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An analysis of genetic differentiation and geographical variation of spinach germplasm using SSR markers

Kouhei Kuwahara†, Reimi Suzuki†, Yusuke Ito†, Tetsuo Mikami and Yasuyuki Onodera*

Laboratory of Genetic Engineering, Research Faculty of Agriculture, Hokkaido University, N-9, W-9, Sapporo 060-8589, Japan

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Abstract

To assess the genetic diversity of spinach germplasm, 250 individuals of 50 accessions collected from geographically diverse regions (West Asia, East Asia, Japan, Europe and the USA) were analysed using simple sequence repeat (SSR) markers. A total of 39 polymorphic alleles were identified, with an average of 6.5 alleles per locus for six loci. The overall gene diversity (0.62) in the entire set of individuals suggests that the germplasm has high genetic variability. The West Asian accessions showed the highest gene diversity, with a value of 0.57, followed by the East Asian accessions. These results help confirm the notion that spinach originated from West Asia. Hierarchical analysis of molecular variance revealed significant genetic differentiation among the geographical regions, which accounts for 26% of the total variation detected. Furthermore, pairwise Φ_{st} values indicate low genetic differentiation between the East Asian and Japanese germplasm accessions, both of which showed high genetic differentiation from the European accessions. The differentiation between the East Asian and European gene pools may be attributed to the founder effect associated with crop dissemination, as well as to the selection and genetic drift that occurred during the breeding process.

Keywords: genetic diversity; germplasm; spinach; *Spinacia oleracea* L; SSR

Introduction

Spinach (*Spinacia oleracea* L.), one of the most nourishing leafy vegetables, is produced in more than 50 countries, primarily in China, the USA, Japan and Europe (FAO statistics; http://faostat.fao.org/site/567/default.aspx#ancor). This crop is thought to be native to West Asia, most probably northern Iran, Afghanistan and Turkmenistan (Sneep, 1982; Vavilov, 1992; Hammer, 2001; van der Vossen, 2004). According to Chinese records, which provide the first mention of spinach outside of its native land, spinach was brought to China through Nepal around the seventh century (Ryder, 1979; Sneep, 1982; Nonnecke, 1989; van der Vossen, 2004). The first Japanese reference to spinach appears in the Japanese encyclopedia *Tashikiben*, which was written in 1631. This suggests that the crop was introduced to Japan through China approximately 400 years ago (Ishiguro, 1982). Spinach was also brought westward from its native region to North Africa, arriving in Spain *c.* the twelfth century. The crop then spread throughout Europe. The colonists brought spinach to North America, and it was frequently cultivated in the early nineteenth century (Ryder, 1979; Ware and McCollum, 1980; Nonnecke, 1989; van der Vossen, 2004).

* Corresponding author. E-mail: onodera@abs.agr.hokudai.ac.jp
† These authors contributed equally to this work.

Traditional spinach cultivars of East Asia are often early bolting and bear spiny fruits and hastate/toothed leaves with long petioles and red bases. However, European cultivars are often characterized by late bolting, round fruits and entire leaves with short, non-pigmented petioles (van der Vossen, 2004). Spiny fruit is considered to be a primitive characteristic, while characteristics such as round fruits, entire leaves and non-pigmented petioles are associated with the domestication process and may have arisen independently in various growing areas (Smith, 1976).

Spinach germplasm accessions collected from many different countries are stored in gene banks in Japan, the USA, the Netherlands, Germany and other countries (see The International Spinach Database; http://documents.plant.wur.nl/cgn/pgr/spinach/con_cont.asp). These accessions, which may have originated from local landraces and old/traditional cultivars, are important resources for future spinach breeding. In fact, spinach germplasm accessions exhibit desirable characteristics for spinach improvement, such as resistance to downy mildew and leaf miner, as well as low oxalate content (Brandenberger *et al.*, 1992; Mou, 2007a, b; Mou, 2008a, b).

Understanding the genetic diversity and relationships among spinach germplasm collections would be useful for the efficient utilization of these accessions as breeding material. Such information would be helpful for preserving existing diversity and for collecting new accessions to increase genetic diversity. Hu *et al.* (2007) used dominant target region amplification polymorphism (TRAP) markers to examine the genetic diversity among 38 spinach accessions originating from over 20 countries. This technique could discriminate between the accessions and revealed a high level of genetic variability between accessions. However, the genetic structure of spinach germplasm remains obscure.

Here, we examined spinach germplasm accessions from five geographical regions (West Asia, East Asia, Japan, Europe and the USA), which represent the land of origin and the major growing regions of spinach, to assess genetic diversity and relatedness among accessions based on polymorphisms at six simple sequence repeat (SSR) loci.

