Research Note

# PCR-SSCP: A method to fingerprint sequence variability in plant gene pools using the Iranian wild diploid *Triticum*

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## Abstract

As a case study, the genotypic polymorphism of the Iranian material of diploid *Triticum* was screened based on internal transcribed spacer 2 (ITS2) of the ribosomal DNA (rDNA) using single strand conformation polymorphism (SSCP) analysis. This is a simple and cost effective technique for genotyping and investigating the allelic polymorphism among plant populations. Performing SSCP analysis among 21 accessions, two distinctive migration profiles associated with the two validly recognized diploid *Triticum* species i.e., *T. monococcum* subsp. *aegilopoides* and *T. urartu* were detected. Nucleotide sequencing confirmed the distinction of two types of observed profiles. This study suggested that SSCP is an applicable diagnostic molecular tool for screening the genotypic variability before the costly direct genomic sequencing is begun.

**Keywords:** diploid, Iran, ITS2, SSCP, *Triticum*

## Introduction

Single strand conformation polymorphism (SSCP) is a simple and useful method for detecting the sequence variants and polymorphisms (Hayashi, 1991). Principle of this technique relies on electrophoretic mobility of the single strand DNA molecule on a non-denaturing gel according to its size and conformation (Rodriguez *et al*., 2011). Small nucleotide sequence changes result in alteration of conformation of single strand DNA which in turn alters the electrophoretic mobility and consequently reveals different SSCP patterns (Hayashi, 1991). Size of the fragment, base composition of the sequence, electrophoresis temperature, pore size and cross linking of gel are the effective parameters for polymorphism detection in SSCP technique (Gasser *et al*., 2006).

Since its invention by Orita *et al*. (1989) SSCP approved to be an effective method in different fields of both basic and applied biology (Sunnucks *et al*., 2000; Gasser and Chilton, 2001), briefly its utilization in plant science is summarized in Table 1. In addition, in the laboratories with a minimum of facilities, SSCP can be a suitable alternative for direct analysis of sequence variation.

DNA barcoding is an increasingly popular specific identifying tool with respect to its affordable cost, speed and objectivity (Crautlein *et al*., 2011). ITS2 region can be used as a barcode in plants because of its valuable characteristics, including the availability of conserved regions for designing universal primers, the ease of its amplification, and enough variability to distinguish even closely related species (Yao *et al*., 2010; Chen *et al*., 2010). In order to initiate and set up the utilizing this method (SSCP) in plant taxonomy in our lab, we select *Triticum* diploid species as a case study using ITS2 amplicons.

## Materials and methods

### *Plant materials*

Our plant materials include 21 seed accessions collected from the wild (Table 2) which were belonged to precisely identified diploid species of *Triticum* i.e., *T. urartu* Thumanjan ex Gandilyan and *T. monococcum* subsp. *aegilopoides* (Link) Thellung. The accessions were grown in the research filed of the Isfahan University in 2011. All the examined accessions were deposited with at least one specimen in the herbarium of the University of Isfahan.



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Table 1. A summarized review of using SSCP in plant biology.

| SSCP application in: | Reference |
|---|---|
| Point mutations detection | To *et al*., 1993 |
| Genome mapping | Fukuoka *et al*., 1994 |
| Population genetic, DNA biogeograghy | Watano *et al*., 1995 |
| Gene mapping, genetic diversity | Bodenes *et al*., 1996 |
| Rare polymorphisms detection | Dumolin-Lapegue *et al*., 1996 |
| Plasmon analysis | Wang *et al*., 1997 |
| Comparative genomic mapping | Plomion *et al*., 1999 |
| Phylogeny | Kita and Ito, 2000 |
| Single nucleotide mutations detection | Martins-Lopes *et al*., 2001 |
| Phylogeography | Meusnier *et al*., 2002 |
| Mutation detection | Sato and Nishio, 2003 |
| Genetic structure of hybrid zones | Watano *et al*., 2004 |
| Genome diversity and gene haplotypes | Salmaso *et al*., 2004 |
| Biological status of complex fern | Ebihara *et al*., 2005 |
| Comparative genomic mapping | Castelblanco and Fregene, 2006 |
| Cytoplasmic DNA characterization of somatic hybrids | Olivares-Fuster *et al*., 2007 |
| Genetic diversity | Kuhn *et al*., 2008 |
| Polymorphism detection | Lu *et al*., 2009 |
| Marker assisted selection | Borchert and Hohe, 2009 |
| Phylogeny | Rousseau-Gueutin *et al*., 2009 |
| Polymorphism detection | Karatas *et al*., 2010 |
| Diversity analysis and varietal identification | Swapna *et al*., 2011 |
| Separation of alleles from polyploid accessions | Rodriguez *et al*., 2011 |

***DNA extraction***

DNA was extracted from fresh and young leaves of a single plant, using the CTAB method (Gawel and Jarret, 1991).

