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SSR-based molecular profiling of 237 persimmon (*Diospyros kaki* Thunb.) germplasms using an *ASTRINGENCY*-linked marker

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Abstract

Pollination-constant non-astringent (PCNA) trait is desirable in persimmon production because it confers natural astringency loss in mature persimmon fruit. Expression of the PCNA trait requires six homozygous recessive PCNA (*ast*) alleles at the single *ASTRINGENCY* (*AST*) locus in hexaploid persimmon. When crossing non-PCNA accessions to breed PCNA offspring, knowledge of *ast* and non-PCNA (*AST*) allele dosage in the parental accessions is important, because more PCNA offspring can segregate from a non-PCNA parent with more *ast* and fewer *AST* alleles. Previously, we have demonstrated that a region linked to the *AST* locus has numerous fragment size polymorphisms with varying numbers of simple sequence repeats. Here, we reveal the polymorphisms in this region in a broad collection of persimmon germplasms. Among 237 accessions, we distinguished 21 *AST*- and 5 *ast*-linked fragments with different sizes. Based on the number of fragments detected per individual, we identified 21 non-PCNA accessions with three different *ast* alleles; by crossing these with a PCNA parent, we obtain PCNA offspring under autohexaploid inheritance. Furthermore, *AST* and *ast* allelic combination patterns in hexaploid persimmon were shown to be applicable to cultivar identification of non-PCNA accessions. We directly sequenced *ast*-linked fragments from 48 accessions with one-size peak of *ast*-linked fragment, and found two distinctive groups of fragments based on single nucleotide polymorphisms. This result suggests that a bottleneck event occurred during *ast* allele development. We conclude that our fragment size profile can be used to accelerate PCNA breeding that uses non-PCNA parents and to study *ast* allele accumulation in persimmon.

Keywords

Astringency, Breeding, Fruit tree, Genetic resources, PCNA, Polyploidy

Introduction

The genus *Diospyros* L. consists of 400 species, among which oriental persimmon (*Diospyros kaki* Thunb.) is the most economically important. The origin of oriental persimmon is believed to be China, and it is a popular fruit particularly in countries of East Asia such as China, Korea, and Japan (Yonemori et al. 2000). Recently, persimmon has become popular in many other countries. According to FAO (Food and Agriculture Organization of the United Nations) statistics, worldwide persimmon production in 2014 was 5,200,000 tons in total, with 71.9% from China, 8.3% from Korea, 4.7% from Spain, 4.6% from Japan, and 3.5% from Brazil. Because many persimmon cultivars have strong astringency owing to soluble tannin in the flesh, deastringency treatment such as postharvest treatment with carbon dioxide is frequently used to make the flesh palatable. Depending on the effect of seed formation on the natural astringency loss in flesh at harvest time, persimmon is classified into four types: (1) pollination-constant non-astringent (PCNA), (2) pollination-variant non-astringent (PVNA), (3) pollination-variant astringent (PVA), (4) pollination-constant astringent (PCA) (Kajiura 1946). PCNA fruit naturally lose astringency at maturity and become edible without deastringency treatment. However, PVNA fruit sometimes retain astringency in the flesh, because loss of astringency in the whole flesh is dependent on seed formation. PVA fruit lose astringency only in a small portion of flesh around the seeds. PCA fruit always retain astringency in the whole flesh irrespective of the number of seeds. In contrast to the non-PCNA (i.e., PVNA, PVA and PCA) types, which can retain astringency at harvest time, PCNA type accessions lack the ability to accumulate large amounts of soluble tannins during fruit development (Yonemori et al. 2000); this natural astringency loss is highly desirable for commercial production. Therefore, breeding superior PCNA cultivars with high eating quality and productivity is a major goal of our

breeding program.

The PCNA trait is recessive to the non-PCNA trait and is qualitatively inherited (Ikeda et al. 1985). Expression of the PCNA trait is under the control of a single locus, *ASTRINGENCY (AST)*, and requires the presence of the recessive *ast* allele at all copies of the *AST* locus on each of the six corresponding chromosomes (Akagi et al. 2009; Akagi et al. 2010). *D. kaki* cultivars are hexaploid (2n = 6x = 90), with the exception of a few nonaploid (2n = 9x = 135) cultivars, such as 'Hiratanenashi' and 'Miyazakitanenashi' (Tamura et al. 1998, Zhuang et al. 1990). *AST* and *ast* alleles generally show autohexaploid inheritance, but segregation of these alleles is sometimes slightly distorted from the ratio expected from the autohexaploid inheritance model (Akagi et al. 2012; Kanzaki et al. 2008; Kono et al. 2016; Mitani et al. 2014b). Although it had been previously believed that PCNA trait originated only in Japan, Wang (1982) reported 'Luotian-tianshi', the PCNA cultivar of Chinese origin. Subsequently, other PCNA cultivars, including 'Tianbaogai' (former name, 'Baogai Tian Shi'), were found in the area where 'Luotian-tianshi' was found (Yonemori et al. 2005). The PCNA trait of Chinese origin (C-PCNA) is genetically dominant to the non-PCNA traits, and the locus responsible for the C-PCNA trait is different from the *AST* locus (Ikegami et al. 2004, 2006).

Owing to the recessive inheritance of the PCNA trait in the hexaploid persimmon, breeders need to mainly cross PCNA accessions to each other to efficiently acquire PCNA offspring. So far, except for bud sports, only 18 local PCNA cultivars that have been found in the central part of Japan are preserved at the Grape and Persimmon Research Station, NIFTS (Yamada et al. 2012), while more than 1000, 900, and 180 cultivars mostly consisting of non-PCNA types have been reported in Japan, China, and Korea, respectively (Agricultural Research Station 1912; Cho and Cho 1965;

Wang et al. 1997). In the persimmon breeding program at the National Agriculture and Food Research Organization (NARO) in Japan, repeated crosses within a small PCNA gene pool resulted in inbreeding depression, represented by reduced fruit weigh, vigor, and productivity (Yamada 1993; Yamada et al. 1994). To avoid inbreeding, we have incorporated non-PCNA cultivars, whose genetic background differs from that of PCNA cultivars (Guo and Luo 2011; Kanzaki et al. 2000; Naval et al. 2010; Parfitt et al. 2015), into the breeding program.