Materials and methods

Plant material

A total of 40 spinach germplasm accessions collected from West Asia (Afghanistan, Iran, Iraq and Syria; Table S1, no. 1–10 (available online)), East Asia (China, Hong Kong, Mongolia, Nepal, Thailand and South Korea; no. 11–20), Europe (Belgium, France, Germany, Hungary, Italy, the Netherlands, Poland, Spain, Sweden and the UK; no. 31–40) and the USA (no. 41–50) were obtained from the United States Department of Agriculture (USDA) National Plant Germplasm System. Ten accessions derived from Japan (Table S1 and Fig. S1, no. 21–30 (available online)) were also obtained from the National Institute of Agrobiological Sciences Genebank (Tsukuba, Japan).

The plants were grown in a plastic house at a temperature ranging from 5 to 30°C. Five plants were randomly sampled from each accession and were used for chromosomal SSR analysis. Total cellular DNA was prepared from individual plants using the method of Doyle and Doyle (1990).

SSR genotyping

Six primer pairs designed to amplify spinach nuclear SSRs, i.e. SO3, SO4, SO7, SO10, SO29 and SO48 (Groben and Wricke, 1998; Khattak *et al.*, 2007), were used to assess genetic diversity and the relationships between accessions. SO7 and SO29 were mapped to linkage group 2 with a 25 cM distance from each other, and SO4 and SO48 were located on linkage group 3 with a 47 cM distance from each other, while the remaining two SSRs were not assigned to any group (Khattak *et al.*, 2006). The forward primers for the SSR markers (except for SO29) were labelled with 6-FAM™, VIC™ and NED™ fluorescent dye (Life Technologies, Carlsbad, CA, USA). Amplification reactions were carried out as described by Khattak *et al.* (2007), being repeated at least twice to avoid mistyping and polymerase chain reaction (PCR) errors. PCR products amplified with fluorescent-labelled primers were analysed on an Applied Biosystems 3130 (Life Technologies, Carlsbad, CA, USA) Genetic Analyzer. PCR products amplified with SO29 primer pairs were loaded onto a 5% native polyacrylamide gel.

Statistical analysis of nuclear SSR data

Basic statistics, including the number of alleles and gene diversity (expected heterozygosity), were calculated using PowerMarker v3.25 software (http://statgen.ncsu.edu/powermarker/; Liu and Muse, 2005).

In the analysis of molecular variance (AMOVA), Φ-statistics were calculated to measure genetic differences among regions, among accessions and within accessions. High positive statistical values indicated large genetic differences. The significance of Φ-statistics was tested based on 9999 permutations. GenAlEx 6.41 software (http://www.anu.edu.au/BoZo/GenAlEx/new_version.php, Peakall and Smouse, 2006) was used to compute AMOVA

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Table 1. Genetic diversity statistics for the 50 spinach germplasm accessions collected from the five geographical regions

Accession groups	*N*	*n*	A_l	H_e
West Asia	10	50	4.667	0.571
East Asia	10	50	4.833	0.548
Japan	10	50	4.167	0.512
Europe	10	50	3.833	0.414
USA	10	50	3.500	0.523
Overall	50	250	6.500	0.615

N, number of accessions per group; *n*, number of individuals; A_l, average number of alleles per group for all six loci; H_e, average genetic diversity within the accession group.

and to calculate pairwise genetic differentiation (pairwise Φ_{st}) between regions.

A pairwise genetic distance matrix (C-S chord distance; Cavallis-Sforza and Edwards, 1967) was constructed based on allelic frequencies among accessions, which were calculated using PowerMarker v3.25. The C-S chord distance can be used to generate correct tree topologies regardless of the microsatellite mutation model (Takezaki and Nei, 1996; Gao and Innan, 2008). The genetic distance matrix was subjected to principal coordinate analysis (PCA) using GenAlEx 6.41.

Results

SSR polymorphism

In a previous study (Khattak *et al.*, 2007), 13 SSRs were used to evaluate genetic diversity among spinach hybrid cultivars. However, half of the SSRs were located in coding regions, which showed relatively low polymorphism. In a preliminary experiment using about 40 spinach germplasm accessions, fewer alleles (two or three) were found at each of the SSR loci in coding regions than at each of the SSR loci in non-coding regions (data not shown), suggesting that the SSRs in coding regions are not necessarily appropriate for the evaluation of genetic diversity. Therefore, six SSRs (SO3, SO4, SO7, SO10, SO29 and SO48) located in non-coding regions were chosen from among 13 SSRs to evaluate genetic diversity and relationships in a set of 50 spinach germplasm accessions originating from West Asia (Table S1, no. 1–10 (available online)), East Asia (eastern Eurasian countries except for Japan; no. 11–20), Japan (no. 21–30), Europe (no. 31–40) and the USA (no. 41–50). We re-examined the sequences of SSR loci from several individual plants with homozygous alleles of interest. When two alleles of different sizes were sequenced, the relationship between the repeat motifs and the repeat numbers could be found. We confirmed that the repeat motifs of all but one (SO29) coincided with previously published data (Khattak *et al.*, 2007). Although the SO29 locus was previously reported to be a polymorphic dinucleotide microsatellite, we found that this region represents a variable number of 85 bp tandem repeats (Fig. S2, available online). Hence, SO29 may be referred to as a minisatellite. However, in this study, we describe SO29 as an SSR, according to the nomenclature proposed by Khattak *et al.* (2007).

