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RESEARCH ARTICLE

EST-SSR cross-amplification and genetic similarity in *Onobrychis* genus

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Abstract EST-SSR from *Medicago truncatula* Gaertn. and *Glycine max* (L.) Merr. were tested for transferability in various species of *Onobrychis* (*O. pyrenaica* Sennen, *O. argentea* Boiss. *and O. viciifolia* Scop.). Repeatable amplification was obtained for 81% of the microsatellites and 52% were polymorphic. Six selected SSRs from *M. truncatula* were used to fingerprint and estimate the genetic similarity of a set of 23 accessions of *O. viciifolia.* PCA analysis discriminated among the different *Onobrychis* species and the sainfoin accessions were clustered in a single major group. This grouping is discussed in terms of the history of cultivation of sainfoin in Spain. The selected SSRs will allow fingerprinting and genetic studies in *Onobrychis* species, solving the lack of available SSR markers in this species.

Keywords Cultivar identification · Fingerprinting · Microsatellites · Molecular markers · *Onobrychis* · Sainfoin · SSR transferability

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Introduction

Sainfoin (*Onobrychis viciifolia* Scop.) is a traditional European fodder legume that thrives on well-drained alkaline soils in many parts of Central and Southern Europe. Cultivated sainfoin is an allotetraploid species ($2n = 4x = 28$, Hayot-Carbonero et al. 2010) obtained from wild botanical forms (Badoux 1965). Sainfoin domestication occurred at the end of the XVI century in the French part of the Rhine Valley. Soon, the common and giant types were selected: the first is characterized by its rusticity and persistence, the second by its higher production level and vigour (Gasparin 1846).

Despite their wide spread in Europe, a decline in the area of sainfoin, along with other temperate legumes, was mainly due to the expansion of grass production based on cheap inorganic nitrogen fertilizers in the 1970s. This intensive ruminant production system has contributed to high air and water pollution within the EU (Gill et al. 2009; Rochon et al. 2004). More recently, the move to more extensive and sustainable production systems has increased the interest for forage legumes since they offer a potential to mitigate these effects while providing local protein sources that are becoming of increasing importance in animal feeds. In this new scenario, sainfoin is appearing as a clear home-grown alternative for fodder production. Sainfoin has some anthelmintic (Hoste et al. 2006) and nutritional advantages in comparison with other forage legumes,

most notably due to its condensed tannins content (Mueller-Harvey 2006), which may lead to a better dietary protein utilisation (Min et al. 2003), lower methane emissions and risk of bloat (Puchala et al. 2005; Ramirez-Restrepo and Barry 2005) in ruminant livestock. Agronomically, it's positive characteristics include a deep taproot, that allows the plant to be very resistant to drought and of course, being a legume, there is a high level of residual fertility after sainfoin ley has been ploughed (Koivisto and Lane 2001).

While advances in plant breeding have led to improved lucerne varieties, sainfoin production has remained relying on old cultivars. Characterisation of existing germplasm seems to be necessary in order to preserve these genetic resources and provide alternative approaches for developing further breeding programmes. While morphological characterization is a necessary step in plant breeding, several molecular tools are being used to complement plant variety characterization and identification. Among these, microsatellites or simple sequence repeats (SSRs) have become one of the most useful molecular marker systems in plant breeding. They are widely used in cultivar fingerprinting, genetic diversity assessment, molecular mapping, and marker assisted breeding due to their high polymorphism, repeatability and because they are codominant.

In legumes, the availability of SSRs has allowed to make progress in the characterization and assessment of the genetic diversity of species like *Medicago* (Falahati-Anbaran et al. 2004), SSR cross-species amplification has been assessed (Peakall et al. 1998) and cross-amplified SSRs have been used for genetic mapping in *Trifolium repens* (L.) (Zhang et al. 2007). While there are not microsatellites available for *O. viciifolia*, the screening of SSRs identified in *Medicago* and *Glycine* spp. offers the possibility to find similar regions on the *Onobrychis* genome that would allow a quick and reliable characterization of the sainfoin material.

