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N. Favarisova, A. Kusmangazinov, D. Karelova,
M. Yermagambetova, S.I. Abugalieva*
Institute of Plant Biology and Biotechnology, Almaty, Kazakhstan
*e-mail: absaule@yahoo.com

Optimization of PCR conditions for *Agriophyllum, Haloxylon* and *Salsola* microsatellite markers

Abstract. Microsatellites or Simple Sequence Repeat (SSR) are among the most informative and popular types of molecular markers for assessment of genetic diversity, population structure, construction of genetic maps, phylogenetics, phylogeography, systematics, etc. Hence, for reliable inference of results, optimization of various conditions involved in polymerase chain reaction (PCR) is a pre-requisite. The usage of the essential PCR components in optimal concentrations, as well as PCR conditions, determines the success of amplification. The present study was carried out to optimize conditions of PCR for further assessment of the genetic diversity of eight species in genera *Agriophyllum*, *Haloxylon,* and *Salsola* that belong to the *Chenopodiaceae* Vent. family. The list of species includes *Agriophyllum squarrosum*, *Agriophyllum minus*, *Haloxylon aphyllum*, *Haloxylon persicum*, *Haloxylon ammodendron, Salsola arbuscula*, *Salsola arbusculiformis*, and *Salsola chiwensis*. These selected species are widespread in desert regions of Kazakhstan and have great importance for sand dunes fixing, animal feeding, and other purposes.
Key words: *Haloxylon*, *Agriophyllum*, *Salsola*, polymerase chain reaction, microsatellite markers.

Introduction

Climate change poses a challenge to the ecological environment, and dune migration may pose a severe hazard to settled and agricultural areas and are considered as one of the main factors causing desertification. Desertification has a notable impact on human economic and political stability, and in the long term, will threaten human survival and development. Global warming is increasing the prevalence of drought, drying up waterholes, and relocating local species. High temperatures increase the number of wildfires, which may alter landscapes eliminating trees and shrubs that are growing. Understanding of these vulnerabilities of desert plants and their communities can then be developed into technologies for conservation and restoration of the flora in dry regions. “At the heart of these complex problems are several key principals: (1) recognizing changes in plant species and communities and whether or not climate is the root cause, (2) understanding species vulnerabilities under climate change, (3) accurate prediction of the movement of plant communities to plan for the future, and (4) mitigating these changes by fostering regeneration or assisting the dispersal of appropriately adapted plant materials” [1].

In Kazakhstan, the most territory is the steppe (26%), semidesert (14%), and desert (up to 44%) areas. The prolonged droughts and strong winds in such areas are common, which affects the yield of agricultural land. The territory of the country is almost entirely part of the arid zone, and its two-thirds already subject to varying degrees of desertification [2].

The major representatives of wild flora in the desert are herbaceous plants, subshrubs, shrubs, and subtrees. In spring, a variety of ephemeris (up to 150 species) blooms in the deserts for a few weeks. Later on, after drought stress and increasing mean daily temperatures, xerophytes, and psammophytes dominate the landscapes, among others, many scrub species of the genera *Calligonum*, *Ammodendron* or *Aristida* [3]. One of the special features of sandy deserts is sites of saxaul thickets, mainly black saxaul (*Haloxylon aphyllum* Minkw.) Iljin and white saxaul (*Haloxylon persicum* Bunge ex Boiss. et Buhse), which are usually interspersed with *Tamarix, Salsola, Artemisia,* and many other plant species.

Haloxylon, Salsola, and Agriophyllum, belonging to *Chenopodiaceae* Vent. (*Amaranthaceae* Juss.) family of flowering plants, are represented by several species that grow in Kazakhstan and have importance for dunes fixing, animal feeding, water conservation, and other purposes. Plants of genus *Haloxylon* Bunge are psammophytic shrubs or small trees. Saxaul has a strong root system fixed in the sands, which can reach up to 8 m in height and, in exceptional cases, even more. Both black and white saxaul constitute the principal arboreal cover of the cold continental deserts of Central Asia, while white saxaul is a rain-fed shrub distributed on sand dunes. The former is a ground-water phreatophyte mainly found on alluvial terraces [4; 5]. Saxaul has an important role as a fodder plant, also used as firewood by local herders [6]. Black and white saxaul have a habitat-forming role, pasture protective, and economically importance. *Haloxylon ammodendron* (C.A. Mey.) Bunge is widely distributed across a range of habitats, including gravel desert, clay desert, fixed and semi-fixed sand, and saline land in Asian and African deserts [7]. For Kazakhstan, it is a rare species with habitat forming, soil protective, pasture protective significance.

Agriophyllum comprises six West and Central Asian species of annual herbs and belongs to the subfamily *Corispermoideae* [8]. Two species, *Agriophyllum squarrosum* (L.) Moq. and *Agriophyllum minus* Fisch. et C.A. Mey. grow in Western and South-eastern Kazakhstan [9]. Sand rice (*A. squarrosum)* is an annual shrub-like plant adapted to mobile dunes in the desert and semi-desert regions of Central Asia [10]. Sand rice has evolved many strategies to adapt to dune surface environment, including rapid root growth after germination, long hypocotyl, and pronounced drought and heat tolerance. It is a good candidate species for domestication to provide a food crop resilient to future climate change [10].

