Analysis of genetic diversity of the critically endangered *Onobrychis conferta* subsp. *conferta* using cross-genera transferability of SSR markers developed from related legume species

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Abstract

The use of transferable cross-species/genera SSR markers is an alternative strategy to ensure availability of markers in genomic resources-limited crops, such as critically endangered species. Eighteen microsatellite markers derived from the genomes of *Medicago truncatula* Gaertn., *Phaseolus vulgaris* L. and *Onobrychis viciifolia* Scop. were tested for transferability and used to study the genetic diversity of the three remaining populations of the critically endangered species *Onobrychis conferta* subsp. *conferta*, collected from their natural habitats. All pairs of primers tested were found to be polymorphic and reproducible. A total of 257 alleles were obtained from 134 loci, resulting in an average of 1.93 alleles per locus. The average number of alleles per accession was 51.4, yielding an average of 14.3 alleles per SSR marker and accession. The lowest number of alleles was recorded in *O. conferta* from Aïn Dyssa, with 37 alleles, while the highest was observed in *O. viciifolia*, with 63 alleles. Each SSR amplified 3-16 alleles. The MTIC343 primer yielded the highest number of loci (16 loci). The mean Polymorphism Information Content (PIC), Marker Index (MI), and Resolving Power (Rp) were respectively 0.36, 2.22, and 4.58, indicating a high level of polymorphism in the studied SSR markers. UPGMA cluster analysis grouped genotypes into two main clusters in corroboration with the morphological distinction of sections. Our study demonstrated that *O. conferta* subsp. *conferta* genomes could be successfully examined using other legume SSR markers, providing a valuable tool to detect polymorphism for future genetic studies, breeding programs and conservation strategies, addressing the lack of available SSR markers in this genus. These microsatellite loci may help to further survey the adaptive evolution and genetic variation of *Onobrychis conferta* conservation.

Keywords: Genetic diversity, *Medicago truncatula*, *Onobrychis conferta*, *O. viciifolia*, SSR transferability

INTRODUCTION

The genus *Onobrychis* Miller belongs to the tribe *Hedysareae* (Fabaceae) and comprises nearly 170 species distributed worldwide (Sakhraoui *et al.,* 2023a). *Onobrychis* species are mainly found in the north temperate regions, with the most important diversity centres in the eastern Mediterranean and West Asia. The genus includes annual and perennial species, predominantly caulescent herbs (rarely spiny shrubs) with an indumentum of simple hairs or glabrous (Lock and Simpson, 1991, Yakovlev *et al.,* 1996, Mabberley, 1997).

Wild species of the genus *Onobrychis* have some interesting agronomic and ecological features such as perenniality, a deep root system and high-stress tolerance, which make them suitable for future cultivation under climate change (Sakhraoui *et al.,* 2023a). Wild *Onobrychis* species can be considered advantageous in crop rotation, as they positively influence weed control by limiting the growth and reproduction of herbaceous species through their procumbent and compact canopy (Erfanzadeh *et al.,* 2020). They are multipurpose species that can be utilized for fodder and honey production even under harsh environmental conditions. *Onobrychis* species provide valuable browse for livestock and are essential wildlife grazing sources throughout the Mediterranean Basin. In arid and semiarid areas, especially during the dry season, they can serve as an alternative food source for livestock when other forage yields are insufficient (Le Houérou, 1995). Moreover, these species play a crucial role in the conservation and recovery of degraded areas (Niknam *et al.,* 2018). Additionally, extracts from wild *Onobrychis* species exhibit a wide range of pharmacological effects, including antioxidant, antidiabetic, anti-inflammatory, antitumor, antimicrobial, and anti-stressor properties (Sakhraoui *et al.,* 2023a).

In comparison to cultivated forage legumes, wild *Onobrychis* species remain underexploited although they have the potential to address forage and food insecurity, either through domestication or by contributing novel alleles for the breeding of the cultivated species *Onobrychis viciifolia* (Sakhraoui *et al.,* 2023a). Wild *Onobrychis* species possess a very rich genetic diversity to adapt to high temperatures, drought, salinity, disease and pests, and can be integrated into breeding programs to create resistant varieties (Hart, 2001). Therefore, targeted breeding activities are needed to select individuals better adapted to a broad range of environmental conditions. However, breeding activities for *Onobrychis*

species have been rather scarce and the species has only recently gained broader interest (Bhattarai *et al.,* 2016). For targeted improvement of *Onobrychis* species, diverse and well characterised genetic resources are needed. Novel breeding approaches must be developed based on knowledge of the mode of inheritance of key breeding targets and the development of molecular genetics tools to assist phenotypic selection.