***PCR amplification***

The ITS2 regions were amplified by PCR using primers PITS3 (5’- ACG CCA AAA CAC GCT CCC AAC -3’, wheat specific primer) (Blatter *et al*., 2002) and ITS4 (5’-TCC TCC GCT TAT TGA TAT GC-3’) (White *et al*., 1990). The thermal and $MgCl_2$ concentration gradient were optimized and the final PCR of ITS2 region was carried out in a total volume of 25 µl containing 40–50 ng of DNA template, 200 µM of each dNTPs, 0.4 µM for each of the primers, 2.5 µl 10 × PCR buffer, 3 mM $MgCl_2$ and 1 U *Taq* DNA polymerase. PCR conditions were: 1 cycle of 94ºC for 5 min followed by 30 cycles of 94ºC for 1 min, 52ºC for 1 min and 72ºC for 1 min and finally 1 cycle of 72°C for 10 min. The PCR products were electrophoretically separated in 1.2% agarose gel.

***SSCP and sequencing analyses***

The SSCP analysis of amplified gene fragments is carried out as described by Rodriguez *et al.* (2011) with some modifications. Briefly, 1–2 µl of PCR products (according to the band intensity on agarose gel) were mixed with 10 µl of a denaturing buffer (95% formamide, 10 mM EDTA, 10 mM NaOH, 1% xylene cyanol and 1% bromophenol blue). After a short spin, mixtures were heated at 100°C for 5 min, chilled on ice for 5 min and then loaded onto a 10% acrylamide: bis acrylamide (39: 1) non-denaturing polyacrylamide gel (25 cm × 20 cm × 1 mm) containing of 5% glycerol. Before sample loading, the gel was pre-run in 1 × TBE for about 1 h at 8°C. Electrophpresis was performed at 200 volts for 17–18 h at 8°C. SSCP bands were visualized by silver staining as described by Sanguinetti *et al*. (1994). In order to corroborate the SSCP results five PCR products from each species were randomly selected and sequenced. The sequence data were aligned manually using BioEdit ver.7 (Hall, 1999). The editing of sequences was checked through ChromasPro ver.1.41 (Technelysium Pty Ltd., Tewantin, Australia). The analysis of sequences was performed using DnaSP ver.5.10 (Liberado and Rozas, 2009).

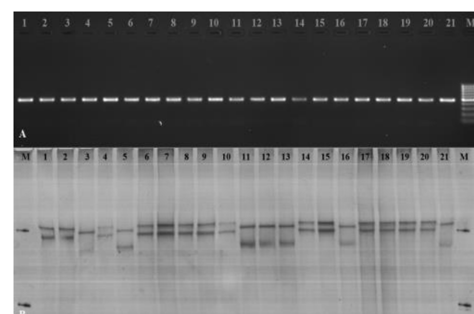

Figure 1. PCR products on agarose gel (A) and SSCP profiles on the non- denaturing acrylamide gel (B) based on the ITS2 region of wild diploid Triticum species. For the number of each line see Table 2. Lane M is 50 bp Ladder.

Table 2. Locality of seed samples of wild diploid *Triticum* species in this study.

