

Latitudinal adaptation and dispersal pathway of foxtail millet suggested by geographical distribution of transposable elements inserted in the *SiPRR37* gene

Kenji Fukunaga^{1*}, Akira Abe², Kazue Ito², Kaori Oikawa², Masaya Tsuji³ and Makoto Kawase⁴

¹ Faculty of Bioresource Sciences, Prefectural University of Hiroshima, Shobara, Hiroshima 727-0023, Japan

² Iwate Biotechnology Research Center, Kitakami, Iwate 024-0003, Japan

³ Graduate School of Comprehensive Scientific Research, Graduate School of Prefectural University of Hiroshima, Shobara, Hiroshima 727-0023, Japan

⁴ Faculty of Agriculture, Tokyo University of Agriculture, Atsugi, Kanagawa 243-0034, Japan

(Received 24 January 2024, accepted 17 May 2024; J-STAGE Advance published date: 24 May 2024)

We investigated the variation and geographical distribution of the *Pseudo-regulator response 37* (*Setaria italica* *PRR37*; *SiPRR37*) gene, which is involved in heading time (photoperiodism) in foxtail millet. An allele of the *SiPRR37* gene, in which an approximately 4.9-kb transposable element (designated TE1) is inserted (a loss-of-function or reduction-of-function type), is distributed sporadically in East Asia and broadly in Southeast Asia and South Asia, implying that this gene is important in latitudinal adaptation. In addition, we found a new allele of *SiPRR37* with an insertion of a 360-bp TE (TE2) at this locus and investigated the geographical distribution of this new type. This *SiPRR37* allele with TE2 is distributed in Japan, Korea, Nepal, Iran and Turkey. Both TE1 and TE2 are useful markers for tracing foxtail millet dispersal pathways in Asia.

Key words: adaptation, foxtail millet, heading time, *PRR37* gene, transposable elements

Foxtail millet (*Setaria italica* (L.) P. Beauv.) is an ancient cereal in Eurasia. This millet has been cultivated broadly in Eurasia and parts of Africa and has adapted to various ecological conditions (Fukunaga et al., 2015), e.g., from low latitude to high latitude and from low altitude to high altitude. As foxtail millet has some advantages for genetic studies, it has become a model for species in the subfamily Panicoideae (Doust et al., 2009), and its genome was sequenced by Bennetzen et al. (2012) and Zhang et al. (2012). As this millet shows high variability in several

agronomic traits, it should also be a good resource for studying domestication and crop evolution (Fukunaga and Kawase, 2024).

Heading time is one of the most important traits for adaptation to local environments (Izawa, 2007). Takei and Sakamoto (1987, 1989) reported wide variation in heading time in foxtail millet landraces due to their variety in basic growth period and different sensitivity in their response to changing day length. This trait has been investigated in other plant species, particularly in two model plants, rice and *Arabidopsis* (Izawa, 2007). In foxtail millet, QTL analyses of heading time have been performed (Mauro-Herrera et al., 2013; Yoshitsu et al., 2017). In particular, the *Heading date 1* (*HD1*) gene has been analyzed and a splicing variant of this gene was found to be distributed broadly in Eurasia (Fukunaga et al., 2015; Liu et al., 2015), but it was not clear how this gene alters heading time.

Recently, two groups found that the foxtail millet *Pseudo-response regulator 37* (*Setaria italica* *PRR37*; *SiPRR37*) gene plays an important role in the latitudinal

Edited by Jeffrey Fawcett

* Corresponding author. fukunaga@pu-hiroshima.ac.jp

DOI: <https://doi.org/10.1266/ggs.24-00023>



Copyright: ©2024 The Author(s). This is an open access article distributed under the terms of the Creative Commons BY 4.0

International (Attribution) License (<https://creativecommons.org/licenses/by/4.0/legalcode>), which permits the unrestricted distribution, reproduction and use of the article provided the original source and authors are credited.