Although novel approaches such as genotyping by sequencing (Elshire *et al.,* 2011) may be readily applied to any species, most approaches require the availability of molecular markers or genome sequence information. Such resources are scarce for *Onobrychis* species and most of genetic studies have been largely based on universal marker systems such as internal transcribed spacer regions (ITS) (Hayot-Carbonero *et al.,* 2012), inter simple sequence repeats (ISSR) (Bhattarai *et al.,* 2016), sequence-related amplified polymorphism (SRAP) markers (Kempf *et al.,* 2015; Kempf *et al.,* 2017) or sequence specific simple sequence repeats (SSR) markers derived from other species (Demdoum *et al.,* 2012). The development of highly informative, specific markers for *Onobrychis* species is indispensable to create a genetic knowledge base and assist breeding by marker assisted selection (MAS) (Sorrells and Wilson, 1997).

SSR markers show codominance 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 *Onobrychis* species genome, and especially the lack of knowledge about its genetic information and maps, SSRs are the markers of choice. These markers are multi-allelic in contrast to next generation high-throughput sequencing (NGS) derived single-nucleotide polymorphism (SNP) marker that 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 closely related taxa (Chen *et al.,* 2015; Ziya, 2016). Transferability is an important property for further studies that have been hampered by the limited availability of species-specific molecular markers (Avci *et al.,* 2014).

Onobrychis conferta subsp. *conferta* (Desf.) desv. is a Critically Endangered species native to arid and semi-arid *Pinus halepensis* forests in North Africa and has only three remaining populations in Tunisia, facing an extremely high risk of extinction in the wild (Sakhraoui *et al.,* 2023b). This endemic species is currently in sharp decline on the calcareous skeletal soils of the Western Tunisian Ridge and the Saharan Atlas (Le Houérou, 1995). Consequently, it is very urgent to understand the genetic diversity and develop a conservation strategy for *O. conferta* subsp. *conferta*.

To develop a set of *O. conferta* specific SSRs markers which reliably detected polymorphism among the three remaining populations of the critically endangered species *Onobrychis conferta* subsp. *conferta*, we explored cross species/genera transferability of 18 SSR markers from *M. truncatula*, *P. vulgaris* and *O. viciifolia* genomes. We hypothesized that these microsatellite markers derived from related legume species would represent a valuable tool to successfully differentiate *O. conferta* populations and to study their genetic diversity.

MATERIALS AND METHODS

Plant material

Based on data published by Pottier-Alapetite (1979) on the range of Tunisian wild species of the *Onobrychis* genus, prospecting missions were carried out during May and June 2018 in North-western Tunisia. Three populations of the critically endangered species *Onobrychis conferta* subsp. *conferta* were found and leaf samples were collected from each population. The geographic locations of the collection sites were recorded using a GPS (Garmin 72H receiver, Olathe, Kansas, USA) (Table 1). Herbarium specimens of the sampled *O. conferta* subsp. *conferta* populations were preserved at the Herbarium of Higher School of Agriculture of Mograne.

Genomic DNA extraction

Thirty plants per population were randomly selected and young fresh healthy leaves from each plant were collected for DNA extraction as described by Reyes-Valdés *et al.* (2013). Genomic DNA extraction was carried out according to Saghai-Maroof *et al.* (1984) with slight modifications. DNA quality and quantity were assessed using gel electrophoresis, spectrophotometry and diluted to 50 ng/µl before PCR amplification.

