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ORIGINAL PAPER

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Phylogenetic and evolutionary relationships between *Elymus humidus* and other *Elymus* species based on sequencing of non-coding regions of cpDNA and AFLP of nuclear DNA

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Abstract Species of the genus *Elymus* are closely related to some important cereal crops and may thus serve as potential alien genetic resources for the improvement of these crops. *E. humidus* is indigenous to Japan and is well adapted to a humid climate. However, the phylogenetic and evolutionary relationships between *E. humidus* and other *Elymus* species are unclear. To elucidate these relationships, we examined the sequences of three non-coding regions of chloroplast DNA (cpDNA) and the amplified fragment length polymorphism (AFLP) variation of nuclear DNA in *E. humidus* and other related species. A total of 15 sequence mutations from the three non-coding regions, *trnL-trnF*, *trnF-ndhJ*(C), and *atpB-rbcL*, covering approximately 1,800 bp, were detected in the *Elymus* species. A phylogenic tree resulting from the cpDNA sequence data revealed that all the species containing the St nuclear genome (St, StH, StY, and StHY) formed a well-supported clade that is remote from the *Hordeum* species (H). This result strongly supports the finding that *Pseudoroegneria* is the maternal genome donor to the genus *Elymus.* In addition, *E. humidus* showed the closest relationship with the cpDNA genome of the *Pseudoroegneria* species. The AFLP analysis detected 281 polymorphic bands with 11 AFLP primer combinations. The AFLP result showed that *E. humidus* is relatively closer to *E. tsukushiensis*. However, the cpDNA sequencing results indicated that *E. humidus* and *E. tsukushiensis* have different cytoplasmic origins. Our results suggest that the evolutionary process between *E. humidus* and *E. tsukushiensis* is not monophyletic, although the two species have similar morphological characters and adaptability.

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Introduction

The genus *Elymus* is the largest genus in the tribe Triticeae with about 150 species distributed in most temperate regions of the world (Dewey 1984). Cytologically, this genus contains an St (from *Pseudoroegneria*) genome combined with an H (from *Hordeum*) genome, a Y (from an unknown donor) genome, a P (from *Agropyron*) genome, or a W (from *Australopyrum*) genome (Jensen 1990). *Elymus* species are closely related to some important cereal crops, such as wheat, barley, and rye, and may thus serve as potential alien genetic resources for the improvement of these crops. *E. humidus* is a species that is indigenous to Japan and that has not been found in other countries. It is well adapted to a humid climate and is usually found around paddy fields. In this respect, *E. humidus* differs from wheat, which originated in a relatively dry climate. Ban (1997) examined the resistance of some *Elymus* species to *Fusarium* head blight (FHB). He found that an *E. humidus* accession, AG91-35, and an *E. racemifer* accession, AG91-24, had higher resistance to penetration of FHB than the resistance found in wheat-resistant cultivars Sumai 3 and Nobeokabouzu-komugi. *Roegneria ciliaris* (*E. racemifer*) is a perennial tetraploid species that is distributed throughout Asia and that is well adapted to high-humidity environments. Eight of 14 chromosomes of *R. ciliaris* have been transferred into wheat for improvement of FHB resistance (Wang et al. 2001). Therefore, such species might be useful as tertiary genetic resources for introduction of useful characters into wheat.

E. humidus is morphologically similar to *E. tsukushiensis* and *E. dahuricus*. The latter two species were widely distributed in Asia. These three species have identical genome constitutions (2*n*=6*x*=42, StStHHYY). *E. humidus* can be distinguished from *E. tsukushiensis* by its wingless keels, but this is difficult when the two species are dried as specimens. Moreover, these two species can produce a natural F_1 hybrid, although the F_1 plants are highly sterile (Sakamoto 1966; Sakamoto and Matsumura 1966). However, it is unclear how *E. humidus*

is genetically differentiated from *E. tsukushiensis* and other *Elymus* species growing in East Asia.

