGTTACTC
SSR	OVK093	CCAAGTGTTTGAAAGTCTCAG	OVK093	TGAGAGTTCGTTCAAGGTAGA
SSR	OVK096	GAGCGTTGCATTTACATTTAC	OVK096	CATCCTCCTTTACACCCTAAT
SSR	OVK097	TCTATAGAGATGAGGCGACAA	OVK097	CGCCCCTAACTAACCTACTAC
SSR	OVK101	GTTGAGTTTCAGACACAGAGC	OVK101	AATAGCTCCCACAATAACTCC
SSR	OVK102	CCAAAGGGTGTTTTATTTTCT	OVK102	GGAAGAAATTAAGCAAATGGT
SSR	OVK107	AAGTTAAAACTTTGCGTTGTG	OVK107	GACGTTGTTCTGGATTTCTTC
SSR	OVK111	TATAGACCTTCTCCTCCCAGA	OVK111	GTGAAAGTCACAAATCCAAAG
SSR	OVK119	ACCCTCCTTCTCTCCTTATTT	OVK119	GACGAGAGAACTCGTTTATGA
SSR	OVK123	CACCCATTAACTATCATGGTC	OVK123	CAAGCCCTTTGTGAGATACTA
SSR	OVK124	GCCTTTTCTGTGACTCGTAA	OVK124	GCTCCATTCCCATTTATAGTT
SSR	OVK125	AAATTTAAGCACCGGAATAAC	OVK125	AAAGCAAAAGGGCTACTAAAG
SSR	OVK126	CGACAAAACTATTTAGGCAAA	OVK126	GGGAAGAGATCATAAACCCTA
SSR	OVK127	GCCCAAAATGTATTATCCTTC	OVK127	AGAACAGACAGATATGCAAGC
SSR	OVK133	TGCTTCAGCATTATTGTAACAT	OVK133	TGCACTTCTCCATACTTCCTA
SSR	OVK138	TAATATGGTGCAAGTTCCAAT	OVK138	TTCTACGCTTAGCTCAAACC
SSR	OVK141	GAGGAGGTACATACAGCACAG	OVK141	CAACCTCCTCGTTATCTTTTT
SSR	OVK142	AACATGACTACTGTGAACAAGG	OVK142	CGAACATGTAATTGATCCAAG
SSR	OVK155	CAGGTTTGAAGTAGCAGAGAA	OVK155	GTAGACCACGCATACTGAATC
SSR	OVK158	TCAGAGTGTGTTGTGTTGTGT	OVK158	AGTGAAGCAAATGTGTGATTT
SSR	OVK159	CATTATTGCCTAGCATTGTTC	OVK159	ATTTCACCATCAAGTATGCAC
SSR	OVK161	AAAGCTTTCTACACGTTGGTA	OVK161	TGGGTTTTTACACTCTGTGAT
SSR	OVK165	TTTCAAACACTCACTCACTCC	OVK165	TCGGATTTGTGACCTAACTC
SSR	OVK168	AATTATCACCCACTGCTATGA	OVK168	GGTTTCCATCACTGTTTGTTA
SSR	OVK172	TTATTAAACCTGCGTCTTCTG	OVK172	GTAGAGCTGTGGGCTTTATCT
SSR	OVK173	TCGTTCTCGTGATTATTCTGT	OVK173	CCTCTATTCAAATAGGGCAAT
SSR	OVK174	ACATGATCGTGAATATGAAGC	OVK174	CAGCAGCAATCAATATATCATC
SSR	OVK175	GTAAAATATCAAGCAGGAGCA	OVK175	AAACTATGCAGACACCCTGTA
SSR	OVK177	TCTGTTGATTTAAGGAGACGA	OVK177	CTCTTGCTCATATTTTCCTCA
SSR	OVK183	GAGGGTAAGAGAGAGTGGAAG	OVK183	CTTGCCTGATATCTTCTCAAA
SSR	OVK196	TTTTGAGAGTGTGGAAGGTTA	OVK196	AGTATGAGCCTGATGATGATG
SRAP	me1	TGAGTCCAAACCGGATA	em1	GACTGCGTACGAATTAAT
SRAP	me2	TGAGTCCAAACCGGAGC	em2	GACTGCGTACGAATTTGC
SRAP	me3	TGAGTCCAAACCGGAAT	em3	GACTGCGTACGAATTGAC
SRAP	me4	TGAGTCCAAACCGGACC	em4	GACTGCGTACGAATTTGA

Table S4.2. Pearson's correlation coefficients among phenotypic traits assessed in offspring derived from self-fertilization of parent 1. Agronomic traits assessed are vigor (Vigor), plant height in spring (HSp) and summer (HSu), flowering time (FT), seed number per plant (SN), sensitivity to rust (Rust), SPAD, leaf area(LA) and leaf length (LL), with the number in subscript giving the year of measurement (2012, 2013). Quality traits are concentration of condensed tannins (CT), share of prodelphinidines on total CT (PD-share) as well as concentration of quercetin, kaempferol, myricetin, quninic acid and gallic acid. Numbers highlighted in green and red indicate that the corresponding correlation coefficient in offspring derived from cross-fertilization between parent 1 (P1) and parent 2 (P2) was at minimum 0.30 higher or lower, respectively.

	Correlated Trait														
Trait	HSp	HSu	FT	ln(SN)	Rust	SPAD	LA	LL	CT	PD-share	Quercetin	Kaempferol	Myricetin	Quinic acid	Gallic acid
Vigor	-0.14	0.67**	-0.50**	0.80**	0.28	0.21	0.27	0.24	-0.14	0.16	-0.06	0.03	-0.09	-0.58**	0.38
HSp		0.10	-0.04	-0.23	0.11	-0.07	0.30	0.24	0.16	0.01	0.02	0.20	0.00	-0.21	0.07
HSu			-0.31	0.27	0.21	0.11	0.41*	0.32	-0.19	0.01	-0.03	-0.08	0.01	-0.34	0.29
FT				-0.42*	-0.45*	-0.20	-0.19	-0.04	-0.27	-0.56**	-0.18	-0.14	-0.26	0.19	-0.31
ln(SN)					0.17	0.10	0.04	-0.06	-0.04	0.24	0.05	0.17	-0.16	-0.44*	0.27
Rust						-0.15	0.11	0.16	-0.26	0.22	-0.30	-0.20	-0.25	-0.32	0.02
SPAD							0.12	0.07	'0.20	-0.26	0.22	-0.46*	0.30	0.02	-0.08
LA								0.87**	-0.14	0.02	0.02	0.03	0.13	-0.18	0.09
LL									-0.18	-0.14	-0.10	-0.17	0.03	-0.18	-0.09
CT										0.35	0.65**	0.01	0.53**	0.17	-0.17
PD-share											0.14	0.25	0.34	-0.05	0.09
Quercetin												-0.02	0.39*	0.33	-0.18
Kaempferol													-0.23	0.04	0.28
Myricetin														0.21	-0.14
Quinic acid															-0.38*

Table S4.3. Pearson's correlation coefficients among phenotypic traits assessed in offspring derived from self-fertilization of parent 2. Agronomic traits assessed are vigor (Vigor), plant height in spring (HSp) and summer (HSu), flowering time (FT), seed number per plant (SN), sensitivity to rust (Rust), SPAD, leaf area(LA) and leaf length (LL), with the number in subscript giving the year of measurement (2012, 2013). Quality traits are concentration of condensed tannins (CT), share of prodelphinidines on total CT (PD-share) as well as concentration of quercetin, kaempferol, myricetin, quninic acid and gallic acid. Numbers highlighted in green and red indicate that the corresponding correlation coefficient in offspring derived from cross-fertilization between parent 1 (P1) and parent 2 (P2) was at minimum 0.30 higher or lower, respectively.

	Correlated Trait														
Trait	HSp	HSu	FT	ln(SN)	Rust	SPAD	LA	LL	CT	PD-share	Quercetin	Kaempferol	Myricetin	Quinic acid	Gallic acid
Vigor	0.45**	0.47**	-0.17	0.73**	0.18	0.25*	0.33**	0.32**	0.15	0.12	0.07	-0.06	-0.04	-0.08	0.00
HSp		0.61**	-0.12	0.28*	0.34**	0.15	0.09	0.12	0.20	0.26*	0.24*	0.11	0.15	-0.11	-0.07
Hsu			-0.11	0.28*	0.10	0.33**	0.16	0.19	0.21	0.06	0.29**	-0.04	0.09	0.00	-0.10
FT				-0.34**	-0.18	-0.17	-0.11	-0.09	-0.16	-0.20	-0.11	-0.01	-0.23*	0.03	0.15
ln(SN)					0.23*	0.14	0.20	0.21	0.07	0.15	0.02	0.00	0.00	-0.15	0.01
Rust						0.08	0.17	0.10	0.34**	0.37**	0.29*	0.19	0.30**	-0.15	-0.06
SPAD							0.10	0.06	-0.13	-0.10	0.06	0.03	-0.10	0.12	-0.24*
LA								0.87**	0.15	0.09	-0.08	-0.09	0.01	-0.05	-0.11
LL									0.17	0.06	0.00	-0.12	0.03	-0.10	-0.03
CT										0.55**	0.55**	-0.17	0.58**	-0.04	-0.15
PD-share											0.35**	0.42**	0.52**	-0.05	-0.14
Quercetin												0.03	0.70**	-0.04	-0.13
Kaempferol													0.12	-0.03	-0.11
Myricetin														-0.07	-0.16
Quinic acid															-0.16

Chapter 5 A first linkage map for tetraploid sainfoin (*Onobrychis viciifolia*)

5.1 Introduction

5.1.1 Use and construction of linkage maps

Availability of information on the position of different markers within the genome is a prerequisite for the application of advanced quantitative trait loci (QTL) mapping techniques like simple interval mapping (SIM, Marquez-Cedillo et al. 2001) or composite interval mapping (Doligez et al. 2002). Such methods allow precise determination of QTL locations, e.g., also between two observed marker loci. Thereby it is also possible to infer if different markers showing significant association to the same trait are associated with the same QTL or different QTLs, what is not possible by simple marker regression as performed in chapter 4. The sequence order and the actual positions of genetic loci and their distances among each other can be identified if a reference genome, i.e., the haploid representation of a genome as DNA sequence assembled from sequence information from one (e.g. B73 reference genome "B73 RefGen_v3" of maize, Andorf et al. 2016) or several donor genotypes (e.g. human reference genome, http://www.ncbi.nlm.nih.gov/
projects/genome/assembly/grc/human/), is available. That information could be transferred into a physical map of the chromosomes giving the actual distances of genetic loci in base pairs (bp). However, with the absence of a reference genome as the case for sainfoin, the establishment of physical maps is not feasible. Here, genetic linkage maps are a good alternative to gain a first insight into the structure of a genome, giving the positions and order of known genetic markers and genes relative to each other on plant chromosomes. Ideally, the position of the markers in a genetic linkage map reflects their actual physical position.