Our strategy is based on a pseudo-backcross (Bouquet 1986; Ruengphayak et al. 2015), in which F_1 non-PCNA offspring from a cross between non-PCNA (Aaaaaa, AAaaaa, AAAaaa, AAAAaa, AAAAAa, or AAAAAA) and PCNA (aaaaaa) cultivars are backcrossed to a PCNA cultivar. However, this strategy still yields only a limited proportion of PCNA offspring in the BC_1 generation: the expected proportion of PCNA offspring from a non-PCNA F_1 parent with Aaaaaa, AAaaaa, or AAAaaa is 50%, 20%, or 5%, respectively, under an autohexaploid model with non-chromatid segregation (Allard 1960). To avoid unnecessary cultivation of non-PCNA offspring in the selection field, we apply DNA marker-assisted selection (MAS) for the PCNA trait. Kanzaki et al. (2010) identified the sequence of an *AST*- and *ast*-linked region by screening a genomic library with a 5R probe that showed restriction fragment length polymorphisms (RFLPs) between PCNA and non-PCNA cultivars. Finally, they produced sequence-characterized amplified region (SCAR) markers for this region (termed the '5R adjacent region'). The reliability of a SCAR marker that amplifies the 5R adjacent region has been demonstrated in practical MAS for offspring of 'Taiten' × 'Kanshu'. Discrepancy between phenotypic evaluation by a sensory test and estimated genotype was found in only 3 out of 251 offspring (Mitani et al. 2014a), confirming tight linkage of the region to the *AST* locus. Therefore, we call this region the *AST* locus–linked

region. Multiplex PCR using a set of three primers (AST-F, PCNA-F, and 5R3R) that hybridize to the 5R adjacent region simultaneously amplifies both *AST* and *ast* allele–linked DNA (Kanzaki et al. 2010). We have tested more than 5,000 individuals by multiplex PCR (Sato and Yamada 2016), and have found this to be a labor-saving and reliable system for MAS. In the pseudo-backcross strategy with MAS, identifying the copy number of *AST* and *ast* alleles in non-PCNA parents is particularly important because this critically determines the segregation rate of PCNA offspring. We can obtain PCNA offspring even at the F_1 generation when a non-PCNA parent with a genotype of AAAaaa, AAaaaa, or Aaaaaa is used. In contrast, pseudo-backcross is necessary to obtain PCNA offspring when a non-PCNA parent with the genotype of AAAAaa, AAAAAa, and AAAAAA is used. Therefore, determination of the copy number of *AST* and *ast* alleles among a wide range of non-PCNA germplasms would accelerate efficient PCNA breeding.

Akagi et al. (2010) reported a method to directly estimate the copy number of *AST* and *ast* alleles based on quantitative real-time polymerase chain reaction (qPCR) of the *ast* allele-linked region. They estimated quantitative genotypes at the *AST* locus in 63 non-PCNA cultivars/selections, among which 15 cultivars were either AAAaaa, AAaaaa, or Aaaaaa. Detection of one copy difference of *ast* allele by qPCR, especially for accessions with higher copy number of *ast* allele, would require high levels of technical skill. This methodological limitation of qPCR resulted in significant calculation errors in measurements for some accessions. Furthermore, the method elaborately uses three genomic regions to standardize quantity of amplified products linked to *ast* allele. However, inconsistency of the estimated allele dosage among the references was observed for 32% (20/63) of the accessions. This implies that copy number in the genome and/or sequence of the references can be slightly different among

the accessions used. Therefore, estimating exact copy number for any persimmon accessions would be technically difficult even by this ingenious method. As another option to estimate allele dosage at the *AST* locus, we have previously presented a fragment analysis that detects high polymorphisms in fragment size at the *AST* locus–linked region (Kono et al. 2016), technically less labor-intensive method than qPCR. We found a total of 12 *AST* allele–linked fragments of various sizes caused mainly by a simple sequence repeat (SSR) in 14 non-PCNA cultivars. The detected number of *AST* fragments indicated the minimum *AST* allele copy number, because we could not determine which allele(s) occurred in more than one copy when the number of detected alleles was fewer than six in hexaploid persimmon. Segregation data of the *AST* allele and/or progeny genotypes would be required to identify the precise copy number of *AST* alleles in non-PCNA individuals. However, we considered that the high-throughput fragment analysis using a capillary sequencer could be informative enough to find non-PCNA parents with fewer *AST* and more *ast* alleles from a wide range of non-PCNA germplasms, because an accession with a highly polymorphic *AST* locus–linked region would display more alleles.

In addition to allele dosage estimation, the fragment size analysis described above could be applicable to cultivar identification because of high polymorphism at the *AST* locus–linked region (Kono et al. 2016). Various types of DNA markers have been applied to persimmon cultivar identification: e.g., random amplified polymorphic DNA (RAPD; Badenes et al. 2003; Luo et al. 1995; Yamagishi et al. 2005), RFLP (Maki et al. 2001), retrotransposon-based (Du et al. 2009), and SSR (Naval et al. 2010) markers. A marker technology that reveals a high amount of polymorphism with minimum cost is optimal for practical cultivar identification. Fragment size analysis using multiplex PCR (Kanzaki et al. 2010) could potentially be such as system because it can detect various

alleles simply by single multiplex PCR and fragment size analysis (Kono et al. 2016).

The objectives of the present study were (1) to determine the sizes of *AST*- and *ast*-allele–linked fragments (hereafter termed *AST* and *ast* fragments) at the *AST* locus–linked region in 237 persimmon accessions of widely varying genetic backgrounds; and (2) to test whether *AST* and *ast* fragment size analysis can be applied successfully for cultivar identification. During the fragment analysis, we identified non-PCNA accessions with a single *ast* fragment peak, which enabled us to identify the sequence of each *ast* fragment. Based on the fragment sizes and sequence polymorphisms, we discuss the possible origin of *ast* alleles.

Materials and Methods

Plant materials

Supplemental Table S1 shows the 237 *D. kaki* Thunb. accessions used in this study: these comprise 148 non-PCNA, 3 C-PCNA, and 86 PCNA accessions. The sample population comprised 202 local cultivars, 18 crossbred cultivars, and 17 selections. Bud mutants and synonyms were each counted as a separate accession. The origins of the accessions were Brazil (1), China (19), Israel (2), Italy (12), Japan (193), Japan–China (1), Korea (4), New Zealand (2), Spain (1), and Turkey (2). We treated the selection 310-24 (C-PCNA) as a Japan–China hybrid, because its origin is both Japan (‘Taishuu’; PCNA) and China (‘Luotian-tianshi’; C-PCNA). All plant materials were obtained from the Grape and Persimmon Research Station, NIFTS, Hiroshima, Japan.

