Genetic structure of landraces in foxtail millet (Setaria italica (L.) P. Beauv.) revealed with transposon display and interpretation to crop evolution of foxtail millet

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Genetic structure of landraces in foxtail millet [*Setaria italica* (L.) P. Beauv.] revealed with transposon display and its interpretation to crop evolution of foxtail millet.

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Abstract

Origin and domestication process of foxtail millet [*Setaria italica* (L.) P. Beauv.] have been studied by several groups but still in discussion with ambiguity. It is cardinal to elucidate the issue by studying large number of accessions with enough number of markers covering the whole genomic regions. Genetic structures were analyzed by transposon display (TD) using 425 landraces of foxtail millet and 12 accessions of the wild ancestor, green foxtail. We applied three recently-active transposons (*TSI-1*, *TSI-7* and *TSI-10*) as genome-wide markers and succeeded in demonstrating geographical structures of foxtail millet. A neighbor-joining dendrogram based on TD grouped the set of foxtail millet accessions into eight major clusters each of which consisted of accessions collected from geographically adjacent areas. Eleven out of twelve green foxtails were

grouped separately from the clusters of foxtail millet. These results pointed that the strong regional differentiations and long history of the cultivation in each region. Relationship between foxtail millet and green foxtail regarding intraspecific hybridization and suggested monophyletic origin of foxtail millet domestication were also discussed.

Keywords: Crop Evolution, Transposon Display (TD), Foxtail millet, Population Structure, Setaria italica

Abbreviations: transposon display (TD), transposon elements (TEs), miniature inverted-repeat transposable elements (MITEs), short interspersed transposable elements (SINEs), Cetyl trimethylammonium bromide (CTAB), Neighbor-Joining (NJ), Principal Coordinate Analysis (PCoA), linkage disequilibrium (LD)

Introduction

Foxtail millet, *Setaria italica* (L.) P. Beauv. ssp. *italica* is one of the oldest cereals in Eurasia. Archaeological remains of this crop were found at Peiligang and Cishan sites near the Yellow River in China dating back to 5,000-6,000 BC (Li and Wu 1996), at prehistoric sites in Europe (Küster 1984) and in the Transcaucasus (Lisitsina 1976). It has been traditionally consumed in various ways among cultures and regions in Eurasia and is thought to have played an important role in early agriculture of the Old World (Sakamoto 1987). The geographical origin of foxtail millet, however, remains a controversial issue.

Cytological and genetic studies had provided *S. italica* ssp. *viridis* as the presumed wild ancestor of this crop (Kihara and Kishimoto 1942; Li et al. 1945; Le Thierry d'Ennequin et al. 2000; Wang et al. 1995). However, geographical origin of domestication could not be elucidated simply due to the wide distribution of this wild species throughout Europe and Asia and even in the New World (Wang et al. 1995; Le Thierry d'Ennenquin et al. 2000). Vavilov (1926) inferred that the primary center of diversity of foxtail millet is East Asia, including China and Japan. Harlan (1975) suggested independent domestication in China and Europe according to archaeological evidence, which is supported by several studies based on archaeological, morphological or molecular evidences (de Wet et al. 1979; Jusuf and Pernes 1985; Benabdelmouna et al. 2001; Li et al. 1995a, b, 1998). Li et al. (1995b) suggested another independent and recent domestication in Afghanistan and Lebanon, where the landraces have primitive characteristics such as numerous tillers with small panicles. In contrast, Sakamoto (1987) reported foxtail millet in Central Asia, Afghanistan, Pakistan and north west India have not only primitive morphological traits but relatively higher cross compatibility with those from

other regions (Kawase and Sakamoto 1987) and thus proposed that foxtail millet originated somewhere in these regions, and China might be the secondary center of diversification.

Such controversies might be partly due to lack of sufficient tools in genetic analyses and effective experimental sizes in the past research Thus, 1) more genetic markers covering the whole genome and 2) a large-scale analysis might be a key to elucidate this entangled issue.

Transposon display (TD), which is a modified AFLP, has been shown to be a suitable marker system for phylogenetic studies and linkage analyses (Casa et al., 2000, Kwon SJ, 2006), because some transposon elements (TEs) such as long terminal repeat (LTR)-retrotransposons are enriched in centromeric and pericentromeric regions while others like miniature inverted-repeat transposable elements (MITEs) are often in euchromatic, gene-rich regions. More importantly, plant genomes are comprised of TEs (Feschotte et al. 2002). Especially, TEs that are active or were recently active may display higher level of polymorphism (Casa et al. 2000), which has been clearly shown in a linkage analysis between very closely related genomes using an active TE as markers in rice (Monden et al. 2009).