Table 1: Code, taxon name, origin, elevation (m above sea level), geographical coordinates and bioclimatic zone for analysed taxa

Code	Taxon	Source (Location, province and country)	Elevation (m)	Latitude	Longitude	Bioclimatic zone[a]
OC1	*Onobrychis conferta*[b]	Dyr El Kef, Kef, Tunisia	930	36°12'35.55"N	8°44'32.90"E	USATW
OC2	*Onobrychis conferta*[b]	Aïn Dyssa, Siliana, Tunisia	868	35°57'47.89"N	9°15'22.16"E	USACW
OC3	*Onobrychis conferta*[b]	Fedj El Hdoum, Siliana, Tunisia	532	36°20'28.70"N	9° 7'50.53"E	USATW
OV1	*Onobrychis viciifolia*[b]	France	-	-	-	-
MT	*Medicago truncatula*	Dyr El Kef, Kef, Tunisia	930	36°12'35.55"N	8°44'32.90"E	USATW

[a] *Bioclimatic zone are defined according to Emberger's (1976) coefficient: USATW upper semiarid variant at temperate winter, USACW upper semiarid at cool winter,* [b]subsp. *conferta*

SSR genotyping

Eighteen microsatellites' loci originally developed for *M. truncatula* (15) *O. viciifolia* (2) and *P. vulgaris* (1) were tested for transferability and to identify genetic diversity in *O. conferta* (Table 2). PCR amplification reactions were performed in a final volume of 25 µl containing 2.5 µl of genomic DNA (50 ng), 2.5 µl each of primer, 1.5 µl of $MgCl_2$ (25 mM), 0.2 µl of Taq DNA polymerase (5 U/µl), 2.5 µl each dNTP (0.2 mM), 5 µl of 10X Taq buffer and 8.3 µl of sterilized distilled water. PCR was carried out using an Applied Biosystems™ 2720 Thermal Cycler (Thermo Fisher Scientific Inc., Illkirch, France). The PCR conditions were optimized with a 4 min initial denaturation at +94 °C followed by 35 cycles of +94 °C for 1 min, with the annealing temperature optimized for each SSR locus for 1 min and an extension at +72 °C for 2 min and final extension step at +72 °C for 3 min before cooling to +4 °C. PCR products were separated by electrophoresis using 3% PhileKorea agarose gels in 1x TBE buffer at 5 V/cm, stained with Cleaver Scientific Runsafe Stain CSL-RUNSAFE and visualized under UV light using the Gel-Documentation Unit (Consort Bvba, Turnhout, Belgium) and photographed. Band scoring was carried out using DNA size standards (100 bp plus DNA Ladder) depending on the SSR amplification range. Bands of a particular molecular weight were scored manually as presence (1) or absence (0).

Data analysis

The percentage of transferability of the markers was calculated by determining the presence of target SSR loci to the total number of the SSR loci analysed. POPGENE 1.32 software (Yeh *et al.,* 1999) was used to generate the pairwise genetic distance (GD) matrix and to calculate the effective number of alleles (ne), observed number of alleles per locus (na), Nei's (1973) gene diversity (h) and Shannon's information measure (I) (Lewontin, 1972). A similarity coefficient among species was calculated according to Nei (1972). Jaccard's coefficient of similarity

Table 2: List and origin of microsatellites tested for transferability and polymorphism in *Onobrychis conferta* subsp. *conferta*