DNA extraction

Approximately 1.0-cm^2 leaves or one to five dormant buds, whose outer scales were peeled by one or two layers (Kono et al. in press), were frozen in liquid N_2 and stored at −20°C until use. Samples were homogenized under liquid N_2, and 500 µl of pre-wash buffer (0.1 M HEPES-NaOH (pH 8.0), 0.1% (w/v) soluble polyvinylpyrrolidone, and 10 mM dithiothreitol) was added and mixed thoroughly by vortexing; dithiothreitol was added to the buffer just before use. After centrifugation at 20,000 × *g* at 4°C for 5 min, the supernatants were discarded. Pellets were resuspended in 1 ml of pre-wash buffer and centrifuged at 20,000 × *g* at 4°C for 3 min; the process was repeated three times in toal. The final pellets were subjected to DNA extraction with a Nucleon Phytopure Plant extraction kit (GE Healthcare UK Ltd., Little Chalfont, UK). DNA concentration was measured by using a Qubit 3.0 fluorometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Fragment size analysis

Multiplex PCR of the *AST* locus–linked region was performed with two fluorescent-labeled forward primers HEX-AST-F (5′-GTTGCATCGCATAGCGGGTTTGAGG-3′), FAM-PCNA-F (5′-CCCCTCAGTGGCAGTGCTGC-3′), and unlabeled reverse primer 5R3R (5′-GAAACACTCATCCGGAGACTTC-3′) (Kanzaki et al. 2010). The 10-μL PCR reaction mixture contained 5 μL of 2× Gotaq Master Mix (Promega, Madison, WI, USA), 0.2 μM of the two forward primers and the reverse primer, and 10–20 ng of genomic DNA. The PCR cycling conditions were as follows: 95°C for 3 min for initial denaturation, followed by 27 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and polymerization at 72°C for 1 min, and final extension at 72°C for 10 min. We increased the number of PCR cycles to 31 when amplification was insufficient. Amplified PCR products were separated by using an ABI 3130xl Genetic Analyzer (Thermo Fisher Scientific Inc.) . The size of each amplified fragment was calculated based on the GeneScan 500 ROX Dye Size Standard (Thermo Fisher Scientific Inc.) by using the GeneMapper ver. 5.0 software (Thermo Fisher Scientific Inc.).

For additional SSR markers, we first screened 19 markers (Naval et al. 2010) developed by Soriano et al. (2006) and selected six (ssrdk10, 14, 16, 17, 29, and 30) that showed clear peaks with few stutter bands. PCR was performed by using a specific forward primer for each marker with an M13(-21) tail at the 5′-end, the universal FAM-labeled M13(-21) primer (Schuelke 2000), and a specific reverse primer with a pigtail (5′-GTTTCTT-3′) added to its 5′-end to reduce non-adenylated products (Brownstein et al. 1996). The 10-μL PCR reaction mixture contained 5 μL of 2× Gotaq Master Mix (Promega), 0.05 μM of the specific forward primer, 0.2 μM of the specific

reverse primer and the universal primer, and 5–10 ng of genomic DNA. PCR conditions were as follows: 95°C for 2 min for initial denaturation, followed by 26 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 45 s, and polymerization at 72°C for 45 s, and final extension at 72°C for 10 min. We increased the number of PCR cycles to 31 when amplification was insufficient. Separation of PCR products and size analysis were performed as described for the multiplex PCR for the *AST* locus–linked region. PCR amplification and fragment analysis of *AST* locus–linked and ssrdk markers were carried out at least twice to ensure the reproducibility of the produced bands.

Direct sequencing

Genomic DNA of 48 non-PCNA accessions (45 genotypes) that showed only one-size peak of *ast* fragment, and two PCNA accessions of 'Gosho' ($a^{347}a^{349}a^{355}$) and 'Gosho-Gose' ($a^{349}a^{355}$) were used. To avoid the production of chimeric fragments from similar template sequences during PCR, which was observed in our previous study on grape (Kobayashi et al. 2001), we did not clone *ast* fragments from accessions with variably-sized *ast* fragments. PCR to generate *ast* fragments was performed using forward primer PCNA-F and reverse primer 5R3R (Kanzaki et al. 2010). The 30-μL PCR reaction mixture contained 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM each primer, 1× ExTaq buffer, 0.25 U ExTaq polymerase (Takara, Kyoto, Japan), and 10–20 ng DNA. The PCR cycling conditions were the same as described for multiplex PCR of the *AST*-linked region in the *Fragment size analysis* section. Amplified *ast* fragments were precipitated with ethanol and dissolved in 10 μL of TE. Aliquots (1 μL) of purified PCR products were sequenced by using a BigDye terminator v3.1 cycle sequencing kit (Thermo Fisher Scientific Inc.) with either PCNA-F or 5R3R primer. For the direct

sequence analysis of the *ast* fragments of 'Gosho' and 'Gosho-Gose', we used a^{349} (5′-TAAAGCAATGAACCTTTTGG-3′)- and a^{353} (5′'-TAAAGCAATGAACCTTTTGT-3′)-specific forward primer.

For the direct sequence analysis of *ast* fragments of 'Aizumishirazu', 'Ichidagaki', and 'Zenjimaru', which have both a^{349} and a^{353} alleles, we performed PCR using either a^{349}-specific forward primer, as described above, and reverse primer 5R3R, or a^{353}-specific forward primer, as described above, and reverse primer 5R3R. Amplified fragments were purified and sequenced using a^{349}-specific forward primer, a^{353}-specific forward primer, or the 5R3R reverse primer. Nucleotide sequences were analyzed by using GENETYX v. 9 software (GENETYX Corp., Tokyo, Japan).