In foxtail millet, several TEs including LTR-retrotransposons and MITEs have been identified in the mutant alleles of *Waxy* (*GBSS1*) gene, which controls amylose content in the endosperm starch (Fukunaga et al. 2002a; Kawase et al. 2005). This fact indicated that people in early Asia favored and repeatedly selected for the sticky endosperm trait of *waxy* phenotype generated by TE insertions (Kawase et al. 2005). Thus, these TEs might have been still active after the domestication event (less than 10,000 years ago) and be useful for markers.

In this paper, we developed a TD marker system utilizing three TEs identified in *waxy* alleles and performed a large scale analysis with 425 accessions of foxtail millet

and 12 green foxtails worldwide, including Asia, Europe, and Africa. The following phylogenetic analysis revealed clear clustering of geographically adjacent foxtail millet accessions but most green foxtails were clustered into one group, which is often observed in crops with monophyletic origin. The evolution and dissimilation of foxtail millet will be further discussed.

Materials and methods

Plant materials and transposon display

A total of 425 foxtail millet accessions covering the major traditional geographical distribution and 12 accessions of green foxtail were used in this study (Table 1). Genomic DNA was extracted from the leaf samples of 1-month-old seedlings or from 20 mature seed grains from each landrace, using the CTAB method of Murray and Thompson (1980) with some modifications.

Three TEs {*TSI-1* (Tourist MITE), *TSI-7* (LTR-retrotransposon) and *TSI-10* (SINE)} were selected from different classes and characteristics on the basis of the transposon sequences found in *GBSS1* gene of foxtail millet reported by Kawase et al. (2005). Sequences of the element-specific primers were shown in Table 2. Transposon display was carried out following the method of Casa et al. (2004) with following modifications. Selective amplification using a “touchdown” protocol: 94°C for 3 min followed by 95°C for 30 sec, 64°C for 45 sec, and 72°C for 45 sec. In subsequent cycles, the annealing temperature was reduced from 63°C to 58°C in 1°C increments each cycle. Twenty-nine cycles were performed at the 58°C annealing temperature, followed by a final extension of 72°C for 3 min. The PCR products were separated by capillary electrophoresis, using an automated DNA sequencer ABI model 3130 (Applied Biosystems Inc.). Two accessions (SI85 from Turkey and SI200 from Korea) were failed

in amplification and excluded. Genotyping was performed by Gene Mapper 3.0 software (Applied Biosystems Inc). The threshold for allele calling was set at 500 relative fluorescent unit (RFU) based on the stable call from reference samples.

Data analysis

Genotype data were analysed using AFLPsurv1.0 (Vekemans 2002) to evaluate the genetic variation. Cluster analysis was performed using distant matrix with Neighbor-Joining (NJ) method in Phylip package (Felsenstein, 2004) and then visualized with the TreeExplorer (Kumar et al. 2004). Statistical testing for the clustering was performed with pooled individual which grouped into the same cluster in the NJ tree. This smaller number of group, it was possible to perform statistical testing via 1,000 times bootstrap resampling. We also performed Principal Coordinate Analysis (PCoA) to determine major pattern within a multivariate dataset by GenAlEx 6 (Peakall and Somuse 2006). First, we calculated the binary genetic distance and made a distance matrix (Huff et al. 1993). Principal coordinates elements were calculated using the distance matrix, then the calculated first two principal coordinates were plotted on the graph.

Results

Insertion and clustering pattern of the three TEs with different characteristics

Three different types of TEs showed 356 polymorphic bands and 129 of them were polymorphic between accessions at 5 % level (allelic frequency of 0.05 - 0.95). The numbers of polymorphic/scored bands were 67/149 (*TSI-1*), 17/93 (*TSI-7*), and 45/114 (*TSI-10*) (Table 3). One hundred seventeen one band were found in foxtail millet (spp. *italica*) but not in green foxtail (spp. *viridis*), whereas 17 bands were exclusively found in

green foxtail. Statistical test (student t-test) for copy number of each TEs between foxtail millet and green foxtail showed significant differences in *TSI-7* ($p<0.00$) and *TSI-10* ($p<0.03$) but not in *TSI-1*. (Fig. 1).

Clustering pattern of Neighbor-Joining dendrogram

We analyzed the genetic relationships among the 423 accessions of foxtail millet with 12 accessions of green foxtail using the NJ dendrogram. The foxtail millet accessions were grouped into eight major groups and each of them consisted of accessions collected from geographically adjacent areas (Fig. 2). Accessions from East Asia (cluster I, II and III) formed three large clusters. The cluster I was mainly comprised of accessions from Japan and Korea, while accessions from Nansei islands, Japan (the south most islands of the country) was grouped into the cluster II with those from Taiwan and the Philippines. Interestingly, seven Nepal accessions were also included in this cluster. Most of the other East Asian accessions (from China, Japan and Korea) were grouped into the cluster III. Three accessions from China (SI97) and Japan (SI157; SI132) grouped together with one green foxtail accession collected from Pakistan (cluster IV). Accessions from East Europe (Uzbekistan, Ukraine, Turkey, Bulgaria, Poland, Hungary and Czech Republic), one from Morocco, and four from China formed the cluster V. The cluster VI was the second largest and comprised of accessions from South Asia, Southeast Asia, China, Taiwan, Africa, West Europe and Middle East. Three subclusters were observed in this cluster, each of which represents (a) mainly Nepal, (b) Southeast Asia including Pakistan and India, and (c) South Asia and Myanmar plus Africa. Accessions in this cluster seem to be distributed surrounding the Indian Ocean. The accessions of Western Europe (Germany, France, Belgium and Spain), Western Asia (Turkey, Lebanon and Georgia), and South Asia (Iran, Afghanistan, and Pakistan) formed the cluster VII. One cluster was consisted exclusively of accessions from Northeast