No.	Primers	Sequences 5'-3'	Repeated motif	Donor specie	Ta* (°C)	References
1	AL79	F: CCCCATTGACGCATTCTTAC R: TCCTCAACCAACCACTTCCT	$(CTT)_8$	*M. truncatula*	52	Zhang *et al.,* (2007)
2	BI74	F: TGTACCAAGCGAATGAAGTGTT R: GGGTTGCATCTAACAACAGACA	$(TGAG)_9$	*M. truncatula*	52	Zhang *et al.,* (2007)
3	BM175	F: CAACAGTTAAAGGTCGTCAAATT R: CCACTCTTAGCATCAACTGGA	$(AT)_5$-$(GA)_{19}$	*P. vulgaris*	55	Blair *et al.,* (2009)
4	B14B03	F: GCTTGTTCTTCTTCAAGCTC R: ACCTGACTTGTGTTTTATGC	$(CA)_9$	*M. truncatula*	55	Julier *et al.,* (2003)
5	FMT13	F: GATGAGAAAATGAAAAGAAC R: CAAAAACTCACTCTAACACAC	$(GA)_2GG(GA)_9$	*M. truncatula*	52	Julier *et al.,* (2003)
6	MAA660456	F: GGGTTTTTGATCCAGATCTTAA R: GGTGGTCATACGAGCTCC	$(TTC)_8$	*M. truncatula*	50	Julier *et al.,* (2003)
7	MTIC21	F: GGTGATTGACTGTGGTGTCG R: TCCGGTCTCCCAGGTTCTA	$(AAG)_5$	*M. truncatula*	51	Julier *et al.,* (2003)
8	MTIC27	F: CGATCGGAACGAGGACTTTA R: CCCCGTTTTTCTTCTCTCCT	$(AAG)_6$	*M. truncatula*	52	Julier *et al.,* (2003)
9	MTIC82	F: CACTTTCCACACTCAAACCA R: GAGAGGATTTCGGTGATGT	$(TC)_{11}$	*M. truncatula*	55	Julier *et al.,* (2003)
10	MTIC84	F: TCTGAGAGAGAGACAAACAAAAC AAR: GGGAAAAGGTGTAGCCATTG	$(TC)_{11}$	*M. truncatula*	52	Julier *et al.,* (2003)
11	MTIC235	F: CCTTTGGTTGATTCAGTTTC R: CCAATATGTCACTCCTTGCT	$(ATT)_7$	*M. truncatula*	54	Julier *et al.,* (2003)
12	MTIC300	F: GGTGATTGGTGTTTTCTGTC R: AGCAAAACTATCACCACCAG	$(ATG)_7$	*M. truncatula*	55	Julier *et al.,* (2003)
13	MTIC338	F: TCCCCTTAAGCTTCACTCTTTTC R: CATTGGTGGACGAGGTCTCT	$(CTT)_5$	*M. truncatula*	56	Julier *et al.,* (2003)
14	MTIC343	F: TCCGATCTTGCGTCCTAACT R: CCATTGCGGTGGCTACTCT	$(GAA)_8$	*M. truncatula*	56	Julier *et al.,* (2003)
15	MTIC432	F: TGGAATTTGGGATATAGGAA R: GGCCATAAGAACTTCCACTT	$(AG)_6$	*M. truncatula*	55	Julier *et al.,* (2003)
16	MTIC451	F: GGACAAAATTGGAAGAAAAA R: AATTACGTTTGTTTGGATGC	$(TC)_{11}$	*M. truncatula*	52	Julier *et al.,* (2003)
17	OVM130	F: GCAAATTATCACCATGCAC R: CGTGAAGAAAATCGGTACTTA	$(AG)_9$	*O. viciifolia*	50	Kempf *et al.,* (2016)
18	OVK158	F: TCAGAGTGTGTTGTGTTGTGT R: AGTGAAGCAAATGTGTGATTT	$(CACT)_6$	*O. viciifolia*	49	Kempf *et al.,* (2016)

**Ta = annealing temperature*

(Jaccard, 1908) was measured, and a dendrogram was constructed based on the similarity coefficient generated by Unweighted Pair Group Method using Arithmetic average (UPGMA) according to Nei and Li (1979) using statistical package NTSYS-pc v2.02 (Applied Biostatistics Inc., New York, USA) (Sneath and Sokal, 1973; Rolf, 2002). To characterize genetic variation, the information content (PIC), resolving power (RP), and marker index (MI) of SSR markers were calculated as follow: $PIC_i = (2f_i \times (1 - f_i))$ (Roldán-Ruiz *et al.,* 2000), $Rp_i = \sum [1 - (2 \times | 0.5 - f_i |)]$ (Prevost and Wilkinson, 1999), $MI_i = PIC_i \times N_i \times \beta_i$ (Powell *et al.,* 1996), where the subscript i represents the i^{th} primer, f_i is the frequency of the amplified allele, $(1 - f_i)$ is the frequency of the null allele, PIC_i is the information content of the i^{th} primer, N_i is the total band for the i^{th} primer, and β_i is the percentage of the i^{th} primer's polymorphic band.

RESULTS

The eighteen pairs of SSR primers were polymorphic, reproducible, and showed 100% transferability despite the various genetic origin (Table 3). A total of 257 alleles were obtained from the 134 loci with an average of 1.93 alleles per locus. Each SSR amplified 3-16 alleles with an average number of alleles per SSR marker of 14.3. The average number of alleles per taxa was 51.4 over all SSR markers, leading to an average of 2.85 alleles per SSR marker and taxa. The lowest number of alleles was recorded for population OC3 with 37 alleles and the highest for OV1 with 63 alleles.

