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Multiplex PCR effectively identifies tetraploid *Triticum* AABB – or AAGG-genome species

Ken-Ichi Tanno[1], Ayaka Takeuchi[2], Eri Akahori[2], Keiko Kobayashi[2], Taihachi Kawahara[3] and Kyoko Yamane[2]*

[1]*Graduate School of Sciences and Technology for Innovation, Yamaguchi University, Yoshida 1677-1, Yamaguchi City, Yamaguchi 753-8515, Japan,* [2]*Faculty of Applied Biological Sciences, Gifu University, 1-1, Yanagido, Gifu City, Gifu 501-1193, Japan and* [3]*Graduate School of Agriculture, Kyoto University, Nakajo, Mozume, Muko City, Kyoto 617-0001, Japan*

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Abstract

We developed a multiplex PCR DNA marker for quick and easy identification of the AAGG-genome timopheevii lineage, including *Triticum timopheevii*, *Triticum araraticum* and hexaploid *Triticum zhukovskyi* (AAA^{m}A^{m}GG), and the AABB-genome emmer wheat lineage, including *Triticum durum*, *Triticum dicoccum* and *Triticum dicoccoides*. Distinguishing between tetraploid AAGG- and AABB-genome wheat species based on morphology is known to be difficult. This multiplex PCR system is based on the simultaneous PCR amplification of two chloroplast regions, *matK* and *rbcL*. The *matK* region molecularly distinguishes the two lineages, whereas the *rbcL* region is a positive control amplicon. We also examined whether the simple sequence repeat is a fixed mutation within species, using genetic resources in the collection of KOMUGI, Kyoto University, which comprises accessioned species collected across diverse geographical areas. The multiplex PCR marker distinguished AAGG from AABB species with complete accuracy.

Keywords: emmer, genotyping, *matK*, *rbcL*, timopheevii, *Triticum*

Introduction

The genus *Triticum* is an allopolyploid complex that is extremely important in agriculture. *Triticum dicoccum* (emmer wheat), *Triticum dicoccoides* and *Triticum durum* are within the AABB-genome emmer lineage, while *Triticum timopheevii*, *Triticum araraticum* and hexaploid *Triticum zhukovskyi* (AAA^{m}A^{m}GG) are within the AAGG-genome timopheevii lineage; the AAGG genome was assigned to *T. timopheevii* due to chromosome pair mismatching with emmer wheat (AABB) (Lilienfeld and Kihara, 1934; Love, 1941; Sachs, 1953; Wagenaar, 1966). Distinguishing individuals with AAGG from those with AABB more simply has been achieved using the morphology of hairy leaves in the timopheevii lineage (AAGG). However, checking hairy leaves and chromosome pairing is often complicated and actually impracticable for partial or sterile specimens, such as seed remains from archaeological sites.

Boscato *et al.* (2008), in their quest to identify *T. timopheevii* from charred fossil remains, designed ribosomal internal transcribed spacer primers discriminating *T. timopheevii* and *T. dicoccum* but were unsuccessful in retrieving ancient DNA. We also applied this approach to modern timopheevii, but in vain. Golovnina *et al.* (2007) studied phylogenetic relationships in *Triticum*. In their alignment data (p. 206), we emphasized a single-nucleotide polymorphism (SNP) in the chloroplast *matK* region, which discerns timopheevii from emmer. Species-specific multiplex PCR is one of the most frequently used assay, as it is easy to perform, quick and cost-effective (Dubey *et al.*, 2009; Mendonca *et al.*, 2010). Against this background, in the present study, we established a multiplex PCR marker to distinguish timopheevii from emmer and confirmed its accuracy using retained genetic resources.

*Corresponding author. E-mail: kyamane@gifu-u.ac.jp

Table 1. Plant materials of *Triticum* and results of PCR bands presented in this study