Pakistan (cluster VIII). All the green foxtail accessions were outgrouped, except one from Pakistan. Two accessions (SI74, SI101) were located between clusters of foxtail millets and green foxtails. It is noteworthy that Chinese accessions were present in almost all the clusters except the cluster II, VII and VIII. Bootstrapping provability of this clustering revealed closer genetic relationship between cluster I and III (97 %), and cluster II and VI (86 %) which is shown in Fig. 2b (supplemental electronic figure). We plotted the geographical distribution of foxtail millet and green foxtail accessions on a map with cluster indications (Fig. 3).

Principal coordinate analysis

Principal coordinate analysis (PCoA) was performed to further explore the relationships among foxtail millet accessions with TD data. Principal coordinates represent 33.22 % and 21.19% for PC1 and 2 respectively (Fig. 4). The pattern of geographic diversification of the foxtail millet accessions overall corresponds to what was observed in the dendrogram. Foxtail millet accessions were scattered on the graphic area of PCoA diagram with clear grouping of geographic structures, while all the green foxtail accessions were gathered around the center of the diagram.

Discussions

Diversity and dissimilation of foxtail millet illustrated by recently active transposons

Lack of enough markers or genotypes has always limited the resolution of genetic analysis of foxtail millets. For example, AFLP analysis with 39 foxtail millet accessions did not reveal any geographic structure (Le Thierry d'Ennequin 2000).

Better results have been obtained by some other studies using RFLP (Fukunaga et al. 2002b), RAPD (Schontz and Rether 1999) or other molecular markers (Fukunaga et al. 1997; Justuf and Pernes 1985; Li et al. 1995b; Nakayama et al. 1999), but no more than five genetic groups have been identified in these studies. However, in this study, the large scale analyses with TD markers have successfully depicted genetic relationship of foxtail millet, providing eight clusters with very clear geographic structures as well as evolutional history of foxtail millets and green foxtails with higher resolution than ever. Our results also indicated the merit of TD as a tool for studying genetic variations. TD mainly detects variations in insertions of TEs and these variations are rapidly generated by active TEs. As such, the use of TEs which had been active even after domestication event (Kawase et al. 2005) as markers might have revealed dynamics of variation during the history of cultivation. Thus, TD would be a suitable marker system for elucidating crop dissimilation after domestication, if active or recently-active TEs were available.

Although geographical structures of foxtail millet have been indicated in previous studies (Li et al. 1995b; Schontz and Rether 1999; Fukunaga et al., 2002b, 2006; Kawase et al. 2005), the NJ tree drawn in this study has provided more detailed image because of the higher number of accessions and the higher number and diversity of the marker used. East Asia was the region of the largest genetic diversity of foxtail millet, as mentioned by Vavilov (1926). Indeed, Chinese landraces were present in six of the eight clusters detected in this study. Although not all the information on the collection localities was available, these accessions were grouped together with accessions which share the originated habitat.

Some of the landraces from Nepal was revealed to be in the cluster II, which is mainly comprised of landraces in Taiwan and Japanese Nansei Islands but not in China or anywhere in between. The separate distribution of this group might be because only a few accessions from south China were available in this study. This sub-region may be

intriguing and unique spots.

Accessions collected from northeast China (Heilongjiang Province) were grouped with the accessions originated East Europe in cluster V. The landraces in the cluster V was also in Russia and Mongolia and genetically close to those in East Asia, suggesting that the close genetic relationship between East European strains and northeast China. Close genetic relationship within cluster V indicate that adaptation to the similar habitat rather geographical vicinity not like other clusters.

There was another genetic border between East Europe and West Europe. All but one accessions from West Europe belonged to the cluster VII while all the East European landraces were in the cluster V. The West European landraces were genetically distinct from East Asian landraces and formed a cluster with those from Middle East and Pakistan. Although foxtail millet is known from remains of ~5000 years ago in Europe (Austin 2006), West Europeans' landraces showed the least genetic diversity. It indicates that West Europe is the periphery of foxtail millet cultivation, where genetic diversity comes to a close. Moreover, the dramatic decline of foxtail millet cultivation in 19th century (de Wet 1995; Jarman et al. 1982) may accelerate the decline of the genetic diversity.