The PIC values in this study varied from 0.32 to 0.48, with an average of 0.36 ± 0.09 (Table 3). The highest PIC value was recorded for MTIC84 marker. Marker index mean value was 2.22 ± 0.52. The highest value was

Table 3: Characterization of 18 polymorphic microsatellites

No.	Marker	No. of allele	Polymorphic band	Band/Locus	PIC	MI	RP
1	**AL79**	18	9	2.00	0.35	1.95	5.4
2	**BI74**	16	7	2.28	0.36	1.76	3.8
3	**BM175**	15	5	3.00	0.32	1.17	2.0
4	**B14B03**	18	11	1.63	0.36	2.44	7.4
5	**FMT13**	17	8	2.12	0.36	1.86	4.6
6	**MAA660456**	11	9	1.22	0.33	3.04	6.8
7	**MTIC21**	11	9	1.22	0.33	3.04	6.8
8	**MTIC27**	23	12	1.91	0.42	2.44	7.4
9	**MTIC82**	17	9	1.88	0.39	2.27	5.6
10	**MTIC84**	9	4	2.25	0.48	2.34	2.2
11	**MTIC235**	11	5	2.20	0.32	1.60	2.8
12	**MTIC300**	15	7	2.14	0.34	1.76	4.0
13	**MTIC338**	18	8	2.25	0.36	1.76	4.4
14	**MTIC343**	30	16	1.87	0.40	2.34	10
15	**MTIC432**	3	3	1.00	0.32	3.52	2.4
16	**MTIC451**	3	3	1.00	0.32	3.52	2.4
17	**OVK158**	15	6	2.50	0.32	1.41	3.0
18	**OVM130**	7	3	2.33	0.37	1.76	1.6
	Total	257	134	-	-	-	-
	Mean	14.27	7.44	1.93	0.36	2.22	4.58

PIC: Polymorphism Information Content, MI: Marker Index, RP: Resolving Power.

Table 4: Summary of genetic variation statistics for all loci

Locus	na*	ne*	I*	h*
OC1	2.0000	1.1637	0.2692	0.1407
OC2	2.0000	1.2863	0.3817	0.2226
OC3	2.0000	1.2412	0.3445	0.1943
OV1	2.0000	1.2117	0.3178	0.1747
MT	2.0000	1.2661	0.3656	0.2102
Mean	2.0000	1.2338	0.33576	0.1885
St. Dev	0.0000	0.0481	0.04424	0.0321

** na = Observed number of alleles * ne = Effective number of alleles (Kimura and Crow, 1964) * h = Nei's (1973) gene diversity * I = Shannon's Information index (Lewontin 1972).*

recorded for MTIC432 and MITC451 (3.52), while the lowest was recorded for MTIC432 (1.4). The 18 markers used in the study exhibited a Rp value ≥ 1.0, ranging from 1.6 for MTIC343 and 10 for OVM130 (average of 4.58 ± 1.08) (Table 3).

In general, the populations included in this study showed a relatively high level of genetic diversity. *O. conferta* populations showed a slightly lower level of genetic variation than *O. viciifolia*. Within *O. conferta* populations, OC2 had the highest level of effective alleles, Shannon's Information index (I) and Nei's gene diversity (h), whereas population OC1 exhibited the lowest value for these parameters. The observed number of alleles (na) for all loci was 2.0000 and the effective number of alleles (ne) ranged between 1.1637-1.2863 (Table 4). Similarly, I and h ranged between 0.2692-0.3817 and 0.1407-0.2226, with a mean value of 0.3358 ± 0.044 and 0.1885 ± 0.032, respectively.

Nei's genetic distance between pairs of populations (GD) ranged from 0.0292 to 0.2513, with an average number of 0.138 ± 0.043. The GD estimation revealed that the most genetically related pair of populations (excluding the outgroup) were OC1 and OC2 (GD = 0.0349). The accessions of OC3 and OV1 were the most distant (GD = 0.1224) (Table 5). The UPGMA dendrogram displayed two primary clusters with a similarity coefficient of 0.89, exhibiting various degrees of sub-clustering that aligned with the morphological distinctions of sections (Figure 1). The three *O. conferta* populations were closely grouped, sharing a 0.94 similarity. OC1 and OC2 appeared particularly related, sharing a 0.97 similarity. *O. viciifolia* presented 0.91 and 0.79 similarity coefficient with the three *O. conferta* populations and with *M. truncatula*, respectively.

