Aegilops section Sitopsis species contains the introgressive PolA1 gene with a closer relationship to that of Hordeum than Triticum–Aegilops species

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The section Sitopsis in the genus Aegilops includes five species, Ae. speltoides, Ae. longissima, Ae. sharonensis, Ae. searsii, and Ae. bicornis, which share the SS genome. Although extensive molecular studies have indicated Ae. speltoides as a donor of BB or GG genome to polyploid wheat species, the precise relationships among SS, BB, and GG genomes remain unclear. PolA1 is a single-copy nuclear gene encoding the largest subunit of RNA polymerase I. Highly polymorphic PolA1 exon 20 sequences were analyzed for 11 Triticum–Aegilops, 13 Hordeum and three related species. Phylogenetic analyses of the PolA1 gene showed that Triticum–Aegilops and Hordeum species were distinctly separated into two clades. Two related species, Secale cereale and Dasypyrum villosum, were grouped into Triticum and Hordeum clades, respectively. Interestingly, seven accessions of the Sitopsis species were clustered into the Hordeum clade whereas two accessions belonged to the Triticum clade. In contrast, all accessions of Sitopsis species shared the same haplotype of plastid PSID sequences with Triticum–Aegilops species. This inconsistency in phylogeny between nuclear and chloroplast sequences suggested that the Sitopsis species probably originated through introgressive hybridization between ancestral species of Triticum–Aegilops and Hordeum.

Key Words: Aegilops, hybrid speciation, introgression, PolA1 gene, Sitopsis, PSID.

Introduction

Triticeae is a tribe within the Pooidae subfamily of grasses that includes more than 15 genera and 300 species, including major crop plants such as common wheat (Triticum aestivum L.), rye (Secale cereale L.), and barley (Hordeum vulgare L.). The genus Triticum along with Aegilops contains 36 species (Eig 1929, Hammer 1980, Tanaka 1983, van Slageren 1994), and the genus Hordeum is comprised of 32 species (Bothmer et al. 1995). These species not only include several important crops, but also provide an excellent model system for studying the evolution of allopolyploids. Kihara (1944) reported that common wheat (AABBDD) originated from allopolyploidization between T. turgidum L. (AABB) and Ae. tauschii (DD). However, the origins of two tetraploids, T. turgidum and T. timopheevii Zhuk. (AAGG), remain unresolved (Gill and Chen 1987, Kilian et al. 2007).

Riley et al. (1958) proposed that ancestral Ae. speltoides was the most likely diploid species for the donor of either BB and / or GG genomes to polyploid wheat species. This was supported by analyses of crossability (McFadden and Sears 1946), chloroplast DNA (Ogiwara and Tsunewaki 1988, Miyashita et al. 1994, Wang et al. 1997, Yamane and Kawahara 2005), mitochondrial DNA (Terachi et al. 1990, Mori et al. 1997), nuclear polymorphic DNA sequences (Dvorak and Zhang 1990, Sasanuma et al. 1996, Salina et al. 2006), and sequence analysis of genes (Huang et al. 2002, Provan et al. 2004, Petersen et al. 2006, Kilian et al. 2007, Golovnina et al. 2007, Chalupska et al. 2008, Salse et al. 2008). Despite decades of intensive research, the detailed relationship between BB and GG genomes has not been clarified, and therefore, the genome symbol of Ae. speltoides was designated as SS (Huang et al. 2002).

Although earlier studies (e.g. Stebbins 1956) suggested that Aegilops and Triticum species should be considered members of the same genus, van Slageren (1994) argued that these species belonged to two distinct genera, and Ae. speltoides was classified into the section Sitopsis (Jaub. & Spach) Zhuk., which also included Ae. longissima, Ae. searsii, Ae. bicornis, and Ae. sharonensis. Five Sitopsis species were classified into two subclasses, Ae. speltoides and four species (Sasanuma et al. 1996, 2004, Salina et al. 2006). The relationships of Sitopsis species with Triticum and other Aegilops species have been controversial (Salleres and Brown 2004). Although Petersen et al. (2006) recently proposed that Ae. speltoides should be excluded from the genus Aegilops and classified into the genus Sitopsis (Jaub & Spach) Á. Löve, none of these reports suggested that

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Sitopsis species originated through hybrid speciation. Hybridization between species has been considered one of the driving forces for speciation in plants (Mallet 2007). Amphidiaplodization provides clear evidence for the involvement of hybridization in speciation (hybrid speciation), namely, interspecific hybridization and subsequent chromosome doubling of a hybrid, although it is difficult to prove hybrid speciation without chromosomal doubling (homoploid hybrid speciation). Furthermore, if backcrossing (introgressive hybridization) is involved, it is more difficult to define the genetic material introduced by the original hybridization.