Although most of the accessions from South Asia and Southeast Asia belonged to only one cluster (VI), they were divided into three geographic subclusters; one was mainly comprised of Nepal landraces, another formed by those from India, Pakistan and Southeast Asia except Myanmar, and the other with those from Myanmar, India, Sri Lanka and Africa. These results indicated that Myanmar is South Asia rather than Southeast Asia according to genetic structure of foxtail millet, and that the African foxtail millets had been introduced from South Asia, maybe from India. Two accessions originated from Southwest China (Yunnan Province) were also grouped with this group.

In this study, landrace groups of a cluster and/or sub-cluster are often endemic

to a small geographical region. Reproductive isolation has been shown between landraces from different area (Kawase and Sakamoto 1987). This might reflect the characteristics of foxtail millet as a minor crop. Foxtail millet germplasm has not experienced modern breeding in global scale like bread wheat. The limited exchange of germplasm has preserved the historical distribution of the landraces. The differentiation of a landrace group in foxtail millet might have occurred along with the dispersal from the place of domestication as is the case for Asian rice (Khush 1997). The strong geographical vicinities and genetic differentiation from one another imply a long history of cultivation of foxtail millet in each region, because the establishment of landraces is the result of the adaptation of the crop for the local ecological environment and agricultural practices (Sakamoto 1987).

Relationship of foxtail millet and green foxtail

The Significant differences in detected copy number of two types of TEs (*TSI-7* and *TSI-10*) between green and foxtail millet indicate the higher activities of these TEs in foxtail millet but not in green foxtail. These two TEs are retrotransposons, the sequence at the insertion sites are retained as they transpose via the replication mechanism, and increased the copy number. On the other hands, *TSI-1* is a DNA transposon which transposes copy and paste system. We observed excess of copy number of TEs in green foxtail compared with the foxtail millet. TEs used in this study are assumed to be activated after the domestication of foxtail millet; thus, we expected larger copy number of TEs in foxtail millet than that in green foxtail. However, number of bands which found exclusively in green foxtail was larger in proportion to the sample size differences between foxtail millet and green foxtail. It has been reported that green foxtail, which is a wild progenitor of foxtail millet, shows greater genetic diversity than the genetic distance between green foxtail and foxtail millet from the same region (Wang et al. 1995; Le

Thierry d'Ennequin et al. 2000). As indicated in the previous studies, the bands found only in green foxtail might transposed in green foxtail rather indicate the broader genetic background of green foxtail and genetic bottle neck which experienced foxtail millet than transposition independently after the differentiation of these sub species.

Previous studies on intraspecific hybridization of green and foxtail millet have indicated repeated genetic introgression (Darmency et al. 1987; Wang et al. 1995; Jarvis and Hodgkin; 1999). However, the TD marker system in this study has provided different image from the previous studies. Eleven of the twelve green foxtail accessions collected from various areas of Eurasia were grouped into one cluster separated from all the foxtail accessions. In addition, green and foxtail millet accessions originated Japan and Korea distributed separately on PCoA plot, whereas those from China were overlapped with each other (Fig. 4). This result has indicated that interspecific hybridization between green and foxtail millet had not been so frequent as had been expected in particular region. The low genetic relationships between green and foxtail millet in Korea and Japan might be explained by intensive selection and adaptation of foxtail millet in the region and strict isolation of crop and the weedy form by cultivation practices. Human selection for agricultural use has probably accelerated formation of region-specific populations of foxtail millet.

Interestingly, there is a small cluster including one green foxtail accession from Pakistan (Cluster IV) in between the clusters of East Asia and the clusters of South Asia and Europe. The three foxtail millets accessions belonging to this cluster stretched from China and Japan. Given the origins of the foxtails and the green foxtail in this cluster were geographically distant; this clustering did not seem to be a result of a recent hybridization event. As such, this cluster might represent the idea of Sakamoto (1987) that foxtail millet originated somewhere in Central Asia, Afghanistan, Pakistan, and northwestern India spread to East Asia and later diversified. Comparing the sequence

of the TE shared within this cluster could offer a check for the molecular lineage.

It should also be noted that the phylogenetic tree in this study exhibited a monophyletic pattern of foxtail millet from green foxtail, contrasting with many previous studies which have supported polyphyletic origin of foxtail millet (Harlan 1975; de Wet et al. 1979; Jusuf and Pernes 1985; Li et al. 1995a, b, 1998; Schontz and Rether 1998; Le Thierry d'Enequin et al. 2000; Benabdelmouna et al. 2001). However, Allaby and Brown (2003) addressed monophyletic pattern is erroneously inferred by genotyping of anonymous marker and NJ analysis when linkage disequilibrium (LD) is not assured carrying out a simulation study of imaginary crop with several different multiple domestication scenarios. Since our monophyletic assumption was based on an anonymous marker with low LD, we could not avoid the chance of erroneous interpretation. Thus, to discuss domestication origin of the foxtail millet, it is necessary to test larger number of the green foxtail millet collected from wider range of Eurasia. In addition, even a crop species with sequenced genomes such as rice, the origin is still under discussion (Vaughan et al. 2008). Particular genes related to the domestication traits will allow us to further elucidate the phylogeny and evolution of the foxtail millet. Integrated genome sequence data would accelerate disentangling complex network lineages of this species.