DISCUSSION

As we hypothesized, the microsatellite markers derived from related legume species (*M. truncatula*, *P. vulgaris* and *O. viciifolia*) successfully differentiated *O. conferta* populations, indicating their usefulness for molecular studies. This implies that genomes of related legumes could be sources of SSR markers useful for dissecting *O. conferta* genomes that have nil SSR markers developed so far. Amplified markers from the donor species yielded banding patterns in the genome of the three studied *O. conferta* populations, suggesting that they originated from the same loci and that these allelic regions of the primer binding sites were conserved (Saini *et al.,* 2023).

Several attempts have been made to transfer markers among legume species such as *M. truncatula*, *Pisum sativum* L. and *Trifolium pratense* L. to *Lens culinaris* Medik. (Reddy *et al.,* 2010), and *M. truncatula* to six Mediterranean *Hedysarum* species (Zitouna *et al.,* 2013). However, there has been a limited attempt to transfer SSR markers to *Onobrychis* species (Băcilă *et al.,* 2023). In this study, the proportion of transferable microsatellites between the three genera *Medicago*, *Phaseolus* and *Onobrychis* to *O. conferta* was higher than previously reported. Intra-genera amplification was usually about 50% (Peakall *et*

Table 5: Nei's original measures of genetic identity (above diagonal) and genetic distance (below diagonal) (Nei, 1972)

Pop ID	OC1	OC2	OC3	OV1	MT
OC1	****	0.9712	0.9259	0.9156	0.7881
OC2	0.0292	****	0.9300	0.9074	0.8004
OC3	0.0770	0.0725	****	0.9033	0.8169
OV1	0.0881	0.0972	0.1017	****	0.7778
MT	0.2382	0.2226	0.2023	0.2513	****

