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M. Le Thierry d'Ennequin · O. Panaud · B. Toupance
A. Sarr

Assessment of genetic relationships between *Setaria italica* and its wild relative *S. viridis* using AFLP markers

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Abstract AFLP markers were used to assess genetic diversity and patterns of geographic variation among 39 accessions of foxtail millet (*Setaria italica*) and 22 accessions of green foxtail millet (*S. viridis*), its putative wild progenitor. A high level of polymorphism was revealed. Dendrograms based on Nei and Li distances from a neighbour joining procedure were constructed using 160 polymorphic bands. Bootstrap values revealed that no specific geographic structure can be extracted from these data. The high level of diversity among Chinese accessions was consistent with the hypothesis of a centre of domestication in China. The results also showed that accessions from Eastern Europe and Africa form two distinct clusters. The narrow genetic basis of these two gene pools may be the result of local-adaptation.

Keywords Domestication · Genetic diversity · Geographical structure · Gene flow

Introduction

Foxtail millet, *Setaria italica*, belongs to the family *Poaceae*. This ancient cereal has been cultivated in Northern China since Neolithic times (8000 years ago), and it remains a staple food crop (Fogg 1983). Archaeological data (de Wet et al. 1979; Li and Wu 1996) show that it has also been cultivated since ancient times (6000 years ago) in Eurasia. Nowadays foxtail millet has been replaced by other cereals, and in Europe it has become a minor crop in Europe. Green foxtail millet, *Setaria viridis*, is its closest wild relative (de Wet et al. 1979). It is a weedy annual commonly found in habitats such as roadsides, gravel pits and waste lands and it also occurs as a weed in crop fields. Its diversity, wide geographical distribution and strong competitiveness in disturbed habitats makes the green foxtail millet a highly successful weed. It spontaneously hybridises with *S. italica* (Li et al. 1945) and is thought to be the putative wild progenitor of the cultivated form. Furthermore, analyses based on interspecific crosses support the idea that *S. italica* and *S. viridis* belong to the same biological species (Till-Bottraud et al. 1992).

Using isozyme markers both Jusuf and Pernès (1985) and Wang et al. (1995) showed that *S. viridis* presents a larger diversity overall than *S. italica*. In addition, these analyses have shown that genetic distances between the cultivated and the wild forms are sometimes smaller than the distances between 2 accessions of *S. italica* from distinct localities. Variation in the primary gene pool of *Setaria* thus seems to be regional rather than taxonomical (Darmency et al. 1984; Jusuf and Pernès 1985). This finding is consistent with evidence of hybrid weakness and partial sterility in offspring derived from intraspecific crosses between landraces from China and Europe (Crouillebois et al. 1988).

These findings support the hypothesis, first suggested by de Wet and Harlan (1975), that foxtail millet was domesticated in China and in Europe independently. The occurrence of foxtail millet cultivation 4000 years ago, itself supports the hypothesis of a second centre of domestication of the crop in Europe, since no archeological evidence of migration or exchange between Europe and China during the Neolithic period has ever been discovered. The crop in Europe, however, is more recent than that in China and is associated in European archeological sites with cereals coming from the Middle East. In addition, the use of different enzymatic systems such as esterase isozymes (Kawase and Sakamoto 1984) did not lead to any clear conclusion regarding the history of foxtail millet domestication (Wang et al. 1995). Furthermore, Li et al. (1995), using morphological traits, suggested that there was a third centre of domestication in an area ranging from Afghanistan to Lebanon.

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M. Le Thierry d'Ennequin (✉) · O. Panaud · B. Toupance · A. Sarr
Laboratoire Evolution et Systématique, UPRESA-CNRS 8079,
Université PARIS XI, Bâtiment 362, 91405 Orsay cedex, France
e-mail: Maud.Le-thierry@esv.u-psud.fr
Fax: +33(0)1 69157353

Foxtail millet is a particularly well-studied species, especially in the context of comparative genomics, with its genetic map (Wang et al. 1998) aligned with other cereal maps (Devos et al. 1998). DNA markers can be used effectively as a complement to isozymes to refine the study of genetic relationships among gene pools. The objective of the study presented here was to use amplified fragment length polymorphism (AFLP) markers to assess the amount and structure of genetic diversity within the primary gene pool of foxtail millet and to further investigate the origins of foxtail millet.