Analyses of chloroplast DNA (cpDNA) genomes have been employed to elucidate the phylogenetic relationships of different taxa from the maternal side (Olmstead and Palmer 1994). Compared with coding regions, non-coding regions may provide more informative characters in phylogenetic studies at the species level because of their high variability due to the lack of functional constraints. Non-coding regions of cpDNA have been assayed for phylogenic studies of *Elymus* and other plant species either by direct sequencing (Manen and Natall 1995; McDade and Moody 1999; Xu et al. 2000; Mason-Gamer et al. 2002) or by restriction site analysis of PCR products (PCR-RFLP) (Wolfe et al. 1997; Cipriani et al. 1998; Xu et al. 2001; McMillan and Sun 2003).

On the other hand, analysis of nuclear DNA genome may elucidate other aspects of the phylogeny and evolutionary relationships of *Elymus* species. In this study, we used amplified fragment length polymorphism (AFLP) to study the nuclear DNA variations of the *Elymus* species. The usefulness and efficiency of AFLP for discriminating related species and for assessing their genetic variation have been demonstrated by previous studies (Mackill et al. 1996; Le Thierry d'Ennequin et al. 2000; Sasanuma et al. 2002). Combining the results of organelle and unclear DNA may give a clear picture of the genetic relationships between *E. humidus* and other *Elymus* species.

Materials and methods

Plant materials

The plant materials used in this study included 12 hexaploid *Elymus* species (StHY) accessions, four tetraploid *Elymus* species (StH and StY) accessions, and diploid species including three *Pseudoroegneria* species (St) accessions and two *Hordeum* species (H) accessions (Table 1). In addition, some species of the genus *Aegilops* and *Triticum* were included as out-groups for cpDNA sequencing. DNA extraction was carried out as described by Doyle and Doyle (1990).

cpDNA non-coding regions sequencing

Three non-coding regions, *trnL-trnF*, *trnF-ndhJ* (C), and *atpB-rbcL*, were selected for sequencing. *trnL-trnF* and *atpB-rbcL* have been extensively used for phylogenetic studies of plant species (Manen and Natall 1995; McDade and Moody 1999; Xu et al. 2000). To amplify the three non-coding regions, three primer pairs were designed utilizing wheat cpDNA sequences (Ogihara et al. 2000) (Table 2). The PCR reaction mixture contained 50 ng of total genomic DNA, 0.25 μM of each primer, 100 μM of dNTPs, 0.5 units of *Taq* polymerase (Toyobo, Osaka, Japan), and PCR buffer containing 50 μM of KCl, 10 μM of Tris-HCl pH 8.3, and 1.5 mM $MgCl_2$ in a total volume of 20 μl. PCR was performed on a thermocycler GeneAmp PCR system 9700 (Perkin Elmer/Applied Biosystems, Foster City, Calif., USA) using the following program: 30 cycles of 94°C for 20 s, 58°C for 30 s, and 72°C for 2 min. PCR products were cloned into vectors with the pGEM-T Easy kit (Promega, Madison, Wis., USA). Cloned fragments were subjected to sequencing with the CEQ sequencing kit (Beckman Coulter, Fullerton, Calif., USA) on a Beckman2000 Sequencer following the