Salsola is a genus of the subfamily Salsoloideae in the family Chenopodiaceae. A common name of various members of this genus and related genera is saltwort, for their salt tolerance. *Salsola arbuscula* Pall. and *Salsola chiwensis* M.Pop. have great environmental importance since they perform sand-fixing and rock-strengthening functions. *S. arbuscula* is a widespread species in the desert regions of Central Asia [11]. *S. chiwensis* is listed in the Red Book of Kazakhstan (2014) as a rare endangered species (category II) occurring in the limited areas and suffers from livestock grazing [12]. *S. arbuscula*, *S. gemmascens* Pall., and *S. rigida* Pall. were used to improve and create long-term pastures in the sandy desert [13]. Also, *S. arbuscula* is used as a year-round feed by camels as well as winter feed by other animals. It is also applied as a fuel, for tanning leathers, and dye for wool.

Due to overgrazing and overexploitation for fuel during the past fifty years, the once-dominant saxaul and other desert species vegetation have considerably degraded [14]. It is important to develop and continue strategies for the conservation of plant genetic resources, combating desertification, measures to restore desert fertility, the reintroduction of rare and endangered native plant species. It is necessary to study the biodiversity of the desert plant species both on inter- and intraspecific levels in terms of preservation strategy and reintroduction of valuable and endangered species. For assessment the genetic diversity, phylogenetic relationships, for molecular taxonomy of wild species there is many various classes of widely used molecular markers, such as RAPD or Random Amplified Polymorphic DNA [15; 16], ISSR or Inter Simple Sequence Repeats [17], IRAP or retrotransposons [18], SSR or Simple sequence repeats [19-21], AFLP or Amplified Fragment Length Polymorphism [22; 23], SNP or Single Nucleotide Polymorphism [24; 25], DNA barcoding markers [9; 26-31]. Among listed markers, SSR markers, or microsatellites, are considered to be one of the most informative and reliable classes of PCR based DNA markers for genetic diversity studies, to estimate gene flow and crossing over rates, in evolutionary studies, above all to infer intraspecific genetic relations. Microsatellites have been the most widely used markers for genotyping plants over the past 20 years because they are highly informative, codominant, multi-allele genetic markers that are experimentally reproducible and transferable among related species. However, for reliable inference of the results of these studies, the optimization of various conditions involved in PCR amplification is a pre-requisite. The utilization of the essential PCR components in optimal concentrations, as well as conditions of amplification, determines the success of amplification.

High amplification success within and between genera in many groups of animals and plants indicates a great potential to use microsatellites and their flanking regions as a source of single- or low-copy nuclear sequences, as suggested by Zhang & Hewitt [32]. Sometimes flanking regions are highly conserved across taxa, allowing cross-species amplification of microsatellite loci from primers developed from other species in the same genus or even family. Since the flanking region of SSR loci are evolutionary well conserved, computational prediction of such

loci has practical significance. Such SSR loci can cater yo the need for molecular markers even in the absence of whole-genome sequence data. Thus, in cases when microsatellite markers have not been developed for a particular plant species a good alternative would be the use of a set of primers to obtain cross-species transferability. Barbará et al. [33] discussed the implications of these findings and close with an outlook on potential alternative sources of cross-species transferable markers. The potential for successful cross-species transfer appears highest in species with long generation times, mixed or outcrossing breeding systems, and where genome size in the target species is small compared to the source [33]. In the transferability or cross-amplification procedure, PCR primers developed for a studied (source) species are used to amplify microsatellites from closely [34; 35] or sometimes quite distantly related species [36].

SSRs can be developed from specific species and potentially used for related species, which lack the source of genome sequences to develop species-specific SSRs. Many reports are describing SSR markers that successfully developed and used for the genetic diversity studies of other species or genera of *Chenopodiaceae* (*Amaranthaceae*) family [7; 37; 38]. The aim of this study was the optimization of PCR conditions for microsatellite analysis to analyze the genetic diversity of desert plant species of three genera *Agriofillum*, *Haloxylon*, and *Salsola* in the family *Chenopodiaceae* using cross-species and cross-genus transferability.

Materials and methods

Plant materials. The objects of this study were wild desert species of three genera *Haloxylon*, *Salsola,* and *Agriophyllum,* which belong to Chenopodiaceae family: *Haloxylon persicum* Bunge ex Boiss. & Buhse, *Haloxylon aphyllum (Minkw.)* Iljin*, Haloxylon ammodendron* (C.A.Mey.) Bunge ex Fenzl.*, Salsola arbuscula* Pall.*, Salsola arbusculiformis* Drobow, *Salsola chiwensis* M.Pop.*, Agriophyllum squarrosum* (L.) Moq and *Agriophyllum minus* Fisch. &Mey.

Three populations of *A. squarrosum* and one population of *A. minus* were sampled in Moyynkym Sands in Zhambyl region on South-east Kazakhstan [9]. Three *Salsola* species were collected in Mangistau region of Western Kazakhstan (by Dr. A. Imanbayeva, Mangyshlak Experimental Botanical Garden), populations of three *Haloxylon* species both in Western (by Dr. A. Imanbayeva) and south-eastern parts of Kazakhstan.