Genome	Taxon (number of entry)	Accession no. (KU-)	Country	Band type: *matK* positive/negative	Band type: *rbcL* positive
AABB	*T. durum* Desf. (*n* = 36)	128-2, 1156, 1354, 3654, 3658, 3661, 3673, 3674, 3675, 3678, 3680, 3685, 3688, 3697, 3706, 3714, 3738, 7342, 7371, 9169, 9246, 9339, 9415, 9695, 9745, 10010, 10042, 10077, 10169, 10466, 10508, 11342, 11731, 11811, 11820, 11836	Afghanistan, China, Egypt, Ethiopia, Greece, Iran, Iraq, Italy, Jordan, Lebanon, Syria, Turkey	0/36	All
	T. dicoccum Schuebl. (*n* = 55)	117, 118, 119, 120, 122, 123, 462, 491, 492, 495, 1582, 3722, 3723, 4541, 7301, 7302, 7303, 7304, 7305, 7306, 7307, 7308, 7309, 7310, 7311, 7312, 9001, 9002, 9005, 9006, 9009, 9010, 9012, 9014, 9015, 9016, 9017, 9018, 9019, 9020, 9025, 9026, 9027, 9028, 9031, 9032, 9763, 9764, 9769, 9782, 9784, 9792, 9793, 10492, 10501	Ethiopia, India, Iran, Turkey, USSR	0/55	All
	T. dicoccoides Koern. (*n* = 50)	108-1, 108-3, 108-4, 109, 110, 195, 198, 1921, 1945, 1951, 1972|B, 1974, 1976B, 1991, 8536, 8537, 8538, 8539, 8541, 8736A, 8736B, 8737, 8804, 8805, 8806, 8808, 8809, 8811, 8812, 8815, 8816A, 8816B, 8817, 8821A, 8821C, 8915A, 8935, 8941, 8942, 13441, 14401, 14417, 14428, 14441, 14451, 14464, 14475, 14490, 14507, 14523	Iran, Iraq, Israel, Syria, Turkey	0/50	All
AAGG	*T. timopheevii* Zhuk. (*n* = 9)	107-1, 107-2, 107-3, 107-4, 107-5, 1818, 1819, 1820, 1821	Turkey, USSR	9/0	All
	T. araraticum Jakubz. (*n* = 47)	1903, 1913, 1914, 1925,1933, 1937, 1958, 1969, 1978|A, 1982, 1983, 8454, 8468, 8478, 8506, 8528A, 8561, 8567, 8593, 8625, 8675, 8683, 8701, 8707, 8711, 8714B, 8718B, 8723, 8727, 8739, 8774, 8779, 8795, 8799B, 8802, 8819, 8824A, 8827, 8863, 8880, 8885, 8889, 8890, 8914, 8926, 8940, 8947	Iran, Iraq, Turkey, USSR	47/0	All
AAAAGG	*T. zhukovskyi* Men. et Er. (*n* = 2)	1822A, 1822B	USSR	2/0	All

Species names in *Triticum* and *Aegilops* are often written as per van Slagen's criteria; this study, however, used the names based on the catalogue list of Kyoto University (Kawahara, 1998). Detailed passport data for each accession can be found at: http://www.shigen.lab.nig.ac.jp/wheat/komugi/top/top.jsp. Underlined and double underlined accession no. represent multiplex PCR result in Fig. 1. Double underlined accessions no. were sequenced for confirmation for SNPs, and their nucleotide sequences were shown in online Supplementary Fig. S3.

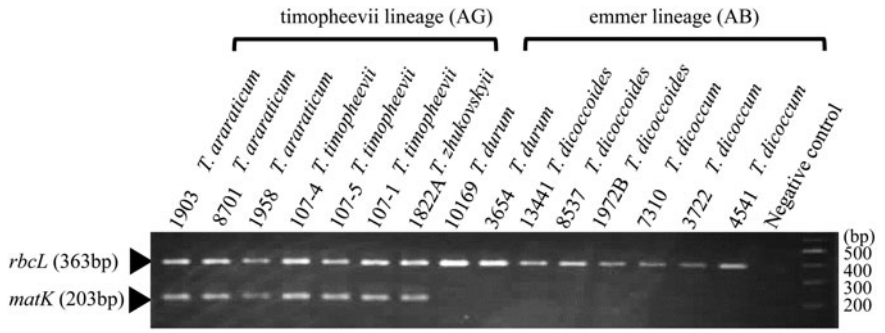

Fig. 1. Agarose gel electrophoresis of amplified multiplex PCR products. The bands below represent the *matK* amplicon, which differentiates AAGG (positive band) and AABB (negative) species. The upper bands represent *rbcL*, which is a positive control for confirming the validity of the experiment.

Experimental

Plant samples

Five tetraploid taxa, *T. durum*, *T. dicoccum*, *T. dicoccoides*, *T. timopheevii* and *T. araraticum*, and one hexaploid species, *T. zhukovskyi*, were examined. All 199 accessions used in this study were from the collection of KOMUGI, Kyoto University (see Table 1; Kawahara, 1997, 1998), and represented nearly the entire geographical ranges of each species. Extraction of genomic DNA using cetyltrimethylammonium bromide was conducted in accordance with Escaravage *et al.* (1998).