Table 1 Plant materials used in this study

Species[a]	Accession	Origin	Nuclear genome constitution
Elymus humidus	AG98-7	Japan	StStHHYY, 2*n*=6*x*=42
E. humidus	AG98-6	Japan	StStHHYY, 2*n*=6*x*=42
E. humidus	AG98-10	Japan	StStHHYY, 2*n*=6*x*=42
E. humidus	AG98-12	Japan	StStHHYY, 2*n*=6*x*=42
E. humidus	EB12	Japan	StStHHYY, 2*n*=6*x*=42
E. tsukushiensis	AG98-1	Japan	StStHHYY, 2*n*=6*x*=42
E. tsukushiensis	AG98-3	Japan	StStHHYY, 2*n*=6*x*=42
E. tsukushiensis	EB18	Japan	StStHHYY, 2*n*=6*x*=42
E. tsukushiensis	EB22	Japan	StStHHYY, 2*n*=6*x*=42
E. dahuricus	98E-10	Pakistan	StStHHYY, 2*n*=6*x*=42
E. dahuricus	98E-16	China	StStHHYY, 2*n*=6*x*=42
E. dahuricus	98E-32	Russia	StStHHYY, 2*n*=6*x*=42
E. strictus	EB51	China	StStYY, 2*n*=4*x*=28
E. barbicallus	EB43	China	StStYY, 2*n*=4*x*=28
E. caninus	PI564915	Russia	StStHH, 2*n*=4*x*=28
E. mutabilis	PI564954	Kazakhstan	StStHH, 2*n*=4*x*=28
Pseudoroegneria spicata	PI236668	Canada	StSt, 2*n*=2*x*=14
P. stripifolia	PI313960	Russia	StSt, 2*n*=2*x*=14
P. stripifolia	PI325181	Russia	StSt, 2*n*=2*x*=14
Hordeum violaceum	PI531775	China	HH, 2*n*=2*x*=14
H. bogdanii	PI531761	China	HH, 2*n*=2*x*=14
T. monococum	AG01-1 (KT3-1)	–	AA, 2*n*=2*x*=14
Aegilops speltoides	AG01-3 (#D)	–	SS, 2*n*=2*x*=14
Ae. squarrosa	AG01-4 (KU2126)	–	DD, 2*n*=2*x*=14
T. durum	AG01-7 (Langdon)	–	AABB, 2*n*=4*x*=28
T. dicoccoides	AG01-8 (Israel A line)	–	AABB, 2*n*=4*x*=28
T. macha	AG01-9 (#627)	–	AABBDD, 2*n*=6*x*=64
T. aestivum	AG01-10 (Chinese Spring)	–	AABBDD, 2*n*=6*x*=42

[a] *Elymus strictus* and *E. barbicallus* were provided by R. von Bothmer, Department of Plant Breeding Research, The Swedish University of Agricultural Sciences, Sweden. *Pseudoroegneria spicata* and *Hordeum violaceum* were provided by Dr. Richard R.C. Wang, USDA-ARS, Forage and Range Research Laboratory, Utah State University, USA. Species of the genus *Aegilops* and *Triticum* were provided by Dr. N. Watanabe, Faculty of Agriculture, Gifu University, Japan

Table 2 Primers used to amplify the three non-coding regions of cpDNA

Region	Primer sequence (5′ to 3′)
trnL-trnF	CCGTCGACTTTATAAGTTGTG
	CACGAGGATTTTCAGTCCTC
trnF-ndhJ(C)	GAGGACTGAAAATCCTCGTG
	CTGGCCCTTACGTAAGGATT
atpB-rbcL	TTCTTCAATTGTGGAAGCCC
	GCTTTAAATCCAACACCTGC

manufacturer's instructions. DNA sequences of the cloned fragments were aligned using the program GENETYX-MAC (version 8.0, Software Development, Tokyo, Japan). Kimura's two-parameter estimates of genetic distance were calculated using the DNADIST program of PHYLIP software package (version 3.573c, Felsenstein 1995). The phylogenetic tree was constructed with the neighbor-joining (NJ) method based on the distance matrix by using the NEIGHBOR program of the PHYLIP software package.