Data analysis

Polymorphism information content (PIC) value is widely used to measure the information content of molecular markers based on the number and frequency distribution of alleles. The PIC value for each marker having *l* alleles was calculated as:

$$\mathrm{PIC} = 1 - \left(\sum_{i=1}^{l} Pi^2\right) - \sum_{i=1}^{l-1}\sum_{j=i+1}^{l} 2P_i^2 P_j^2,$$

where P_i and P_j are the population frequency of the *i*th and *j*th allele (Botstein et al. 1980). P_i or P_j for the *AST* marker was calculated as the occurrence of the given allele divided by the sum of occurrences of *AST* and *ast* alleles in 184 genotypes.

Results

The *AST* locus–linked region of 237 persimmon accessions (Supplemental Table S1) was analyzed by using the multiplex PCR system. Since our samples may include unknown bud mutants and/or synonyms, we additionally analyzed these accessions with six SSR markers (ssrdk10, 14, 16, 17, 29, and 30; Naval et al. 2010) to uncover unique genotypes and thus avoid complexity of the sample population. Multiplex PCR for the *AST* locus–linked region and uniplex PCR for each of the six ssrdk markers yielded a total of 101 different alleles; the total allele number per individual accession was 19–32 (Table 1). The combination of the *AST* locus–linked marker and ssrdk markers identified 184 different genotypes (127 non-PCNA, 3 C-PCNA, and 54 PCNA) (Table 1). Accessions sharing identical genotypes at the set of 7 markers were categorized into 21 groups (Table 2). These groups consisted of known bud-sports having the same origin and/or morphologically similar cultivars with different names, most likely synonyms. Of the 12 accessions introduced from Italy, only ‘Shogatsu-Italy’ and ‘Cioccolatino’ exhibited unique genotypes, while the others were categorized into either group 3, 4, 8, 12, 16, or 19 (Table 2). The Korean cultivar ‘Chung Nam’ showed the same genotype as the Japanese cultivar ‘Okayamaokugosho’ (Group 6). ‘Flat Fuyu’ and ‘Fuyu-New Zealand’, which were introduced from New Zealand, showed the same genotype as the Japanese cultivars ‘Okugosho’ (Group 15) and ‘Fuyu’ (Group 14), respectively (Table 2). The other non-Japanese accessions, Brazil (1), China (19), Israel (2), Japan–China (1), Korea (3), Spain (1), and Turkey (2), showed unique genotypes among the 237 accessions.

Among the 184 unique genotypes, 21 *AST* fragments (size, 220–254 bp) and 5 *ast* fragments (size, 347–355 bp) were detected by the multiplex PCR system (Tables 3, 4). Given the tight link between the 5R adjacent region and *AST* locus in previous

300 studies (Mitani et al. 2014a; Kanzaki et al. 2010), we regarded polymorphisms of the amplified fragments as polymorphisms of *AST* and *ast* alleles at the *AST* locus. We detected 12 rare *AST* (A^{220}, A^{230}, A^{232}, A^{234}, A^{236}, A^{238}, A^{242}, A^{244}, A^{248}, A^{250}, A^{252}, and A^{254}) alleles and one rare *ast* (a^{347}) allele (frequency, <0.02; Naval et al. 2010). At least one rare allele were found in 14 out of 19 Chinese (IDs: 17, 72, 74, 75, 77, 78, 88, 89,
305 113, 114, 115, 122, 124, 129), 3 out of 4 Korean (IDs: 29, 107, 190), and 1 out of 1 Brazilian accessions (ID: 63) (Table 3, Supplemental Table S1), suggesting uniqueness of Chinese, Korean, and Brazilian accessions among the 237 accessions. Cultivars from Israel, Italy, New Zealand, Spain, and Turkey did not have any rare alleles (Tables 3, 4).

The total number of different *AST* and *ast* alleles per individual genotype was
310 2–7 with an average of 4.4 (Table 1). Our samples included a few nonaploid cultivars: e.g., ‘Miyazakitanenashi’ and ‘Hiratanenashi’ (Tamura et al. 1998, Zhuang et al. 1990). A total of seven different *AST* and *ast* alleles were detected in ‘Miyazakitanenashi’ ($A^{224}A^{231}A^{233}A^{240}a^{349}a^{353}a^{355}$) only; and a total of six different *AST* and *ast* alleles were observed for ‘Hiratanenashi’ ($A^{224}A^{228}A^{231}A^{250}a^{349}a^{353}$), its four known bud sports, and
315 ‘Hiratanenashi-Italy’, and ‘Tone Hiratanenashi’ (group 8 in Table 2; Table 3). Among the 182 genotypes with hexaploidity, 31 non-PCNA genotypes showed a total of six *AST* and *ast* fragments, suggesting the presence of one copy of each allele in these genotypes (Tables 3). The C-PCNA locus differs from the *AST* locus (Ikegami et al. 2004), and three C-PCNA accessions, 310-24 (Kono et al. 2016), ‘Tianbaogai’, and
320 ‘Luotian-tianshi’ (Akagi et al. 2010) had *AST* alleles (ID: 19, 40, and 72 in Table 3). In this study, we focused on the genotype at the *AST* locus–linked region, and thus we treated the three C-PCNA accessions the same as non-PCNA type. Among the 127 genotypes of hexaploid and nonaploid non-PCNA accessions and 3 genotypes of C-PCNA accessions, the number of *AST* alleles was 1–6 with an average of 3.4 (Table

3). We identified 40, 13, and 6 genotypes having 4, 5, and 6 different *AST* alleles per individual, respectively. *ast* alleles were detected not only in PCNA genotypes, but also in a wide range of non-PCNA genotypes. Of the 127 non-PCNA and 3 C-PCNA genotypes, 109 had at least one *ast* allele (Table 3), which is consistent with the prevalence of *ast* alleles in the non-PCNA population studied in Akagi et al (2010). We identified 19 genotypes composed of 21 accessions (IDs: 2–4, 19–28, 67–71, 111, 190, 203) with 3 different *ast* alleles per individual. Because *ast* alleles were less polymorphic than *AST* alleles, the detected number of *ast* alleles per individual was less than that for the *AST* allele: the number of *ast* alleles in non-PCNA accessions was 0–3 with an average of 1.5 (Table 3).