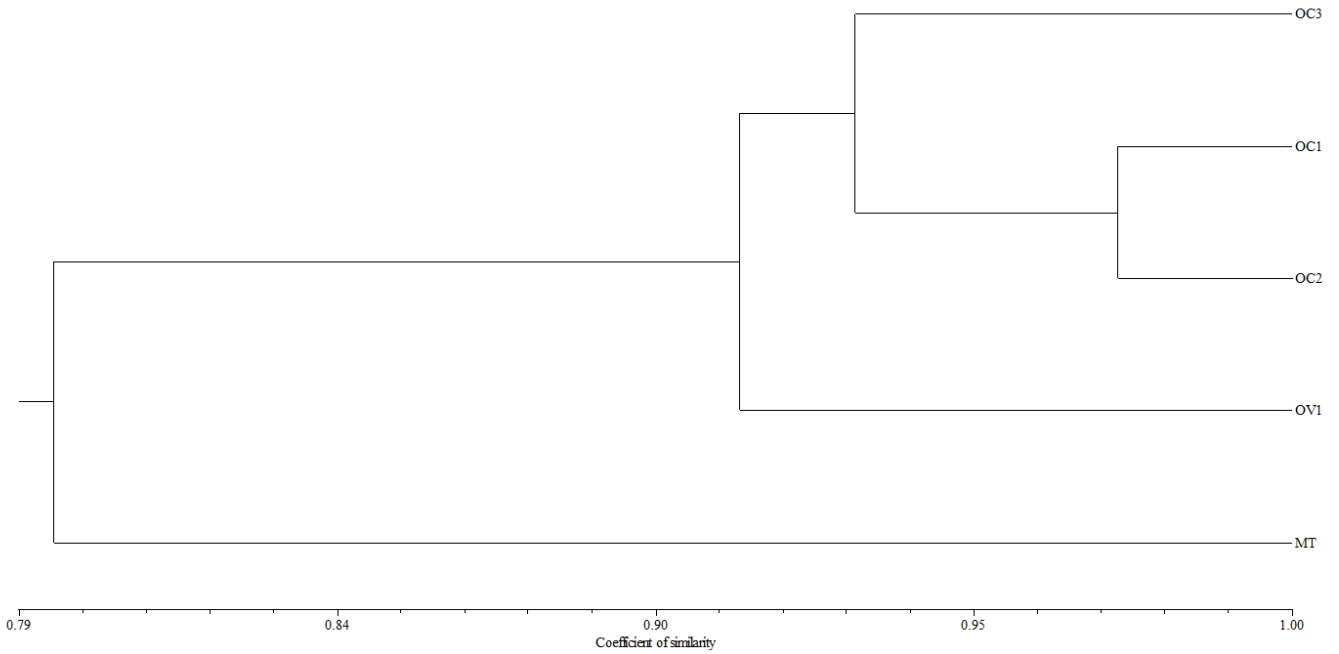

Figure 1: ***UPGMA dendrogram of similarity based on genetic distance***

al., 1998; Eujayl *et al.,* 2004) and it decreased rapidly when it became inter-genera. However, Demdoum *et al.* (2012) reported 81% of SSR markers transferability from *Medicago* and *Glycine* to several *Onobrychis* taxa. In addition, cross genera transferability from *Iris* to *Gladiolus* was 48% (Hiremath *et al.,* 2023). Only 19% of transferability from *M. truncatula* and *Glycine max* (L.) Merr. to *Onobrychis* taxa genome was reported (Avci *et al.,* 2014; Zhang *et al.,* 2007) reported a transferability rate of 18-22% from *Medicago* to *Trifolium*, while Peakall *et al.* (1998) found only 1 to 3% transferability from *Glycine* to other legume genera.

The success of cross-transferability depends upon the evolutionary distance between the source and the target species. The higher the genomic homology, the greater the conservation of SSR-flanking regions and, hence, the transferability of SSR markers (FitzSimmons *et al.,* 1995; Saini *et al.,* 2023). Moreover, it is also worth considering that the degree of transferability and the level of polymorphism of a given SSR marker could be influenced by the level of ploidy, as well as by mutational events (Dirlewanger *et al.,* 2002). Differences in amplification success for SSR markers observed among species are due to the genetic variability between the different species in the Fabaceae Family, where the SSRs were developed. It has been shown that closely related species are more likely to share SSR priming sites than more distantly related ones, but it is possible to transfer functional SSR primers from more distantly related species in some cases (Lorieux *et al.,* 2000). In this sense, these results indicate that *O. conferta* is genetically close to *M. truncatula*, *P. vulgaris* and *O. viciifolia*. The main reason for the high-level transferability of SSR markers taken from the above-mentioned species to *O. conferta* may be attributed to reshuffling of regions in genomes and variations incorporated through mutation and crossing during evolution, where some part of genome might have got modified and resulted in speciation (Singh *et al.,* 2014).

In the attempt of Demdoum *et al.* (2012) to transfer microsatellites from *M. truncatula* and *Glycine max*, a total of 35 alleles have been detected using the six SSRs among 25 *Onobrychis* populations. However, Avci *et al.* (2014) found a higher allele number than the previous study by amplifying 725 alleles from 18 SSR markers in diverse *Onobrychis* subspecies using markers from *P. vulgaris* and *M. truncatula*. The higher number found by this author compared to the reported results could be explained by the larger diversity of germplasm used and collected from different subspecies. The number of polymorphic bands ranged from 6 to 14, which is higher than previously reported (5 to 7) by Demdoum *et al.,* (2012) in several *Onobrychis* taxa using a bulk of 10 individual for DNA extraction, Avci *et al.,* (2014) using only one plant per population (2 to 7 polymorphic bands), and Falahati-Anbaran *et al.,* (2004) in *Medicago sativa* L. (4 to 14 polymorphic bands). This superiority is probably because the studied *O. conferta* populations were analysed in a bulk of 30 individuals. Indeed, the number of detected alleles can be underestimated. This could be caused by various reasons. First, the detection method used (agarose gel electrophoresis) is not sensitive enough to differentiate alleles with small size differences and may not detect all the alleles that have been amplified. More sensitive detection methods, such as capillary electrophoresis, would provide even more difficult-to-label fragment motifs due to stuttering of the markers. On the other hand, the swelling of DNA samples and their co-amplification by PCR are responsible for the loss of rare alleles and only detect the most common alleles. This may result in the non-detection of several present alleles, leading to an underestimation of detected number of alleles per locus in each population. The generation of multiple products during interspecific amplification could occur by mutation, rearrangements, and duplications in the flanking region and / or changes in the number of repeats (Peakall *et al.,* 1998; Gutierrez *et al.,* 2005).