AFLP analysis

About 500 ng of total genomic DNA was simultaneously digested with *Eco*RI and *Mse*I at 37°C for 6 h. Adaptors were ligated to the digested fragments with T4 DNA ligase. Pre-selective amplifications were performed with an *Eco*RI+A/*Mse*I+CA primer combination. After pre-selective amplification, selective amplifications were carried out with 11 *Eco*RI+3/*Mse*I+4 primer combinations (AAC/CAAC, AAC/CAGT, AAC/CATA, AAC/CATC, ACG/CAAG, ACG/CAGT, ACG/CATC, AGT/CAAG, AGT/CATC, ATC/CATC, and ATC/CAAG). Following the selective PCR, electrophoresis was performed on an ABI373 DNA sequencer (Perkin Elmer/Applied Biosystems). AFLP data were analyzed with GeneScan software (version 3.4). Each accession was scored for the presence (1) or absence (0) of each polymorphic band. Genetic distances between any pair of accessions were calculated using the RESTDIST program of the PHYLIP software. The genetic distance matrix was subjected to cluster analysis with the NJ method as described above.

Results

cpDNA sequencing

A total of 15 sequence mutations, including 11 single-base substitutions, two insertion/deletions, and two sequences with different numbers of single-base repeats, were detected in the three non-coding regions covering approximately 1,800 nucleotides in the *Elymus* species (Table 3). Sequence variations were detected between and within species. Combining the variants at the 15 mutations gave six cpDNA haplotypes that were designated as *cpI-1*, *cpI-2*, *cpII-1*, *cpII-2*, *cpIII*, and *cpIV*. Two cpDNA haplotypes (*cpI-1* and *cpI-2*) that differed by two mutations were detected in *E. tsukushiensis,* whereas three cpDNA haplotypes (*cpI-2*, *cpII-1*, and *cpII-2*) were found in *E. humidus*. One *E. humidus* accession, AG98-7, has a cpDNA haplotype (*cpI-2*) that is identical to that of two *E. tsukushiensis* accessions, EB18 and EB22. No cpDNA sequence variation was detected among or within *E. dahuricus*, *E. strictus*, and *E. barbicallus*, although their

Table 3 Sequence mutations detected in the three non-coding regions in the *Elymus* species

Species	Accession	cpDNA haplotype	*trnL-trnF*			*trnF-ndhJ*(C)						*atpB-rbcL*					
			#1[b]	#2	#3	#1	#2	#3	#4	#5	#6	#1	#2	#3	#4	#5	#6
E. tsukushiensis (2)[a]	AG98–1, AG98–3	*cpI-1*	-	$(C)_8$	T	G	A	C	C	A	$(T)_{10}$	A	A	A	-	A	T
E. tsukushiensis (2)	EB18, EB22	*cpI-2*	-	$(C)_8$	T	G	A	C	C	A	$(T)_9$	A	G	A	-	A	T
E. humidus (1)	AG98–7	*cpI-2*	-	$(C)_8$	T	G	A	C	C	A	$(T)_9$	A	G	A	-	A	T
E. humidus (3)	AG98–6, AG98–10, AG98–12	*cpII-1*	-	$(C)_9$	T	T	C	C	A	A	$(T)_8$	A	G	A	TGGAATTCCTATAG	A	C
E. humidus (1)	EB12	*cpII-2*	-	$(C)_9$	T	T	C	C	A	A	$(T)_9$	A	G	A	TGGAATTCCTATAG	A	C
E. dahuricus (3)	98E-10, 98E-16, 98E-32	*cpIII*	-	$(C)_8$	T	T	A	T	A	A	$(T)_9$	A	G	A	TGGAATTCCTATAG	C	C
E. strictus (1)	EB51	*cpIII*	-	$(C)_8$	T	T	A	T	A	A	$(T)_9$	A	G	A	TGGAATTCCTATAG	C	C
E. barbicallus (1)	EB43	*cpIII*	-	$(C)_8$	T	T	A	T	A	A	$(T)_9$	A	G	A	TGGAATTCCTATAG	C	C
E.caninus (1)	P1564915	*cpIV*	TT	$(C)_{10}$	G	T	A	T	A	C	$(T)_8$	C	G	G	TGGAATTCCTATAG	A	C
E. mutabilis (1)	P1564954	*cpIV*	TT	$(C)_{10}$	G	T	A	T	A	C	$(T)_8$	C	G	G	TGGAATTCCTATAG	A	C

[a] Numbers in parentheses indicate the number of accessions
[b] The first mutation detected in this non-coding region

ploidy number and genome constitution are different. These three *Elymus* species thus have the same cpDNA haplotype (*cpIII*). The two StH genome accessions (*E. caninus* and *E. mutabilis*) have the same cpDNA haplotype *cpIV*.