Microsatellites isolation can be time and money consuming in the absence of abundant DNA sequences in a species, restricting sometimes their use to a few important agricultural crops. Still, the amount of SSRs identified is increasing at a steady pace, especially in legumes model species, and the development of SSR markers through data mining has also become an efficient and low cost option for many plant species. An alternative approach consists in the utilization of SSR loci from related species (Smulders et al. 1997). The transferability of SSR loci often depends on genetic relatedness, and while high rates of transferability across species within the same genus (>50%) has been reported in tree species (Liewlaksaneeyanawin et al. 2004; Wünsch and Hormaza 2002) and among legumes (Eujayl et al. 2004; Gaitán-Solís et al. 2002; Peakall et al. 1998), the transferability across genera and beyond seems to be lower (Peakall et al. 1998; White and Powell 1997). On the other side, SSRs identified in ESTs (EST-SSR) markers show higher transferability rates and are expected to be more conserved than genomic SSR markers (Scott et al. 2000).

In the absence of microsatellite loci available in the genus *Onobrychis*, in this work the transferability of microsatellite markers from *Medicago* and *Glycine* to *Onobrychis* species was evaluated. A group of microsatellites were selected and these were used to provide an initial characterization of a collection of sainfoin accessions and to carry out a preliminary evaluation of the genetic diversity among them.

Materials and methods

Plant material and genomic DNA extraction

Twenty-three accessions of sainfoin were used in this study (Table 1), among them 12 European accessions represent the commercial material available, nine are accessions collected from seed production growers in the North-eastern part of Spain and the remaining two are selections obtained aiming respectively for persistency and for production from the CITA (Zaragoza, Spain). Two related wild species from the Pyrenees range, *O. pyrenaica* Sennen and *O. argentea* Boiss., were included to check their relatedness to *O. viciifolia* and between themselves, and *Trifolium repens* was used as an outgroup (Table 1). Additionally, *Medicago truncatula* Gaertn. and *Trifolium repens* L. accessions were used as positive controls when testing SSR transferability.

Ten plants per population were randomly selected and young healthy leaves from each plant were collected for DNA extraction. Genomic DNA was extracted using the modified (Doyle and Doyle 1987) DNA extraction protocol described by Hormaza

Table 1 List and origin of the 27 accessions analysed in this work

Species	Accession name	Number	Origin	Provided by
O. viciifolia	Ambra	5	Italy	Caussade semences (Fr)
O. viciifolia	Cotswold common	1	United Kingdom	Cotswold seeds (UK)
O. viciifolia	Esparcette	3	Poland	Cotswold seeds (UK)
O. viciifolia	Fakir	6	France	INRA (Fr)
O. viciifolia	Incoronata	8	Italy	INEA Bari (It)
O. viciifolia	Korunga	11	Turquey	Agricultural Faculty, Izmir
O. viciifolia	Polonia	12	Poland	Rocalba S.A. (Spain)
O. viciifolia	Sepial	4	France	Caussade semences (Fr)
O. viciifolia	Somborne	2	United Kingdom	Cotswold seeds (UK)
O. viciifolia	Ukrania	7	Ukraine	Rocalba S.A. (Spain)
O. viciifolia	Visnovsky	9	Czech Republic	Research Inst. for Fodder Crops
O. viciifolia	Yubileyna	10	Bulgaria	Forage Research Inst., Pleven
O. viciifolia	Graus	21	Spain	Collected landrace
O. viciifolia	Lagueruela	17	Spain	Collected landrace
O. viciifolia	Loarre	18	Spain	Collected landrace
O. viciifolia	Mezquita	16	Spain	Collected landrace
O. viciifolia	Reznos	14	Spain	Collected landrace
O. viciifolia	Selection 1	15	Spain	Selection
O. viciifolia	Selection 2	13	Spain	Selection
O. viciifolia	Tartareu	22	Spain	Collected landrace
O. viciifolia	Torrecilla de Cameros	20	Spain	Collected landrace
O. viciifolia	Villahermosa del Río	23	Spain	Collected landrace
O. viciifolia	Villahoz	19	Spain	Collected landrace
O. argentea			Spain	Wild species, collected
O. pyrenaica			Spain	Wild species, collected
Medicago truncatula			Spain	Unspecified cultivar
Trifolium repens			Spain	Wild species, collected

(1999). Extracted DNA was quantified spectrophotometrically and diluted to 10 ng/µl before PCR amplification. For testing SSR transferability, individual DNAs were used, for variety characterization an equivalent amount of diluted DNA of the ten plants was pooled and used for SSR amplification.