AFLP markers (Zabeau and Vos 1993) show considerable variation among varieties and have been used to establish genetic relationships based on the estimation of genetic similarities in *Lens* (Sharma et al. 1996) and *Glycine* (Maughan et al. 1996). Furthermore, genetic similarity matrices obtained with AFLPs and other types of markers such as restriction fragment length polymorphisms (RFLPs) and simple sequence repeats (SSRs) are highly congruent (Powell et al. 1996), although Russel et al. (1997) have pointed out a lack of correlation between SSRs and the other markers within species. Our study is the first application of AFLP markers in *Setaria*.

Material and methods

Plant material

Twenty-two accessions of *S. viridis* and 39 accessions of *S. italica* were included in the AFLP survey (Table 1). Five individuals of each accession were grown under standard conditions in the greenhouse at Orsay University (France) before being transferred to an experimental field. The accessions were chosen to cover a wide range of geographical origins. Most of the green foxtail millet accessions sampled originated from Europe and China. In China, samples originated from the following regions: Liaoning, Shandong, Jinan, Shanxii, Hunan, Pekin and Shanga. Accessions from France originated from several areas from the southwest (519-84) and the southeast (130-86).

DNA extraction and quantification

DNA extraction was performed on leaf tissue ground in liquid nitrogen and incubated in 50 ml tubes containing 25 ml of 0.1 *M* TRIS-HCl (pH 8.0), 0.5 *M* NaCl, 50 m*M* EDTA (pH 8.0) and 1.25% SDS, supplemented with 3.8 g/l of sodium bisulfite before use, for 30 min at 60°C. Twenty-five milliliters of chloroform/isoamyl alcohol (24/1) was added, and the tubes were shaken for 30 min in an orbital shaker at 1500 rpm and then centrifugated for 30 min at 3500 rpm. The supernatant was transferred to a new tube containing 4 ml isopropanol to precipitate DNA. DNA pellets were collected by centrifugation at 3500 rpm for 10 min, then washed in 70% ethanol, dried and resuspended in 1 ml TE buffer (1×). RNAse was added at a concentration of 50 µg/ml (15 min at 37°C). DNA was precipitated by adding 500 µl of 7.5 *M* ammonium acetate and 2 ml of absolute ethanol. After a further centrifugation of 10 min (3500 rpm), the DNA pellet was washed, dried and resuspended in 100 µl of TE buffer (1×).

AFLP procedure

The AFLP procedure was performed according to Zabeau and Vos (1993) with some modifications. Briefly, 250 ng of genomic DNA was digested with *Eco*RI and *Mse*I (MBI, Fermentas, Lithuania) in a final volume of 25 µl. After inactivation (15 min at 70°C), ligation of the adapters corresponding to the sticky ends of both enzymes was performed. A ligation mixture of 15 µl, each aliquot containing 1 µl of 5 µ*M* *Eco*RI adapters (5′CTCGTAGACTG CGTACC3′ and 5′AATTGGTACGCAGTC3′), 1 µl of 50 µ*M* *Mse*I adapters (5′GACGATGAGTCCTGAG3′ and 5′TACTCAGGACTC AT3′) and 1 U of T4 ligase enzyme (MBI, Fermentas, Lithuania) in 40 mM TRIS-HCl (pH 7.8), 10 m*M* MgCl2, 10 m*M* DTT and 0.5 m*M* dATP, was added to the digested DNA. The resulting reaction mix was incubated for 2 h at 20°C.

This was followed by a pre-amplification step using non-selective primers and performed in a total volume of 50 µl containing 5 µl of ligation mixture (diluted 10 times in TE). The polymerase chain reaction (PCR) programme was the same as that described by Zhu et al. (1998) except that only 20 cycles were per-

Table 1 Geographical origin of plant material. In grey, accessions of *S. viridis*; the others belong to *S. italica* species