The samplings of the wild species plant material were done in the framework of the Program 0237 (2015-2017) and project AP05131621 (2018-2020) supported by MES RK [27] and the International collaborative project REF: 2016YFE0203400 (2018-2020) by Chinese Ministry of Science and Technology.

Young leaves of collected plant material dried in silica gel were used for DNA extraction.

DNA extraction. The total genomic DNA was isolated from the dry leaf tissues using CTAB (cetyl trimethylammonium bromide buffer) method [39].

The quality and concentration of extracted DNA was assessed on NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) and observed for its intactness using 1% agarose gel electrophoresis.

The DNA concentration for all samples was normalized to 10-50 ng/μl for further analysis.

PCR amplification. PCR amplification using different SSR primer pairs for each above-mentioned species was performed in a thermal cycler Veriti (Thermo Fisher Scientific, USA). A temperature gradient was used via six innovative VeriFlex™ Blocks for precise control over PCR conditions optimization for each template-primer pair combination.

Presently, different software packages are available to calculate the melting temperature (T_m) as well as the annealing temperature (T_a) for a particular PCR reaction using particular Taq polymerase. Tm is a temperature at which half of the DNA strand denatured or opened.

In many cases, a known simple formula for Tm that usually works is:

$$T_m = 4\,(G + C) + 2\,(A + T)$$

Commonly, the appropriate annealing temperature is 5-7 °C lower than T_m. Still, it is just an assumption not correctly work all the time. Most of the time, this value is not optimal, and it is necessary to determine Ta empirically. Thus, it should be matched for a particular primer pair for particular conditions to get appropriate bands. For the most primers, the T_a between 55 °C to 65 °C should be ideal for PCR reaction, deviation of Ta above or below this range can cause non-specific bindings or reaction failure. If the T_a is too high, the primer can not bind properly to the template DNA. On the other side, if the annealing temperature is too low, it facilitates more bindings, more bands, and non-specific amplification during the PCR reaction.

Below is the information on primers and polymerase chain reaction conditions for three plant genera.

A. squarrosum and *A. minus*. Eighteen EST-SSR markers were selected from the list of 6150 SSR primer sequences derived from RNA_seq data of *A. squarrosum* genome reported by Zhang et al. [37]. Information on chosen primers concerning forward and reverse primers sequences, repeated motif, and expected size of amplified products are given in Table 1.

Primers had tri- (8), tetra- (5), and pentanucleotide (3) simple repeat motifs. There were compound repeats, which are composed of two successive sets of perfect repeats, such as $(CA)_6(AAT)_5$ and $(TTG)_6(TTC)_6$ (Table 1).

Table 1 – SSR markers developed for *A. squarrosum* [37] used in this study

Primer	F & R primers sequence (5'- 3')	Motif	Expected size (bp)*	Expected T_a (°C)**	T_a (°C)***
Ags02	F: AGCATCGGATGTGAGGAATC R: TCCTTCAACTCCTCCGTGTC	$(CAT)_6$	237	54-60	54
Ags03	F: AGGGAAATCAAGGGCTAGGA R: ATCCGACCTCTTACACGACG	$(CTT)_6$	280	54-60	60
Ags05	F: CTATGCCCATTCGTCATCCT R: GGCCGTTAGCTGAGTTGAAG	$(TCC)_6$	280	54-60	48
Ags07	F: AGGAGCAGCAGTAGAGGCAG R: CAACAGAAAAGAAGGCGGAG	$(AGC)_7$	242	54-60	54
Ags09	F: CAAGTTTTAATCTTTTAGCACCCTTT R: CCCCCTTTTCCCTCTTTCTA	$(AGA)_7$	280	54-60	54
Ags10	F: TTGGCTGTGGTTTGCATTTA R: AGAAGGCGTGAGCAATCTGT	$(GAT)_7$	280	54-60	54
Ags11	F: CCAATGCAGTGAATGTGGAG R: TCCTCTTTCTGGCCTTCTGA	$(CAG)_8$	217	54-60	54
Ags13	F: TTGGGAGTAGGGAAAAGAGGA R: GGAGGAGATGGTTGAAGCAC	$(TTG)_8$	275	54-60	48
Ags21	F: TCCTTCCCCTCTCACCTTCT R: TGTTTGGGAGGAGAAACTGG	$(TGTA)_5$	125	54-60	57
Ags22	F: AGTGGTGTTTGTTGCTGCTG R: ACTCCCTCACCCCTCACTCT	$(CTTT)_5$	124	54-60	54
Ags23	F: CAATGGGGTTTGAGCATTTC R: TTCCGGATGAATGATGGAAT	$(ATTA)_6$	254	50-56	54
Ags24	F: AAAGACAGGTCGTGAGTGGG R: AAAAACAGGTCTGATCCCCC	$(AGAT)_6$	108	54-60	51
Ags25	F: ACAACAAAATTGCCGAGGAC R: CGCCTCTCCCTCTTCTTTTT	$(AATC)_6$	279	48-54	42
Ags26	F: GAATTTTGATCGAAAAGGCG R: TCTCTCTCCTCCATTGCCAC	$(TTATT)_5$	185	48-54	54
Ags27	F: TTGGGCTACAACATTGGTGA R: GGCAGGTTACAACCTTTGGA	$(CCACT)_5$	182	54-60	45
Ags28	F: ACCAGCACCAAAACCTATGC R: ATAGCTGCTTCACGGTCGTC	$(AACCA)_5$	246	54-60	48
Ags29	F: TAAGTTCATCCTTGGCCCAT R: CCTCTTGCTGGACATGTGTTT	$(CA)_6(AAT)_5$	270	54-60	42
Ags30	F: TTGAGAGGGCTTGTTTGACA R: ACAATAACGACAACCCCAGC	$(TTG)_6(TTC)_6$	250	54-60	51