The six nuclear SSR loci were successfully amplified for all samples examined (250 individuals), and no null alleles were detected. All loci were polymorphic; the number of alleles per locus ranged from 13 (SO4) to 4 (SO3), with a mean of 6.50, and a total of 39 alleles were identified (Table 1 and Table S2 (available online)). Gene diversity (H_e, expected heterozygosity) was highest in the West Asian accessions ($H_e = 0.571$), followed by the East Asian accessions ($H_e = 0.548$), US accessions ($H_e = 0.523$) and Japanese accessions ($H_e = 0.512$). The European accessions exhibited the lowest gene diversity ($H_e = 0.414$). The gene diversity over the entire set of accessions was 0.615 (Table 1).

AMOVA

Table 2 summarizes the results of AMOVA for the SSR dataset. Half of the total genetic variance was found among individuals within accessions, while the other half

Table 2. AMOVA for the 50 accessions collected from the five geographical regions (West Asia, East Asia, Japan, Europe and the USA)

Source	df	SS	MS	Estimated variance	Percentage of the total variance	Φ-statistics	*P*
Among regions	4	303.260	75.815	1.339	26	0.261	<0.001
Among accessions	45	398.580	8.857	1.266	25	0.333	<0.001
Within accessions	200	506.200	2.531	2.531	49	0.507	<0.001
Total	249	1208.040	87.203	5.136	100		

SS = Sums of Squares, MS = Mean Sums of Squares.

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Table 3. Pairwise Φ_{st} values among the five regions

	West Asia	East Asia	Japan	Europe
East Asia	0.257^{***}			
Japan	0.283^{***}	0.091^{***}		
Europe	0.185^{***}	0.414^{***}	0.438^{***}	
USA	0.137^{***}	0.343^{***}	0.302^{***}	0.228^{***}

$^{***} < 0.001$.

was found among accessions ($\Phi_{st} = 0.333$, $P < 0.001$) and among five geographical regions (West Asia, East Asia, Japan, Europe and the USA; $\Phi_{st} = 0.261$, $P < 0.001$), indicating that significant genetic differences existed at three levels (among individuals within accessions, among the accessions and among the regions). Pairwise comparisons were made between the different geographical regions based on the Φ_{st} values. The results indicate apparent geographical differentiation (Table 3). In particular, the germplasm accessions from Japan and Europe showed the highest genetic differentiation (0.438), followed by the East Asian and European accessions (0.414). By contrast, the lowest differentiation (0.091) was displayed by neighbouring regions, i.e. East Asia and Japan.

Genetic relationships of spinach germplasm accessions

Genetic differentiation among the geographical regions was further indicated by the first two principal components of PCA of the pairwise genetic distance matrix for the SSR dataset. The coordinate axes 1 and 2 explained 36.94 and 18.99% of the total genetic diversity, respectively (Fig. 1).

In general, the locations of the accessions on the coordinate plane match their geographical distribution. The East Asian and Japanese accessions co-occur on the left side of the coordinate plane. The European accessions appear on the upper, right quadrant, whereas most of the American accessions are scattered in the lower, right quadrant. The two American accessions (NSL 6093 and NSL 32 629) are mixed with the European accessions. The accessions from West Asia are clustered in the centre, as a number of these are related to either the European or US accessions.

Discussion

Understanding the genetic structure and relationships of germplasm accessions of a given crop species is important for the conservation of biological diversity and the exploitation of natural genetic resources for crop improvement. In this study, we used SSR markers to study the genetic variation among spinach germplasm accessions that originated from many different regions. A total of 39 alleles were detected for six SSR loci. These six loci were previously employed to estimate the genetic diversity between 38 commercial spinach F1 cultivars grown in Asia, Europe and the USA, resulting in the detection of 24 alleles (Khattak *et al.*, 2007). A relatively low genetic variation in F1 cultivars has also been reported by Hu *et al.* (2007), who used TRAP markers to study spinach germplasm accessions and commercial hybrids. Such results are expected, as most modern cultivars have a limited germplasm base.