Origin	Accession number	Origin	Accession number	Origin	Accession number	Origin	Accession number
Afghanistan	PI231732[a]	France	519-84[b]	Taiwan	73-81[b]	China	292-84[b]
Botel-Tobago	79-81[b]	Hungary	017398[d]	Taiwan	75-81[b]	China	373-83[b]
China	2–92[b]	Hungary	017400[d]	Taiwan	77-81[b]	France	130-86[b]
China	21-92[b]	Iran	PI250025[a]	Slovakia	449-86[b]	France	151-81[b]
China	22-92[b]	Iran	PI430147[a]	ex-USSR	PI314741[a]	France	177-86[b]
China	24-92[b]	Japan	00078242[c]	Afghanistan	PI212625[a]	France	369-83[b]
China	00080622[c]	Japan	00078273[c]	China	27-84[b]	France	371-83[b]
China	00080629[c]	Japan	00078293[c]	China	PI408811[a]	Germany	1–96[b]
China	31-92[b]	Kenya	PI464556[a]	China	81-79[b]	Switzerland	505-84[b]
China	33-97[b]	Kenya	PI464553[a]	China	96-79[b]	Turkey	PI204624[a]
China	34-97[b]	Korea	534-80[b]	China	99-79[b]		
China	4-92[b]	Morocco	PI517051[a]	China	128-86[b]		
China	PI391643[a]	Nepal	00040601[c]	China	129-86[b]		
Ethiopia	PI464544[a]	Pakistan	00080580[c]	China	287-84[b]		
Ethiopia	PI464545[a]	Poland	PI531438[a]	China	288-84[b]		
France	115-80[b]	South Africa	PI464547[a]	China	289-84[b]		
France	24-82[b]	Spain	PI283988[a]	China	291-84[b]		

[a–d] Origin of material: [a] seeds from North Central Regional Plant Introduction Station (Iowa State Univ., USA), [b] seeds from millet collection of Orsay (University of Paris, France), [c] seeds from National Institute of Agrobiological Resources (Japan), [d] seeds from Institute of Agrobotany (Hungary)

formed. Selective amplifications were performed in a 50-µl final volume containing 5 µl of pre-amplification products (diluted 50× in TE) with primers having three additional 3′nucleotides. Amplification reactions were performed according to Zabeau and Vos (1993). Eight primers associated with *Eco*RI (E+AAC; E+AAG; E+ACA; E+ACC; E+ACG; E+ACT; E+AGC; E+AGG) were used in combination with 8 primers associated with *Mse*I (M+ CAA; M+CTT; M+CAC; M+CAG; M+CAT; M+CTA; M+CTC; M+CTG).

Five microliters of amplification products was loaded onto a 3–6% denaturing polyacrylamide gel and electrophoresed in 1× TBE for 1.5–3 h depending on the primer combination. Bands were detected using the silver staining protocol described by Cho et al. (1996).

Data analysis

One accession (369-83) was tested for all primer combinations in order to select the 4 combinations with the largest number of bands. The visible and reproducible bands were scored for all the accessions as presence (1) or absence (0). We used the PHYLIP software package (Felsenstein 1993) for a bootstrap analysis (Felsenstein 1985) on all data matrices. We drew 100 bootstrap samples. The procedure DIST was modified to calculate similarity indices (F) according to Nei and Li (1979), which were converted to genetic distances (1-F). Distances between all pairs of genotypes were calculated for each bootstrap sample, and the neighbour-joining procedure (Saitou and Nei 1987) was then used to estimate dendrograms. Consensus dendrograms were obtained according to the CONSENSE procedure of PHYLIP. Correlations between distance matrices obtained from each of the 4 combinations used were evaluated and tested by a Mantel test (Raymond and Rousset 1995).

Results

AFLP methodology

Data (Table 2) based on the analysis of 1 accession (369-83) revealed that many bands are generated on a single PCR reaction: 38 combinations among 64 revealed more than 30 bands. Only 4 combinations, E-AAC/M-CTC, E-AAC/M-CTT, E-ACC/M-CTT, E-AGC/M-CTT, were used for the analyses. Three to five individuals (number indicated in parenthesis) were then tested for each primer combination from each of the following 12 accessions: 536-80(4), 159-96(5), 129-96(4), 534-80(3), 130-96(4), 154-81(3), 15-96(5), 129-86(4), 75-81(4), 128-86(4), 11-96(3), 2-92(3). AFLP methodology gave highly reproducible bands (no differences were encountered on the AFLP profiles from replicate samples of the same individual). Also, the polymorphism among individuals within accessions was very low (data not shown). Therefore, we used a bulk sample of fresh leaf tissue of 5 plants per accession for all the analyses. The 4 primer combinations used revealed 160 visible polymorphic bands. Results from the Mantel test revealed that four of the six distance matrices obtained are highly correlated except for the combination E-ACC/M-CTT with E-AAC/M-CTC and E-AAC/MCTT (Table 3).