Design of PCR primers

Sequences including an SNP site that varies between *T. timopheevii* and *T. turgidum* in the chloroplast *matK* region, which was first presented by Golovnina *et al.* (2007), were aligned using ClustalW (Thompson *et al.*, 1994) (online Supplementary Fig. S1). Since Golovnina *et al.* did not search for polymorphisms within species, we examined whether this SNP is a fixed mutation within species. Initially, we designed the *matK* reverse primer using the *T. timopheevii*-specific site, which was located at the 3′ end of the primer (online Supplementary Fig. S1). The resulting PCR band was positive in both AAGG and AABB species. This means that a single mismatch primer would be inadequate for discriminating between these species. Therefore, a second artificial mismatch was introduced at the third base from the 3′ end of the reverse primer (matK_416R: 5′-GAAAGAATCGCAATAAAGGT-3′) in order to increase specificity (online Supplementary Fig. S1) (Bottema and Sommer, 1993). The expected band size (203 bp) of the *matK* product was found in AAGG-genome species as well as two accessions of hexaploid *T. zhukovskyi*. A subset of accessions double underlined in Table 1 were sequenced for confirmation using the matK_233F primer (5′-TTGTCCGAAAGAAAAAGAAA-3′) as a forward primer and the matK_1534R primer as a reverse primer at an outboard to the SNP (online Supplementary Fig. S1).

An additional primer set for the *rbcL* gene was developed as a positive control amplicon, which enables a check of whether the PCR reaction was successful. A primer set (RUBISCO_834F: 5′-AAATACTACTTTGGCTCATT-3′, RUBISCO_1197R: 5′-CACCAAATTGTAATACAGAA-3′) was designed at conserved positions based on the alignment sequences from GenBank [No. LN626622 (*T. timopheevii*), LN626619 (*T. aestivum*) and KM352501 (*T. turgidum*)] (online Supplementary Fig. S2). The results showed that an approximately 363-bp band of the amplified *rbcL* product was detected in all PCR reactions.

Multiplex PCR amplification

The multiplex PCR reaction using a mixture of *matK* and *rbcL* primers was carried out as follows: 30 cycles of 45 s at 95°C for denaturation, 30 s at 47.5°C for annealing and 15 s at 68°C for polymerization (*Taq* DNA polymerase; New England Biolabs, Frankfurt am Main, Germany), with a final extension of 5 min at 68°C. The *matK* and *rbcL* primers were mixed at a ratio of 6:7. PCR products were observed using a 2% agarose gel, ethidium bromide and UV illumination. It was confirmed at least twice for each accession. As shown in Fig. 1, all of the AABB and AAGG species investigated could be distinguished (Table 1).

Discussion

Although there is still a risk of miss-identifying the species when we use only one SNP as a marker, the multiplex PCR marker presented here distinguished between AAGG from AABB species with complete accuracy. Multiplex PCR is rapid and affordable, allowing simultaneous detection of multiple loci, and thus, has been applied in various species, such as grapevine (Merdinoglu *et al.*, 2005; Migliaro *et al.*,

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2013), Chinese egret (Huang *et al.*, 2012) and for the typing of high molecular weight alleles in wheat (Ma *et al.*, 2003).

Our multiplex PCR marker should also be useful for the identification of ancient remains as well as modern genetic resources. The so-called 'new-glume wheat', featuring charred spikelets with a characteristic morphology as found at archaeological sites in Europe (Jones *et al.*, 2000; Kohler-Schneider, 2003; Toulemonde *et al.*, 2015), could be timopheevii, but its identity has remained unclear, mainly because its morphology is not exactly the same as that of modern cultivated timopheevii and its wild progenitor, *T. araraticum*. The process of domestication of timopheevii is mysterious due to the strong resemblance between charred seed remains and those of emmer.

Wild *T. araraticum* is distributed across large parts of West Asia, especially Iraq, east Turkey and Armenia (Zohary and Hopf, 2000), but the genetic resources of this species have not been well collected, due to the inaccessibility of these areas to researchers. Recently, the situation in some areas has improved, and thus, archaeological and genetic resource investigations have been initiated or restarted (Gasparyan and Arimura, 2014; Tanno *et al.*, 2015, personal communication with M. Arimura). The multiplex PCR technique presented here is a novel approach for the rapid and easy identification of two important tetraploid wheat species.

Supplementary material

The supplementary material for this article can be found at https://doi.org/10.1017/S1479262117000181.

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Author Contribution Statement

K.-I.T. planned and coordinated the study, and wrote the paper. K.Y. planned and performed the experiment and analyses, and wrote the paper. A.T. and E.A. performed the experiment. K.K. analyzed the data. T.K. was responsible for plant materials.

References

Boscato P, Carioni C, Brandolini A, Sadori L and Rottoli M (2008) Molecular markers for the discrimination of *Triticum turgidum* L. subsp. *dicoccum* (Schrank ex Schübl.) Thell. and *Triticum timopheevii* (Zhuk.) Zhuk. subsp. *timopheevii*. *Journal of Archaeological Science* 35: 239–246.