Conclusion

In this study, geographical genetic structure of foxtail millet was successfully demonstrated by large scale phylogenetic analysis using TE insertion polymorphism as DNA markers. Two clear genetic borders were identified: 1) between accessions from East Asia and those from other regions including Central, South, or Southeast Asia and the Middle East, and 2) between West Europe and East Europe. Almost all green foxtail

accessions were outgrouped; indicating intercrossing intensity between foxtail millet and it wild ancestor differs among regions. The domestication origin still remained unclear, but our results might require reconsidering of the widely-accepted argument that this crop has multiple origins of domestication. However, this long-standing issue will be elucidated by further studies such as in silico approach using upcoming complete genome sequence (Doust et al. 2009) with larger number of green foxtail accessions.

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Figure titles

Fig. 1 Box plot of copy number of each transposable element in foxtail millet and green foxtail. The box comprised 50 % of the data and bold line within the box represents the median value. Open circles indicate outside values.

Fig. 2Neighbour joining dendrogram based on transposon display. Black bars indicate the clusters. Asterisks identify those accessions of Nansei islands, south most islands of Japan. Colours of branches indicate geographic origin of each accession.

Fig. 3 Geographical distribution of foxtail millet and green foxtail accessions. The colours of circles on the map indicate the cluster which each accession belongs to. Country/area name, cluster ID and number of accessions belong to each cluster are indicated in boxes.

Fig. 4 Graph of first two axes from a principal coordinate analysis of foxtail millet accessions and green foxtail accessions. The first two coordinates explains 33.22 % and 21.19 % of the total variation.

Tables

Table 1 Number of accessions and their origins of foxtail millet (*S. italia* spp. *italica*) and green foxtail (*S. italia* spp. *viridis*) used in the study.

Countries and areas	No. of Accs.	Countries and areas	No. of Accs.
***Setaria italica* spp. *italic*a (Total 425)**			
East Asia		**West Asia**	
China	23	Georgia	2
Japan	125	Lebanon	6
Korea	41	Turkey	6
Taiwan	21	**Europe**	
Mongolia	1	Belgium	3
Southeast Asia		Bulgaria	1
Indonesia	7	Czech	1
Laos	1	France	3
Myanmar	30	Germany	1
Philippines	9	Hungary	5
Thailand	2	Poland	1
South Asia		Russia	2
Afghanistan	5	Spain	1
Bangladesh	3	Switzerland	1
Bhutan	1	Ukraine	1
India	44	**Africa**	
Iran	3	Ethiopia	1
Nepal	25	Kenya	2
Pakistan	36	Morocco	1
Sri Lanka	6	South Africa	2
Central Asia			
Kyrgyzstan	1		
Uzbekistan	1		
***Setaria italica* spp. *viridis* (Total 12)**			
China	4	Korea	1
Iran	1	Pakistan	1
Japan	5		

Table 2 Primer sequences for transposon display used in the diversity study of *Setaria italia*

Element	**PCR**	**Specific primer**	***Mse*I primer**
TSI-1	Primary	CTCCTAATTAGTGTCCAAACATTCG	GACGATGAGTCCTGAGTAAG
(AB21023)	Secondary	ATGGGACGGGGGCTAAAAT	GACGATGAGTCCTGAGTAAGTA GACGATGAGTCCTGAGTAAGTC GACGATGAGTCCTGAGTAAGTG
TSI-7	Primary	GCTAGCAGGTTCAAGGGTGT	GACGATGAGTCCTGAGTAA
(AB210216)	Secondary	TACCCCTCCTCTCATCGAAA	GACGATGAGTCCTGAGTAA
TSI-10	Primary	TCCCTCCAAATTGTCCATGT	GACGATGAGTCCTGAGTAAG
(AB210219)	Secondary	CTCCTGCTCTCATCGACCAC	GACGATGAGTCCTGAGTAAGTA GACGATGAGTCCTGAGTAAGTC GACGATGAGTCCTGAGTAAGTT

Table 3 Number of bands detected by transposon display in *Setaria spp.*

Selective base	***TSI-1***	***TSI-7***	***TSI-10***
0	-	93	-
+gta	47	-	32
+gtc	54	-	47
+gtg	48	-	-
+gtt	-	-	35
Total	149	93	114
(Average per accession)	(24.3)	(11.0)	(30.89)