The cpDNA sequences of the *Pseudoroegneria* species showed a high level of similarity to the *Elymus* species sequences, especially to the *E. humidus* sequences. Of the three *Pseudoroegneria* accessions (two *P. stripifolia* accessions and one *P. strigosa* accession), the two *P. stripifolia* accessions have sequences identical to those of *E. humidus* (*cpII-2*), and the one *P. strigosa* accession differs from those of *E. humidus* (*cpII-2*) by a single base substitution out of the 1,800 cpDNA nucleotides. In contrast to the high level of sequence similarity between the *Pseudoroegneria* species and the *Elymus* species, many mutations were detected between the *Hordeum* species and the *Elymus* species.

A cpDNA phylogenetic tree based on the cpDNA sequences is shown in Fig. 1. All the species containing the St genome (St, StH, StY, and StHY) formed a well-supported clade that is remote from the *Hordeum* species (H). This result strongly suggests that *Pseudoroegneria* (St) is the maternal genome donor to *Elymus*, although we cannot rule out the possibility that an unknown species, which is thought to contribute the Y genome of *Elymus*, is the maternal genome donor. One *E. humidus* accession, AG98-7, is located in the *E. tsukushiensis* cluster. The remaining four *E. humidus* accessions, together with the two *P. stripifolia* accessions, formed a clade and connected with one *P. strigosa* accession, indicating that *E. humidus* has the closest relationship with the cpDNA genome of the *Pseudoroegneria* species. The phylogenetic relationships of the cpDNA genomes of the species of *Aegilops* and *Triticum* obtained in this study are in accordance with those determined in previous studies (Tsunewaki and Ogihara 1983; Ogihara and Tsunewaki 1988).

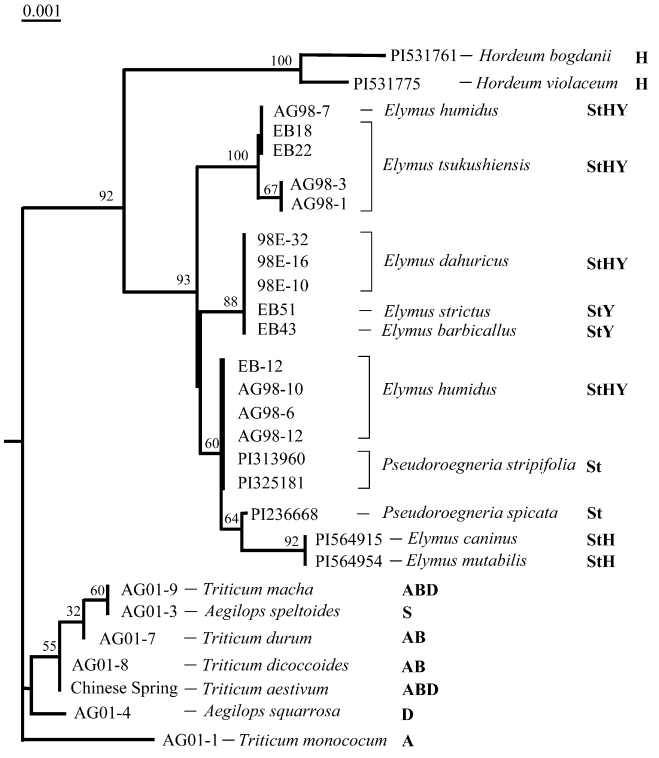

Fig. 1 Neighbor-joining tree based on sequence data of three non-coding regions of cpDNA with *Triticum monococum* as an outgroup species. *Scale bar* indicates the Kimura's two-parameter genetic distance. Bootstrap values (%) based on 100 replicates are shown beside *nodes*. Nuclear genomes of each species are given in *bold*