Nakamura et al. (1997) reported a plastid subtype identity (PSID) sequence, which is a linker between rpl16 and rpl14 genes on plastid DNA. PSID sequences of most land plants are 100–200bp in length and can be analyzed using a pair of common primers. Hebert et al. (2003) proposed a DNA barcoding system using the mitochondrial cytochrome oxidase I (COI) sequence as a tag of eukaryotic species. Although these cytoplasmic DNA markers are useful for species identification of plants, it is impossible to resolve hybrid speciation without analyzing nuclear DNA markers. Many researchers now prefer phylogenetic analysis using the combined sequences of many genes but such analysis will neglect small numbers of alien genes introduced through introgressive hybridization. Phylogenetic analyses based on the diffusion equation have been developed for population genetics and neutral evolution of molecular sequences (Kimura 1968) but not for hybrid speciation. Therefore, to obtain evidence of hybrid speciation by introgression, it is important to find species-specific sequence variations of single-copy nuclear genes per haploid genome (Sang 2002).

Recently, we found that the PolA1 gene contained highly polymorphic sequences in plants. PolA1 encodes the largest subunit of the RNA polymerase I complex, which synthesizes 45S ribosomal RNA, one of the most important parts of the machinery in living cells. Takahashi et al. (2009a) reported that the DNA sequence of intron 19 shows species- and genus-specific variations in the genus Oryza. Sequence variability in the same region successfully distinguished closely related accessions in Petunia axillaris and P. integrifolia complexes, and therefore, this is a promising DNA marker for discrimination before blooming (Zhang et al. 2008). Furthermore, based on analysis of the intron 19 sequences, Takahashi et al. (2009b) reported that wild and cultivated subspecies of T. timopheevii had originated by divergence. They also found that it was difficult to perform phylogenetic analysis of the intron 19 sequences between Triticum monococcum (112 bp) and Hordeum vulgare (244 bp) together because of their high divergence. Interestingly, the Sitopsis species were found to contain the long intron 19 (242bp).

This study was performed to resolve the relationship of the Sitopsis species with related Triticum, Aegilops, and Hordeum species through comparative analyses of nuclear PolA1 exon 20 and plastid PSID sequences.

Materials and Methods

Plant materials and DNA extraction

Seeds or DNAs of all the accessions of Triticum, Aegilops and Hordeum species used in this study were provided by Dr. T. Kawahara, National Bio-resources Project, Kyoto University, Mozume, Japan, Dr. R.v. Bothmer, Swedish University of Agricultural Sciences, Alnarp, Sweden, Dr. K. Takeda, Research Institute for Bioresources, Okayama University, Kurashiki, Japan, and Dr. K. Kato, Faculty of Agriculture, Okayama University. Seeds of H. bulbosum GBC77-1 was kindly provided by Dr. M. Furusho, Fukuoka Agriculture Research Center, Chikushino, Fukuoka, Japan. DNAs of Lolium elongatum were gifts from Dr. H.W. Cai, Japan Grassland Farming and Forage Seed Association, Forage Crop Research Institute, Japan.

Nucleotide sequences of exon 20 in PolA1 gene were analyzed for 26 accessions of 11 Triticum–Aegilops species (T. monococcum, T. urartu, Ae. uniaristata, Ae. comosa, Ae. markgrafii, Ae. umbellulata, Ae. tauschii, Ae. bicornis, Ae. speltoides, Ae. searsii, Ae. longissima), 22 accessions of 13 Hordeum species (H. vulgare, H. bulbosum, H. marimum, H. murinum, H. brevisubulatum, H. roshevitzii, H. euclaston, H. intercedens, H. erectifolium, H. chilense, H. pubiflorum, H. patagonicum, H. pulilflorum) and 2 accessions of 2 related species (Secale cereale, Dasyopyrum villosum). Two accessions of Lolium elongatum were analyzed as an out-group (Table 1).