Investigating diversity structure

The dendrograms take into account the wild accessions on one hand (Fig. 1a) and the cultivated accessions on the other (Fig. 1b). We chose to separate them because a dendrogram that included them both (Fig. 2) showed that accessions are intermingled. Both dendrograms (Fig. 1a, b) indicated that no clear geographical structure could be detected. Wild accessions from China and Western Europe could not be distinguished, and most bootstrap values were very low. Cultivated accessions revealed no apparent geographic pattern either, although accessions from Eastern Europe and Africa formed two clusters supported by bootstrap values of 72 and 47, respectively. Both clusters were further investigated in order to study their genetic relationships with the wild accessions

Table 2 Number of bands obtained for each of 64 combinations of primers (*Eco*RI primers and *Mse*I primers)

*EcoR*I→ *Mse*I↓	AAC	AAG	ACA	ACC	ACG	ACT	AGC	AGG
CAA	35	60	20	34	39	60	35	18
CAC	23	31	22	30	25	25	26	29
CAG	50	40	26	32	30	36	20	16
CAT	36	45	43	49	30	43	36	33
CTA	30	29	25	26	20	31	25	25
CTC	40	27	18	27	22	11	36	30
CTG	36	40	22	28	20	39	31	25
CTT	47	50	30	50	32	40	45	30

Table 3 Correlations between different combinations of primers. Significance was tested by a Mantel test

Primer combinations	E-AAC/M-CTC	E-AAC/M-CTT	E-ACC/M-CTT	E-AGC/MCTT
E-AAC/M-CTC				
E-AAC/M-CTT	0.587***			
E-ACC/M-CTT	0.139	0.171		
E-AGC/MCTT	0.536***	0.458**	0.327**	

*, **, ***: Significant after Bonferroni corrections at 5%, 1% and 1%, respectively

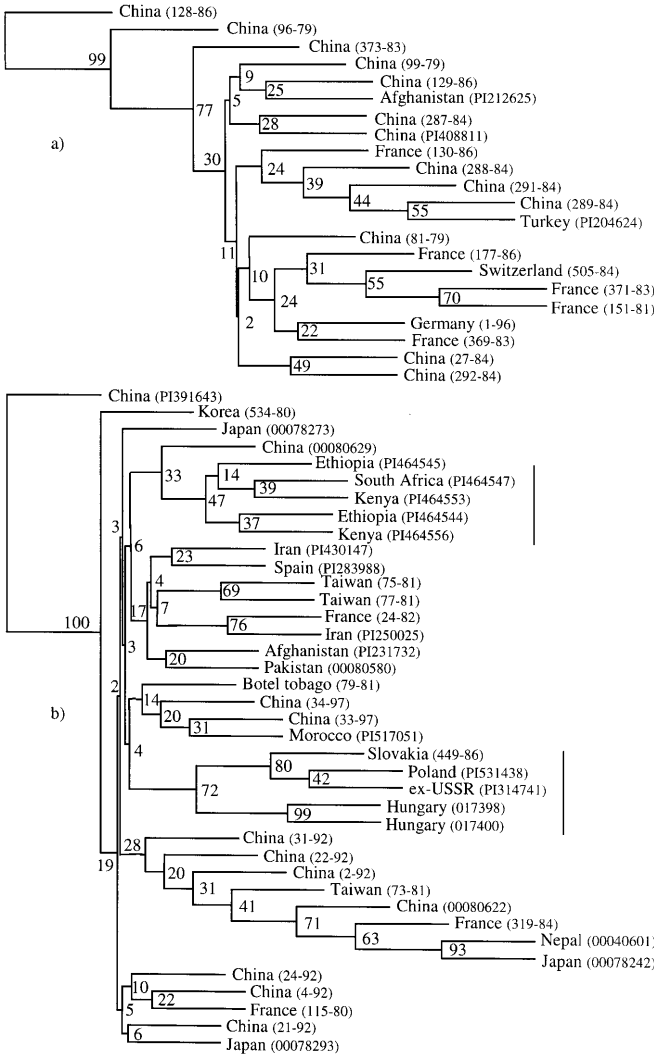

Fig. 1a, b Neighbour-joining unrooted tree based on Nei and Li distances obtained from the analysis of the raw presence/absence matrix showing the genetic relationships among 22 accessions of *S. viridis* (**a**) and among 39 accessions of *S. italica* (**b**). *Numbers* at nodes represent the bootstrap values of the consensus tree obtained