* – Zhang et al. [37]; ** – Expected T_a (°C) calculated by formula; *** – T_a (°C) – Annealing temperature, optimized in this study

All PCR reactions were performed in total 16 µl volumes, including 4 mM of each dNTP, 2mM of $MgCl_2$, 6.4 mM of primer mix (forward + reverse), 1.6 U of Taq polymerase and 10 ng of DNA.

The reactions were performed with an initial denaturation step at 94 °C for 3 min, followed by 40 cycles of 94 °C for 30 sec, T_a °C for 45 sec and 72 °C for 1.5 min. The final extension step was at 72 °C for 10 min. Annealing temperature (T_a) for individual primer pairs and a common concentration of reagents in a PCR reaction mix were determined empirically using Veriflex.

H. persicum, H. aphyllum, H. ammodendron. Ten primer pairs developed for *H. ammodendron* [7] were used for PCR optimization for further genetic diversity studies of this and other saxaul species. Table 2 contains the information on primers used in this study.

Table 2 – Characteristics of SSR primers developed for *H. ammodendron* [7] in this study for three *Haloxylon* species

Primer	Forward and reverse primers sequences (5′-3′)	Expected size (bp)[1]	Expected T_a (°C)[2]	T_a (°C)[3]	Alleles number[4]
Hal27760	F: AACTGCTGGGGATGGGAATG R: CAGCCCAATACTGCCCCTTT	242	62	58	7
Hal32182	F: AAAGGAGCAGAGTGAGTGCA R: TGCCTGCCTTTGTGTAGTGT	249	62	58	3
Hal42802	F: AACCCTAGAAAGCTTCGCCC R: TTTGGGAAAGCAGCGGAGAT	280	60	43	4
Hal45535	F: AACATCAACAGCGCCCACTA R: GGCCTATGATGCTGCACTCT	212	60	43	5
Hal47234	F: AACACAACATCCGCACCTCA R: GGATTTGGGTACGGGTCAGG	277	60	43	2
Hal60072	F: TGCACACACAGTTGCACTTG R: TGGGGTTTTGGGAGGAGAGA	268	60	62	3
Hal62940	F: TAACAACCGTGGCTGAAGCA R: GCGCGATGATGCCTTCTTTT	214	60	62	3
Hal63650	F: AAGAAGGTGGTGGTGGTTGG R: GCGGACGGTTGAAATTCACC	270	62	62	5
Hal64839	F: AAGAGGAAGACGAGGGTGGT R: TTGCGGAAGGAAAGTGGGAG	271	60	59	3
Hal65094	F: CTTGGAGCAGTGCCCTAGTG R: TTTGACTTCGGCGGCTACAT	277	60	53	3

Note: 1 – Long et al. [7]; 2 – Expected Ta (°C) calculated by formula; 3 – Ta (°C) – Annealing temperature, optimized in this study; 4 – Alleles number observed in this study

PCR was performed in a volume of 20 µL, containing genomic DNA (about 50 ng), 0.1 µM of each primer, 2 U Taq DNA polymerase (Fermentas, USA), 0.2 mM of each dNTP, $MgCl_2$ (1.5, 2.0 or 2.5 mM).

The PCR program consisted of denaturing the template DNA at 94 °C for 4 min, followed by 35 cycles, each at 94 °C for 30 s, T_a for 45 sec, and 72 °C for 1 min, followed by 72 °C for 10 min. The annealing temperature for each primer pair was selected due to the gradient PCR conditions.

S. arbuscula, *S. arbusculiformis* and *S. chiwensis.* Eleven SSR markers developed for other plant genera/species in the *Chenopodiaceae* family [7; 37; 38] were used for *Salsola* species.

The common reaction mixture (10 µl) contained 3.7 µl ddH_2O, 1 µl Taq buffer, 1 µl dNTP, 1.2 µl $MgCl_2$, 2 µl primer, 0.1 µl Taq polymerase and 50 ng of genomic DNA.

The PCR was performed with an initial denaturation step at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 sec, T_a °C for 45 sec and 72 °C for 1.5 min. The final extension step was at 72 °C for 10 min. The annealing temperature was individually optimized for each primer pair and varied from 43 °C to 58 °C.

Electrophoresis. The PCR products of wild species DNA were separated on 6% denaturing polyacrylamide gel (PAG) in 0.05M Tris-EDTA-borate

buffer, pH 8.0. The gel solution was applied to the assembled gel plates (0.35 mm thick) using the TV400-DGGE Sequencing electrophoresis apparatus (Scie-Plas, UK). The gels were stained with ethidium bromide, and SSR patterns images were captured using the GelDoc XR gel documentation system (Bio-Rad, USA). The size of the fragments was estimated based on a 100 bp ladder (Fermentas, USA).