| No. | Taxon | Locality |
|---|---|---|
| 1 | *T. monococcum*. subsp. *aegilopoides* | Ardabil, 35 km to Givi |
| 2 | *T. monococcum*. subsp. *aegilopoides* | Ilam, Gardaneh Reno |
| 3 | *T. urartu* | Chaharmahal and Bakhtiari, Cheshmeh Ali |
| 4 | *T. monococcum*. subsp. *aegilopoides* | East Azarbaijan, Maragheh |
| 5 | *T. urartu* | Chaharmahal and Bakhtiari, Ardal to Dashtak |
| 6 | *T. monococcum*. subsp. *aegilopoides* | Isfahan, Bouin to Aligodarz |
| 7 | *T. monococcum*. subsp. *aegilopoides* | Kermanshah, Taghi abad |
| 8 | *T. monococcum*. subsp. *aegilopoides* | Kurdistan, 5 km to Kamyaran |
| 9 | *T. monococcum*. subsp. *aegilopoides* | Kermanshah, Abolvafa |
| 10 | *T. monococcum*. subsp. *aegilopoides* | Kermanshah, after Kohdasht to Eslam abad |
| 11 | *T. urartu* | Isfahan, Bouin to Aligodarz |
| 12 | *T. urartu* | Kermanshah, 10 km to Songhor |
| 13 | *T. urartu* | Kohgiluyeh and Boyer-Ahmad, near Yasouj |
| 14 | *T. monococcum*. subsp. *aegilopoides* | West Azarbaijan, Piranshahr |
| 15 | *T. monococcum*. subsp. *aegilopoides* | Kurdistan, Marivan |
| 16 | *T. urartu* | Kurdistan, Marivan |
| 17 | *T. monococcum*. subsp. *aegilopoides* | Kurdistan, Sanandaj |
| 18 | *T. monococcum*. subsp. *aegilopoides* | Lorestan, 35 km to Khoram abad |
| 19 | *T. monococcum*. subsp. *aegilopoides* | Lorestan, Khoram abad to Sefid dasht |
| 20 | *T. monococcum*. subsp. *aegilopoides* | Lorestan, Sefid dasht |
| 21 | *T. urartu* | Kurdistan, Jenyan |

## Results

The PCR products (double strand DNA) of the amplified fragments in both Triticum species were aligned for a length of 270 bp (Figure 1- A). The two banded SSCP profiles among the 21 accessions examined revealed two species specific patterns associated with *T. urartu* and *T. monococcum* subsp. *aegilopoides* (Figure 1- B). The patterns were reproducible in repeated experiments. All the five repeated PCR products sequenced in each species were identical and comparing the patterns among the two species revealed two different haplotypes separated on two SNPs (Figure 2).

## Discussion

This study showed that prior to a costly direct genomic sequencing SSCP can be a cost effective method when researchers are facing with a large number of accessions. In addition, compared with the other molecular fingerprinting methods it is simple and efficient. While fragments with the same size but different in their sequences migrate to the same position on agarose gel, using the non-denaturing acrylamide, these fragments have different mobility shift and consequently turn in allelic sequence variations (Cotton, 1997) (Figure 1: - A and -B) which can be interpreted easily.

On the basis of the two banded profiles detected it can be concluded that all the accessions studied were homozygote for ITS2. SSCP profiles divided the studied accessions into two groups which were exactly coincidence with our taxonomic species, i.e., *T. monococcum* subsp. *aegilopoides* and *T. urartu*. Therefore ITS2 can be used for species identification. This result is in agreement with Chen *et al*. (2010) and Yao *et al*. (2010).

We found no geographical subdivision or intraspecific variation which can be taken as evidence for the highly conserved nature of ITS2 in these taxa.

Plant species are primarily distinguished by their morphological features; however poor constructed keys or flawed specimens can cause misidentifications or doubtful determinations. In diploid *Triticum* species, leaf blade (vegetative stage) and anther size (reproductive stage) are two key characters (Rahiminejad and Kharazian, 2005; Salimi *et al*., 2005). The belonging of SSCP profiles of our flawed diploid *Triticum* specimens to one of the two SSCP profiles was confirmed by their complete samples which were provided by growing their seeds. This study proved that SSCP to be a useful tool to separate different taxonomic entities at species level.

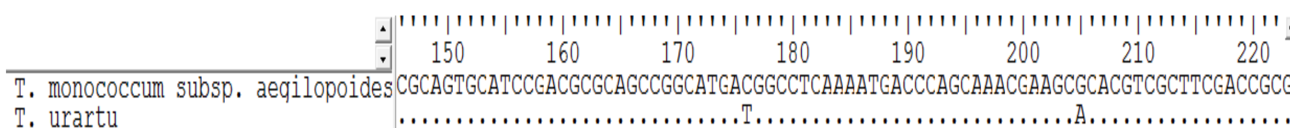


**Figure 2.** Comparison of ITS2 sequences among wild diploid Triticum material used in this study.

As the final conclusion, this first report of using SSCP among the Iranian *Triticum* materials suggested this method as an effective and distinctive applicable tool for the taxonomy of other plant taxa and represents another step forward toward routine use of DNA sequence data as a tool for identification at the specific level.

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