Young leaves (ca. 100 mg) of seedlings were frozen in 2-ml plastic tubes with liquid nitrogen and crushed into fine powder using a MULTI-BEADS SHOKKER (Yasu Ki Kikai Co., Kyoto, Japan). Total genomic DNA was extracted according to the CTAB method (Doyle and Doyle 1987)

PCR amplification and direct sequencing

The DNA fragments containing PolA1 exon 20 sequences were amplified using PCR with a pair of primers, 19ex5P: 5′-AGGGGACATGAAATGTACCTGG-3′ and 21ex3P: 5′-ACCTTATTGTTTCTGGGCAATCT-3′, which are located on exon 19 and exon 21 of PolA1 genes, respectively (Fig. 1). For the sequencing of the exon 20, an internal sequencing primer 20ex5P (5′-ATCCAGGAAAACAGGG GTAAAG-3′) were used. ExTag DNA polymerase (TaKaRa Co., Shiga, Japan) was used for PCR according to the manufacturer’s instructions. The PCR amplification was set as 36 cycles of 1 min. at 94°C for denaturation, 1 min. at 58°C for annealing, and 2 min. at 72°C for elongation in a PTC200 thermocycler (MJ Research Inc., MA, USA).

The amplified PCR products were subjected to 1.2% agarose gel electrophoresis and purified using a PCR purification kit (QIAquick, Qiagen Inc., CA, USA). DNA sequences of the purified PCR products were determined by direct sequencing with three sequencing primers, 19ex5P, 20ex5P and 21ex3P, using an automated DNA sequencer ABI310 (Applied Biosystems, CA, USA) with a Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, USA).
Phylogenetic analyses of PolA1 exon 20 sequences

The determined nucleotide sequences of PCR products were analyzed to assign the positions of donor and acceptor sites of the exon 20 in the PolA1 gene using the NCBI web-based Blast server (http://blast.ncbi.nlm.nih.gov/Blast.cgi, Altschul et al. 1990) and software (Genetyx Software ver. 6.0; Software Development Co., Tokyo, Japan). A rooted phylogenetic tree using nucleotide sequences data of the PolA1 exon 20 was constructed from evolutionary distance matrices by a Neighbour-Joining (N-J) algorithm using the maximum composite likelihood method with bootstrap analysis using 1,000 replicates in the MEGA4.0 program (Tamura et al. 2007).

Comparison of amino acid sequences in PolA1 exon 20

The deduced amino acid sequences of exon 20 were aligned among Triticum, Aegilops, and Hordeum using web server (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/) of Mafft ver. 6.0 (Kato and Toh 2008).

Analysis of plastid subtype identity (PSID) sequences

DNA fragments (ca. 350 bp) containing PSID sequences were amplified by PCR from the total DNA extracts as a template using a pair of common primers, PSID5P: 5′-GTAGC CGTTGTTAAACCAGTCGAATCTTGTGTAATGAAAT-3′, PSID3P: 5′-ACACGCAACACTGCTCACAATGTCGATATCCG-3′ (Nakamura et al. 1997, Takahashi et al. 2009b). PCR was performed for 35 cycles of 30 sec. at 94°C for denaturation, 30 sec. at 55°C for annealing, and 1 min. at 72°C for elongation. Nucleotide sequences of DNA