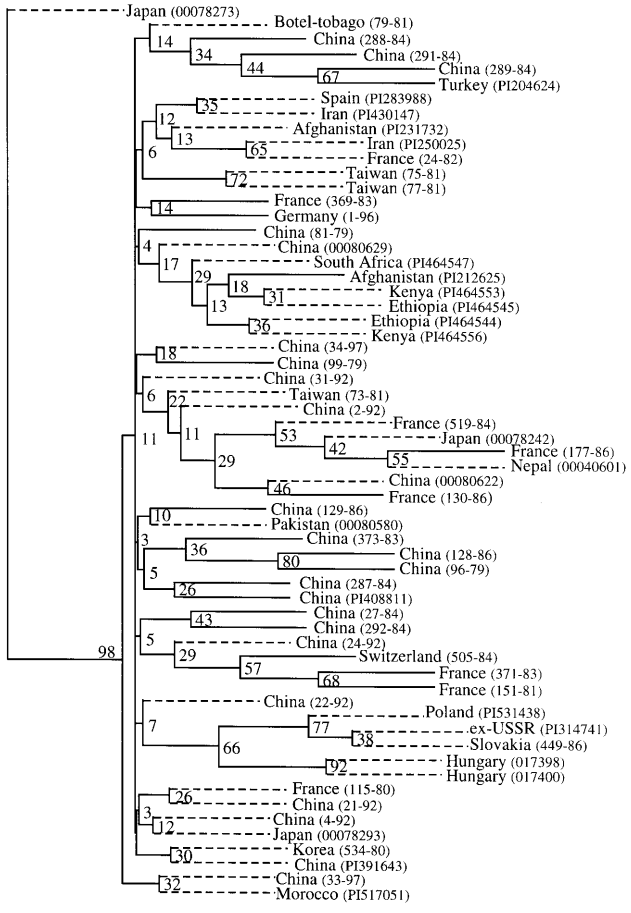

Fig. 2 Neighbour-joining unrooted tree based on Nei and Li distance obtained from the analysis of the raw presence/absence matrix showing the genetic relationships among 22 accessions of *S. viridis* and among 39 accessions of *S. italica* (*dashed lines*). *Numbers* at nodes represent the bootstrap values of the consensus tree obtained

(Fig. 2). The results showed that cultivated accessions from Eastern Europe formed a coherent group independent of any wild accession and that cultivated accessions from Africa clustered with 1 wild accession coming from Afghanistan.

Discussion

AFLP markers

The AFLP procedure allowed us to produce a large amount of data in a short time period, as has been found in other species. The construction of an AFLP-based linkage map of foxtail millet in our laboratory (data not shown) did not reveal any clustering of loci. This implies that our data may have provided a genome-wide estimation of the genetic diversity. Results from the Mantel test (Table 3) showed that the primer combinations used provided complementary information. A similar result was obtained from an AFLP analysis of a bean core collection, where the lower correlation coefficient between combinations was estimated to be 0.27 (Tohme et al. 1996).

Assessing diversity structure within a species

Our results showed that while there was little variation within accessions, there was a great deal of variation among accessions. This is in agreement with the results presented by Wang et al. (1995) and, more generally, with the genetic structure usually observed in self-pollinated plants (Hamrick and Godt 1997).

Data from our study revealed no clear geographical structure of diversity, unlike several studies based on the analysis of morphological traits (Li et al. 1995). Morphological differentiation is the result of selection caused either by the natural environment or by agricultural prac-

tices, or both. Agro-morphological markers should therefore be used with caution in systematic studies, especially when investigating relationships between accessions originating from a wide geographical area.

Studies based on isozymes often show a clear partition of diversity. This is also the case in foxtail millet (Jusuf and Pernès 1985), but the pattern could not be confirmed by our DNA studies. A similar result was noticed in wild beans by Tohme et al. (1996). An independent analysis of a wild bean core collection using isozymes (Koenig and Gepts 1989) showed that accessions from the Andean and the Meso-American groups are distinct. The resulting dendrogram exhibited short internodes, however, revealing a very low level of differentiation. Tohme et al. (1996) noted that the distinction between gene pools shown by AFLPs is not as clear as had been reported previously and that statistical analysis using a bootstrap procedure reveals that the species varies continuously over its range. These authors, proposed that this continuum may be caused by the introgression of local populations, seeing that an average outcrossing rate of 1.5% could alter wild population structure.