The PIC value provides an estimate of the discriminatory power of a locus or loci, by taking into consideration of number of alleles (Saini *et al.,* 2023). The PIC value will be almost zero if there is no allelic variation and it can reach a max of 0.5 if a genotype has only new alleles, which is a rare phenomenon. In our study, the PIC values varied from 0.32 to 0.48 with an average of 0.36, which indicated a high level of polymorphism in the studied SSR markers. Average PIC value was found higher than obtained in 32 *O. viciifolia* genotypes (Kempf *et al.,* 2016). Nevertheless, PIC mean value was comparable to the values detected in other genera such as *Catharanthus*, *Cuminum* and *Rheum* (Shaw *et al.,* 2009; Rostami-Ahmadvandi *et al.,* 2013; Tabin *et al.,* 2016), indicating that the microsatellite flanking regions were conserved and highly useful in inferring the phylogenetic relationship between several species. High PIC values outlined the high discriminatory power of these applied SSR and their considerable molecular polymorphism (Ben Romdhane *et al.,* 2018)

The resolving power of a marker is an indication of the utility of the markers in assessing genetic differentiation and diversity. A resolving power ≥ 1.0 is considered more informative (Azevedo *et al.,* 2012). All the markers used in this study, exhibited a Rp ranging from 1.6 to 10 (average of 4.58), which indicates that they are useful for genetic discrimination and diversity studies in *O. conferta*. The high genetic diversity and polymorphism observed in the present study are in concordance to results of Demdoum *et al.,* (2012) and Avci *et al.,* (2014). Marker index elucidates the discriminatory power of a marker and therefore it was calculated for all the markers. Marker index values ranged between 1.4 and 3.52, showing that transferred markers are a valuable tool in revealing polymorphism in *O. conferta*.

The average number of observed alleles (na), effective alleles (ne), Shannon's Information index (I) and Nei's gene diversity (h) were used to demonstrate the level of population genetic diversity. In general, the populations included in this study showed a relatively high level of genetic diversity. *O. conferta* populations showed a slightly lower level of genetic variation than *O. viciifolia*. This observation could be due to populations decline due to bottleneck effect generated by several faceted threats linked to habitat deterioration and overgrazing

as recently discussed by Sakhraoui *et al.,* (2023b). Based on the genetic diversity indexes, the set of transferable SSRs from *M. truncatula*, *P. vulgaris* and *O. viciifolia* to *O. conferta* genomes showed an untapped potential in the discrimination and analysis of genetic diversity of *O. conferta* populations, forming a promising molecular tool, which could be used in further genetic studies.

All the studied taxa shared a similarity coefficient of 0.80, which is equal to the findings of Demdoum *et al.,* (2012) using SSR markers and slightly lower than those obtained by Sardaro *et al.,* (2003) using Amplified Fragment Length Polymorphism (AFLP) technique who reported a similarity of 0.73 between several *Onobrychis* accessions. The shortest genetic distance was between OC1 and OC2 (0.0349). These two populations are close situated. The high similarity indicates that even having cross-pollination and seed dispersal mechanisms, somehow this species is maintaining homozygosity in its populations, which requires further investigations. According to the clustering analysis, *O. conferta* was well separated from the other taxa. The studied taxa could be divided into three subgroups based on Nei's original GD. The pattern of clustering of *O. conferta* populations could correspond to their geographic distribution (Zhao *et al.,* 2011). The two collections from Dyr el Kef (OC1) and Fedj el Hdoum (OC2) clustered separately from accessions collected from Ain Dyssa (OC3), which reflected the geographic separation by mountains.

CONCLUSION

In conclusion, the selected genetically mapped SSR markers were conserved and allowed successful cross-amplification between the three genera *Medicago*, *Phaseolus* and *Onobrychis* belonging to the same family (Fabaceae). The high level of transferability detected indicates that SSR selection for transferability to new species is a good strategy for identifying SSR markers. The transferable markers detected a considerable level of inter/intraspecific genetic diversity among the studied taxa. Although additional SSRs could be used, the highly informative markers studied here could be considered for assessing accession identification in *O. conferta*. The results undertaken for the first time on the critically endangered species *O. conferta* subsp. *conferta* would be of great interest to establish strategies aiming at rational management and conservation strategies, genetic diversity evaluation, gene mapping and molecular breeding facing up to environmental changes.

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