Bottema CD and Sommer SS (1993) PCR amplification of specific alleles: rapid detection of known mutations and polymorphisms. *Mutation Research* 288: 93–102.

Dubey B, Meganathan PR and Haque I (2009) Multiplex PCR assay for rapid identification of three endangered snake species of India. *Conservation Genetics* 10: 1861–1864.

Escaravage N, Questiau A, Pornon D, Boche B and Taberlet P (1998) Clonal diversity in a *Rhododendron ferrugineum* L. (Ericaceae) population inferred from AFLP markers. *Molecular Ecology* 7: 975–982.

Gasparyan B and Arimura M (2014) Studies of the Stone Age in the Republic of Armenia: achievements and perspectives. In: Gasparyan B and Arimura M (ed.) *Stone Age of Armenia: A Guide-Book to the Stone Age Archaeology in the Republic of Armenia*. Japan: Kanazawa University Press, pp. 13–33.

Golovnina KA, Glushkov SA, Blinov AG, Mayorov VI, Adkison LR and Goncharov NP (2007) Molecular phylogeny of the genus *Triticum* L. *Plant Systematics and Evolution* 264: 195–216.

Huang XH, Zhou XP, Lin QX, Peng ZN, Fang WZ and Chen XL (2012) A novel multiplex PCR assay for species identification in the Chinese Egret (*Egretta eulophotes*) and Little Egret (*E. garzetta*). *Conservation Genetics Resources* 4: 31–33.

Jones G, Valamoti S and Charles M (2000) Early crop diversity: a "new" glume wheat from northern Greece. *Vegetation History and Archaeobotony* 9: 133–146.

Kawahara T (1997) *Catalogue of Aegilops-Triticum Germ-Plasm Preserved in Kyoto University 2*. Kyoto, Japan: Laboratory of Crop Evolution, Kyoto University.

Kawahara T (1998) *Catalogue of Aegilops-Triticum Germ-Plasm Preserved in Kyoto University 2*, supplement. Kyoto, Japan: Laboratory of Crop Evolution, Kyoto University.

Kohler-Schneider M (2003) Contents of a storage pit from late Bronze Age Stillfried, Austria: another record of the "new" glume wheat. *Vegetation History and Archaeobotany* 12: 105–111.

Lilienfeld FA and Kihara H (1934) Genome analyse bei *Triticum* und *Aegilops*. V. *Triticum timopheevii* Zhuk. *Cytologia* 6: 87–122.

Love RM (1941) Chromosome behavior in F_1 wheat hybrids. I. Pentaploids. *Canadian Journal of Research* 19: 351–369.

Ma W, Zhang W and Gale KR (2003) Multiplex-PCR typing of high molecular weight glutenin alleles in wheat. *Euphytica* 134: 51–60.

Mendonca FF, Hashimoto DT, De-Franco B, Porto-Foresti F, Gadig OBF, Oliveira C and Foresti F (2010) Genetic identification of lamniform and carcharhiniform sharks using multiplex-PCR. *Conservation Genetics Resources* 2: 31–35.

Merdinoglu D, Butterlin G, Bevilacqua L, Chiquet V, Adam-Blondon AF and Decroocq S (2005) Development and characterization of a large set of microsatellite markers in grapevine (*Vitis vinifera* L.) suitable for multiplex PCR. *Molecular Breeding* 15: 349–366.

Migliaro D, Morreale G, Gardiman M, Landolfo S and Crespan M (2013) Direct multiplex PCR for grapevine genotyping and varietal identification. *Plant Genetic Resources* 11: 182–185.

Sachs L (1953) Chromosome behaviour in species hybrids with *Triticum timopheevi*. *Heredity* 7: 49–58.

Tanno K-I, Kawahara T and Takata K (2015) Archaeobotanical studies at and around Qalat Said Ahmadan (Appendix 3: excavation of Qalat Said Ahmadan, Slemani, Iraq-Kurdistan). *Al-Rafidan* 36: 59–63.

Thompson JD, Higgins DG and Gibson TJ (1994) ClustalW—improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22: 4673–4680.

Toulemonde F, Durand F, Berrio L, Bonnaire E, Daoulas G and Wiethold J (2015) Records of "new" glume wheat in France: a review. *Vegetation History Archaeobotany* 24: 197–206.

Wagenaar EB (1966) Studies on the genome constitution of *Triticum timopheevi* ZHUK. II. TheT. *timopheevi* complex and its origin. *Evolution* 20: 150–164.

Zohary D and Hopf M (2000) *Domestication of Plants in the Old World*. NY: Oxford Press, p. 45.

https://doi.org/10.1017/S1479262117000181 Published online by Cambridge University Press