The *AST* locus–linked marker in this study showed a PIC value of 0.90 (Table 1), which is considered highly informative according to the criteria of Botstein et al. (1980) (highly informative, PIC > 0.5; moderately informative, 0.25 <PIC < 0.5; and relatively uninformative, PIC < 0.25), suggesting that multiplex PCR of the *AST* locus–linked region could be used for cultivar identification. Of the 127 non-PCNA and 3 C-PCNA genotypes, 113 showed a unique *AST* and *ast* allele pattern, whereas only 5 out of 54 PCNA genotypes showed a unique *ast* allele pattern (Table 1). Table 1 shows the discrimination ability of the *AST* locus–linked marker compared with six ssrdk markers developed by Soriano et al. (2006). A total of 75 alleles was detected by the six ssrdk markers. Single ssrdk markers had 9 to 20 alleles, including 2 to 11 rare alleles. Observed allele number per individual for each ssrdk marker was 1–5, 1–6, or 2–6 with an average of 2.5 to 4.0. PIC values ranged from 0.70 to 0.86. As a result, a unique genotype based on a single ssrdk marker was found for only 11 to 62 out of 127 non-PCNA and 3 C-PCNA genotypes, and 0 to 14 out of 54 PCNA genotypes (Table 1). The set of six ssrdk markers discriminated all genotypes except for two non-PCNA and

four PCNA genotypes that could be discriminated by the *AST* locus–linked marker (Table 1): 'Tamopan' ($A^{224}A^{226}A^{229}A^{236}a^{349}$) had A^{236}, whereas 'Mopanshi' ($A^{224}A^{226}A^{229}A^{238}a^{349}$) had A^{238} (Table 3); 'Misatogosho' ($a^{347}a^{349}a^{355}$) did not have a^{353}, which was present in 'Fukurogosho', 'Hazegosho', and 'Zennosuke' ($a^{347}a^{349}a^{353}a^{355}$) in group 17; 'Gosho' ($a^{347}a^{349}a^{355}$) had a^{347}, but this was absent in 'Gosho-Fukushima', 'Gosho-Gose', 'Izushikogosho', and 'Kaibaragosho' ($a^{349}a^{355}$) in group 18 (Table 4).

Analysis of *ast* fragments identified 45 non-PCNA genotypes with only one-size peak of *ast* fragment (22, 2, 19, and 2 genotypes for a^{349}, a^{351}, a^{353}, and a^{355}, respectively) (Tables 3, 5). To gain insight into the *ast* allele, we directly sequenced the amplified *ast* fragments of the 48 accessions with these genotypes. We found that differences in the sizes of the *ast* fragments were caused by different numbers of AT-dinucleotide SSRs beginning at the 259th nucleotide of the fragment (Fig. 1), which corresponds to the region that was previously reported to cause allele size polymorphisms at *AST* fragments (Kono et al. 2016). The sequences of all a^{349} fragments from 19 Japanese and 5 Chinese accessions were identical (Table 5). Interestingly, the a^{353} fragment contained several single nucleotide polymorphisms (SNPs), resulting in four types of a^{353} ($a^{353\text{-}1}$, $a^{353\text{-}2a}$, $a^{353\text{-}2b}$, and $a^{353\text{-}2c}$; Fig. 1). The allele $a^{353\text{-}1}$ was found in six Japanese, one Turkish, one Israeli, and one Chinese accession (Table 5). The alleles $a^{353\text{-}2a}$, $a^{353\text{-}2b}$, and $a^{353\text{-}2c}$ were found in eight, four, and one accession, respectively, all of which were Japanese (Table 5). Sequences of the a^{351} fragment from two Chinese accessions, 'Raotianhong' ($A^{222}A^{226}A^{229}a^{351}$) and 'Gong-cheng-shui-shi' ($A^{228}A^{230}A^{231}A^{242}A^{250}a^{351}$), were identical, and sequences of the a^{355} fragment from two Japanese accessions, 'Hegurogaki' ($A^{225}A^{228}A^{231}A^{240}a^{355}$) and 'Yukineri' ($A^{226}A^{228}A^{231}A^{233}a^{355}$), were identical (Table 5). The sequence difference between $a^{353\text{-}2a}$ and a^{351}, and between $a^{353\text{-}2b}$ and a^{355} fragments, was one AT repeat of the

SSR (Figs. 1, 2). Fragments a^{351}, $a^{353\text{-}1}$, $a^{353\text{-}2a}$, $a^{353\text{-}2b}$, $a^{353\text{-}2c}$, and a^{355} (a^{353} group) were more similar to each other than to a^{349}, which differs from the a^{353} group at >10 SNPs (Fig. 2). Consequently, the above seven *ast* alleles were categorized into two groups based on their sequence similarity to a^{349} (Fig. 2).

Sequences of the a^{353} fragments of 'Amahachiya' ($A^{224}A^{226}A^{228}a^{353}$), 'Omidanshi' ($A^{224}A^{225}A^{228}A^{231}a^{353}$), and 'Shimofuri' ($A^{224}A^{225}A^{228}A^{231}a^{353}$) were consistent with $a^{353\text{-}2a}$ and $a^{353\text{-}2b}$ except for the 212th nucleotide, where sequence chromatograms showed heterozygous C/T (Fig. 3a). Since the difference between $a^{353\text{-}2a}$ and $a^{353\text{-}2b}$ is one SNP (C or T) at this position (Fig. 1), these alleles can be notated as 'Amahachiya' ($A^{224}A^{226}A^{228}a^{353\text{-}2a}a^{353\text{-}2b}$), 'Omidanshi' ($A^{224}A^{225}A^{228}A^{231}a^{353\text{-}2a}a^{353\text{-}2b}$), and 'Shimofuri' ($A^{224}A^{225}A^{228}A^{231}a^{353\text{-}2a}a^{353\text{-}2b}$) (Table 5). The a^{353} fragment of 'Otani' ($A^{224}A^{225}A^{226}a^{353}$) was consistent with $a^{353\text{-}2a}$ except for the 101th nucleotide, where sequence chromatograms again showed heterozygous C/T (Fig. 3b). We denoted the a^{353} fragment with C nucleotide at 101th position as $a^{353\text{-}2c}$; hence the allele present in 'Otani' would be $A^{224}A^{225}A^{226}$ $a^{353\text{-}2a}a^{353\text{-}2c}$ (Table 5). Sequences of the a^{353} fragments of 'Rojo Brillante' ($A^{224}A^{228}a^{353}$) and 'Kanzo' ($A^{224}A^{231}a^{353}$) showed heterozygous chromatograms at several positions (data not shown), indicating the existence of at least two types of a^{353} fragment in these accessions. The sequences of these a^{353} fragments could not be determined because SNPs at multiple sites gave several possible sequence patterns (Table 5).