Linkage maps are built upon the theory that recombination events via crossing over (gene exchange between homologous chromosomes) during meiosis occur more frequently between genes or markers located distant to each other than between genes or markers at closer distance on a chromosome (Sturtevant 1921). This phenomenon of linkage between markers violates the second Mendelian law of independent assortment of genes to offspring after crossing two heterozygous parents (Mendel 1866). The order and distance of linked markers can be

estimated by the recombination frequency (RF), which gives the probability of recombination between two markers. Linkage is indicated by RF < 0.5, with smaller values indicating shorter distances between the marker loci. Unlinked marker loci show RF of 0.5, meaning they are at the two ends of the same chromosome or on two different chromosomes. Groups of markers with low RF among each other are denoted as linkage groups. Ideally, each linkage group is representative for a chromosome, with the total number of linkage groups corresponding to the haploid chromosome number of the individual. The RF among the different linkage groups corresponds to the maximum value of 0.5. Positions of markers within a linkage group are usually given in the genetic map distance (D) using the unit centimorgan (cM), where 1 cM corresponds to one recombination event between two marker loci in 100 meiosis (1% recombination). Double crossing over (two crossing over events occurring between two marker loci), of which the chance increases with distance between markers, results in no recombination between the two markers. Therefore, RF tends to underestimate the distance between two linked genes, especially if they are at larger distance. Hence, RF and D show a logarithmic relationship and the Haldane ($D = 0.5 \times \ln(1-2\times RF)$ (Haldane 1919) or Kosambi function ($D = 0.25 \times \ln((1+2RF)/(1-2RF))$ (Kosambi 1943) might be used to translate RF into D, correcting for effects of double crossing over. A further common value to assess linkage is the logarithm (base 10) of odds (LOD) score, which is a likelihood statistic for obtaining the marker combinations by chance, or due to linkage. The critical LOD value for indication of linkage is ≥ 3 (Page et al. 1998).

5.1.2 Populations for creation of linkage maps

A basic requirement to create a linkage map is the establishment of an experimental mapping population with segregation of genes in the offspring. Typical examples are F_2 populations, backcross populations (BC) or recombinant inbred lines (RIL) developed from the biparental cross of two inbred parents (Armstead et al. 2002; Sindhu et al. 2014). These populations have to be genotyped with a sufficient number of markers. Generally, the number of markers required increases with the number of recombination events having happened among the individuals of the mapping population, e.g., is larger for a

RIL population where several recombination events happened during repeated self-fertilization starting from F_2 progenies of the initial biparental cross, compared to the F_2 population itself.

However, all these populations rely on inbred parental plants, which are difficult to obtain for outbreeding species where self-fertilization is often difficult or impossible. Therefore, F_1 full sib families, generated by crossing two heterozygous parental plants, are often used in such species for linkage mapping (Pereira et al. 2013). However, linkage analysis is more complicated in full sub families from outbreeding species, due to different heterozygosity levels in the parents, markers with varying number of segregating alleles and unknown linkage phases of marker pairs (Maliepaard et al. 1997). Taking these problems into account, the so-called pseudo-testcross approach is used in mapping F_1 populations, only including markers segregating in a 1:1 ratio (Margarido et al. 2007) in the offspring. This refers to presence of the marker allele in one parent (heterozygous for the allele) and to absence in the other parent (null allele). With sainfoin being an outbreeding species, application of the pseudo-testcross approach to F_1 progenies of biparental crosses as determined in chapter 2 and employing genetic markers described in chapter 3, segregating with a 1:1 ratio in F_1 progenies, would be the best approach to create a first linkage map for this species.

5.1.3 Mapping software for polyploid species

For diploid species, linkage mapping is well-established with many mapping software packages like Mapmaker (Lander et al. 2009), Joinmap (van Oijen 2006) or OneMap, the latter being particularly developed to handle F_1 populations (Margarido et al. 2007). However, sainfoin is a tetraploid species of which the allo- or autopolyploid origin yet has to be determined. Hence, special software packages have to be employed, accounting either for the auto- or allotetraploid case.

In case of allotetraploid species, i.e., polyploidy arisen from hybridization of two different ancestor genomes (Leitch and Bennett 1997), chromosome pairing and crossing over during meiosis mainly occurs between the two homologous chromosomes (chromosomes from the same diploid ancestor species), resulting in

a so-called disomic behavior (Butruille and Boiteux 2000). In this case, homeologous chromosomes (chromosomes from the different ancestor species) could be treated like different chromosomes of a diploid species and the same statistics and software packages could be applied. The applicability of this approach has been shown by the successful use of MapMaker and JoinMap software packages for mapping hexaploid wheat, an allotetraploid species (Buerstmayr et al. 2002; Risser 2010). Ideally, the number of linkage groups corresponds to the sum of the haploid chromosome numbers of the subgenomes in allotetraploids.

In autotetraploids, like patato (*Solanum tuberosum*) where the polyploidy developed by the combination of genomes within a diploid species (Otto and Whitton 2000), TetraploidMap (Hackett et al. 2007) is the only software available so far for this type of polyploids. Issues that have further to be taken into account in autotetraploids are multivalent chromosome pairing (Fig. 5.5), double reduction and homology discrepancies between chromosomes. Double reduction can occur as consequence of multivalent chromosome pairing when crossing over occurs between two sets of sister chromatids that subsequently migrate to the same pole. Under this situation, it is possible for a chromatid and its recombinant copy to end up in the same gamete, e.g., genotype A000 can produces the diploid gamete AA in autotetraploids (Mather 1935; Bourke et al. 2015). Double reduction is, hence, exclusive for autoteraploid species, whereby the outcome are departures from supposed random pairing and distorted gene segregation ratios (Gar et al. 2011), violating the biological assumptions that are the base for linkage mapping statistics. TetraploidMap software indicates the likelihood for double reduction, whereby markers showing significant double reduction have to be rechecked for genotyping errors or must be discarded from the analysis. So far, linkage maps have been successfully created in autotetraploid orchard grass (*Dactylis glomerata*, Xie et al. 2012), chokecherry (*Prunus virginiana*, Wang et al. 2014), cut rose (*Rosa hybrida*, Gar et al. 2011), switchgrass (*Panicum virgatum*, Liu et al. 2012) and potato (Hackett et al. 2013). The number of linkage groups in autotetraploids refers to the sum of the haploid chromosome number of the single ancestor genome.

In sainfoin, no linkage map has been created so far, mainly due to the limited availability of genetic markers. Most investigations have been based on dominant markers such as inter simple sequence repeat (ISSR) and randomly amplified polymorphic DNA (RAPD) marker (Rasouli 2013; Zarrabian et al. 2013), which have the disadvantage that heterozygous marker loci are not readily identified. A few investigations used co-dominant SSR markers derived from other legume species such as barrelclover (*Medicago truncatula*) and soybean (*Glycine max,* Demdoum et al. 2012; Avci 2014). The adaptation and application of dominant sequence related amplified polymorphism (SRAP) markers in sainfoin (Kempf et al. 2015, chapter 2) as well as the recent development of sainfoin specific, co-dominant SSR marker through Ilumina based next generation sequencing (Mora-Ortiz et al. 2016) have opened the route for the creation of a first linkage map in sainfoin. However, the origin of polyploidy is still not known and the unknown segregation patterns of sainfoin chromosomes have to be kept in mind, demanding the examination of different approaches. Hence, the aim of the present research was the use of co-dominant SSR and dominant SRAP markers in a first attempt for linkage mapping in sainfoin. Thereby, software designed for autotetraploid as well as allotetraploid species was used due to the unknown origin of polyploidy in sainfoin.

5.2 Material and Methods

5.2.1 Plant material and marker genotyping

For the creation of a linkage map, we used a F_1 population (n=122) from a biparental cross between two plants of the varieties Brunner (P1; Agroscope, Zurich, Switzerland) and Perdix (P2; Agroscope, Nyon, Switzerland), respectively. This corresponds to the same F_1 population as used for simple marker regression presented in chapter 4, excluding S_1 progenies (originating from self-fertilization of one of the two parental plants) from analysis. The population is expected to show gene segregation due to heterozygosity in the parental plants. DNA of the two parental plants and the 122 F_1 individuals was extracted from freeze dried, grounded leaf material using the illustra™ DNA Extraction Kit PHYTOPURE

(GE Healthcare, Little Chalfont Buckinghamshire, United Kingdom) according to the manufacturer's instructions. DNA concentration was measured by gel electrophoresis with a mass standard (High DNA Mass Ladder, Invitrogen™, Life Technologies, Carlsbad, USA). Marker resources comprised 16 SRAP primer combinations based on four fluorescently labeled forward and reverse primers (Li and Quiros 2001) as well as 48 fluorescently labeled, sainfoin specific SSR markers (see Table S4.1). For both marker systems, 10 ng genomic DNA was used for PCR as described in chapter 2.

PCR amplicons were analyzed with the fragment analysis function on the Applied Biosystems 3500/3500XL Genetic Analyzer (AB applied biosystems, Forster City, USA) using 1 µl of the undiluted PCR product, 0.5 µl LIZ 600 (GeneScanTM-600LIZ® Size Standard; AB, applied biosystems) and 10 µl Formamide (Hi-Di™ Formamide; AB, applied biosystems). The mix was heated at 94 °C for 5 min and subsequently cooled down before fragment analysis. The resulting data were analyzed with GeneMarker software (Softgenetics, V2.4.0 Inc., State College, USA). SSR marker alleles found in the parental plants were scored for presence (1) and absence (0) in the offspring. For SRAP markers, only alleles present in one parent and absent in the other parent were scored in the offspring.