Table 1. Triticeae accessions used in this study

<table>
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<th>Accession</th>
<th>Genome</th>
<th>PSID</th>
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<td>P1</td>
</tr>
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<td>KT3-1, KT3-4</td>
<td>AA</td>
<td>P1</td>
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<tr>
<td><em>T. urartu</em> Tumanian ex Gandilyan</td>
<td>PI428181, PI487270</td>
<td>AA</td>
<td>P2</td>
</tr>
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<td>KU2760, KU4018</td>
<td>UU</td>
<td>P3</td>
</tr>
<tr>
<td><em>Ae. comosa</em> Sm. in Sibth. &amp; Sm.</td>
<td>KU17-3, KU5804</td>
<td>MM</td>
<td>P1</td>
</tr>
<tr>
<td><em>Ae. markgrafii</em> (Greuter) K.Hammer</td>
<td>KU6-2, KU11422</td>
<td>CC</td>
<td>P1</td>
</tr>
<tr>
<td><em>Ae. uniaristata</em> Vis.</td>
<td>KU19-1, KU11479</td>
<td>NN</td>
<td>P1</td>
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<tr>
<td><em>Ae. tauschii</em> Coss.</td>
<td>AT07, AT13, KU20-9</td>
<td>DD</td>
<td>P4</td>
</tr>
<tr>
<td><em>Ae. bicornis</em> (Forssk.) Jaub. &amp; Spach.</td>
<td>KU3-2, KU14610, KU14615</td>
<td>SS</td>
<td>P1</td>
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<td><em>Ae. longissima</em> Schweinf. &amp; Muschl.</td>
<td>KU14642, KU14648</td>
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<td><em>Ae. scabrii</em> Feldman &amp; Kisselev ex K. Hammer</td>
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<td>SS</td>
<td>P1</td>
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<td><em>Ae. speltoides</em> Tausch.</td>
<td>KT115-6, KT115-8</td>
<td>SS</td>
<td>P1</td>
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<td><em>H. vulgare</em> L. ssp. vulgare</td>
<td>BA04, BA17</td>
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<td>P5</td>
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<tr>
<td>ssp. spontanum (C. Koch) Thell.</td>
<td>OUH602, OUH743</td>
<td>SS</td>
<td>P5</td>
</tr>
<tr>
<td><em>H. bulbosum</em> L.</td>
<td>GBC77-1</td>
<td>SS</td>
<td>P5</td>
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<td><em>H. maritinum</em> Huds. ssp. maritum</td>
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<td>P6</td>
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<td>XX</td>
<td>P6</td>
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<td><em>H. maritinum</em> L.</td>
<td>H551, H798</td>
<td>XX</td>
<td>P6</td>
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<td><em>H. roshevitzii</em> Bowden.</td>
<td>H7434, H10070</td>
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<td><em>H. brevissulatulum</em> (Trin.) Link.</td>
<td>H8788, H10056 (autotetraploid)</td>
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<td><em>H. pulbiborum</em> Hook.f.</td>
<td>H1236</td>
<td>HX</td>
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<td><em>H. intercedens</em> Nevis</td>
<td>H1941</td>
<td>HX</td>
<td>P7</td>
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<td>H2024</td>
<td>HX</td>
<td>P7</td>
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<tr>
<td><em>H. euclaston</em> Steud.</td>
<td>H2148</td>
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<td><em>H. patagonicum</em> (Hauman) Covas</td>
<td>H6852</td>
<td>HX</td>
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<tr>
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<td>KU4017</td>
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<td>P1</td>
</tr>
<tr>
<td><em>Dasypyrum villosum</em> (M. Bieb.) Maire</td>
<td>KU3905</td>
<td>VV</td>
<td>P1</td>
</tr>
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<td><em>Lolium elongatum</em> L. (outgroup)</td>
<td>IR10-4, PRwz19</td>
<td>EE</td>
<td>nt</td>
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</table>


nt–not tested.
Introgressive PolA1 gene of Aegilops section Sitopsis species

fragments were determined by direct sequencing using the same primer for the initial amplification.

Results

Amplification of DNA fragments containing PolA1 exon 20
DNA fragments (1.5–2.0 kb) containing PolA1 exon 20 were amplified by PCR using a pair of primers (19ex5P and 21ex3P; Fig. 1), and fractionated by agarose gel electrophoresis. As exon 20 DNA sequences were 806 bp in length in all accessions of Triticum, Aegilops, and Hordeum species as well as Secale, Dasypyrum, and Lolium species with the exception of two accessions (KU3-2, KU14610) of Ae. bicornis (803 bp), size variations of the amplified DNA fragments were due to differences in length of introns 19 and 20 in the PolA1 genes (Fig. 2).

Sequence information for PolA1 genes obtained in this study was deposited to DDBJ data bank as accession Nos. AB501216–AB501270.