Results and discussion

Considering the limited information on the new studied primers, and cross transferability of primers for related genera, the T_a for several sets of primer pairs was selected empirically as the result of gradient PCR on VeriFlex™ Blocks of Verity^{Tm} (Thermo Fisher Scientific, USA).

A. squarrosum and *A. minus.* Eighteen SSR primer pairs developed for *A. squarrosum* reported by Zhang et al. [37] with available information on the expected size of PCR product (Table 1) were used to optimize PCR amplification for two *Agriophyllum* species in our conditions. Figure 1 shows an example of PCR patterns of *Agriophyllum* samples as a result of T_a optimization for Ags21.

As shown in Table 1, T_a for primers with trinucleotide motifs was expected in the range of 54-60 °C. Due to the gradient, T_a for *A. squarrosum* and *A. minus* samples was different for three groups: 48 °C, 54 °C, or 60 °C, respectively. T_a for four primers with tetranucleotide repeated motif, was 42 °C for Ags25, 51 °C for Ags 24, and 54 °C for primers Ags22, and Ags23, and 57 °C Ags21, respectively. PCR product sizes were coincided with expected sizes for primers Ags03, Ags05, Ags11, Ags13, Ags16, Ags21, Ags25, Ags26, Ags27, and Ags29.

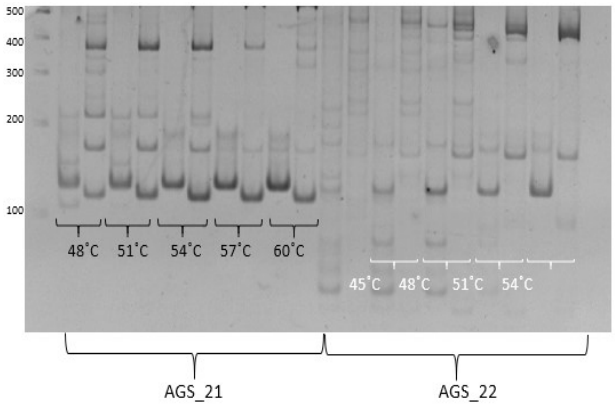


Figure 1 – Optimization of annealing temperature for Ags21 primers pair for *A. squarrosum* and *A. minus*

The optimized conditions were used for SSR analysis of populations of *A. squarrosum* and *A. minus* collected in the south-eastern region of Kazakhstan. Figure 2 represents SSR patterns on marker *Ags*26 of individuals representing different populations of these two species amplified on the 54 °C.

These conditions revealed distinguishing the species and individuals inside populations or species later on.

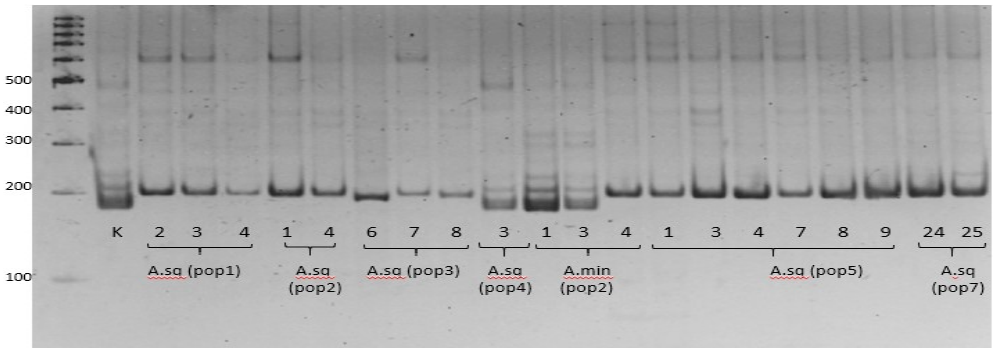
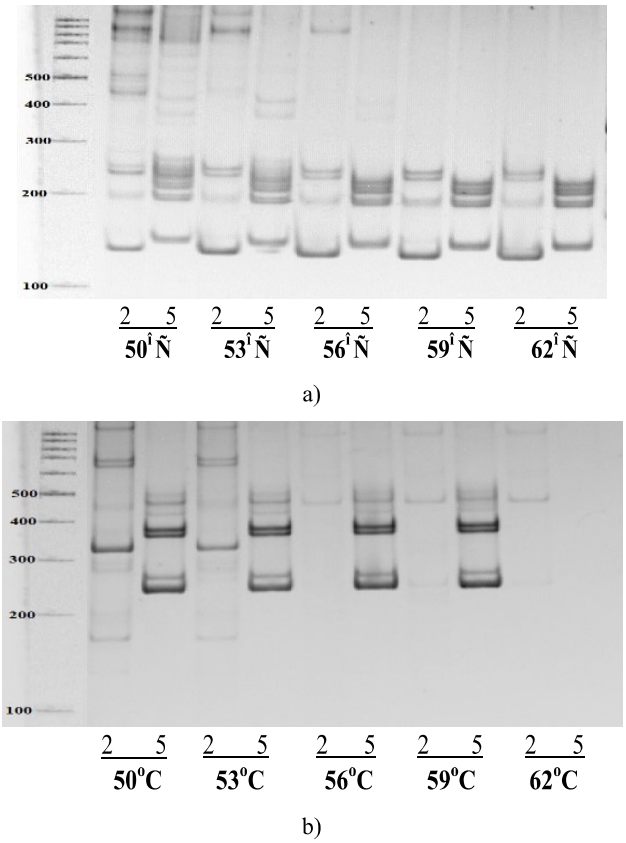