SSR analysis

Twenty seven selected microsatellites from *Medicago truncatula* and *Glycine max* (L.) Merr. (Gutierrez et al. 2005; Peakall et al. 1998; Zhang et al. 2007) previously cross-amplified in other legume species (Gutierrez et al. 2005; Peakall et al. 1998; Zhang et al. 2007) were tested for transferability in *O. viciifolia* (Table 2). PCR reactions were performed in 20 µl volumes containing 20 mM Tris–HCl, pH 8.4, 50 mM KCl, 4 mM $MgCl_2$, 0.1 mM each dNTP, 0.2 mM each primer, 40 ng genomic DNA and 0.45 units Taq polymerase (Life Technologies, USA). Reactions were carried out on a iCycler thermocycler (BioRad, USA) using the following temperature profile: an initial step of 2 min at 94°C, 35 subsequent cycles of 45 s at 94°C, 45 s at Ta and 1 min at 72°C, and a final step of 5 min at 72°C. Ta being the annealing temperature, optimised for each SSR locus. PCR products were separated by electrophoresis using 3% 'Metaphor' (FMC Bioproducts) agarose gels in 1× TBE buffer at 5 V/cm, stained with ethidium bromide and visualised under UV

Table 2 List of microsatellites analysed in this work for transferability and polymorphism in *Onobrychis*

SSR locus	Reference (of cross-amplification)	Original species	Cross-amplification in other species	Transferability and polymorphism in Onobrychis
AG81	Peakall et al. (1998)	*G*	*V*, *P*, *L*	p
AW567861	Zhang et al. (2007)	*G*	*T*	p
BE608020	Zhang et al. (2007)	*G*	*T*	na
MtBA01B04R2	Gutierrez et al. (2005)	*M*	*V*, *P*, *C*	p[a]
MtBA27D09F1	Gutierrez et al. (2005)	*M*	*V*, *P*, *C*	p
MtBA29B03F1	Gutierrez et al. (2005)	*M*	*V*, *P*, *C*	np
MtBA25E01F1	Gutierrez et al. (2005)	*M*	*V*, *P*, *C*	np
MtBA28E09F1	Gutierrez et al. (2005)	*M*	*V*, *P*, *C*	np
MtBC27H11F1	Gutierrez et al. (2005)	*M*	*V*, *P*, *C*	na
MtBA09E01R1	Gutierrez et al. (2005)	*M*	*V*, *P*, *C*	na
MtBB36F05F1	Gutierrez et al. (2005)	*M*	*V*, *P*, *C*	p[a]
MtBA04C08R1	Gutierrez et al. (2005)	*M*	*V*, *P*, *C*	p
MtBA32A12R1	Gutierrez et al. (2005)	*M*	*V*, *P*, *C*	np
MtBA37D08F1	Gutierrez et al. (2005)	*M*	*V*, *P*, *C*	na
MtBB36E02F1	Gutierrez et al. (2005)	*M*	*V*, *P*, *C*	np
MtBB22G10F1	Gutierrez et al. (2005)	*M*	*V*, *P*, *C*	p
MtBC47B06F1	Gutierrez et al. (2005)	*M*	*V*, *P*, *C*	p[a]
MtBA02H03F3	Gutierrez et al. (2005)	*M*	*V*, *P*, *C*	np
MtBB44F02R1	Gutierrez et al. (2005)	*M*	*V*, *P*, *C*	p[a]
BI74	Zhang et al. (2007)	*M*	*T*	p[a]
AL79	Zhang et al. (2007)	*M*	*T*	p[a]
BG178	Zhang et al. (2007)	*M*	*T*	p
BE74	Zhang et al. (2007)	*M*	*T*	na
AW64	Zhang et al. (2007)	*M*	*T*	np
AL46	Zhang et al. (2007)	*M*	*T*	p
AW306	Zhang et al. (2007)	*M*	*T*	np
AW265	Zhang et al. (2007)	*M*	*T*	p

na no amplification, *np* not polymorphic, *p* polymorphic

[a] selected SSRs for *O. viciifolia* characterization. *Medicago. truncatula* (*M*), *Glycine max* (*G*), *Vicia faba* (*V*), *Pisum sativum* (*P*), *Cicer arietinum* (*C*), *Lupinus angustifolium* (*L*), *Trifolium repens (T)*

light. *M. truncatula* and *T. repens* DNA was used as positive controls in the PCR reactions. Band scoring was carried out using DNA size standards (10 bp or 1 kb, Life Technologies) depending on the SSR amplification range. Those SSRs that were amplified in *Onobrychis* and that allowed a clear scoring of SSR alleles were selected for the characterization of all the accessions. Thus 6 SSRs (Table 3) were selected and evaluated in the 25 *Onobrychis* accessions and *T. repens*. All PCR amplifications were done at least twice in order to ensure the results reproducibility.