 Phylogenetic analysis of the PolA1 exon 20 sequences

Phylogenetic tree was constructed using nucleotide sequences of PolA1 exon 20 among Triticum, Aegilops, Hordeum and two related species using the N-J method in the MEGA4.0 package (Fig. 3). Phylogenetic analysis of PolA1 exon 20 clearly indicated that Triticum–Aegilops and Hordeum species could be separated into Triticum and Hordeum clades, respectively. Six accessions in three Sitopsis species, Ae. searsii, Ae. longissima, and Ae. speltoides (SS genome), were clustered into the Hordeum clade. Although one accession (KU14615) of Ae. bicornis was related to the Hordeum clade, two accessions (KU3-2, KU14610) of Ae. bicornis were belonged to the Triticum clade. Secale cereale belonged to the Triticum clade whereas Dasypyrum villosum was clustered with the Hordeum clade.

In the Triticum clade, two AA genome species, T. monococcum and T. urartu, belonged to the same subclade, and a group of three species, Ae. markgrafi (CC), Ae. umbellulata (UU), and Ae. tauschii (DD), and another of two species, Ae. uniaristata (NN) and Ae. comosa (MM), were clustered into two different subclades. Secale cereale (RR) was related to two Ae. bicornis accessions. In the Hordeum clade, H. vulgare and H. bulbosum (II genome), H. murinum (XuXu), and H. marinum (XaXa) were clustered
into a single clade. *Dasypyrum villosum* (VV) was related to these species. In contrast, all nine species with the HH genome formed another clade, in which two Eurasian species, *H. brevisubulatum* and *H. roshevitzii*, were closely related to seven American species, *H. erectifolium*, *H. pulbiflorum*, *H. chilense*, *H. intercedens*, *H. pusillum*, *H. euclaston*, and *H. patagonicum*.

Amino acid sequences of PolA1 exon 20 between Triticum and Hordeum groups

The deduced PolA1 exon 20 amino acid sequences could be clearly differentiated at 22 positions (indicated by “X” in Fig. 4) between the Triticum group (*T. monococcum* KT3-1, *Ae. tauschii* SS, *Secale cereale* KU4017, *A. bicornis* KU3-2, and Hordeum group; *H. vulgare* KT115-8, *H. roshevitzii* H7434, *D. villosum* Hv) and the Hordeum group. Asterisk and X indicated identical amino acid between two groups and amino acid substitution specific to each group, respectively. *Ae. bicornis* KU14615 and three other Sitopsis species shared the same amino acid sequence.

Variations of PSID sequences in Triticeae species

Only seven types (P1–P7) of the PSID sequence were found among *Triticum*, *Aegilops* and *Hordeum* species (Fig. 5). One base substitution (T to A) at position 32 was found between *Triticum*–*Aegilops* (P1–P4) and *Hordeum* species (P5–P7). Although one base substitution (C to T) at position 46 was found between *T. monococcum* (P1) and *T. urartu* (P2), the P1-type sequence was contained in four Sitopsis species as well as three other *Aegilops* species, *Ae. comosa*, *Ae. markgrafii*, and *Ae. uniaristata*. The P1-type sequence was also found in *Secale cereale* and *Dasypyrum villosum*. Unique insertions of 1-bp and 7-bp were detected in *Ae. umbellulata* (P3) and *Ae. tauschii* (P4), respectively. *Hordeum vulgare* and *H. bulbosum* contained the same PSID sequence (P5). The P6 type sequence was shared by *H. marinum* and *H. murinum*. *Eurasian H. roshevitzii* contained the P7 type, which was also found in four American species, *H. erectifolium*, *H. pulbiflorum*, *H. pusillum*, and *H. euclaston* (Table 1).

Discussion

Hybrid speciation by allopolyploidization, as in Triticeae and Brassicaceae, is a basic phenomenon in plant evolution (Mallet 2007). Allopolyploid species are reproducibly isolated from their parental species by ploidy and both parental species can be recognized by ordinary molecular genetics. On the other hand, recombinational or homoploid hybrid