adaptation of this crop (Li et al., 2021; Fukunaga et al., 2022). *PRR37* has been reported to be associated with circadian rhythms and is a key gene in latitudinal adaptation in rice and sorghum (Murphy et al., 2011; Koo et al., 2013). In both species, this gene is the major repressor of heading under long-day conditions. Li et al. (2021) investigated Chinese landraces of foxtail millet using a genome-wide association study and found that a transposable element (TE) insertion in *SiPRR37* was important in the adaptation of foxtail millet to northeast China. Fukunaga et al. (2022) also identified the same TE insertion in a Taiwanese landrace by QTL analysis between a Japanese landrace and a Taiwanese landrace, and reported that this TE insertion is predominantly distributed throughout southern Asia, in areas such as South Asia, Afghanistan, Southeast Asia, Taiwan and up to the Nansei Islands of Japan, by genotyping 99 Eurasian and African accessions of foxtail millet landraces.

Here, we report a new TE insertion (designated TE2) of 360 bp, found using Nanopore sequencing, in addition to the previously identified insertion (Fukunaga et al., 2022), which we renamed TE1. The geographical distribution of both TE1 and TE2 among the 480 accessions analyzed reveals a new picture of how foxtail millet has expanded its cultivation latitudinally.

We used 480 foxtail millet landraces and 13 accessions of *S. italica* ssp. *viridis* (L.) Thell., the wild progenitor of foxtail millet (Supplementary Table S1). These samples covered the traditional area of foxtail millet cultivation, from East Asia to Western Europe, and parts of Africa. All foxtail millet samples were maintained in the NARO Genebank (National Agriculture and Food Research Organization), Tsukuba, Japan (https://www.gene.affrc.go.jp/index_en.php). These 480 accessions were used to analyze variation in *rDNA*, *HD1*, *polyphenol oxidase* (*Si7PPO*) and *SiDREB2* genes (Eda et al., 2013; Fukunaga et al., 2015; Inoue et al., 2015; Suehiro et al., 2018).

All ssp. *viridis* accessions were obtained from the Genetic Resources Information Network of the Agriculture Research Service, United States Department of Agriculture (USDA). The 13 accessions included three from Turkey; two each from Russia, Afghanistan, Iran and mainland

China; and one each from Chile and Mongolia. DNA extraction was carried out according to Eda et al. (2013).

We performed *de novo* assembly for three foxtail millet accessions ('Yuikogane', a new cultivar released by Iwate Prefecture; JP 222871, Kochi Prefecture, Japan, which has been used for developing a new mapping population in the Prefectural University of Hiroshima; and JP222980, an Indian landrace, which has been used in our research group for analyzing several traits as a representative for Indian landraces) using Nanopore long reads and Illumina short reads. Libraries were then prepared using a Ligation Sequencing Kit (SQK-LSK-109; Oxford Nanopore Technologies, United Kingdom) according to the manufacturer's instructions. Nanopore sequencing and *de novo* assembly were carried out as described by Fukunaga et al. (2022). The genome sequences of the four accessions (JP71640 from Miyazaki Prefecture, Japan, JP73913 from Taiwan, and two landraces from Iwate Prefecture, 'Nisatai-zairai' and 'Otsuchi 10') obtained previously (Fukunaga et al., 2022) were also used in this study. All genome sequences were deposited in Zenodo (<https://doi.org/10.5281/zenodo.10461727>). The *SiPRR37* gene was retrieved and its sequences in the seven accessions and 'Yugu1', the reference sequence (Bennetzen et al., 2012), were compared by BioEdit (Hall, 1999). In addition to the TE previously reported in 'Yugu1', 'Nisatai-zairai', JP73913 and JP222980 (Fukunaga et al., 2022), we found a 360-bp insertion in intron 3 in 'Otsuchi 10' and 'Yuikogane' (Fig. 1). As 'Otsuchi 10' is one of the parents of 'Yuikogane' (Nakajo, 2015), this *SiPRR37* gene sequence of 'Yuikogane' will have been derived from 'Otsuchi 10'. We renamed the TE found by Fukunaga et al. (2022) TE1 and designated this new 360-bp sequence as TE2. We genotyped the TE1 insertion in intron 3 of the *SiPRR37* gene according to Fukunaga et al. (2022), and checked the absence or presence of TE2 (Fig. 1). For genotyping of TE2, we used the primer set TE2-F and TE2-R (Supplementary Fig. 1). The PCR conditions were as follows: 5 min at 94 °C; 35 cycles of 1 min at 94 °C, 1 min at 60 °C and 1 min at 72 °C; and 5 min at 72 °C. Quick Taq HS DyeMix (Toyobo, Osaka, Japan) was used for amplification. The PCR products were electrophoresed on a 1.2% agarose gel and visualized under UV light with EtBr