SSR markers have shown extensive diversity among, but little variation within, cultivars and/or germplasm accessions of autogamous plant species (e.g. lettuce; van Hintum, 2003; Jansen *et al.*, 2006). In outcrossed species such as spinach, individual plants exhibit considerable heterogeneity that may lead to SSR variations both within and among accessions (Hu *et al.*, 2007). As expected, AMOVA for the SSR dataset revealed that half of the total genetic variance was accounted for by the variation among individuals within accessions. Despite a substantial internal variation, there was measurable divergence among the accessions (see Table 2). This prompted us to perform the PCA using the entire SSR dataset, with the intention of estimating the genetic differentiation of spinach germplasm from different geographical regions. For this purpose, all accessions were provisionally assigned into five groups according to their geographical origin: West Asia, East Asia, Japan,

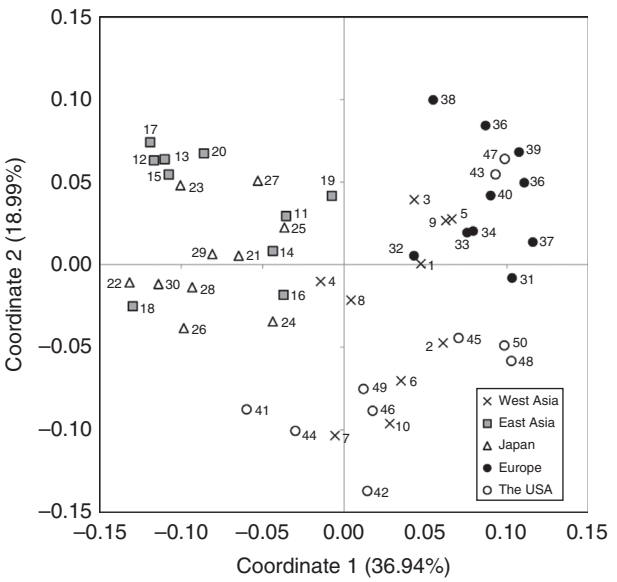

Fig. 1. PCA of the matrix of the C-S chord distance among the 50 spinach accessions. The numbers 1–50 represent the accessions listed in Table S1 (available online).

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Europe and the USA. On the whole, the PCA separated the germplasm accessions geographically, against a background of extensive within-accession polymorphism.

An important finding of the present study was that the West Asian accessions had the highest level of gene diversity. This is consistent with the commonly accepted view that spinach was initially domesticated as a leaf vegetable in regions neighbouring Persia (Iran) (Ryder, 1979; Vavilov, 1992; Hammer, 2001; van der Vossen, 2004). As already mentioned in the Introduction section, spinach reportedly spread eastward and westward from its place of origin to East Asia and Europe. The West Asian accessions are clustered in the centre of the PCA plane, which neighbours the East Asian accessions and is mixed with the European accessions. This probably reflects the origin and dissemination pattern of this crop. We also found a high level of genetic differentiation between the East Asian and European accessions. These results suggest that differences between the East Asian and European gene pools could be due to a founder effect associated with crop dissemination, as well as to the selection and genetic drift that occurred during the breeding process.

Notably, the PCA showed that the US accessions had close relations not only with the European accessions but also with the West Asian accessions. The observation that the US accessions had close relations with the European accessions is consistent with the well-known fact that spinach was brought from Europe to North America relatively recently (Ware and McCollum, 1980; Nonnecke, 1989). The observation that the US accessions had close relations also with the West Asian accessions raises the possibility that a wide array of spinach genotypes were introduced into North America from geographical regions other than Europe. The analysis of additional accessions from various countries will help further elucidate the history of spinach cultivation in the geographical area of interest.

Another important result of this study was the low level of genetic differences between the East Asian and Japanese accessions, which may be a consequence of the recent and multiple introductions of spinach from China to Japan. Spinach had already been brought to Japan from China by the seventeenth century, but it is also well known that a local landrace was introduced into western Japan from China in 1934 (Ishiguro, 1982). This Chinese landrace was grown throughout Japan and was widely used as source material for spinach breeding because of its high yield and heat tolerance.

In conclusion, this study examined the amount and distribution of genetic diversity in spinach germplasm from different regions. The information obtained in this study will be valuable for spinach breeding and germplasm management. The considerable genetic differentiation revealed in spinach from different geographical regions emphasizes the importance of collecting new germplasm from as many different growing areas as possible to obtain additional genetic variability. The regeneration cycles of germplasm resources in small field plots with limited numbers of individuals may be associated with a change and/or loss of the original genetic diversity (Borner *et al.*, 2000; Parzies *et al.*, 2000; van Hintum *et al.*, 2007). As mentioned above, the data show that approximately half of the total genetic diversity in the spinach germplasm collections is present within accessions. This suggests that minimizing the loss of genetic variation in each accession during regeneration is critical for preserving the genetic integrity of spinach germplasm collections over a long period of time.

Supplementary material

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S1479262113000464

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