### Table 1

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</table>

### Fig. 4

Alignment of deduced amino acid sequences of PolA1 exon 20 between Triticum group; Tm: *Triticum monococcum* KT3-1, At: *Aegilops tauschii* AT7, SS: *Secale cereale* KU4017, B1: *Ae. bicornis* KU3-2, and Hordeum group; B2: *Ae. bicornis* KU14615, As: *Ae. speltoides* KT115-8, Hr: *Hordeum roshevitzii* H7434, Dv: *Dasypyrum villosum*, Hv: *H. vulgare*. Asterisk and X indicated identical amino acid between two groups and amino acid substitution specific to each group, respectively. *Ae. bicornis* KU14615 and three other Sitopsis species shared the same amino acid sequence.
speciation without changes in chromosome number have been considered very rare because there is generally no reproductive barrier between hybrids and their parental species (Rieseberg 1997). One well-documented example of hybrid speciation in plants was three species of desert sunflower, Helianthus anomalus, H. deserticola, and H. paradoxus, which were hybrid species derived from hybridization between H. annuus and H. petiolaris (Buerkle et al. 2000, Gross and Rieseberg 2005). These hybrid species are differentiated from their parental species by the abilities to survive under conditions of severe drought. If hybrid and parents compete to occupy the same habitat, one may become extinct, or they may merge into a single species complex.

Most hybrids between distantly related species are reproductively sterile. The sterile hybrids have three choices to maintain their genetic lineages; 1) propagation as clones, 2) recovery of fertility by doubling their chromosome number (amphidiploidy), and 3) repeated backcrossing (introgression) with one of the parental species until self-fertility is restored. In the first and second cases, hybrids can be easily characterized by molecular genetic analysis, but in the third case, identification of the DNA sequences introduced by the original hybridization is very difficult because the genomes of the backcrossing parent and backcrossed hybrids will be identical, except for short stretches of the introduced DNA sequences.

Anderson (1953) postulated speciation through introgression as illustrated in Fig. 6. Hybridization between Species A (female) and Species B (male) occurs, and the hybrid is backcrossed with Species A (or related species), followed by several backcrosses with Species A (introgressive hybridization). If an introduced gene fraction (IGF) from Species B by introgression encodes common proteins such as housekeeping enzymes or storage proteins, the backcrossed progeny will be considered the same as Species A (sympatric introgression), whereas if the IGF creates some divergent properties, the hybrid will be distinguished as a subspecies within Species A (allopatric introgression).

The hybrids arising from hybridization between genera or between distantly related species are generally male-sterile. Therefore, extensive backcrossing with pollen donors is necessary to recover self-fertility of the backcrossed progeny. As the relationship between the two species is less related to each other, although the IGF of the progeny will be smaller, it may have a stronger allopatric effect for the establishment of a new species. Many instances of sympatric and allopatric introgressions between closely related species have been reported but the origin of species by the intergenus hybridization and extensive backcrossing (introgressive homoploid hybrid speciation) has not been reported.

Although it has not been suggested that Aegilops section Sitopsis species originated through hybrid speciation, we found signature sequences from Hordeum species in PolA1 exon 20 of four Sitopsis species (Fig. 3 and Fig. 4). Phylogenetic analysis of PolA1 exon 20 sequences indicated that

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**Fig. 5.** Alignment of four types PSID sequences (P1–P4) in Triticum–Aegilops species and three types (P5–P7) in Hordeum species. One base substitution (T to A) at the position 36 was specific to each group (Arrow). The P1-type sequence was shared by four Sitopsis species, Triticum monococcum, three Aegilops species, Secale cereale, and Dasypyrum villosum (Table 1). Triticum monococcum and T. urartu were differentiated by one base substitution at the position 46 (Asterisk). Hordeum vulgare and H. bulbosum (II genome) had the P5 type. H. marinum (XaXa) shared the P6 type with H. maritinum (XaXu).
two accessions of *Ae. bicornis* (B1 type) were closely related to *S. cereale* in the Triticum clade whereas one accession of *Ae. bicornis* (B2 type) and six accessions of three other *Sitopsis* species shared the identical amino acid sequence, which was closely related to those of HH genome species, such as *H. rosinetii*, in the Hordeum clade (Fig. 3 and Fig. 4). This phylogeny based on the *PolA1* gene was inconsistent with that based on the PSID sequence because four *Sitopsis* species showed the P1 type PSID sequence, which was shared by *T. monococcum*, *Ae. comosa*, *Ae. markgrafii*, and *Ae. uniaristata* (Table 1 and Fig. 5). The *Sitopsis* species are morphologically recognized as species in the genus *Aegilops* (van Slageren 1994). Molecular data indicated that *Sitopsis* species had a close relationship to polyploid species in the genus *Triticum* (Petersen et al. 2006, Salina et al. 2006). These results suggest that the *Sitopsis* species might have originated by ancient hybridization between ancestors of *Triticum–Aegilops* species (female, B1 type *PolA1* in Fig. 4) and *Hordeum* species (male, B2 type), and presumably by backcrossing of the resulting hybrid(s) with ancestors of *Triticum–Aegilops*. In fact, the intergenus hybridization between diploid *Hordeum* and diploid *Triticum–Aegilops* species is impossible today. Even in artificial crossing between hexaploid wheat and *Hordeum vulgare*, successful production of the hybrids requires 2,4-D treatment just after crossing.