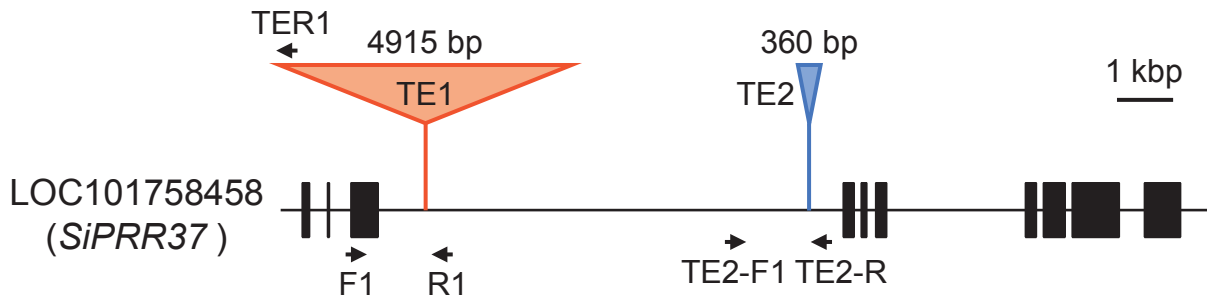


Fig. 1. Structure of the *SiPRR37* gene and insertion of transposable elements (TE1 and TE2). Primers used for the analysis of TE insertions are shown as arrows.

staining.

Censor (<https://www.girinst.org/censor/>) was used for homology searching of known transposable elements and Phytozome (<https://phytozome-next.jgi.doe.gov/>) was also used to determine the distribution of the transposable elements in the foxtail millet genome (The JGI Plant Gene Atlas *Setaria italica* v2.2).

We compared the gene sequences of the seven accessions (JP71640 from Miyazaki Prefecture, Japan; JP73913 from Taiwan; two landraces from Iwate Prefecture, Japan, namely 'Nisatai-zairai' and 'Otsuchi 10'; JP222871 from Kochi Prefecture, Japan; 'Yuikogane', a new cultivar from Iwate Prefecture, Japan; and JP222980, an Indian landrace) and 'Yugul', the reference sequence of foxtail millet (Bennetzen et al., 2012). TE1 (previously reported as TE by Fukunaga et al., 2022) in intron 3 was observed in JP222980 (Indian accessions) but not in 'Yuikogane' or JP222871 of Kochi Prefecture in Japan, as expected by PCR genotyping. In addition to TE1, we found a 360-bp sequence inserted in different positions of intron 3 in 'Yuikogane' and 'Otsuchi 10' (Fig. 1, Supplementary Fig. S1). We carried out a homology search with this 360-

bp sequence using Censor and found homology with harbinger-like sequences of maize (HARB14Zm and HARB N36Zm). We also performed a BLAST homology search of the foxtail millet genome (The JGI Plant Gene Atlas *Setaria italica* v2.2) using Phytozome and found that more than 1,000 sequences throughout the genome show 67–100% identity with this sequence, which is thus highly repetitive in the foxtail millet genome and probably still active.

Twelve of the 13 wild ssp. *viridis* accessions showed no insertion of either TE1 or TE2. PI212625 from Afghanistan was the exception, with TE1 but not TE2. As this accession shows non-shattering grains (Liu et al., 2022), similar to foxtail millet, it may have been misidentified as ssp. *viridis* or derived from introgression between ssp. *viridis* and foxtail millet.