Data analysis

The polymorphism information content (PIC) of each of the analysed SSRs was calculated using the formula: $PIC = 1 - \sum_{j=1}^{n} Pij^2$ (Botstein et al. 1980) where Pij is the frequency of the '*i*th' allele for marker '*i*' in the '*jth*' population and summation extends over n alleles. For the estimation of genetic similarity SSR alleles were scored as presence or absence in all the accessions and a similarity matrix was obtained from the proportion of shared fragments (Nei and Li 1979), these calculations were performed

Table 3 Number of alleles, PIC value and range of amplification of six SSR loci analysed in 23 sainfoin populations

SSR locus	No of alleles	Range of allele size in *M. truncatula* (bp)	Range of allele size in *O. viciifolia* (bp)	PIC value
MtBA01B04R2	7	400–370	420–333	0.84
MtBB36F05F1	5	220–180	220–180	0.82
MtBC47B06F1	6	110	779–466	0.80
MtBB44F02R1	5	140–130	237–143	0.58
BI74	7	177–79	865–79	0.85
AL79	5	240	506–389	0.45
Total	35			

PIC polymorphism information content, *SSR* simple sequence repeat

using the NTSYS-pc 2.02 package (Rohlf 2002). To visualize differences between populations, the similarity matrix was used to run a Principal Component Analysis (PCA) in Genalex 6.1 (Peakall and Smouse 2006). Scatter plot representation was used to represent the first two Eigen values.

Results

The 27 SSRs analysed were correctly amplified in *Medicago truncatula*, their original species, or in *Trifolium repens*, a species in which they had already been tested for cross-amplification (Table 2). In *Onobrychis* species, consistent amplification was obtained for 22 (81%) of the 27 microsatellites tested (Table 2) once an appropriate annealing temperature was optimised for each microsatelite. However, amplification sizes of the different SSRs were usually larger in *Onobrychis viciifolia* (79–865 bp) than that originally described in *Medicago* (79 to 240 bp).

Fourteen of these 22 SSRs were polymorphic in all three *Onobrychis* species (Table 2) and six of them were selected for characterization of the sainfoin genotypes as they allowed the detection of clear bands and easy fragment recognition (Table 2). A total of 35 alleles were detected using the six microsatellites in the 25 *Onobrychis* populations. Each SSR amplified 5–7 alleles in *Onobrychis* and the average number of alleles per SSR was 5.83 (Table 3). The PIC values were calculated for each SSR on the 25 *Onobrychis* accessions (Table 3). This value ranged from 0.45 to 0.85 with the average being 0.72. Microsatellite BI74 was the SSR that revealed the higher polymorphism in the accessions tested.

While *O. viciifolia* accessions showed high genetic similarity, it was lower between *O. viciifolia* and *Onobrychis* wild species, *O. argentea* and *O. pyrenaica* (Fig. 1). These two species seemed to be closely related to each other as their similarity was almost 0.95. However, *T. repens*, was remarkably distinguished, with only a 0.23 of similarity with *Onobrychis* accessions.

The analysis of 6 SSRs in 23 populations of *O. viciifolia* and in *O. pyrenaica*, *O. argentea* and *T. repens*, produced unique fingerprints for each population, allowing the differentiation of all the 26 accessions. The first two Eigen vector of the PCA analysis explained 60.1% of the observed variation (Fig. 1). From this analysis a clear differentiation was observed between *T. repens* and all the *Onobrychis* species. Another group brought together the two wild species *O. argentea* and *O. pyrenaica*, clearly separated from *O. viciifolia* accessions and the *T. repens* genotype.