Figure 2 – SSR patterns of individuals of *A. squarrosum* and *A. minus* populations from Kazakhstan on SSR marker *Ags26.*
Note: M – 100 bp Molecular weight marker (Thermo Fisher Scientific), *A.sq – A. squarrosum, A.min – A.minus*

H. persicum, H. aphyllum, H. ammodendron. *H. persicum, H. aphyllum,* and *H. ammodendron* are closely related species in the genus *Haloxylon* of *Chenopodiaceae* (*Amaranthaceae* Juss.). Several studies were conducted on genetic diversity studies of these species using molecular markers [40-42]. Few SSR markers have been reported for *Haloxylon* species, and their genetic

background is still poorly understood [42]. In this study, ten primer pairs of EST-SSR developed for *H. ammodendron* [7] were used for PCR optimization (Table 2) for further genetic diversity studies of intra- and interspecific diversity of three *Haloxylon* species. To optimize PCR conditions for these primers, the annealing temperature was set either to 40 °C, 43 °C, 46 °C, 50 °C, 53 °C, 56 °C, 59 °C or 62 °C (Figure 3).



2, 5 – individuals of populations 2 (2-8) and 5 (5-3) of *H. aphyllum*

Figure 3 – Annealing temperature optimization using gradient PCR conditions (50-62 °C) on SSR primers *Hal64839* (a) and *Hal 65094 (b)* for *H. aphyllum*

The optimized annealing temperature for ten primer pairs allowed amplifying appropriate PCR products for three saxaul species and observing both intra- and interspecific diversity (data not presented). Figure 4 demonstrates the microsatellite patterns of individuals from populations of *H. ammodendron* and *H. aphyllum* on microsatellite markers *Hal65094* and *Hal64839,* which produced clear amplification of expected sized.

Preliminary data have shown from 2 to 7 alleles per locus while studying populations of three saxaul genera from Kazakhstan (Table 2). Intra- and interspecific diversity of saxaul species will be determined.

Salsola. Transferability of SSR primers developed for other genera. Several reports described the development of microsatellite markers for use in genetic studies in different related genera and cross-genus transferability SSR primers [19; 42-45].

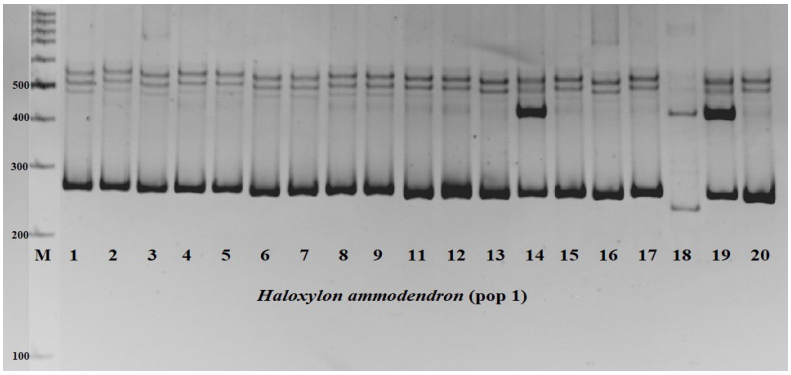
EST-SSR markers derived from transcribed regions of the DNA produce a higher rate of transferability, but less polymorphism [44]. Many EST-SSR primers were shown to be useful in discriminating different genera or species within a particular genus and genomes of the tribe *Triticeae* of the *Poaceae* [43]. Microsatellite markers derived from the functional parts of the bread wheat genome may be successfully used both in cultivated wheat and its wild relatives belonging to *Triticum-Aegilops* species for comparative genomics. Based on wheat genomic SSR markers, the transferability from wheat to rye was found to be only 17%; while, based on EST-SSR markers, the transferability from wheat to 18 *Triticum-Aegilops* species was found as 84% [43], and from Tall fescue (*Festuca arundinacea* Schreb.) to seven grass species was found to be nearly 92% [45].

As for genera of *Chenopodiaceae*, McGray et al. [19] reported that six of twenty SSR primer pairs previously developed for *Beta vulgaris* were found to be useful in molecular classification of five genetically distinct *Salsola* taxa [19]. The transferability (1.8%) of the SSR markers from the other related genera to genus *Haloxylon* was considerably low, suggesting a larger genetic divergence between taxa [42].