Genotyping of TE1 and TE2 in foxtail millet landraces revealed clear geographical distribution patterns, as shown in Fig. 2. No accessions had both of the two TEs (TE1 and TE2) together. Of the 480 accessions, 155 (32.3%) had neither TE1 nor TE2, 238 (49.6%) had only TE1 and 84 (17.5%) had only TE2. For three accessions (two from

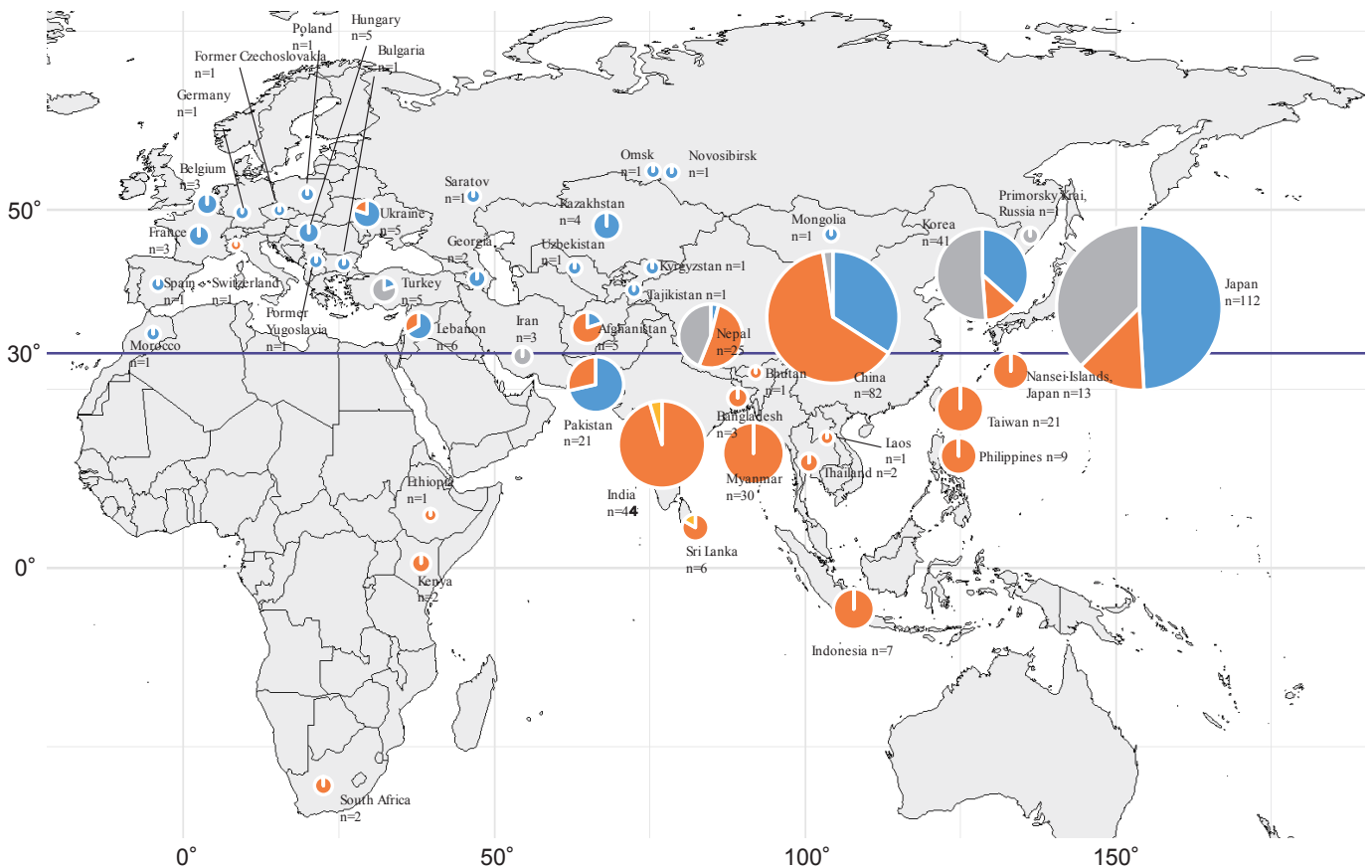


Fig. 2. Geographical distribution of TE1 insertion type, TE2 and non-insertion type (wild type) of *SiPRR37* gene of foxtail millet. Blue, orange, gray and yellow in the pie charts represent non-insertion type, TE1 insertion type, TE2 insertion type and TE1 non-amplified type, respectively. The size of the pie chart represents the number of accessions. South of approximately 30°N, the TE1 insertion type is predominant.