'Gosho' ($a^{347}a^{349}a^{355}$) and 'Gosho-Gose' ($a^{349}a^{355}$) (and others in group 18) shared the same alleles for all six ssrdk markers, but not the *AST* locus–linked marker (Table 4); furthermore, the morphological traits of 'Gosho' were very similar to those of the accessions in group 18 (data not shown). Therefore, 'Gosho' could be derived from one of the accessions in group 18 if a mutation of either a^{349} or a^{355} gave rise to a^{347}. To

400 determine the origin of the a^{347} allele, we directly sequenced *ast* fragments amplified with PCNA-F and 5R3R by using either an a^{349}- or a^{353}-specific sequence primer; these primers, respectively harbor an a^{349}- or a^{353}-group–specific SNP at their 3′-end (G for a^{349} or T for a^{353} group at 47th nucleotide; Fig. 1). The sequence chromatogram for 'Gosho' with the a^{349}-specific primer showed heterozygosity from the 275th nucleotide
405 onward, while that with the a^{353}-specific primer showed homozygosity in this region (Fig. 3c). This result is consistent with the situation where the a^{349}-specific primer yields the sequences of both the a^{349} fragment and a related fragment with an AT dinucleotide deletion in the AT repeat region. Chromatograms for 'Gosho-Gose' sequenced with either the a^{349}-or a^{353}-specific primer showed homozygosity. These results clearly
410 indicate that a^{347} originated from a^{349} (Fig. 2).

Fragment analysis revealed that the set of *ast* alleles in the local non-PCNA cultivars having known ancient origin contained either a^{349} or a^{353} or both: 'Aizumishirazu' (originated in the 1300s; $A^{224}A^{228}A^{231}A^{240}a^{349}a^{353}$) 'Ichidagaki' (originated around 600 years ago; $A^{224}A^{228}A^{231}A^{233}a^{349}a^{353}$), 'Saijo' (originated in 1239;
415 $A^{222}A^{224}A^{226}A^{231}A^{233}a^{349}$), and 'Zenjimaru' (originated in 1214; $A^{224}A^{228}A^{240}a^{349}a^{353}$) (Yamada 1996a–d) (Table 3). We therefore reasoned that revealing the sequences of the a^{349} fragment of 'Saijo', and the a^{349} and a^{353} fragments of the other three ancient cultivars may shed light on the development of *ast* alleles in Japan. Assuming that the a^{349} and a^{353} fragments of 'Aizumishirazu', 'Ichidagaki', and 'Zenjimaru' have a^{349}- and
420 a^{353}-specific SNPs at the 47th nucleotide, respectively (Fig. 1), we conducted PCR with two primer combinations for each cultivar: a^{349}-specific forward and 5R3R reverse primers, and a^{353}-specific forward and 5R3R reverse primers. The size of the PCR products corresponded to that of the region between the specific forward primers and the 5R3R reverse primer (data not shown), supporting the presence of both a^{349} and

425 a^{353}-group fragments. Direct sequencing analysis revealed that the a^{349} fragments of the 3 cultivars were the same as the corresponding regions of the a^{349} fragment of the 24 accessions in Table 5. The sequences of the a^{353}-fragments derived from 'Ichidagaki' and 'Zenjimaru' were the same as that of the corresponding region of $a^{353\text{-}1}$, and those of 'Aizumishirazu' were the same as that of the corresponding region of $a^{353\text{-}2a}$ (Table 5).

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Discussion

Information on the *AST* and *ast* allele dosage in non-PCNA accessions is important for efficient breeding of PCNA progenies from non-PCNA parents, because parents with fewer *AST* and more *ast* alleles have a higher chance of producing PCNA offspring. By conducting high-throughput fragment analysis of the *AST* locus–linked marker in 237 persimmon accessions using capillary sequencer, we identified a total of 21 *AST* and 5 *ast* fragments of different sizes. The number of different-sized fragments indicates the minimum copy number of *AST* and/or *ast* alleles. We identified 21 non-PCNA accessions (19 genotypes) with 3 different-sized *ast* fragments per individual; these included 17 local non-PCNA cultivars (IDs: 2–4, 20–23, 25–27, 67–71, 190, 203); 3 F_1 crossbred cultivars/selection (IDs: 19, 24, and 28) from PCNA × non-PCNA crosses; and 1 nonaploid seedless cultivar (ID: 111). Compared with other non-PCNA cultivars, these 17 local cultivars have great potential for use as parents in our pseudo-backcross strategy, because crosses between any of these cultivars and a PCNA cultivar would produce non-PCNA progenies with fewer *AST* and more *ast* alleles and even PCNA progenies at the F_1 generation. Among these 17 local cultivars, ‘Aosa’, ‘Chung Nam’, ‘Hiroshimashimofuri’, ‘Sakushumishirazu’, ‘Shogatsu-Italy’, ‘Okayamaokugosho’, ‘Tenryubo’, and ‘Yoshidagosho’ bear relatively large fruit (around 300 g) with fewer appearance defects on fruit skin, and thus would be primary candidates for parents in the pseudo-backcross strategy. Furthermore, according to AFLP (Amplified Fragment Length Polymorphism) analysis, ‘Hiroshimashimofuri’, ‘Sakushumishirazu’, and ‘Tenryubo’ have a different genetic background to that of PCNA cultivars (Parfitt et al. 2015), and so their use would avoid inbreeding depression. On the other hand, a total of 59 non-PCNA genotypes with four or more different *AST* alleles were identified. If one of these accessions were to be used in the initial non-PCNA × PNCA cross in the

pseudo-backcross strategy, the breeder would need to choose F_1 non-PCNA individuals with fewer *AST* and more *ast* alleles to backcross to the PCNA parent to efficiently produce PCNA offspring at the BC_1 generation.