5.2.2 Approaches for creation of linkage maps

Accounting for the missing knowledge on the origin of polyploidy in sainfoin, we used TetraploidMap (Hackett et al. 2007) as a tool specifically developed for autotetraploid species and JoinMap (van Oijen 2006), which was developed for diploid organisms, to account for the allotetrapoid case. The procedures for creation of linkage maps, separately for each parent, followed the developer's instructions in the corresponding manuals.

For analysis with TetraploidMap, all available markers were imported. The software automatically infers the possible parental marker genotypes based on the segregation ratios in the offspring. Only markers with a segregation ratio of 1:1 or 1:5 in the offspring, which refers to simplex (1000 x 0000) and duplex (1100 x 0000) crossings in the parents, respectively, were selected for further analysis

because crossing over events are only visible in these two marker classes (Fig.5.6). Markers were distributed into groups after test for random segregation using chi square test of independence. The number of groups was chosen according to the haploid chromosome number of sainfoin (= 7) or according to the most likely group formation as visualized by the cluster dendrograms, the maximum number allowed by the software being 12 (the haploid chromosome number of potato). Furthermore, suspicious markers showing outlying clustering were excluded from the analysis and cluster analysis was repeated to reach more homogenous grouping. Linkage groups (LG) were formed according to a critical recombination frequency (RF_{Crit}) of 0.35, 0.3 and 0.25 as advised by the developers, with marker pairs showing $RF < RF_{Crit}$ belonging to the same and marker pairs showing $RF > RF_{Crit}$ belonging to different LG. Ordering of markers within independent LG was conducted following three steps: (1) initial ordering with a seriation algorithm, (2) two-point analysis calculating the RF value and (3) LOD score for each marker pair and ripple ordering. Before these steps, markers with significant double reduction according to chisquare test-statistics delivered by the program were discarded. Because TetraploidMap could only handle 52 markers per LG, further markers had to be deleted before calculating map distances for large LGs. In this case, markers with the lowest p-value from chi-square tests for double-reduction were discarded.

JoinMap does not infer the possible parental marker genotypes based on the segregation ratios in the offspring. Therefore, markers were previously tested for their segregation ratio and only those showing a 1:1 ratio were imported into the software. The population was classified as outbreeding full sib family (CP) and mapping was conducted for each parent separately. Information on likelihood of double reduction is not provided by JoinMap, but markers are tested for deviation from the Mendelian segregation ratio. Grouping of the marker loci to LGs was carried out based on RF, allowing a direct comparison to TetraploidMap, which also uses RF for formation of linkage groups. RF should be smaller than 0.5 to suggest linkage of marker loci. No markers were removed from the analysis. After determining the number of LG according to RF with the most likely grouping, the linkage maps were calculated using the Monte Carlo maximum likelihood (ML) mapping algorithm in a pseudo-testcross analysis (van

Ooijen 2011). After grouping, strongest cross link information (SCL) of each marker was provided by the software, a parameter indicating linkage with markers outside the respective LG. For JoinMap, all markers were included for creation of linkage maps. Marker information, the corresponding LGs and genetic distances as obtained by both software packages, were exported and linkage maps were drawn using MapChart (Voorrips 2002) software.

5.3 Results

5.3.1 Grouping of markers with TetraploidMap

Preselection of markers with 1:1 and 1:5 segregation ratios yielded in 133 markers for parent 1 (P1) and 136 markers for parent 2 (P2). Double reduction was indicated for 17 (12.8%) markers in P1 and 25 (18.4%) markers in P2. In P1, initial clustering of markers into seven groups (the expected number according to the haploid genome size) produced inhomogeneous groups with 107 markers in one and the remaining 26 markers distributed over the other six groups (Table 5.1). Reducing the number of linkage groups to < 7 (what would correspond to increasing RF_{Crit}) led to an increased size of the largest group and decreased size of the smaller groups. The average linkage cluster dendrogram indicated an optimum number of eight LG (data not shown), which corresponds to an RF_{Crit} of 0.3. If the number of LGs was increased to > 8, the largest group remained unchanged, indicating certain stability of linkage relations within this group. In P2, clustering of selected markers into seven LGs resulted in two large LGs (64 and 47 marker) and five small LGs (Table 5.1). The curve of the average linkage clustering indicated an optimum grouping of markers into two groups (data not shown), which corresponds to an RF_{Crit} between 0.25 and 0.30 (TetraploidMap does not report the actual RF_{Crit} given a certain number of LGs). However, this yielded one large group with 134 and a small group with two markers. The deletion of these two markers of the second LG changed the grouping to 121 markers in LG1 and 13 markers in LG2.

Table 5.1 Distribution of number of markers per linkage group (LG) as dependent on the number of linkage groups formed using the software TetraploidMap

	Markers per linkage group											
No. LGs	LG1	LG2	LG3	LG4	LG5	LG6	LG7	LG8	LG9	LG10	LG11	LG12
							Parent 1					
1	133											
2	127	6										
3	118	6	9									
4	113	6	9	5								
5	113	6	5	5	4							
6	110	6	5	5	4	3						
7	107	6	5	5	4	3	3					
8	78	6	5	5	4	3	3	29				
9	78	3	5	5	4	3	3	29	3			
10	73	3	5	5	4	3	3	29	3	5		
11	73	3	5	5	4	3	3	17	3	5	12	
12	73	3	5	5	4	3	3	17	3	3	12	2
							Parent 2					
1	136											
2	134	2										
3	121	2	13									
4	64	57	9	4								
5	64	59	9	4	2							
6	64	47	9	4	2	10						
7	64	47	9	4	2	8	2					
8	64	27	9	4	2	8	2	20				
9	64	20	9	4	2	8	2	20	7			
10	64	20	9	4	2	5	2	20	7	3		
11	59	20	9	4	2	5	2	20	7	3	5	
12	54	20	9	4	2	5	2	20	7	3	5	5

5.3.2 Grouping of markers with JoinMap

Preselection of markers for a 1:1 segregation ratio to be used with JoinMap yielded 83 markers for P1 and 95 markers for P2. Using an RF_{Crit} value of 0.35, the grouping tree function yielded only one LG for P1 (Table 5.2). Decreasing RF_{Crit} to 0.3, the large group dissociated into one large group with 70 markers, a second LG with five markers and nine small LGs, each containing one marker. With a further decrease of RF_{Crit}, the same pattern of one large LG and an increasing number of very small LGs, each one only harboring few markers persisted. In P2 the grouping tree showed a similar grouping as for P1, with all markers clustering into one large LG at RF_{Crit} of 0.35 (Table 5.2). This single LG dissociated into one large group containing 94 markers and one LG with only one marker when lowering RF_{Crit} to 0.3, or into one large LG comprising 85 markers and eight small LGs of one to three markers when lowering RF_{Crit} to 0.25.

Table 5.2 Number of linkage groups (LG) and distribution of markers among them as dependent on the critical recombination frequency (RF_{Crit}) and obtained by the software JoinMap

	Markers per Linkage group												
RF_{Crit}	LG1	LG2	LG3	LG4	LG5	LG6	LG7	LG8	LG9	LG10	LG11	LG12	LG 13-36
								Parent 1					
0.35	83												
0.30	70	5	1	1	1	1	1	1	1	1			
0.25	29	4	4	3	3	2	2	2	2	2	2	2	1-2
								Parent 2					
0.35	95												
0.30	94	1											
0.25	85	3	1	1	1	1	1	1	1				

5.3.3 Linkage maps

Linkage maps are shown for specific situations in both software packages. In TetraploidMap, the optimum number of LGs as indicated by the average linkage cluster dendrogram was used, with eight LGs (corresponding to RF_{Crit} of 0.3) for P1 (Fig. 5.1) and four LGs (corresponding to RF_{Crit} between 0.25 and 0.3) for P2 (Fig. 5.2). In JoinMap, final linkage maps are shown for an RF_{Crit} of 0.25 in P1 (Fig. 5.3) and P2 (Fig 5.4), whereby LGs with less than three markers were omitted. Observed clustering patterns were not comparable between JoinMap and TetraploiMap. Over all LGs in P1, only 28% of markers grouped by TetraploidMap were also grouped together by JoinMap, with the corresponding number in P2 being 41.9% (data not shown). Linkage maps created by Tetraploid map covered 613 cM for P1, ranging from 30.5 to 151.6 cM among the different LGs, and 420 cM for P2, ranging from 72.1 to 142.2 cM. Linkage maps created by JoinMap were considerable larger, with the four main LGs of P1 already covering 582.5 cM and the two LGs having more than one marker in P2 covering 1763.9 cM.

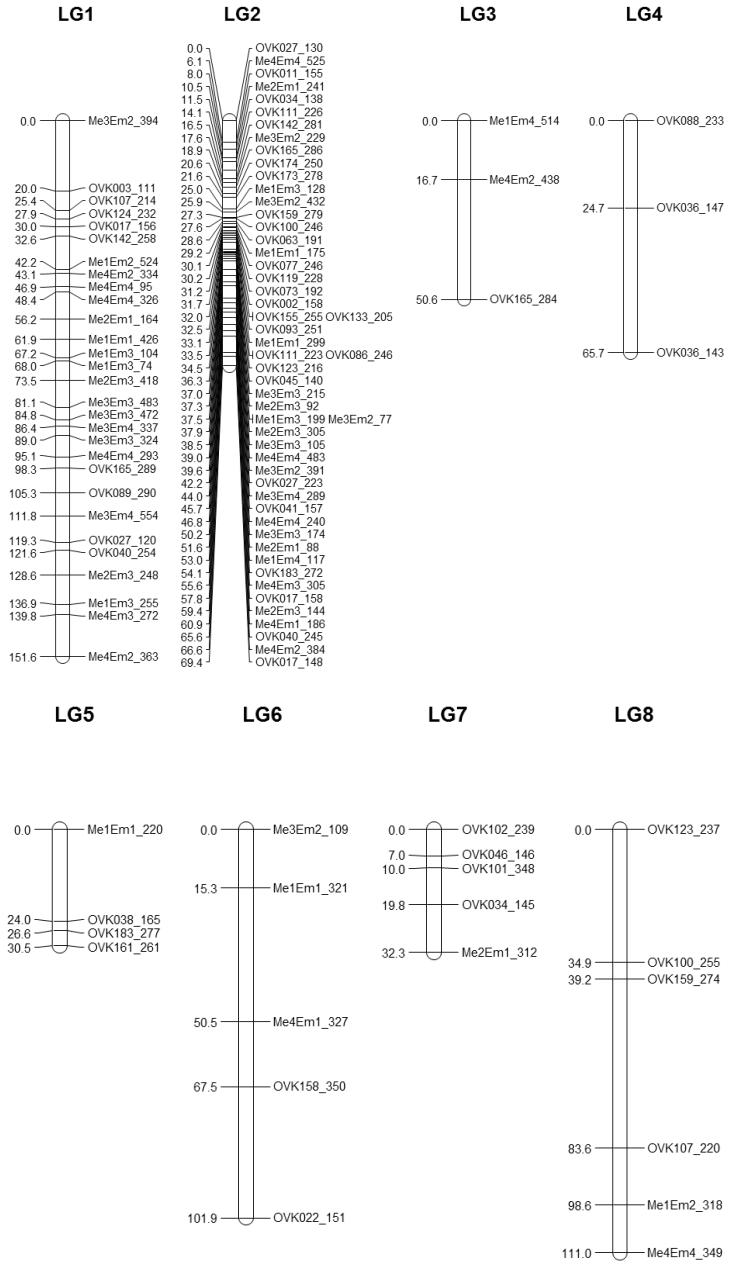

Fig. 5.1 Linkage group 1 (LG1) to linkage group 8 (LG8) of parent 1 (P1) as established with TetraploidMap software.