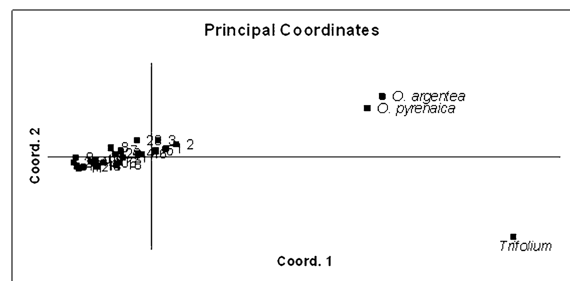

Fig. 1 Scatter plot representing the PCA analysis on the genetic similarities among *O. pyrenaica*, *O. argentea*, *T. repens* and 23 accessions of *O. viciifolia*, based on 6 microsatellite loci (Genalex 6.1)

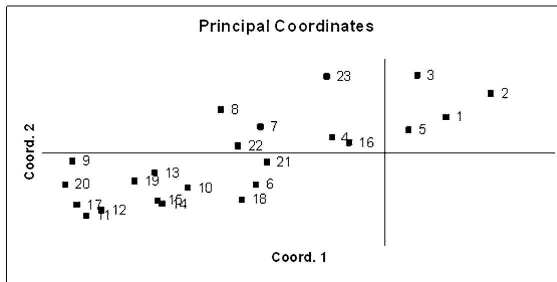

Fig. 2 Scatter plot representing the PCA analysis on the genetic similarities among *O. pyrenaica*, *O. argentea*, *T. repens* and 23 accessions of *O. viciifolia*, based on 6 microsatellite loci (Genalex 6.1), zoom on *O. viciifolia* accessions

All 23 *O. viciifolia* accessions were clustered in a genetically diverse group (Fig. 2). Within these accessions 'Cotswold common', 'Somborne', 'Esparcette', 'Ambra', 'Sepial', 'Mezquita de Jarque' and 'Villahermosa del Rio' scattered in a large clump, that appeared to be distant from the more tightly related 'Ukrania', 'Incoronata', 'Ambra', 'Selection 1', 'Lagueruela', 'Loarre', 'Reznos', 'Visnovsky', 'Polonia', 'Korunga', 'Yubilena', 'Torrecilla de Cameros', 'Villahoz', 'Selection 2', 'Graus' and 'Tartareu'. The British accessions, 'Cotswold Common' and 'Somborne', were the most genetically distant from the East European accessions like 'Visnovsky', 'Polonia' and 'Korunga'. The French and Italian 'Ambra', 'Sepial', 'Incoronata' and 'Fakir' spread the British and East European accessions. Spanish accessions like 'Mezquita de Jarque', 'Selection 1', 'Lagueruela', 'Loarre', 'Reznos', 'Torrecilla de Cameros', 'Villahoz', 'Selection 2', 'Graus' and 'Tartareu'. were distributed among French, Italian and East European accessions.

Discussion

In order to characterize and evaluate the genetic diversity of cultivated sainfoin, microsatellite loci derived from related legume species were evaluated for transferability in *O. viciifolia* and the wild species *O. argentea* and *O. pyrenaica.* Transferable SSR loci were selected on the basis of polymorphism and reproducibility and these were used for the characterization and genetic similarity evaluation of 23 sainfoin accessions.

Cross-genus transferability

The proportion of microsatellites transferable between *Medicago* and *Onobrychis* (81%) was higher than data previously reported for cross-genus amplification. Intra-genus amplification was usually around 50% (Eujayl et al. 2004; Peakall et al. 1998) and declined quickly when reaching out the genus. Zhang et al. (2007) found 18–22% of transferability from *Medicago* to *Trifolium*, but Peakall et al. (1998) reported only 1–3% of transferability of *Glycine*'s SSR among other legumes genus. In this work, EST-SSR were used and these are expected to be more conserved than genomic SSR (Peakall et al. 1998; Scott et al. 2000). Additionally, the SSR loci assayed for transferability had already been shown to be conserved among different genera. Thus the high level of transferability detected here indicates that selecting cross-conserved SSR for transferability across new species is a good strategy for the identification of transferable SSR in a new species. In spite of the conserved nature of the EST-SSRs, which may limit their polymorphism, a high proportion of microsatellites were found to be polymorphic in *Onobrychis* (63% of transferable SSRs). Six of these were used for the analysis and showed high PIC values (average 0.72) indicating their usefulness for molecular studies in *O. viciifolia*.