The minimum numbers of *AST* and *ast* alleles determined here were in good accordance with the allele numbers estimated in 63 non-PCNA accessions by qPCR analysis of an *ast* allele–linked marker (Akagi et al. 2010); of these 63 accessions, 34 were also included in the current study. The numbers of *AST* and *ast* allele fragments were equivalent to or smaller than the estimated number of the corresponding alleles in Akagi et al. (2010) with two exceptions: 'Heixinshi' (AAAAAa in Akagi et al. 2010; $A^{226}A^{254}a^{349}a^{353}$ in current study), and 'Omidanshi' (AAAaaa in Akagi et al. 2010; $A^{224}A^{225}A^{228}A^{231}a^{353}$ in current study). A possible explanation for this inconsistency is that a one-*ast*-allele difference could occur in the quantitative genotyping owing to methodological limitation and depending on the reference site used (Akagi et al. 2010). One of the possible genotypes for 'Heixinshi' and 'Omidanshi' deduced from our fragment analysis is AAAAaa, which is one *ast* allele more, and one *ast* allele less, respectively, than the genotypes determined for these cultivars by Akagi et al. 2010. Since we identified $a^{353\text{-}2a}$ and $a^{353\text{-}2b}$ fragments in 'Omidanshi' in the sequence analysis, the genotype of 'Omidanshi' would be $A^{224}A^{225}A^{228}A^{231}a^{353\text{-}2a}a^{353\text{-}2b}$.

The *AST* and *ast* allele data obtained by the sequencer-based fragment analysis is limited in that it represents minimum copy number rather than actual allelic copy number. Excluding the 2 genotypes of 8 nonaploid accessions, 31 genotypes with a total of 6 different *AST* and *ast* alleles were assumed to have one copy of each allele, because *D. kaki* is generally hexaploid. However, for the other 151 genotypes, which showed fewer than six different-sized fragments, the method could not reveal which alleles were duplicated. One solution to this problem is to examine the segregation of

the alleles in the F_1 generation of a non-PCNA of interest × PCNA cross. We previously successfully estimated the copy number of the *AST* allele of six non-PCNA parents by assessing segregation of each *AST* allele and F_1 progeny genotypes in the non-PCNA × PCNA F_1 population (Kono et al. 2016). A different approach is necessary to determine *ast* allele copy number because the presence of the same *ast* alleles in both non-PCNA and PCNA parents makes it difficult to deduce copy number and inheritance of the *ast* allele in the F_1 generation. Crossing PCNA or non-PCNA cultivars of interest with a non-PCNA parent that has no *ast* alleles would be one solution; here, we successfully identified 23 non-PCNA accessions (21 genotypes) that have no *ast* alleles. In the same way as for the *AST* allele, one could estimate *ast* allele copy number and inheritance in a parent of interest by assessing segregation of each *ast* allele and/or progeny genotype.

Our results indicate that fragment analysis with the *AST* locus–linked marker would be applicable for cultivar identification of non-PCNA accessions. The *AST* locus–linked marker discriminated 87% (113/130) of non-PCNA genotypes, whereas single ssrdk markers discriminated 8% (11/130) to 48% (62/130) of them. PIC value, the number of total and rare alleles, and the mean alleles number per individual of the *AST* locus–linked marker explains its higher discrimination power compared to ssrdk markers. Additionally, the *AST* locus–linked marker displayed allele size polymorphisms within groups of cultivars with different names but practically indistinguishable morphological traits, most likely synonyms: ‘Tamopan’ and ‘Mopanshi’; ‘Gosho’ and cultivars in group 18; ‘Misatogosho’ and cultivars in group 17. It would be possible that a clonal individual derived from the original one was named differently and independently accumulate nucleotide mutations that do not alter phenotype significantly. The fact that the above cultivars shared identical genotypes in the ssrdk markers, but not in the *AST* locus–linked marker, is consistent with the high

discrimination power of the *AST* locus–linked marker. We previously revealed that polymorphisms in the *AST* fragment consist of AT repeats, indels, and SNPs (Kono et al. 2016); and here we observed that polymorphisms in the *ast* fragment consisted of AT repeats and SNPs. These results suggest that the *AST* locus–linked region has a high mutation rate. In practical germplasm identification, breeders would be interested in verifying the identity of a germplasm with the original cultivar. Partly because persimmons are relatively new to non-Asian countries, germplasm exchange and frequent bud mutation have resulted in mislabeling and confusion of persimmon cultivars (Badeness et al. 2003; Yonemori et al. 2000). Under these conditions, highly polymorphic and cost-effective DNA markers would be an ideal tool for cultivar identification. Distinguishing the size differences of *AST* and *ast* alleles by using a capillary sequencer is reproducible and simple. The allele data presented in this study, which covers many persimmon accessions, should work well for screening target accessions. In contrast to the *AST* allele, the *ast* allele revealed only five different sizes, suggesting it is of more recent origin. Low polymorphism of the *ast* allele limits discrimination of PCNA accessions by the *AST* locus–linked marker, and thus addition of other markers, such as ssrdk markers, is indispensable. The genotyping data for each of the six ssrdk markers presented in Supplemental Table S2 are readily available.

There are many synonym groups among persimmon genetic resources (Agricultural Research Station 1912; Yonemori et al. 2000). Here, by using the *AST* locus–linked marker and the six ssrdk markers, we identified several possible synonyms, most of which were suggested as synonyms in previous studies based on morphological traits and isozyme analysis (Fruit Tree Experiment Station of Hiroshima Prefecture 1979; Sugiura et al. 1990; Tao and Sugiura 1987; Tao et al. 1989). Based on AFLP analysis, Yonemori et al. (2008) reported high similarity between Italian accessions

‘Brazzale’, ‘Moro’, ‘Rispoli’, and the Japanese accession ‘Zenjimaru’ (group 4), and between the Japanese accession ‘Amahyakume’ and the Italian accession ‘Kaki Tipo’ (group 3). Since many Italian cultivars are likely to have been imported directly or indirectly (through North America) from Japan in the 19th century (Bellini and Giordani 2005), some Italian accessions could be categorized into the same group as the original Japanese accession. We here newly report the following possible synonyms: ‘Chung Nam’ and ‘Okayamaokugosho’ (group 6); ‘Edoichi’ and ‘Kurokuma’ (group 7); ‘Kubogataobishi’ and ‘Shoujyou’ (group 11); ‘Mizushimagosho-Italy’ and ‘Shogatsu’ (group 12); ‘Yamatogaki’ and ‘Yamatohyakume’ (group 13); and ‘Giant Fuyu’ and ‘Mikado’ (group 16). Similar morphological traits within the above groups support the notion that group members are synonyms to each other (data not shown). Except for bud-sports, no accession was categorized into a group from which its morphological features differed substantially. All known bud mutants of ‘Hiratanenashi’, ‘Fuyu’, and ‘Jiro’ were categorized into the same group as the original cultivar (group 8, 14, and 19, respectively).