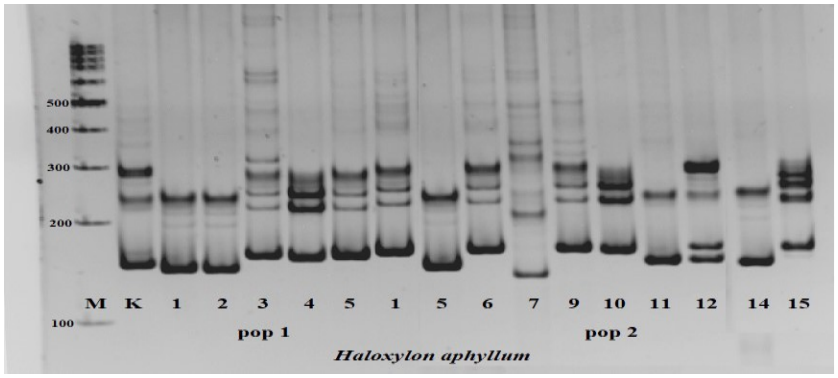
As no novel SSR loci have been reported for Salsola genus, in this study, we tested primers developed for the related species from the same family, namely for *Beta vulgaris, A. squarrosum,* and *H. ammodendron*. The PCR protocols were optimized using a particular primer pair is to change the T_a or the concentration of $MgCl_2$. PCR products with the usage of all studied primers pairs were successfully amplified. The information on the results of PCR amplification of Salsola species at varying annealing temperatures is given in Table 3.

Cureton et al. suggested T_a for sugar beet primer pairs (Bmb3 and Bmb4) used in this study [39] as 55 °C and 43 °C. In our study, the better T_a for Bmb3 and Bmb4 was 46 °C and 50 °C, respectively. McGray et al. [19] showed the successful usage of 6 out of 17 SSR *Beta* primer pairs in good amplification with yielding scorable PCR products and further genetic analysis of 5 Salsola taxa in California: *S. tragus, S. paulsenii, S. kali ssp. austroafricana, S. p. lax,* and Type C [19]. The authors reported that Bmb3 primers worked for all five taxa and revealed polymorphism within 5 species of *Salsola*, while Bmb4 failed to amplify *Salsola's* DNA.

populations of black saxaul.



a)



b)

Figure 4 – SSR patterns of individuals of *H. ammodendron* on SSR marker *Hal65094* (a) and *H. aphyllum* (populations 1 and 2) on SSR marker *Hal64839* (b). Note: M – 100 bp Molecular weight marker (Thermo Fisher Scientific), 1-20 – individuals of the population

Table 3 – Characteristics of SSR primers developed for *Beta vulgaris* (Bmb), *A. squarrosum* (Ags), and *H. ammodendron* for three Salsola species (*S. arbuscula*, *S. arbusculiformis*, and *S. chiwensis* in this study

Primer	F & R primers sequence (5'- 3')	Motif	Exp. size (bp)	T_a (°C)	T_a (°C)***	$MgCl_2$ (µl)	Alleles N
Bmb3	F: CGGTTGCAAGTCGATAAGGT R: CCGGTTGAACAGCAGAACAGG	$(CA)_{42}$	261*	55* 43**	46	3.6	4
Bmb4	F: CCTCTTTATTTCACGAGGTCCC R: CCCAGATTGAAATCAGGATCG	$(CA)_{13}$	212	55*	50	2.9	
Ags3	F: AGGGAAATCAAGGGCTAGGA R: ATCCGACCTCTTACACGACG	$(CTT)_6$	280	N/A	58	1.2	3
Ags9	F: CAAGTTTTAATCTTTTAGCACCCTTT R: CCCCCTTTTCCCTCTTTCTA	$(AGA)_7$	280	N/A	46	1.2	3
Ags29	F: TAAGTTCATCCTTGGCCCAT R: CCTCTTGCTGGACATGTGTTT	$(CA)_6(AAT)_5$	270	N/A	46	1.2	3
Hal34975	F: AACTCGCCCATTATTGCACG R: AGAGGGTCAACGTCGTCAAC	N/A	235	N/A	43	1.0	2
Hal42802	F: AACCCTAGAAAGCTTCGCCC R: TTTGGGAAAGCAGCGGAGAT	N/A	280	N/A	43	1.0	3

Table 3 continued

Primer	F & R primers sequence (5'- 3')	Motif	Exp. size (bp)	T_a (°C)	T_a (°C)***	$MgCl_2$ (µl)	Alleles N
Hal45535	F: AACATCAACAGCGCCCACTA R: GGCCTATGATGCTGCACTCT	N/A	212	N/A	43	1.0	2
Hal47234	F: AACACAACATCCGCACCTCA R: GGATTTGGGTACGGGTCAGG	N/A	277	N/A	43	1.0	3
Note: * – Cureton et al.[39]; ** – McGray, 2008 [19]							

Determining the optimal concentration of different $MgCl_2$ concentrations (even for different primers from the same region of a given template) may have a large impact on the success of the PCR. For Bmb3 primers, two different $MgCl_2$ concentrations were reported to be optimal – 1.5 mM [39] and 3.6 mM [39], while no information was given on this matter for Bmb4 primers [19; 39]. In our case, the concentration 3.6 mM for Bmb4 was more suitable for the better PCR product yield, and less smearing on the gel. Examples of the SSR patterns of individuals of *S. arbusculiformis* on SSR marker *Ags*9 and *Bmb*3 are presented on Figure 5.