We observed a lack of any geographical structure to the wild species *S. viridis* (Fig. 1a), which could be explained by the fact that this species is known to be an important weed all over the world. Thus, numerous episodes of migration followed by successive introgressions may have blurred the distinction among populations over the distribution range of the species, thereby destroying any geographical structure. During the dissemination of *Setaria* seeds all over the world by human migration, seeds could have been exchanged southwards and eastwards from North China to Korea and the Japanese islands; to the west towards Europe via Caucasia; and from India north-eastwards to Taiwan and Japan and north-westwards towards Europe (Nguyen Van and Pernès 1985). An experiment (Till-Bottraud et al. 1992) aiming at estimating the gene flow between foxtail millet and green foxtail millet showed that although outcrossing rates within both species are very low (0.3–4%), spontaneous interspecific hybridisations occur in both directions at a rate that sometimes approaches the outcrossing rate. Furthermore, these authors have found offspring from such interspecific crosses in the fields, strongly suggesting that continuous gene flow between crop and the wild species has indeed occurred. These results are in agreement with the existence of an intermediate type, *S. italica* var. major, that probably originated from a stabilised hybrid between *S. italica* and *S. viridis* (Darmency and Pernès 1987).

These patterns can be compared to those observed in a study of nucleotide variation in the Adh region of 17 ecotypes of *A. thaliana* (Innan et al. 1996). The authors found no association between sequence type and geographical origin. Each sequence type contained samples from different continents, despite the fact that the Himalayas have been regarded as the centre of diversity of the genus (Price et al. 1994). Furthermore, the results demonstrated the existence of divergent parental sequences that could be due to introgression from a related species even though the outcrossing rate is very low, being 0.3% according to Abbott and Gomes (1989).

Foxtail millet domestication

The spread of accessions over the dendrogram (Fig. 2) provides evidence that the Chinese gene pool has a high level of diversity. This result is consistent with the commonly accepted hypothesis that China is the primary centre of diversity of foxtail millet and may therefore have been a main domestication centre (Vavilov 1926).

Accessions from Eastern Europe form a distinct cluster (Fig. 1), suggesting that they constitute a gene pool having a reduced diversity that could have resulted from local adaptation. The African accessions show a similar pattern, however, a lower bootstrap value suggesteds that this group is unstable. The African cultivated forms clustered with 1 wild accession from Afghanistan (Fig.2).

The lack of structure of the wild gene pool does not allow us to infer the origin of domestication based on the genetic relationships of wild and cultivated populations. As mentioned above, genetic relatedness within the *S. viridis* gene pool (Fig. 1a) or between the *S. viridis* and *S. italica* gene pools (Fig. 2) may be a consequence of gene flow between cultivated forms and weed populations that has occurred recently. Our data shows the genetic proximity between a wild accession from Afghanistan and the African cultivated forms but does not allow us to identify a domestication center in this region (Li et al. 1995).

Morphological observations of some cultivated forms originating from Afghanistan and Lebanon show that they are intermediates between *S. italica* and *S. viridis* (Li et al. 1995). These authors proposed classifying these forms in a new race called ‘Nana’. Moreover, based on RFLP analysis of the rDNA region, Fukunaga et al. (1997) have shown that cultivated accessions from Afghanistan exhibit patterns that are different from the rest of the cultivated gene pool. We built a dendrogram (data not shown) regrouping the wild accessions and the cultivated accession from Afghanistan (PI231732) that showed that this cultivated accession clusters with the wild accession coming from the same region (PI212625). This was supported by a bootstrap value of 70. Altogether, those data suggest that accessions from Afghanistan exhibit a particular pattern. More analyses of the diversity of the Eurasian gene pool (both wild and cultivated forms) are needed to draw any conclusions. In addition, particular attention should be given to the sampling of *S. viridis* accessions, especially by sampling wild (rather than weedy) populations in undisturbed areas in order to obtain a more accurate assessment of the diversity of this gene pool.

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