The number of allele per locus observed in *Onobrychis* ranged from 5 to 7, which is slightly lower than previously reported in lucerne (4–14) (Falahati-Anbaran et al. 2004). Since *Onobrychis* is a tetraploid species and accessions were analysed in bulks of 10 individuals the number of alleles detected seem to be low. This could be caused for various reasons. First the detection method used, agarose electrophoresis, is not sensitive enough to differentiate alleles with small size differences and it may not be scoring alleles that are being amplified. Nevertheless, working with a tetraploid species, an initial evaluation of the accessions by horizontal electrophoresis allows the visualization of the amplified loci and facilitates a preliminary evaluation of the markers. Otherwise more sensitive detection methods like capillary electrophoresis would provide fragment patterns even more difficult to score due to the stutter of the markers. On the other hand, the bulking of DNA samples and their co-amplification by PCR cause less frequent alleles to be lost and only most common

alleles to be detected. This may cause that a number of alleles present in the population could not be detected, leading therefore to an under estimation of the number of alleles detected per loci in each population.

In various SSR (like MtBC47B06F1 and MtBB44F02R1) various loci seemed to be amplified. The generation of multiple products during cross-species amplification may occur by mutation, rearrangements and duplications in the flanking region and/or changes in the number of repeats (Peakall et al. 1998), as reported by Gutierrez et al. (2005) using EST-SSR in legumes.

Genetic similarity

The level of polymorphism shown by the six microsatellites selected made it possible to produce a unique fingerprint of each population. The PCA analysis based on the coefficient of similarity differentiated all *O. viciifolia* accessions from *T. repens* and from *O. argentea* and *O. pyrenaica* wild species, that were highly similar amongst themselves. This is surprising because morphologically *O. argentea* is more similar to *O. viciifolia* than to *O. pyrenaica*.

All sainfoin varieties shared a similarity coefficient above 0.80, matching results from Sardaro et al. (2003) that reported by ALFP a similarity level of 0.73 among sainfoin accession using AFLP analysis. The high similarity found among *O. viciifolia* accession suggested that they were more closely related between themselves than varieties of *Trigonella foenum-graecum* L. (0.66, Dangi et al. 2004) or *Phaseolus coccineus* L. or *vulgaris* L. (0.6 and 0.75, Sicard et al. 2005) which are more ancient domesticated legume crops.

The distribution of the *O. viciifolia* genotypes in the PCA analysis was in relation with the geographic distribution of these accessions, with the British accessions more distant from the rest, East Europeans clustered together and distant from the British ones, and French and Italian accessions in between these two groups. The British cultivars 'Cotswold Common' and 'Somborne', were clearly differentiated from the other material and this was probably caused by the isolation of British germplasm and low seed exchanges with the rest of the continent from early 1980 s (Aldrich 1984). Only East European accession 'Esparcette' from Poland, appeared closer to the British accessions, and was provided by the same seed producer, Cotswold Seeds, and this similarity may be due to common ancestry or hybridisation.

On the other side, Spanish accessions showed a high genetic similarity with both East European and French accessions. Sainfoin originally sown in Spain was the "common" type and giant sainfoin was introduced experimentally by the Botanical Gardens of Madrid in 1791 (Muller 1893). Since then there have been successive introductions, the most important being promoted by the Ministry of Agriculture at the end of the 1960 s. Thus, foreign giant sainfoins (mainly introduced from France) could have mixed with native forms (Pujol 1974) given that sainfoin is thought to be an allogamous species. Nowadays, part of the seed demand is met with imports from Eastern Europe countries. This situation together with seed transfer between farmers and commercial enterprises is contributing further to the contamination of local ecotypes (Delgado et al. 2008). This is particularly true for "Selection 1" and 'Selection 2' which were obtained from French and East European material. 'Villahermosa del Rio' was found to be the most distinct accession of all and therefore represent an interesting source of variability for breeding purposes.

The results obtained indicate that conservation of SSR allows successful cross-genus amplification of *Medicago* and *Glycine* microsatellite markers in *Onobrychis* and *Trifolium*, which belong to the same family (Leguminosae) but different genera. Transferable selected microsatellites were useful to fingerprint *Onobrychis* accessions and to estimate their genetic diversity. The availability of these markers will be useful to accelerate the implementation of appropriate germplasm conservation strategies, to assist in the selection process for development of synthetic varieties with high level of heterosis or to assess seed purity in sainfoin collections.

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