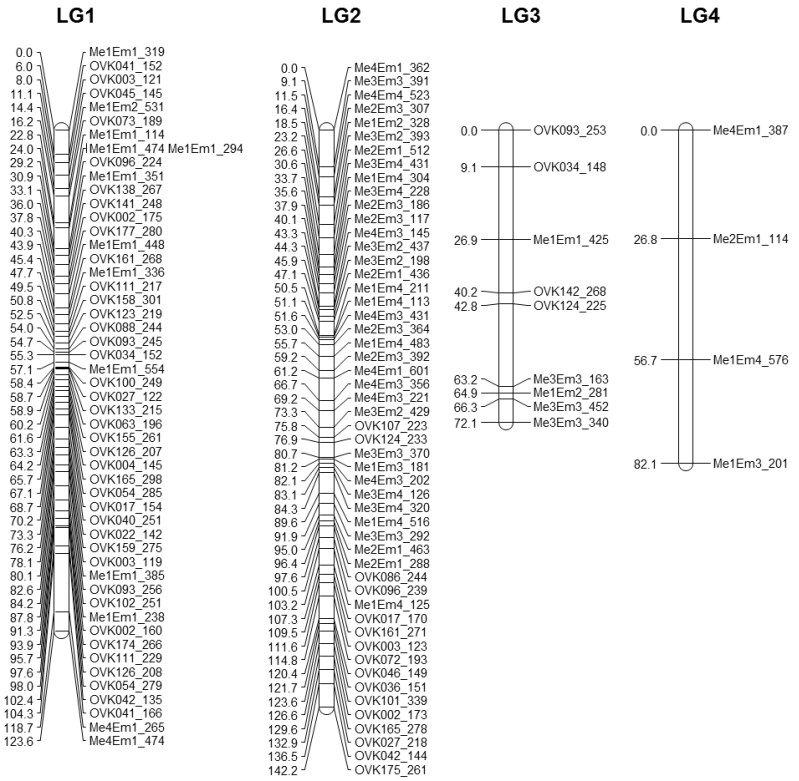

Fig. 5.2 Linkage group 1 (LG1) to linkage group 4 (LG4) of parent 2 (P2), as established with TetraploidMap software.

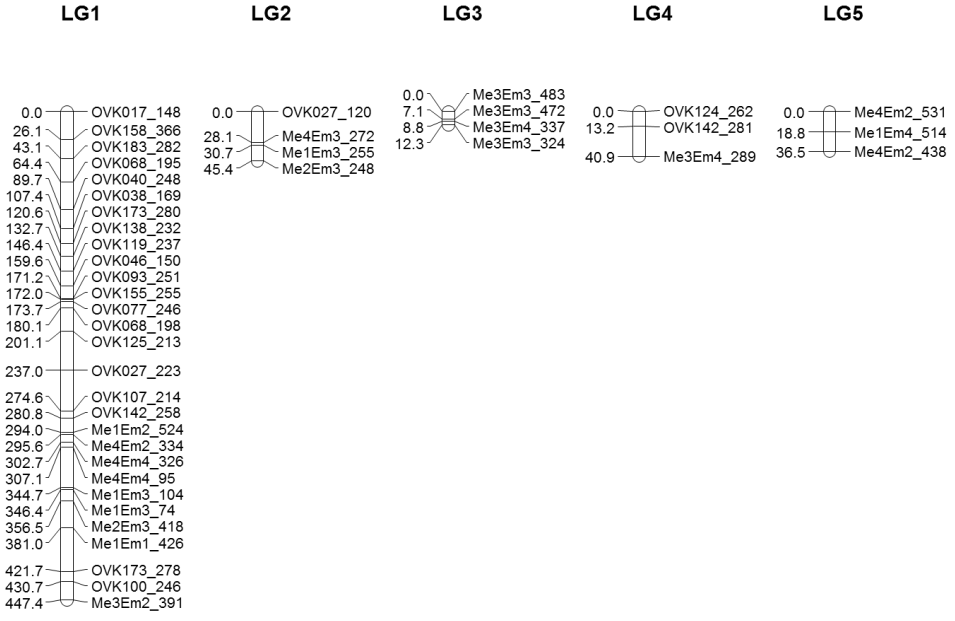

Fig. 5.3 Linkage group 1 (LG1) to linkage group 5 (LG5) of parent 1 (P1), as established with JoinMap software. For better representability, maps of the remaining 31 linkage groups are not shown.

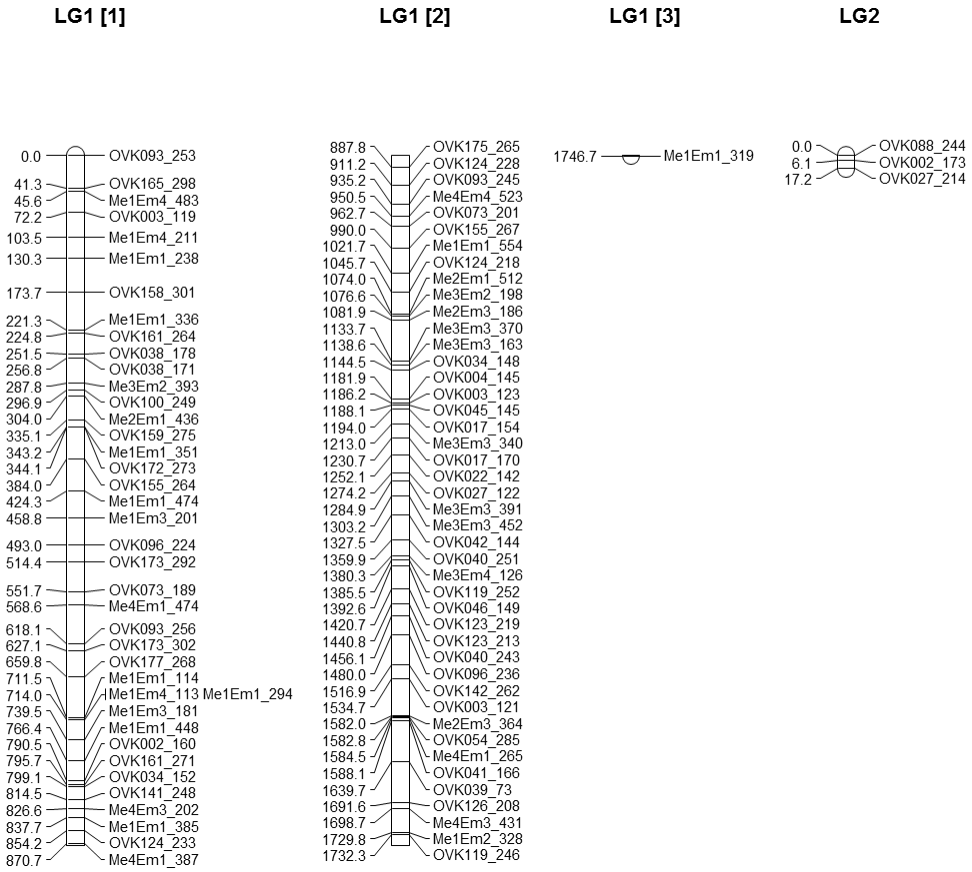

Fig 5.4 Linkage group 1 (LG1) and linkage group 2 (LG2) of parent 2 (P2), as established with JoinMap software. For better representability, maps of the remaining eight linkage groups are not shown.

5.4 Discussion

The markers used in this study within a F_1 full sib family derived from two non-inbred, heterozygous parental plants, were all strongly linked with indications of only one linkage group (LG). Different LGs could only be obtained using stringent (i.e., low) RF_{Crit} values, which resulted in potentially random or erroneous separation into different linkage groups. This is supported by the fact that marker clusters of one LG produced by TetraploidMap software showed a separation into more than one linkage group in JoinMap and vice versa. Furthermore, the LOD scores from the strongest cross linkage as calculated by JoinMap indicate that the linkage between markers of different LGs was as high as linkage between markers within the same LG. Hence, a final definition of LGs and allocation of markers was not possible, with a large number of very small

LGs appearing. Manual rejection of markers showing such a deviant clustering into small linkage groups, as it is also recommended by the software manuals, did not improve the analysis, as the pattern of one large and several smaller LGs persisted. The most likely number of LGs as indicated by both software packages did not reflect the amount of haploid or two haploid chromosome numbers, respectively. For sainfoin, a haploid chromosome number of seven has been verified (Fyfe 1946) and, therefore, seven linkage groups would be expected for TetraploidMap software and 14 linkage groups for JoinMap (according to the sum of the haploid chromosome number in both subgenomes).