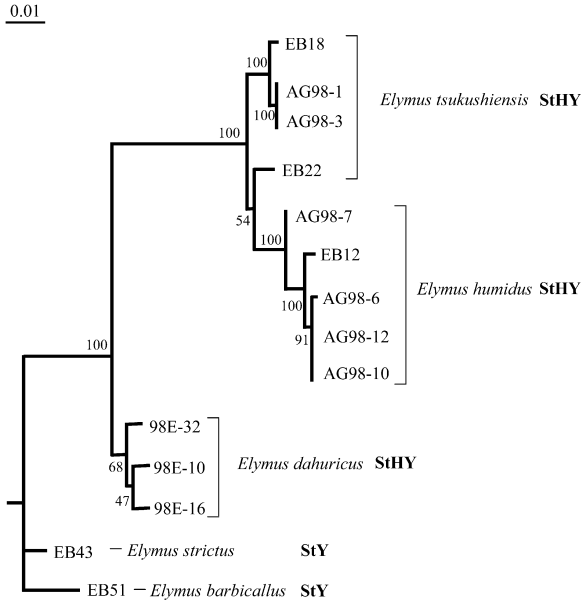

Fig. 2 Neighbor-joining tree based on amplified fragment length polymorphism data with *Elymus strictus* as an outgroup. *Scale bar* indicate the genetic distance. Bootstrap values (%) based on 100 replicates are shown beside *nodes*. Nuclear genomes of each species are given in *bold*

AFLP variation between and within the *Elymus* species

AFLP analysis was performed only on the hexaploid *Elymus* species with two tetraploid *Elymus* species as outgroups. The 11 primer combinations that we used produced a total of 281 AFLP polymorphic bands. AFLP variation was detected between and within species. In addition, many species-specific bands were detected. Two *E. humidus* accessions, AG98-10 and AG98-12, and two *E. tsukushiensis* accessions, AG98-1 and AG98-3, have no polymorphism. This suggests that each of these pairs of accessions were derived from the same origin, although AG98-10 and AG98-12 were collected from different places. One *E. humidus* accession, AG98-7, which has the *E. tsukushiensis* cpDNA haplotype, seems to be a natural hybrid between *E. tsukushiensis* and *E. humidus* because (1) it shares most of the species-specific bands of *E. tsukushiensis* and *E. humidus*, (2) it has almost twice the number of polymorphic bands that the other materials

have, and (3) the intensities of most of the bands of AG98-7 are only half of the intensities of the bands of the other materials. Each of these three reasons also indicates that the *E. humidus* AG98-7 is heterozygous. An NJ tree (Fig. 2) constructed with the AFLP data shows that *E. humidus* and *E. tsukushiensis* formed an upper cluster that is remote from the other three species, *E. dahuricus*, *E. strictus*, and *E. barbicallus*. The putative natural hybrid of *E. humidus* and *E. tsukushiensis*, AG98-7, is located between the clusters of the two species.