India and one from Sri Lanka), TE2 was not inserted, and no bands were amplified by the primer combinations for genotyping TE1 (Supplementary Fig. S2). This may be due to deletions or nucleotide substitutions within the primer sequence(s) for genotyping TE1, which may have prevented amplification.

TE1 was found broadly in landraces from subtropical and tropical regions (south of approximately 30°N), such as the Nansei Islands of Japan, Taiwan, the Philippines, Indonesia, Laos, Thailand, Myanmar, Bangladesh, Bhutan, India, Sri Lanka, Afghanistan, Kenya and South Africa, and sporadically in Japan, Korea, China, Nepal and Pakistan (Fig. 2, Supplementary Table S1). Li et al. (2021) proposed that TE1 insertion into the *SiPRR37* gene plays an important role in adaptation in northeastern China based on a detailed genetic analysis of 299 Chinese accessions, but they did not use material collected outside China. As TE1 insertion is frequently found in East Asia in our study, but the collection sites of our material from China are not clear (most of them are from north China), our data did not indicate so clearly that TE1 insertion into *SiPRR37* is important in adaptation to high latitude in China. Although we used 125 Japanese landraces, we did not find clear differences between the eastern and the western parts of Japan excluding the Nansei Islands (Okinawa Prefecture) (frequency of TE1 insertion was 13.6% (6 of 44 accessions) and 13.2% (9 of 68 accessions), respectively, as shown in Supplementary Table S2). However, this study strongly indicates that TE1 in the *SiPRR37* gene is found in low-latitude (south of approximately 30°N) areas in Southeast and South Asia (Fig. 2 and Supplementary Table S1). The present study indicates that TE1 insertion into the *SiPRR37* gene is important for adaptation not only in northeast China, as Li et al. (2021) suggested, but also in the low-latitude area.

TE2 has also been detected in Japan, Korea, Nepal, Iran and Turkey; two Chinese accessions also had this insertion. There is no evidence that the insertion of TE2 is associated with adaptation, because no genetic analysis has been carried out for the TE2 insertion type, but the geographical distribution of TE2 will be useful as a genetic marker for tracing the dispersal of foxtail millet. As foxtail millet was probably domesticated in China (Fukunaga and Kawase, 2024), this implies a close relationship between foxtail millet landraces among East Asia, Nepal and western parts of Asia (Iran and Turkey) (Fig. 2, Supplementary Table S1).

This suggests that a functional *SiPRR37* gene is necessary for foxtail millet to adapt to the middle latitudinal regions, but is not essential in the high and low latitudinal regions, where changes in day length are not critical for heading. This clock gene probably controls photoperiodism and suppresses flowering under long-day conditions. The wild type having neither TE1 nor TE2 is distributed in the middle latitudinal regions (Fig. 2), where

plants can be affected by changes in day length, although the wild type showed early to late heading and the TE1 type also showed early to extremely late heading under natural day length in Japan (data not shown). Detailed analysis of the effect of TE1 insertion into the *SiPRR37* gene is required using near-isogenic lines. The effect of TE2 on the *SiPRR37* gene in heading time is not clear. Further analysis of effect of TE2 insertion in this gene on heading time is therefore also required, e.g., using near-isogenic lines.

TEs have played important roles in the domestication and diversification of foxtail millet. In our previous study (Fukunaga et al., 2022) and in this study, we identified two transposable elements in the *SiPRR37* gene. TEs are also found in several other genes, such as shattering genes and the *Waxy*, *Si7PPO* and *C* genes (Fukunaga and Kawase, 2024). Genome-wide polymorphisms of TE insertions have been identified by transposon display in the foxtail millet genome (Hirano et al., 2011).