The weak grouping as obtained by both software can be multicausal. The population size of 122 individuals might be too small, especially with regard to F_1 populations. The precise identification of QTL in a population is dependent on the size and kind of mapping population as well as the amount of recombination events due to meiosis between marker and QTL (Mackay 2001). In F_1 populations, only one meiosis event happening during gamete formation in each parent is sampled per offspring plant, offering a limited number of crossing over and, thus, recombination events to happen. Further, tracing the gene segregation in F_1 offspring is difficult with low numbers of individuals, and would be improved with higher numbers of individuals. Hence, the intermixture of the genome due to recombination was small and might lead to marker clustering into one linkage group.

Under the assumption that sainfoin might be autotetraploid, multivalent pairing of chromosomes during meiosis (Fig. 5.5, Otto 2007) occurs to a certain amount. With multivalent pairing, double reduction could occur, which reduces the number of markers in which recombination events (crossing over) can be detected. The rate of possible double reduction identified by TetraploidMap in the present study illustrated that multivalent pairing and double reduction is likely in sainfoin. This consideration is supported by recent findings from chromosome arrangements during meiosis, where multivalent as well as bivalent formation was observed in sainfoin (Fig 5.1, David Kopecký, personal communication, 2015). This is also in line with the large amount of markers showing deviation from Mendelian segregation ratios as found by JoinMap, what would be a consequence of deviation from bivalent chromosome pairing and double

reduction. Hence, multivalent chromosome pairing might be even more significant in sainfoin than in other autotetraploid species like potato, where multivalent pairing is supposed to occur rather seldom compared to bivalent pairing (Bourke et al. 2015), hampering the separation of markers into linkage groups.

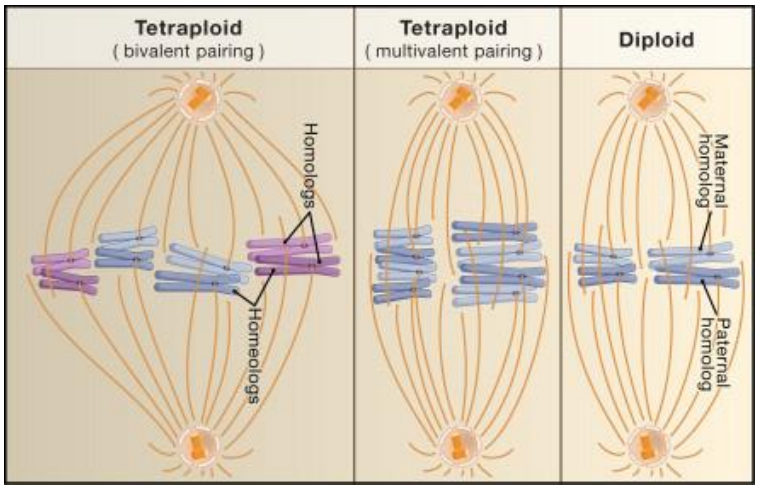

Fig. 5.5 Chromosome arrangement for crossing over during meiosis. In diploids, only bivalent pairing of chromosomes is possible. In autotetraploids, all four homologue chromosomes can combine for a multivalent pairing. Bivalent pairing would be predominantly observed between homologue chromosomes of allotetraploids, but is also possible to occur in autotetraploids. Graph taken from Otto (2007).

The accuracy of markers is reduced by the unknown allele dosage in co-dominant markers, where only the quadruplex state (four different alleles at the locus) would show the exact dosage. Loci showing three or less alleles could carry more copies of the same alleles on homologous or homeologous chromosomes. This aspect complicated the assessment of the segregation ratios for co-dominant markers. TetraploidMap does infer possible dosages, but with small numbers of individuals, the results are barely reliable. Furthermore, the software discards co-dominant markers with odd segregation ratios, as these hinder conclusions about parental marker genotypes and detection of linkage. If SSR markers were coded as co-dominant, most were rejected by TetraploidMap software based on segregation distortion. Therefore, every single SSR allele was coded as a different, dominant marker as proposed by McCord (2014), thereby increasing the number of usable markers. In JoinMap, only simplex marker alleles could be

used due to the 1:1 diploid like segregation behavior at a heterozygous locus (1000 x 0000), whereas TetraploidMap could use 1:1 and 1:5 (i.e. duplex, 1100 x 0000) markers. This explains the larger number of markers used with TetraploidMap compared to JoinMap (133 vs. 83 for P1 and 136 vs. 95 for P2, Table 5.1). Simplex and, to a lower extent, also duplex markers are considered to be the most informative ones because they allow the detection of crossing over events (Fig 5.6). Markers with 3:1 segregation (1000 simplex state in both parents) could only be used as anchor markers for a final merge of the two parental maps into one map but, apart from that, are less informative (Julier et al. 2003; Hackett et al. 2007; Stein et al. 2007).

The size of our F_1 mapping population was comparably small due to a high number of S_1 progenies haven arisen from self-fertilization, an issue so far unknown in sainfoin, and a relatively poor seed set. For future development of linkage maps in sainfoin, it is advisable to drastically increase the size of the F_1 mapping population for a better detection of recombination events, what might be reached via synchronization of flowering time between parental plants. Alternatively, it may be also considered to have multiple F_1 full-sib families, so that markers that do not segregate in one parent may be segregating in another parent. This approach was aimed in our study, but had to be abandoned due to the high amount of inbred offspring reducing the number of plants usable for linkage mapping in other populations. Such maps could then be merged using markers showing a 1:3 segregation ratio as explained above. The use of other populations than F_1 might be also promising, as these do regularly involve a larger amount of crossing over events. For example, F_2 populations may be produced from (partially) inbred parental plants which is feasible in sainfoin (Kempf et al. 2015), whereby the approach of multiple families could also be used for this type of population. A further step could be the development of a physical map after establishment of a reference genome. This would require a largely increased input, but might be more successful in case of a high percentage of multivalent pairing and other factors impeding genetic mapping in sainfoin.

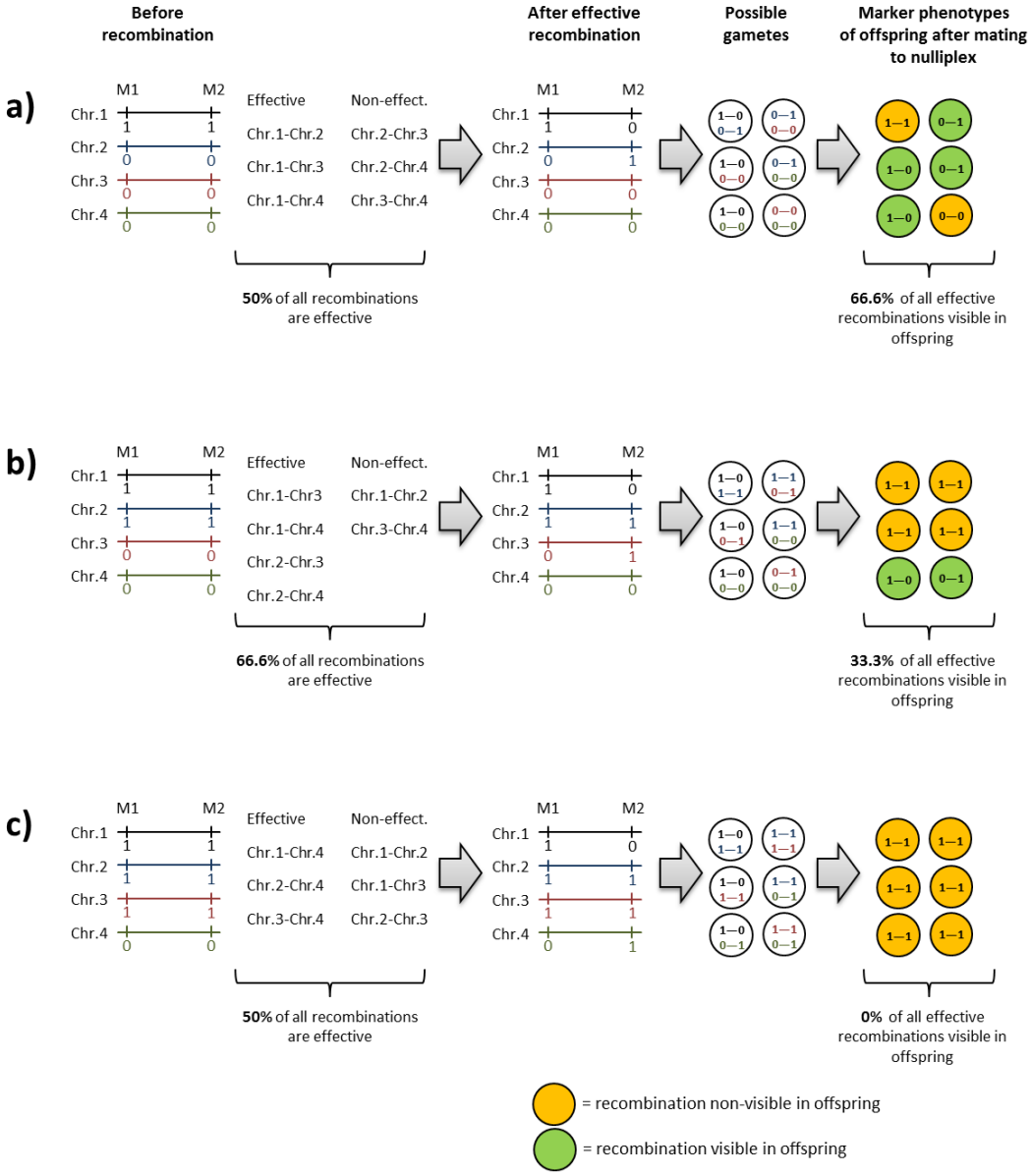

Fig 5.6 Illustration of information content for two dominant markers M1 and M2 (1 for presence, 0 for absence of allele) if both of them are in **a)** simplex, **b)** duplex and **c)** triplex state. A recombination event (crossing over, assuming bivalent chromosome pairing) might be effective (e.g., 1 replaced by 0 or 0 replaced by 1) or non-effective (1 replaced by 1 or 0 replaced by 0), whereby the proportion of effective recombinations differs dependent on the marker state (e.g. 50% for simplex and triplex, 66.6% for duplex). If an effective recombination between markers M1 and M2 has happened, the chance of detecting this effective recombination event in the offspring from mating with a nulliplex (0000) parent also depends on the marker state (e.g., 66.6% for simplex, 33.3% for duplex and 0% for triplex markers). The product of the chance of having an effective recombination and the chance of detecting this effective recombination event is representative for the information content, being highest for a) simplex markers (e.g. 50% x 66.6% = 33.3%), intermediate for b) duplex markers (e.g. 66.6% x 33.3% = 22.2%) an zero for triplex markers (e.g. 50% x 0% = 0%).