Discussion

Based on sequence data of the chloroplast *ndhF* gene, Redingbaugh et al. (2000) found that there is a strong preference for cpDNA inheritance from the St genome-containing parent in hybridization between different Triticeae species. Mason-Gamer et al. (2002) reported that *Pseudoroegneria* is the maternal genome donor to North American tetraploid *Elymus*. A similar result was obtained by McMillan and Sun (2003) with a PCR-RFLP analysis of cpDNA in tetraploid *Elymus* species containing an StH or StY genome. In our study, we investigated the cpDNA sequence of tetraploid and hexaploid *Elymus* with different nuclear genome combinations (StH, StY, StHY) from Asia. Our result further suggested that *Pseudoroegneria* is the maternal genome donor to *Elymus* species, regardless of their genome constitutions and distribution, although we cannot rule out the possibility that an unknown species, which is thought to contribute the Y genome of *Elymus*, is the maternal genome donor. McMillan and Sun (2003) did not clearly separate the StH genome species from StY genome species based on their cpDNA PCR-RFLP analysis. In our phylogenic tree based on cpDNA sequence, however, the two StH genome species (*E. caninus* and *E. mutabilis*) showed divergences from the Y genome containing *Elymus* species (Fig. 1). Because the donor species of the Y genome of *Elymus* is unknown, we can not identify whether the cpDNA differentiation between StH genome species and the Y genome containing *Elymus* species is contributed by the unknown species that is thought to be the donor of the Y genome of *Elymus* species.

E. humidus is very similar to *E. tsukushiensis* in morphological characters. It is difficult to distinguish the two species when they are dried as specimens. Moreover, the two species have identical genome constitutions and can produce a natural F_1 hybrid, although the F_1 plants are highly sterile (Sakamoto 1966; Sakamoto and Matsumura 1966). The present study revealed that the two species are not only differentiated in the nuclear genome as revealed by AFLP analysis, but also in the chloroplast genome. The cpDNA haplotype *cpII-*, which is a representative haplotype of *E. humidus*, differed from the cpDNA haplotype *cpI-*, which is a representative haplotype of *E. tsukushiensis*, by at least six mutation events. Moreover, the *E. humidus* species showed a closer relationship with *Pseudoroegneria* than *E. tsukushiensis* and other *Elymus* species examined. In terms of the cytoplasmic genome, the genetic differentiation between *E. humidus* and *E. tsukushiensis* is comparable with that between *T. aestivum* and *Ae. squarrosa* (Fig. 1), indicating that *E. humidus* and *E. tsukushiensis* have different cytoplasmic genome origins.

Based on a nuclear DNA AFLP analysis, Sasanuma et al. (2002) estimated that *E. humidus* diverged from *E. tsukushiensis* about one million years ago. However, the results obtained in this study do not support the hypothesis that *E. humidus* directly diverged from *E. tsukushiensis*. The fact that the two species have different cytoplasmic origins indicates that the evolutionary relationship of *E. humidus* and *E. tsukushiensis* is not monophyletic but polyphyletic, i.e., one involving out-crossing. The AFLP analysis of nuclear DNA showed that *E. humidus* and *E. tsukushiensis* formed an upper cluster that is remote from other species, whereas the cpDNA result showed that these two species have different cytoplasmic origins and are more distantly related. A discrepancy between the phylogeny based on nuclear DNA and the phylogeny based on cpDNA was also found by Mason-Gamer and Kellogg (1996) and Redingbaugh et al. (2000). This discrepancy may reflect the different histories of the chloroplast and nuclear genomes. Because the chloroplast genome is generally uniparentally inherited, the evolutionary history of chloroplast genome may not reflect the evolutionary history of the organism, especially in a tribe in which out-crossing is so common.

One accession, AG98-7, was regarded as *E. humidus* based on its morphological characters when it was collected from the field. Sasanuma et al. (2002) assumed this accession was a natural hybrid of species *E. humidus* and *E. tsukushiensis* based on their AFLP analysis. The present study further revealed that the AG98-7 accession has the *E. tsukushiensis* cpDNA haplotype and is heterozygous. Thus, *E. tsukushiensis* appears to have served as the maternal parent of the putative natural hybrid. Natural hybrids between wild members of the Triticeae tribe have been frequently reported in plant communities where several species live sympatrically (Stebbins et al. 1946; Sakamoto 1966; Scotti et al. 2002). The genetic characters of the accession AG98-7 show that it is a living example of natural hybridization between different species in the genus *Elymus*. The results obtained in the present study imply that natural hybridization plays an important role in producing new species in the Triticeae tribe.

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