As reviewed above, TE insertions in certain genes have played important roles in domestication, followed by crop evolution with landrace diversification. Further studies of genome-wide TE insertions will be helpful in better understanding the evolution and phylogeny of this millet.

DECLARATIONS

Funding: This research was supported by JSPS KAKENHI (Grant Numbers 20K06098, 23K05279).

Authors' contributions: K. F. designed the research and carried out genotyping. A. A., K. I. and K. O. carried out NGS analyses. M. T. carried out cultivation of plant materials and DNA extraction. K. F. and M. K. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest: The authors declare no conflict of interest.

We thank the NARO Genebank, Japan, UGA and USDA for providing plant materials. In this project, we used the NIG supercomputer at ROIS National Institute of Genetics, Japan, and the supercomputer of ACCMS at Kyoto University, Japan.

REFERENCES

- Bennetzen, J. L., Schmutz, J., Wang, H., Percifield, R., Hawkins, J., Pontaroli, A. C., Estep, M., Feng, L., Vaughn, J. N., Grimwood, J., et al. (2012) Reference genome sequence of the model plant *Setaria*. *Nat. Biotechnol.* **30**, 555–561. DOI: 10.1038/nbt.2196
- Doust, A. N., Kellogg, E. A., Devos, K. M., and Bennetzen, J. L. (2009) Foxtail millet: a sequence-driven grass model system. *Plant Physiol.* **149**, 137–141. DOI: 10.1104/pp.108.129627
- Eda, M., Izumitani, A., Ichitani, K., Kawase, M., and Fukunaga, K. (2013) Geographical variation of foxtail millet, *Setaria italica* (L.) P. Beauv. based on rDNA PCR-RFLP. *Genet. Res. Crop Evol.* **60**, 265–274. DOI: 10.1007/s10722-012-9832-8