Chapter 6 General Discussion

Sainfoin is underrepresented in today's agriculture, despite its undeniable value as an animal health promoting forage crop and its suitability for environments with low water and nutrient availability. This underrepresentation is also noticeable in plant breeding, with only few varieties available and a stagnant number of new ones coming to the market. The knowledge base for breeding sainfoin is limited with only marginal information about its genetics and inheritance of different traits available. The few genetic studies conducted so far did not support an increase of sainfoin breeding activities and further efforts are missing. However, a close interaction between research and applied breeding is vital for the success in genetic progress and cultivation of an agricultural species. Future studies must, thus, be aimed at answering questions and support approaches related to sainfoin breeding. Therefore, the main aim of our study was to fill knowledge gaps related to the mode of reproduction and to provide molecular tools relevant for improvement of sainfoin.

The results of the present study delivered a valid proof of the possibility of self-fertilization in sainfoin to high numbers and the co-occurrence of inbreeding depression, namely for seed yield and plant vigor. In contrast to earlier studies dealing with inbreeding in sainfoin (Knipe and Carleton 1972; Demdoum 2012), these results are based on molecular markers of a dominant (sequence related amplified polymorphism, SRAP) and co-dominant (single sequence repeat, SSR) type that were applied in different populations established through insect pollination. Thereby, conditions favoring self-fertilization, i.e., limited availability of flowers from the same plant as present under artificially directed pollinations (chapter 2), or reducing self-fertilization, i.e., abundant flowers as present under non-directed pollinations, could be identified. This information completes earlier studies dealing with self-fertilization in sainfoin and can be used to optimize conventional breeding methods or to implement hybrid breeding systems that require the development of inbred lines.

To boost genetic studies, 101 recently developed, species specific co-dominant SSR markers were tested for their applicability in sainfoin by measuring allelic diversity in a pool of sainfoin plants from different origins (chapter 3). These SSR markers, together with dominant SRAP markers, proved also to be suitable for the use in genetic association studies, as trait associated markers (TAM) were

detected for vigor related traits (seed yield, plant height, plant vigor) and one relevant chemical compound in a biparental F_1 mapping population of 122 individuals (chapter 4). Finally, creation of a linkage map was tackled using the above mentioned F_1 population and markers, whereby the two software programs TetraploidMap and JoinMap were tested to account for an auto- and allotetraploid character of sainfoin, respectively. However, due to a limited statistical power of the single mapping population, no accurate linkage map could be established, and no indication for the best software to be used of linkage mapping in sainfoin can yet be given (chapter 5).

6.1 Breeding opportunities in sainfoin

6.1.1 Self-fertilization and breeding systems

The high numbers of self-fertilization (48.5 to 64.8%) in our three biparental populations (populations derived from artificial derived pollination: ADP, chapter 2) suggested that inbreeding is not necessarily a rare event in sainfoin. The presence of a self-incompatibility system as proposed by Tasei (1984) can, therefore, be negated. The accompanied inbreeding depression in offspring originating from self-fertilization (hereafter denoted as selfings) compared to offspring origination from cross-fertilization (hereafter denoted as crossings) was strongest for seed yield with reductions of up to 79.1%. This information is of significance, because inbreeding has to be avoided in classical population varieties in order to increase seed yield and performance of individuals. So far, sainfoin was bred based on methods for outbreeding species with population or synthetic varieties as the final product. Reducing self-fertilization and, hence, inbreeding would especially be important when performing a polycross, i.e., the isolated pollination among a limited number of clonally multiplied elite plants for the production of base seeds of a synthetic variety (Posselt 2010). Our results from inbreeding in naturally directed pollinations lead to the suggestion that, with presence of a sufficiently large number of pollinators and mature flowers from different plants, the rate of self-fertilization (up to 3.9%) is negligible. Hence, if the size (number of different elite genotypes) of a polycross is not chosen

drastically small and flowering time is synchronous among the different genotypes, efficient cross-fertilization can, although a strict self-incompatibility system is missing, be assured. In classical polycrosses for forage plants, the number of different genotypes is chosen from 4 to 10 genotypes in replicate numbers of the same range (Humphreys 2000). However, taking into account low seed yields and the limited potential to clonally propagate single genotypes, to assure efficient cross-fertilization and harvest sufficient seeds it might be necessary to slightly increase this number of genotypes per polycross in case of sainfoin. This is crucial because avoiding inbreeding by emasculation of maternal plants in sainfoin is less promising (De Vicente and Arus 1996) and a male sterility system is not established.

Nevertheless, realization of self-fertilization in sainfoin can also be of advantage. For example, it is a necessary prerequisite for the production of inbred lines, enabling hybrid breeding systems where two distinct inbred lines of different heterotic pools are crossed to benefit from the hybrid effect in the F_1 progeny, also known as heterosis (Becker 1993). For sainfoin, hybrid breeding was not considered so far, but it is already aspired for other forage plants like perennial ryegrass (*Lolium perenne*), switchgrass (*Panicum virgatum*) and red clover (*Trifolium pratense*, Riday and Krohn 2010a; Aguirre et al. 2012). Self-fertilization for inbred line development is impeded in most forage plants due to the existence of a genetic self-incompatibility system (Posselt 2010). However, based on our results, this does not apply for sainfoin. If self-fertilization and, consequently, inbreeding is aimed for sainfoin, precautions as presented for ADP populations (chapter 2) must be undertaken to technically enable a hybrid system. If possible, plants designated for self-fertilization could be clonally multiplied in order to increase the number of flowers and, therefore, the chance of successful seed set. These clones would then be subjected to isolated pollination by bumble bees, whereby this process could be repeated for several generations. After production of partially or completely inbred lines, open pollination among these ones with good combining ability among each other could be performed, whereby the predominantly outcrossing fertilization pattern with availability of abundant flowers as observed in NDP populations would preferably results in a high proportion of F_1 hybrid seeds.

Yet, we have proven that one generation of inbreeding can be performed without problems, achieving viable S_1 offspring. However, inbreeding depression might hamper further advanced inbred generations. The observed inbreeding depression in S_1 plants observed for seed yield and general plant vigor in our experiment was remarkable, especially considering that in autotetraploids, the proportion of fully homozygous loci increases 2.8 to 3.9-fold (depending on tetrasomic or disomic inheritance) slower than in diploids (Bever and Felber 1992), i.e., can be assumed to be still small in S_1 plants. This early inbreeding depression could be consequence of a very strong genetic load (accumulation of deleterious alleles) and/or the loss of complementary gene interactions that is more severe during early generations of inbreeding (Bingham et al. 1994). If the later would have been the predominant cause of observed inbreeding depression and taking into account that S_1 plants with sufficiently large seed yield were obtained in this study, production of advanced inbred generations would be a feasible option. Furthermore, the problem of inbreeding depression could also be mitigated by choosing different parental material, since we found significant differences in inbreeding depression dependent on population and parent ($P > 0.001$). Overcoming inbreeding depression by careful selection of parental plants is current practice for today's economically important crops species like maize and alfalfa by improving heterogenic groups, inbred lines and selection of partners with best combining abilities (Argillier et al. 2000; Bhandari et al. 2007).

However, even if a hybrid breeding system would be technically possible, the decision for its implementation in sainfoin must be weighed against its economic gain. Due to the observed inbreeding depression for seed yield (chapter 2), seed multiplication factors during production of parental lines of the potential F_1 hybrid can be expected to be low. Hence, the consumer would have to pay even higher prices than for the already expensive seeds of conventional varieties. The market for sainfoin must increase considerably to justify the labor and costs coherent with development of hybrid varieties, which could last 10 years or more (Becker 1993). With sainfoin being a species being suitable for marginal areas, this would barely be the case.

In addition to a hypothetical hybrid breeding system, the possibility of inbreeding offers purging populations from deleterious alleles. The phenomenon of purging was already discovered by Darwin (1876) when observing plant generations suffering inbreeding depression for traits after initial cycles of inbreeding, but with a re-improvement after continued inbreeding. During first cycles of inbreeding, individual plants getting homozygous for deleterious alleles could die or fail in reproduction, successively eradicating those alleles from the population (Crnokrak and Barrett 2002). Differences in purging could be expected dependent on the effect of the deleterious allele, whereby alleles with a large effect are supposed to vanish faster in contrast to alleles with smaller effects (Wang et al. 1999). There is no information of targeted purging by repeated inbreeding in breeding populations, but the results of inbreeding depression from comparisons between self-compatible and self-incompatible natural populations of different plant species showed that inbreeding depression is higher in self-incompatible plants than in self-compatible plants, the latter already having experienced eradicated of deleterious alleles to some degree (Busch 2005). Hence, purging would be a valuable option for the predominantly outbreeding species sainfoin. To the best of my knowledge, no information of successful purging using classical self-fertilization is available for cultivated crops. However, examples of successful purging are available from maize breeding programs using the so-called doubled haploid technology, a method in which fully inbred offspring can be obtained *in vivo* within one generation (Roeber et al. 2005; Strigens et al. 2013).

6.1.2. Breeding for animal health

The presence of secondary metabolites (thereunder polyphenolic compounds) in sainfoin is a motivating factor to cultivate sainfoin for use as animal forage due to its effectiveness supporting animal health and welfare (chapter 1). Our study wanted to assist sainfoin breeding also by providing molecular tools for the selection of desired chemical properties. Encouraging breeding activities to select for chemical compounds raised the question if and how those beneficial compounds could be influenced and changed by plant breeding. Breeding for generally higher concentrations of supposedly beneficial compounds would be injudicious without knowledge about the effectiveness of the single compounds,

their interactions and the optimum dose to obtain positive effects. Overall, the phenolic profile of sainfoin is highly complex and wide variations for composition and concentrations could be identified between individuals of the same variety (Regos et al. 2009). The effects of those secondary metabolites of sainfoin with respect to composition, concentration and structure were determined in animal studies showing complex effects and interactions (reviewed in Mueller-Harvey 2006). In studies dealing with anthelmintic properties of sainfoin, it was demonstrated that larger polymer sizes of condensed tannins and higher amounts of prodelphinidins caused a reduction of one worm species (Haemonchus contortus) in sheep, whereas another worm species (Trichostrongylus colubriformis) responded only to higher prodelphinidin concentrations (Quijada et al. 2015). Consequently, condensed tannins have different modes of action against different parasites. A multicausal mode of action of tannins was also reported for reduction of methane gas emissions *in vitro*, with no effect of prodelphinidin concentration, but a linear reduction with increasing levels of condensed tannins in total and, moreover, with increased molecule size of condensed tannins (Saminathan et al. 2015; Hatew et al. 2016). Furthermore, effects observed within one variety or species are not always transferable to other varieties or species, respectively, underlining the highly complex and interactive modes of action of the different compounds.