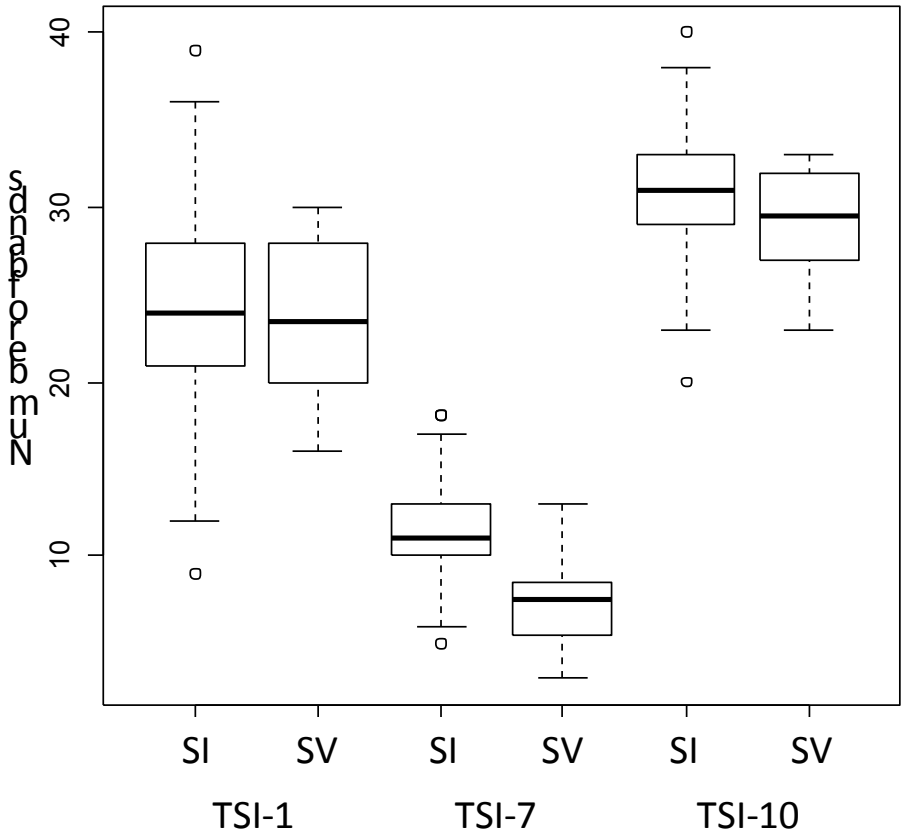

Fig. 1 Box plot of copy number of each transposable element in foxtail millet and green foxtail. The box comprised 50 % of the data and bold line within the box represents the median value. Open circles indicate outside values.