Our results provide insights into the origin of the *ast* allele. Polymorphisms in *ast* alleles have been reported (Akagi et al. 2010; Akagi et al. 2012; Kono et al. 2016), but there has been no systematic analysis of the distribution and sequences of these alleles among persimmon genetic resources. Here, we found five *ast* fragments of different sizes among 237 accessions; *ast* alleles were found not only in Japanese accessions, but also in some accessions from China, Korea (Akagi et al. 2010), and other countries. Excluding accessions with the same allele composition at the *AST* locus–linked marker and six ssrdk markers as Japanese accessions, Chinese local cultivars in particular had *ast* alleles; the *ast* fragment sizes in the Chinese cultivars were either 349, 351, or 353, which is the same as those found in Japanese cultivars.

Our finding that the sequences of the a^{349} allele of 24 accessions (19 Japanese and 5 Chinese) were identical suggests that the a^{349} allele has a common origin in these accessions. The presence of $a^{353\text{-}1}$ in 'Huo-shi' (China), 'Harbiye' (Turkey), and 'Triumph' (Israel) as well as in six Japanese accessions also implies that $a^{353\text{-}1}$ shares the same origin in these accessions.

Depending on sequence similarities of the *ast* fragment, the eight *ast* alleles found in this study could be categorized into two groups: the a^{349} group (a^{347} and a^{349}) and the a^{353} group (a^{351}, $a^{353\text{-}1}$, $a^{353\text{-}2a}$, $a^{353\text{-}2b}$, $a^{353\text{-}2c}$, and a^{355}). We did not find any *ast* alleles that had a moderately similar sequence to both the a^{349} and a^{353} group; therefore, future work to finding the missing link between these two allelic groups is required. In conjunction with the two separated groups of *ast* alleles, the limited existence of the a^{349} and a^{353} groups in the four Japanese local cultivars with ancient origin suggests that a bottleneck event occurred during *ast* allele development. A possible scenario is that *ast* alleles first arose in China and then developed several mutations, and that Chinese cultivars with *ast* allele(s) from the a^{349} and a^{353} groups were by chance introduced to Japan. The same sequence for a^{349} in Japanese and Chinese accessions, and for $a^{353\text{-}1}$ in Japanese and Chinese accessions, is consistent with this scenario. To assess this notion, we need to screen many Chinese accessions and characterize their *ast* alleles. Fragment analysis of the *AST* locus–linked marker would be useful to screen for *ast* allele–containing accessions from among the more than 900 local non-PCNA cultivars in China (Wang et al. 1997).

In this study, we present comprehensive data for the minimum number and characteristics of *AST* and *ast* alleles in 237 persimmon accessions. Based on allelic size polymorphisms, we identified non-PCNA accessions with the larger than average numbers of *ast* alleles. These accessions are important genetic resources that could be

used to accelerate PCNA breeding using our pseudo-backcross strategy, and to extend the genetic background of PCNA cultivars to prevent inbreeding. In addition, we have demonstrated that the displayed allele data is useful for identification of non-PCNA cultivars. Fragment size and direct sequence analysis of *ast* fragments suggest that *ast* alleles likely originated in China. We conclude that the data presented here will help breeders select non-PCNA parents with more *ast* alleles to more efficiently breed PCNA offspring, and that the data provides new insights into how the *ast* allele has developed.

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Conflict of interest

The authors declare that they have no conflict of interest.

Data Archiving Statement

The data obtained by the direct sequence analysis of the 45 accessions with only one-size peak of the *ast* allele–linked fragment was submitted to DNA Data Bank of Japan (DDBJ). The full list of the data was described in Supplemental Table S3.

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Figure Legends

Fig. 1 Schematic representation of the *AST*- and *ast*-allele–linked regions isolated from genomic libraries of 'Nishimurawase' and 'Jiro', respectively (Kanzaki et al. 2010), and multiple nucleotide sequence alignment of seven *ast* fragments amplified with PCNA-F and 5R3R primers. Black boxes indicate the 5R probe that was used for library screening. The grey box indicates a large insertion named Indel-3 in the *ast* allele–linked region. Arrows indicate the positions of primers used for the multiplex PCR. Numbers indicate the positions from the 5′-end of the 5R region. Our fragment analysis detected *AST* allele–linked fragments amplified with AST-F and 5R3R, and/or *ast* allele–linked fragments amplified with PCNA-F and 5R3R. Note that non-proofreading DNA polymerase adds an adenylate to the 3′ end of the PCR fragment, resulting in a longer PCR fragment than the actual size of the amplified region. Because the mobility of the labeled single-stranded fragments depends on several factors (i.e., length, sequence, running conditions), size inconsistency between sequencing and fragment analysis can occur (Pasqualotto et al. 2007). For simplicity, we used "called" sizes in this study

Fig. 2 Diagram of the relationship between *ast* alleles. Sequence differences between *ast* allele–linked fragments connected by double-headed arrows are indicated. Relationship between a^{347} and a^{349} is indicated by a grey double-headed arrow because the complete sequence of the a^{347} fragment was not obtained

Fig. 3 Sequencing chromatograms of *ast* allele–linked fragments. Black arrows indicate the locations of heterozygous nucleotides. a) 'Amahachiya', 'Omidanshi', and 'Shimofuri' show heterozygosity at the 212th nucleotide of a^{353}, whereas 'Kubo' and

‘Dejima’ show homozygosity. b) ‘Otani’ shows heterozygosity at the 101th nucleotide of a^{353}, whereas ‘Kubo’ shows homozygosity. c) Sequencing of ‘Gosho’ and ‘Gosho-Gose’ with a^{349}- and a^{353}-specific primers. Note that a^{349}- and a^{353}-specific primer harbors an a^{349}- and a^{353}-group–specific SNP at its 3′-end. In ‘Gosho’, sequencing with an a^{349} specific primer shows heterozygosity beginning from the 275th nucleotide. The other three chromatograms show homozygosity in this region