These results indicate that breeding exclusively for high condensed tannins concentrations in sainfoin does not consequently exploit the full animal health promoting potential of sainfoin. As synthesis from literature indicates, larger molecule sizes and higher shares of prodelphinidins on total condensed tannins are desirable. However, the respective effectiveness may vary dependent on area of operation e.g. anthelmintic effect, methane gas reduction, bloat prevention and better protein utilization.

Selection of parents for new varieties with higher prodelphinidin shares could be supported by marker assisted selection, as we found one trait associated marker (TAM) for prodelphinidin share. Total condensed tannin content is correlated to prodelphinidin share and, therefore, could be improved simultaneously (chapter 4). However, this TAM has its limitations because its occurrence (by the presence of the SRAP marker allele) is population dependent. Populations established with

the same genoytpes as used in the present study could feature this allele, whereas other genotypes might miss it. Furthermore, the inheritance of tannins has to be studied in detail, due to controversial information from the literature. Dominant gene action for inheritance of tannins was suggested for birdsfoot trefoil (*Lotus corniculatus*, Dalrymple et al. 1984), common bean (*Phaseolus vulgaris*, Ma and Bliss 1977) and fababean (*Vicia faba*, Crofts et al. 1980). Dominant inheritance pattern was also found for tannin composition, i.e., PD-share and chain length of the molecule in F_1 hybrids of poplar (*Populus angustifolia* and *Populus fremontii*, Scioneaux et al. 2011). Contrastingly, other authors reported predominantly additive gene action for inheritance of tannin concentrations in birdsfoot trefoil (Miller and Ehlke 1997) and cotton (*Gossypium hirsutum*, White 1982). Hence, trait inheritance seems to be dependent on the plant material investigated, calling for further studies investigating inheritance of tannin related traits in sainfoin.

Despite the proof of effectiveness of condensed tannins for animal health, also non-tannin polyphenols have positive effects in sainfoin and, therefore, represent potential breeding targets. Anthelmintic properties could be demonstrated at relatively low concentrations of the two flavonol glyocosides rutin and narcissin (Barrau et al. 2005). Rutin belongs to the polyphenolic compound group quercetin, a flavonol, and was correlated to total quercetin concentrations in our dataset with Pearson's correlation coefficient of $r = 0.99$ (chapter 4, data not shown). This relationship makes selection for higher total quercetin concentrations a reasonable target. Generally, flavonols and their glycosides like quercetin, myricetin and gallic acid are characterized as antioxidants, which could be proven in several studies related to human nutrition (Noroozi et al. 1998; Liu 2004; Yao et al. 2004). However, information about the effectiveness of those substances in sainfoin extracts and the optimum concentrations are scarce. Therefore, future definition of breeding aims regarding sainfoin compounds is only possible by tight collaboration with animal scientist.

6.2 Future research tasks

6.2.1 Origin of polyploidy

To understand further parameters influencing plant performance, it will be necessary to clarify whether sainfoin is of allo- or autotetraploid origin. This knowledge is far-reaching, because it provides information about the possibility to retrieve desired gene combinations after crossing or selfing. The relevant difference in parent to offspring gene transfer via gametes in allotetraploid (polyploidy due to hybridization of two diploid ancestor genomes) and autotetraploid (polyploidy arising from a diploid ancestor genome) species happens in meiosis. During meiosis, gene recombination takes places due to non-paternal chromosome combinations in newly formed gametes and by crossing over events, i.e., the exchange of chromosome segments during metaphase. In allotetraploids, bivalent chromosome pairing and crossing over only occurs between the two homologous chromosomes (chromosomes from the same diploid ancestor species) and barely between homeologous chromosomes (chromosomes from different diploid ancestors), resulting in a so-called *disomic* behavior (Butruille and Boiteux 2000). In autotetraploids, more than two homologous chromosomes can pair at meisosis (multivalent chromosome pairing), resulting in a so-called *polysomic* behavior, where more possibilities for gene recombination exists (Otto 2007). For breeding a direct consequence would be that under polysomic inheritance fewer homozygotes would be produced in one generation than under disomic inheritance (Hancock 2012). This complicates the fixation of desired genes in the genome under polysomic inheritance.

However, many plant species exhibit a mixture of polysomic and disomic inheritance. The origin of ploidy and preponderance of disomic or polysomic recombination patterns could be investigated using microscope observations of chromosome arrangements during metaphase (Singh and Hymowitz 1985; Jackson and Hauber 1994). This approach was conducted related to the present study in immature anthers of single sainfoin individuals and revealed the presence of bivalent (occurring in allo- and autotetraploids, disomic inheritance)

as well as quadrivalent (occurring in autotetraploids, polysomic inheritance) formations (Fig.6.1, David Kopecký, personal communication, 2015).

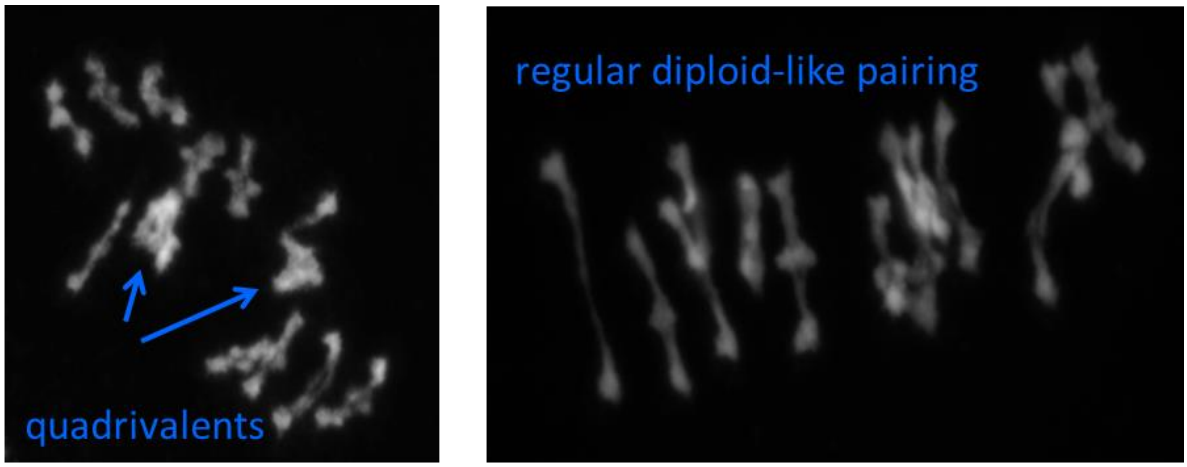

Fig. 6.1 Chromosome arrangement in sainfoin during metaphase 1 of meiosis in immature sainfoin anthers (David Kopecký, personal communication, 2015)

Nevertheless, a final determination of allo- or autotetraploidy in sainfoin is difficult. Bivalent formation could also occur preferentially in autotetraploids as shown for potato (Bourke et al. 2015). Contrastingly, quadrivalent formation also exists in allotetraploids derived from two close ancestor species and due to strong analogy of homeologous chromosomes (Sybenga 1996). Analyzing large panels of individuals under the microscope would allow for an insight into potential mechanisms of gene recombination in sainfoin. Alternatively to chromosome observations, co-dominant marker genotypes from selfings of different sainfoin individuals could be investigated according to proposed ratios for either disomic or polysomic (tetrasomic in tetraploids) inheritance by De Vicente and Arus (1996) due to allelic differences in gamete formation (Fig. 6.2, Comai 2005).

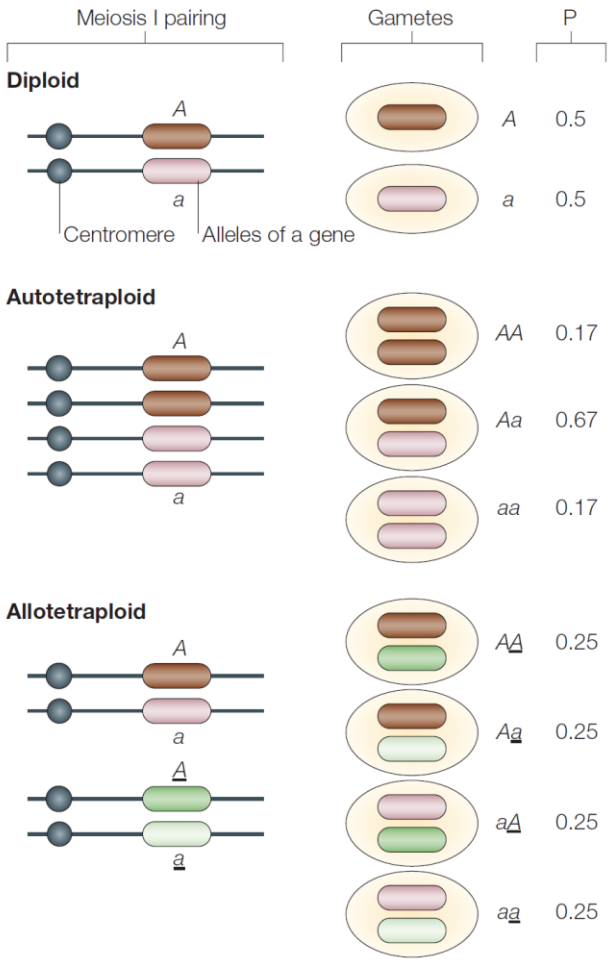

Fig. 6.2 Gamete formation in allo- and autotetraploids taken from Comai (2005)

Here, co-dominant markers have to be quadriallelic (ABCD), triallelic (e.g. AABC) or diallelic (e.g. AABB) in the parental plant to be informative. SSR markers characterized in the present study (Mora-Ortiz et al. 2016) could be a valuable source for such marker genotype assessments in larger groups of sainfoin individuals. The assessment of disomic or tetrasomic segregation by co-dominant markers would also be possible in crossings from two parents sharing no or only one allele at a gene locus (Pairon and Jacquemart 2005). Knowledge about disomic or tetrasomic inheritance of the markers identified in our study could also be directly used for selection of suitable markers for linkage mapping, as marker loci showing disomic inheritance (bivalent pairing) will not exhibit double reduction. Because allele dosages of co-dominant SSR markers could not be estimated due to highly variable DNA concentrations and possible differences

between samples due to efficiency of the PCR (see below), inferences on the type of segregation pattern could not be performed in this study.