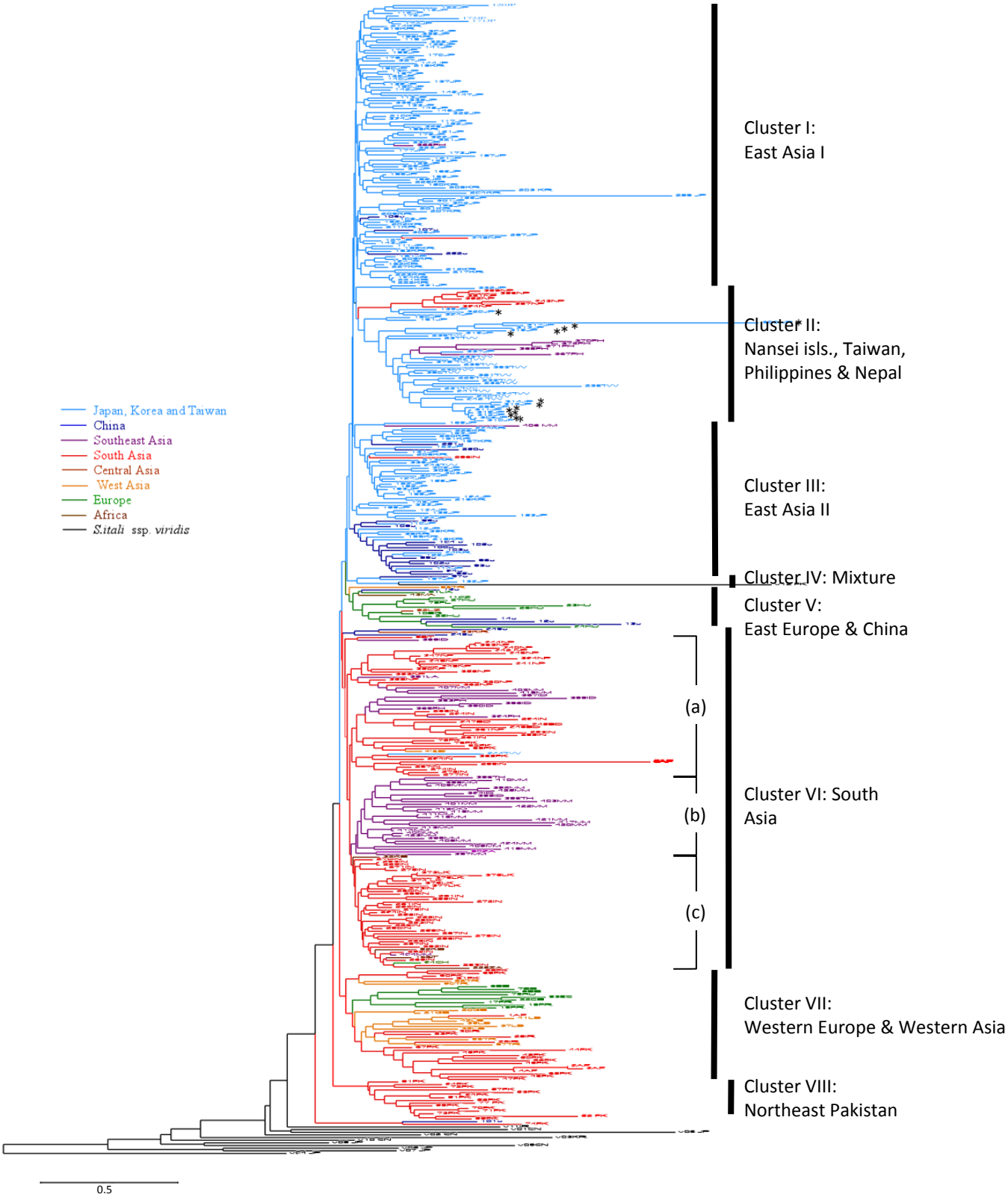

Fig. 2Neighbour joining dendrogram based on transposon display. Black bars indicate the clusters. Asterisks identify those accessions of Nansei islands, south most islands of Japan. Colours of branches indicate geographic origin of each accession.