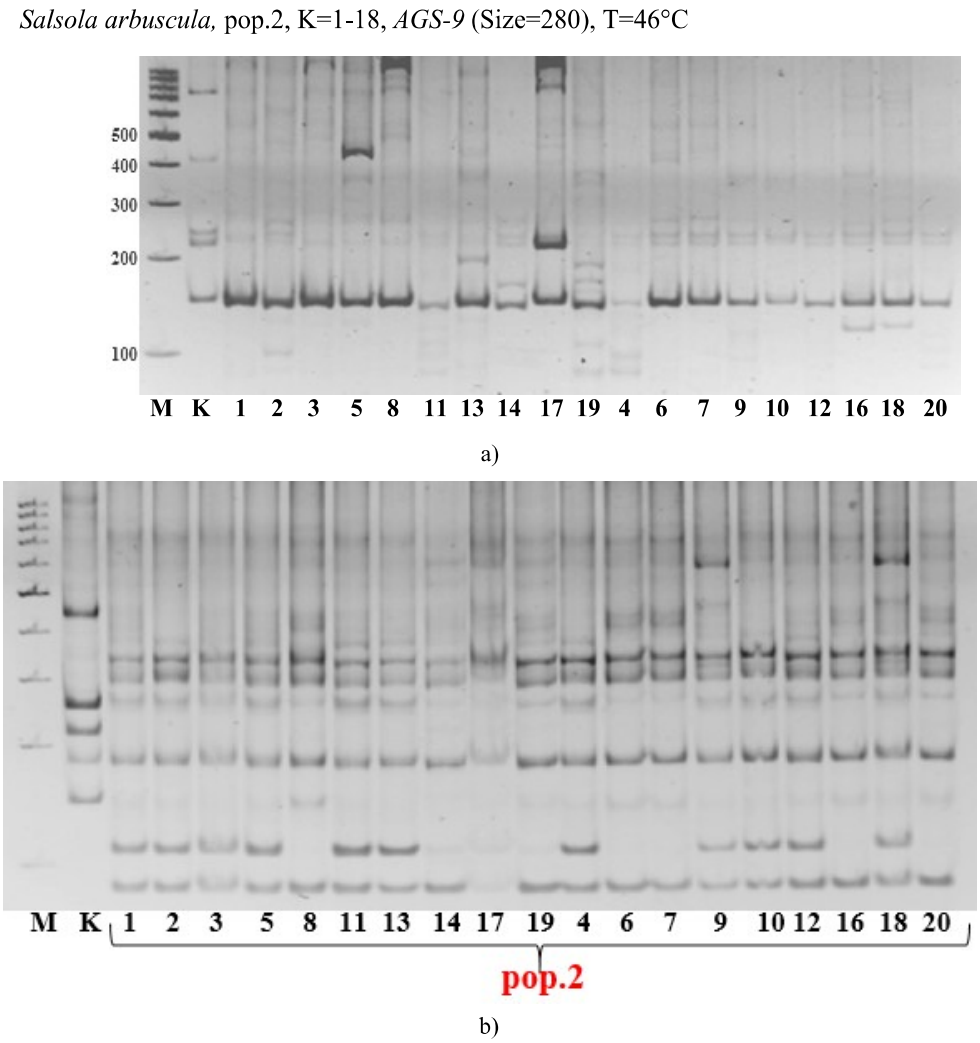


Figure 5 – SSR patterns of individuals of *S. arbusculiformis* on SSR markers *Ags*9 (a) and *Bmb*3 (b). Note: M – 100 bp Molecular weight marker (Thermo Fisher Scientific, USA), K – individual #18 of the population 1 of *S. arbuscula*

Expected sizes of PCR products for other related species are given in Table 3: for Ags3, Ags 9, and Ags29 primers (for sand rice) – according to Zhang et al. [37], and for Hal34975, Hal42802, Hal45535, and Hal47234 (for saxaul) according to Long et al. [7]. Preliminary data showed more than 2 alleles per locus when study focused on *S. arbusculiformis.*

Obtained data showed that all nine primer pairs have their high cross-genera transferability and usefulness in differentiating three *Salsola* taxa – *S. arbuscula*, *S. arbusculiformis* and *S. chiwensis*.

Optimized PCR conditions for the annealing temperature and $MgCl_2$ concentration are reliable to analyze the genetic diversity both between and within eight species of *Agriophyllum, Haloxylon,* and *Salsola* collected in Kazakhstan to the date. The obtained information is important in terms of the development of optimized PCR conditions as a prerequisite for the successful application of SSR markers in population genetics of studied species.

Conclusion

Investigation on the level and pattern of genetic diversity of populations of the rare and valuable species using microsatellite markers is crucial for understanding the structure of the population and assessing the strategies for biodiversity preservation. SSR markers are reliable, informative markers with codominant nature are widely used in molecular genetic studies. An important limitation, however, regarding the use of microsatellites is the prior need for optimization of PCR conditions for each SSR marker.

The annealing temperatures and $MgCl_2$ concentrations were individually optimized for each primer pair for samples of *Agriophyllum, Haloxylon*, and *Salsola*.

Since little information was found in the literature on microsatellite markers for the Salsola genus, we tested the cross-genera transferability of SSR markers previously developed for other members of the Chenopodiaceae family, including *Beta vulgaris, Agriophyllum squarrosum*, and *Haloxylon ammodendron.* PCR conditions for representatives of the other two genera, *Agriophyllum* and *Haloxylon* were also tested in this study. The optimization of PCR conditions for studied Salsola species indicated their good cross-genera transferability via the successful amplification and will be used in population structure analyses.

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