6.2.2 DNA extraction and marker analysis in sainfoin

The present study demonstrated the usefulness of molecular marker assessments in sainfoin to answer questions regarding parenthood, diversity patterns and to identify trait associated markers (TAM). Extracting DNA for those studies from sainfoin was sufficiently feasible with the commercial extraction Kit illustraTM DNA Extraction Kit PHYTOPURE (GE Healthcare, Little Chalfont Buckinghamshire, United Kingdom) as advised by Hayot Carbonero (2011) . In the present thesis, DNA was extracted from freeze dried material stored in the dark at room temperature with silica gel to prevent moistening. After grinding freeze dried material, DNA extraction yielded highly variable DNA concentrations, ranging from 1000 to 10 000 ng DNA in 20 mg plant material as measured by gel band intensity. Up to 20 fold differences in DNA concentrations were also found by Hayot Carbonero (2011). The reasons for this varying DNA yields might be due to the presence of condensed tannins and other polyphenols which are known to hamper DNA extraction in plants rich in these compounds such as cotton (Katterman and Shattuck 1983). Despite varying DNA concentrations, the DNA quality was sufficient for amplification of SRAP and SSR marker loci during fragment analysis with distinguishable peak patterns. To increase DNA yields and to simplify DNA extraction, it is advised to sample fresh leaf material, freeze it immediately with liquid nitrogen followed by freeze drying for 48h. The freeze dried material should be ground directly and be stored at minus -20 °C until DNA extraction. Extraction from plant material stored under this conditions over three years reached DNA yields which were more homogenous with 3500 ng to 5500 ng in 20 mg freeze dried plant material.

SSR marker amplification provided reliable peak patterns in fragment analysis in our study, but had to be repeated in some samples due to the occurrence of stutter peaks. Stutter peaks (or stutter alleles) are peaks differing by one or more repeat units to the original peak and occur due to polymerase slippage during PCR elongation of the synthetized strand (Hauge and Litt 1993; Walsh et al. 1996). In such cases it is crucial to differentiate the stutter peak from the actual

peak to avoid false genotyping results, which would have fatal consequences if allelic diversity has to be estimated. The intensity of such bands increases with degradation of the DNA and the peak signal during fragment analysis could be higher than the original signal. However, if several PCR are run on the same sample, the mentioned problem does not necessarily affect every amplification product (Schmerer 2003). In such cases, it is reasonable to repeat the analysis or, alternatively, replicates should be incorporated for each sample. In some cases sequencing of the loci might be also appropriate to know which allele could be expected. The occurrence of signals with only one base pair difference to the supposed repeat size also blurred the SSR fragment analysis in some of our samples, especially in case of two base pair motifs. This occurred due to non-templated addition of a nucleotide to the newly synthetized product, a problem that could be solved in future studies by primers modified with a sequence impeding base pair addition (PIG-tailing, Brownstein et al. 1996).

Improvement of DNA quality and quantity, together with optimized conditions for PCR are also necessary to enhance confidence of peak patterns during fragment analysis, what might allow identification of the different allele doses amplified by dominant SRAP markers and co-dominant SSR markers by peak size (Qiang et al. 2015). In the present study, allele dose could not be inferred, as the peak sizes from fragment analysis were highly variable over all samples. For inferring the allele dose by peak size intensity, the quality of the experimental data must be high over all samples, e.g., by equal yields of the PCR product overall samples, exclusive amplification of the target sequence and very few errors by polymerase (Cha and Thilly 1995). The occurrence of stutter alleles also hinders the inference of the allele dose (Esselink et al. 2004), what was the case for some samples used in our study. Furthermore, incorporation of standard samples with known allele doses would enable a better estimation of the allele doses of non-characterized samples. Knowing the allele dose of SSR markers would bear several advantages. Regarding diversity studies, polymorphism information content (PIC) values could be calculated directly on inferred allele frequencies for the complete marker instead of separate calculation for each single dominant coded allele as performed in chapter 3. For linkage mapping, availability of allele frequencies would allow to fully exploit the higher

information content of the co-dominant markers. In the case of autotetraploid origin of sainfoin, the knowledge of the allele dose at a locus would allow for the assignment of alleles to the different homologous chromosomes of each chromosome (Hackett et al. 2007).

6.2.3 Towards marker assisted breeding in sainfoin

With testing and application of newly developed, sainfoin specific SSR marker and the proof of usability of dominant SRAP markers in the present study, a door has been opened for linkage mapping and QTL studies in sainfoin. Trait associated markers (TAM) for vigor related traits, seed yield and prodelphinidin-share have been identified in crossings of one biparental sainfoin population by simple marker regression methods (chapter 4). These TAM display a first step towards marker assisted selection (MAS) in sainfoin. Hereinafter, linkage mapping was conducted using two software packages: JoinMap addressing a potential allotetraploid character of sainfoin and TetraploidMap taking possible autotetraploidy into account. 14 linkage groups were anticipated for JoinMap (referring to the sum of haploid chromosomes of two diploid ancestor species) and 7 linkage groups for TetraploidMap (the number of haploid chromosomes in the single diploid ancestor species). Linkage maps provided by both software showed tendencies of marker assignments to single groups, but failed to reach the full coverage of all possible linkage groups. However, tracing the TAM found in our study (chapter 4) in the linkage maps established by JoinMap and TetraploidMap software, showed that at least some could be recovered in single linkage groups calculated with TetraploidMap. Five markers associated with plant height and seed yield (Fig 4.6, OVK063_191, Me1Em1_175, OVK133_205, Me1Em1_299 and OVK111_223) were identified in linkage group 2 of parent 1. The two of these markers showing the largest distance were 4.9 cM apart from each other, indicating that these markers are located actually on the same region of a linkage group. Additionally, one single marker for share of prodelphinidins was located on linkage group 2, but with a smallest distance of 22.5 cM to the closest neighboring marker from the above mentioned group. The close distance of the TAM on linkage group 2 of parent 1 could lead to the suggestion that those markers are associated with the same single QTL. However, in red clover

(*Trifolium pratense*) it could be shown that up to five different QTL for seed yield were located at a distance of 10 cM and QTL of highly correlated traits (r = 0.95) were located at distances of 1 cM (Herrmann et al. 2006). For seed yield in perennial ryegrass (*Lolium perenne*), two markers were considered being associated with the same QTL at a distance of 0.4 cM (Studer et al. 2008). Yet, due to the low confidence of our linkage map, it is difficult to deduce the affiliation of different markers to certain QTL by comparing to results from other studies that are based on more confident linkage maps. The establishment of a more precise linkage map based on alternative populations as discussed within chapter 5 will, therefore, be essential. Concluding, combining such proposed approaches with optimized protocols for DNA handling and marker screening is necessary to further advance breeding and breeding research in a non-model species like sainfoin.

6.3 Conclusion

This thesis gave new insights in the biology of sainfoin by clarifying its reproduction system that was formerly considered as strongly cross pollinated. We confirmed the possibility of self-fertilization via insect pollination in biparental populations established in the greenhouse and field-grown natural sainfoin populations for the first time on the basis of dominant and co-dominant molecular markers. The rates of inbreeding due to successful self-fertilization were high in biparental populations, whereas they could be neglected in field-grown natural populations (chapter 2). We concluded that high inbreeding rates are possible by restriction of foreign pollen and reduced insect pollinators. Inbreeding depression was visible in biparental populations by decreased performance of seed yield, plant height and general plant vigor. These results have to be kept in mind by planning new breeding strategies for sainfoin if outbreeding or inbreeding is desired. Additionally, we were able to test sainfoin specific co-dominant SSR marker which display allelic polymorphisms between sainfoin plants of different origin (chapter 3). Those markers detected the presence of two clusters of sainfoin plants dependent on its geographical origin

leading to the assumption of the existence of different genepools in sainfoin. Thereafter, a set of these newly available SSR markers was amplified in offspring from cross-pollination from one biparental population to detect marker trait associations (chapter 4). Such marker trait associations were detected for plant height, seed yield, vigor and one chemical compound. Additionally, correlations between those markers might point to the existence of two QTL for those traits. Furthermore, the establishment of a linkage map and localization of these QTLs was limited due to clustering of most markers to only one linkage group (chapter 5).

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Curriculum vitae

Personal Data

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Work experience

Project collaborator	**Saatzucht Steinach**
05/2011 - 08/2011	Pyramidisation of resistance genes in the genus Lolium aimed to develop improved fodder plants
Student work assistant	**University of Hoheimheim, Stuttgart, Germany** **Institute of plant breeding**
02/2010 - 04/2010	Selection of doubled haploid maize kernels
Internship	**GAB eurofins, Niefern-Öschelbronn, Germany**
04/2008 - 09/2008	Assistance in eco-toxicological field studies

Education

PhD thesis	**Agroscope, Zurich, Switzerland** **Molecular ecology group**

05/2012 – 06/2016	Self-fertilization and marker- trait associations in sainfoin (*Onobrychis viciifolia*)
Studies	**University of Hohenheim, Stuttgart**
04/2006 – 04/2011	Agricultural biology
Diploma thesis	**Institute of plant breeding**
	Production of doubled haploids in maize (*Zea mays* L.) with colchicine-alternatives
High school (Abitur)	**Mathilde-Planck-Schule, Ludwigsburg**
09/2002 – 07/2005	Gymnasium of nutrition science

Scientific training

	University of Reading, England
06/2012	Training for industry 1
	University of Wageningen, Netherlands
11/2012	Animal Nutrition Course
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Summer semester 2013	Gene Technology in Foods
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11/2013	Training for industry 2
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05/2014	Training in chemistry and grant application writing
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