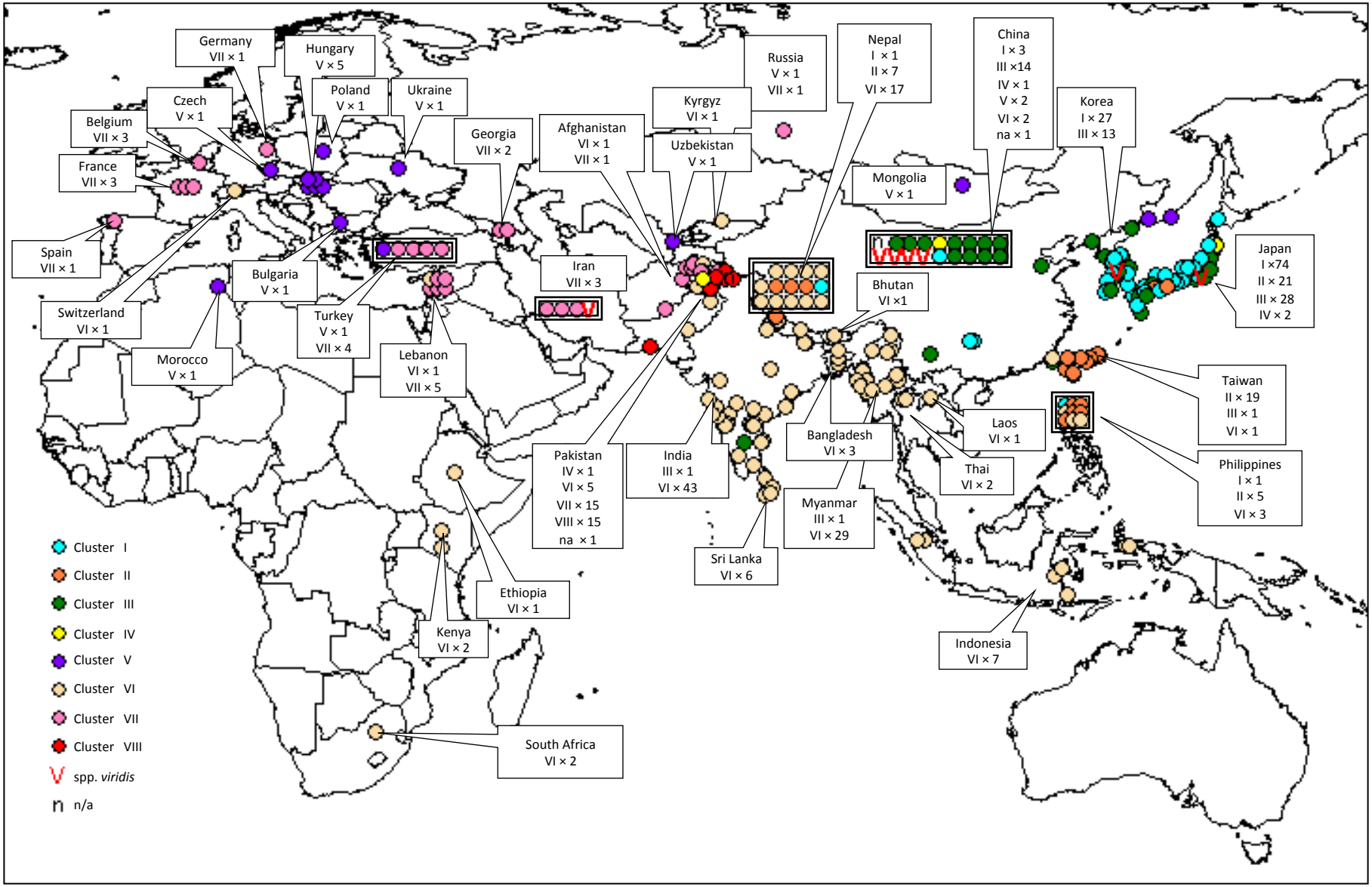

Fig. 3 Geographical distribution of foxtail millet and green foxtail accessions. The colours of circles on the map indicate the cluster which each accession belongs to. Country/area name, cluster ID and number of accessions belong to each cluster are indicated in boxes.

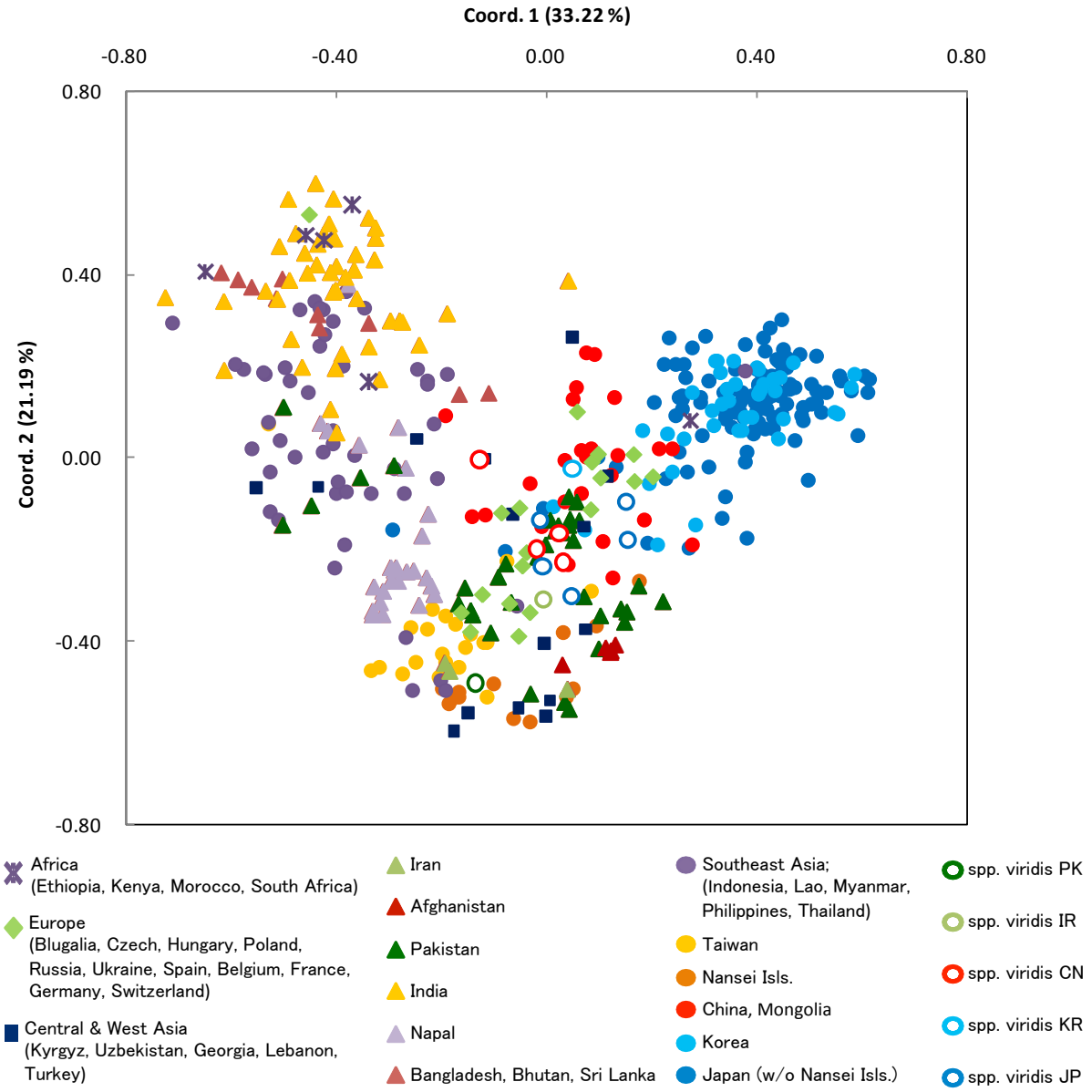

Fig. 4 Graph of first two axes from a principal coordinate analysis of foxtail millet accessions and green foxtail accessions. The first two coordinates explains 33.22 % and 21.19 % of the total variation.