Petersen et al. (2006) analyzed sequences of two single copy nuclear (*DMC1* and *EF-G*) genes and one plastid (*ndhF*) gene, and concluded that one of the *Sitopsis* species, *Ae. speltoides*, had the BB genome and was the cytoplasmic donor of *T. aestivum*. Salse et al. (2002) also reported sequence similarity of the *SPA* gene region between BB and SS genomes. However, Takahashi et al. (2009b) showed that *Ae. speltoides* was the GG genome donor to *T. timopheevii* based on analysis of the *PolA1* gene. Thus, the *PolA1* gene may be a component of IGF, which was probably introduced into an ancestor of *Ae. speltoides* and other *Sitopsis* species from *Hordeum* species by introgression.

Dasyxypyrum villosum may also be an introgressive hybrid species because its *PolA1* gene was related to those of *Hordeum* species, whereas the PSID sequence was of the P1 type found in *Triticum–Aegilops* species. The relationships of *Secale cereale* and some *Aegilops* species should be confirmed from the viewpoint of hybrid speciation because of inconsistency in phylogenies between *PolA1* and PSID sequences (Table 1 and Fig. 4). Sasano et al. (2004) also reported the incongruity between chloroplast and nuclear data in the genus *Aegilops*.

Based on the analysis of *EF-G* gene in *Hordeum*, Komatsuda et al. (1999) reported that II and XuXu genome species were separated from XuXa and HH genome species. Analysis of thioredoxin-like gene and *RPB2* gene showed the same patterns (Kakeda et al. 2009, Sun et al. 2009). In the present study, II, XuXu, and XuXa genome species were clustered into a single clade based on the *PolA1* gene (Fig. 3). As the same P6 type PSID sequence was shared with XuXu and XuXa genome (Table 1), *H. marinum* may be a hybrid species. In the HH genome, two Eurasian species, *H. rosinetii* and *H. brevisubulatum*, and seven American species, *H. erectifolium*, *H. pulibiflorum*, *H. chilense*, *H. intercedens*, *H. pusillum*, *H. euclaston*, and *H. patagonicum*, were separated into two clades; however, these species were closely related to each other to form a single monophyletic group (Komatsuda et al. 1999). PSID sequences of five HH genome species were identical (P7 type). These results suggested that the American HH
genome species were probably introduced from Eurasia (Blattner 2006, 2009) as American black rice was brought from Africa during the slave trade in the 17th century (Carney 2002).

Introgressive homoploid hybrid speciation may be a rather common mechanism responsible for the establishment of a significant number of species in Triticeae. The development of a new methodology will be required to resolve introgressive hybrid speciation. Although full genome sequencing may be a straightforward way to identify chromosomal fragments introduced by introgression, such analysis using limited numbers of species or accessions may lead to misleading phylogenetic reconstruction. Because PolAI gene contains species- or genus-specific variations (Zhang et al. 2008, Takahashi et al. 2009a, Takahashi et al. 2009b), in addition to ordinary phylogenetic analyses using many genes or DNA markers, comparative analysis of nuclear PolAI exon 20 and plastid PSID sequences will be useful to find evidence of introgressive homoploid hybrid speciation. If the tiny IGF in the Sitopsis genome was involved in their speciation, the Sitopsis species will be an excellent model in which to examine a novel mechanism of speciation of plants.

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Literature Cited