- Fukunaga, K., Abe, A., Mukainari, Y., Komori, K., Tanaka, K., Fujihara, A., Yaegashi, H., Kobayashi, M., Ito, K., Ohsako, T., et al. (2022) Recombinant inbred lines and next-generation sequencing enable rapid identification of candidate genes involved in morphological and agronomic traits in foxtail millet. *Sci. Rep.* **12**, 218. DOI: 10.1038/s41598-021-04012-1
- Fukunaga, K., Izuka, N., Hachiken, T., Mizuguchi, S., Ito, H., and Ichitani, K. (2015) A nucleotide substitution at the 5' splice site of intron 1 of rice *HEADING DATE 1* (*HD1*) gene homolog in foxtail millet, broadly found in landraces from Europe and Asia. *Crop J.* **3**, 481–488. DOI:10.1016/j.cj.2015.07.003
- Fukunaga, K., and Kawase, M. (2024) Crop evolution of foxtail millet. *Plants* **13**, 218. DOI: 10.3390/plants13020218
- Hall, A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**, 95–98.
- Hirano, R., Naito, K., Fukunaga, K., Watanabe, K. N., Ohsawa, R., and Kawase, M. (2011) Genetic structure of landraces in foxtail millet (*Setaria italica* (L.) P. Beauv.) revealed with transposon display and interpretation to crop evolution of foxtail millet. *Genome* **54**, 498–506. DOI: 10.1139/g11-015
- Inoue, T., You, T., Ohta, T., Hitomi, E., Ichitani, K., Kawase, M., Taketa, S., and Fukunaga, K. (2015) Multiple origins of the phenol reaction negative phenotype in foxtail millet, *Setaria italica* (L.) P. Beauv., were caused by independent loss-of-function mutations of the *polyphenol oxidase* (*Si7PPO*) gene during domestication. *Mol. Genet. Genomics* **290**, 1563–1574. DOI: 10.1007/s00438-015-1022-x
- Izawa, T. (2007) Adaptation of flowering-time by natural and artificial selection in *Arabidopsis* and rice. *J. Exp. Bot.* **58**, 3091–3097. DOI: 10.1093/jxb/erm159
- Koo, B.-H., Yoo, S.-C., Park, J.-W., Kwon, C.-T., Lee, B.-D., An, G., Zhang, Z., Li, J., Li, Z., and Paek, N.-C. (2013) Natural variation in *OsPRR37* regulates heading date and contributes to rice cultivation at a wide range of latitudes. *Mol. Plant* **6**, 1877–1888. DOI: 10.1093/mp/sst088
- Li, C., Wang, G., Li, H., Wang, G., Ma, J., Zhao, X., Huo, L., Zhang, L., Jiang, Y., Zhang, J., et al. (2021) High-depth resequencing of 312 accessions reveals the local adaptation of foxtail millet. *Theor. Appl. Genet.* **34**, 1303–1317. DOI: 10.1007/s00122-020-03760-4
- Liu, H., Fang, X., Zhou, L., Li, Y., Zhu, C., Liu, J., Song, Y., Jian, X., Xu, M., Dong, L., et al. (2022) Transposon insertion drove the loss of natural seed shattering during foxtail millet domestication. *Mol. Biol. Evol.* **39**, msac078. DOI: 10.1093/molbev/msac078
- Liu, H., Liu, H., Zhou, L., Zhang, Z., Zhang, X., Wang, M., Li, H., and Lin, Z. (2015) Parallel domestication of the *Heading Date 1* gene in cereals. *Mol. Biol. Evol.* **32**, 2726–2737. DOI: 10.1093/molbev/msv148
- Mauro-Herrera, M., Wang, X., Barbier, H., Brutnell, T. P., Devos, K. M., and Doust, A. N. (2013) Genetic control and comparative genomic analysis of flowering time in *Setaria* (Poaceae), G3 (Bethesda) **3**, 283–295. DOI: 10.1534/g3.112.005207
- Murphy, R. L., Klein, R. R., Morishige, D. T., Brad, J. A., Rooney, W. L., Miller, F. R., Dugas, D. V., Klein, P. E., and Mullet, J. E. (2011) Coincident light and clock regulation of *pseudoresponse regulator protein 37* (*PRR37*) controls photoperiodic flowering in sorghum. *Proc. Natl. Acad. Sci. USA* **108**, 16469–16474. DOI: 10.1073/pnas.1106212108
- Nakajo, S. (2015) Breeding of a new foxtail millet variety “Yuikogane”. *Bulletin of the Iwate Agricultural Research Center* **14**, 27–40 (in Japanese with English summary).
- Suehiro, S., Ichitani, K., Doman, E., and Fukunaga, K. (2018) Genotyping of the *SiDREB2* gene in worldwide foxtail millet (*Setaria italica* (L.) P. Beauv.) genetic resources with special attention to Indian landraces. *Genet. Resour. Crop Evol.* **65**, 1559–1564. DOI: 10.1007/s10722-018-0645-2
- Takei, E., and Sakamoto, S. (1987) Geographical variation of heading response to daylength in foxtail millet (*Setaria italica* P. BEAUV.). *Japan J. Breed.* **37**, 150–158. DOI: 10.1270/jsbbs1951.37.150
- Takei, E., and Sakamoto, S. (1989) Further analysis of geographical variation of heading response to daylength in foxtail millet (*Setaria italica* P. Beauv.). *Japan J. Breed.* **39**, 285–298. DOI: 10.1270/jsbbs1951.39.285
- Yoshitsu, Y., Takakusagi, M., Abe, A., Takagi, H., Uemura, A., Yaegashi, H., Terauchi, R., Takahata, Y., Hatakeyama, K., and Yokoi, S. (2017) QTL-seq analysis identifies two genomic regions determining the heading date of foxtail millet, *Setaria italica* (L.) P. Beauv. *Breed. Sci.* **67**, 518–527. DOI: 10.1270/jsbbs.17061
- Zhang, G., Liu, X., Quan, Z., Cheng, S., Xu, X., Pan, S., Xie, M., Zeng, P., Yue, Z., Wang, W., et al. (2012) Genome sequence of foxtail millet (*Setaria italica*) provides insights into grass evolution and biofuel potential. *Nat. Biotechnol.* **30**, 549–554. DOI: 10.1038/nbt.2195