Genetic diversity of three *Elymus* species indigenous to Japan and East Asia (*E. tsukushiensis*, *E. humidus* and *E. dahuricus*) detected by AFLP

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With the aim of broadening the gene pool of Triticeae for crop improvement, the genetic diversity of three hexaploid *Elymus* species indigenous to Japan and East Asia (*E. tsukushiensis*, *E. humidus* and *E. dahuricus*) was evaluated using amplified fragment length polymorphism (AFLP) markers. A total of 34 accessions: five of *E. tsukushiensis*, 13 of *E. humidus*, 15 of *E. dahuricus*, and one tetraploid *Elymus* species as an outgroup, were analyzed. The AFLP analysis was conducted with 12 selective primer combinations. In all, 329 bands were scored. The average number of bands scored per accession was 135.3. Among the 34 accessions, no polymorphism was detected among 10 accessions of *E. humidus* and among three accessions of *E. tsukushiensis*. Each species showed a diagnostic band pattern. One accession of *E. humidus* showed a band pattern intermediate between those of *E. humidus* and *E. tsukushiensis*, suggesting the occurrence of interspecific introgression. The level of intraspecific variation was highest in *E. dahuricus* (1-psb = 0.17, π = 0.010), followed by *E. tsukushiensis* (1-psb = 0.14, π = 0.008) and *E. humidus* (1-psb = 0.03, π = 0.002). The level of interspecific variation was higher than that of intraspecific variation in all the pairs compared. The level of divergence between *E. humidus* and *E. tsukushiensis* was relatively low, but even so it was two times higher than that of the intraspecific variation for each species. The phylogenetic tree constructed here showed clear separations of all the species. The results of this study indicate a clear divergence of *E. humidus* from *E. tsukushiensis* in spite of the morphological similarity between these two species. Based on the values of nucleotide diversity, the time of divergence between *E. humidus* and *E. tsukushiensis* was estimated to be 0.9–1.2 million years ago.

INTRODUCTION

The genus *Elymus* L. is the largest genus in the tribe Triticeae. It comprises about 150 species (Dewey, 1984). The genus is widely distributed in divergent ecogeographical regions of Europe, Asia, Australia, and North and South America. *Elymus* species, tolerant to various biotic and abiotic stresses, have a close relationship to important crops like wheat, barley and rye. This genus is therefore considered to be a potential source for cereal crop improvement.

Elymus tsukushiensis Honda (= *Agropyron tsukushiense* Ohwi, Japanese vernacular name “Kamojigusa”) is distributed in East Asia. This species is common in Japan and well adapted to the humid Japanese climate. *E. humidus* Osada (= *A. humidum* Ohwi et Sakamoto, Japanese vernacular name “Mizutakamoji”) is indigenous to Japan. This species is thought to be more adapted to moist areas than *E. tsukushiensis* since it is commonly found around paddy fields. The adaptation to a humid climate is an important character for the improvement of Triticeae cereals because cereals originating in dry areas are sensitive to humidity and humidity-related biotic stresses such as *Fusarium* head bright (FHB) disease. Several researchers have evaluated and tried to use *E. tsukushiensis* and *E. humidus* as sources of humidity tolerance or FHB resistance (Muramatsu et al., 1983; Weng

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and Liu, 1989; Weng et al., 1993; Ban, 1997). Their findings have confirmed the potential usefulness of *E. tsukushiensis* and *E. humidus* as sources for Triticeae cereal improvement. In spite of the potential importance of these two Japanese indigenous *Elymus* species, there is little information available on the genetic diversity of these species. In fact, *E. humidus* is a taxonomically ambiguous species because of its morphological similarity to *E. tsukushiensis* (Ohwi and Sakamoto, 1964; Sakamoto, 1978). Furthermore, a cytological study indicated that the two Japanese *Elymus* species appear to have identical chromosomes (Sakamoto and Muramatsu, 1966). Sakamoto (1966), on the other hand, found that F_1 hybrids between *E. humidus* and *E. tsukushiensis* are highly sterile.

E. dahuricus Turcz. ex Griseb. is another species widely distributed in East Asia. There is wide morphological and genetic variation within this species (Agafonov et al., 2001). In Japan, *E. dahuricus* is usually found around the seashore and mountainous grasslands (the Japanese vernacular names for the seashore and mountainous grassland types are "Hamamugi" and "Yamamugi", respectively) (Osada, 1993). *E. dahuricus* is genomically close to the Japanese indigenous *Elymus* and can produce F_1 hybrids with *E. tsukushiensis*, although they are sterile (Sakamoto, 1982).

In this study, we evaluated the genetic diversity of three *Elymus* species indigenous to Japan and East Asia by using amplified fragment length polymorphism (AFLP) markers (Vos et al., 1995). The AFLP technique is useful for the evaluation of genetic diversity because of its ability to examine number of loci at one time and its high reproducibility. To date several AFLP analyses have been conducted to evaluate the genetic diversity of grass family species, e.g., rice (Mackill et al., 1996), perennial ryegrass (Guthridge et al., 2001), wild barley (Pakniyat et al., 1997) and wild wheat (Heun et al., 1997; Sasanuma et al., 2002). Here we evaluated the level of interspecific divergence and intraspecific variation of three *Elymus* species, as noted above. Based on the results obtained, the genetic relationship between *E. humidus* and *E. tsukushiensis*, and the potential usefulness of the indigenous Japanese *Elymus* for cereal improvement are discussed.

MATERIALS AND METHODS

Plant materials Thirty-four accessions of *Elymus* were used in this study (Table 1). Among them five accessions were *E. tsukushiensis*, 13 were *E. humidus* and 15 were *E. dahuricus*. All three of these species are hexaploid. As an outgroup species, one accession of tetraploid *Elymus* (*E. yezoensis* Osada) was used. All of the accessions of *E. tsukushiensis*, *E. humidus* and *E. yezoensis* were originally collected in Japan. Regarding *E. dahuricus*,

three accessions originated in Pakistan, six in China and six in Russia. The accessions EB18, EB22, EB12, EB13 and EB35 were provided by Dr. S. Taketa, Research Institute for Bioresources, Okayama University, Japan. The accessions of *E. dahuricus* were provided by Prof. R. von Bothmer, Department of Plant Breeding Research, Swedish University of Agricultural Science, Sweden. The remaining accessions were collected by one of the present authors (T. Ban) in 1998.

AFLP analysis Total DNA was extracted from 200 mg of fresh leaves of each accession by the modified CTAB method (Murray and Thompson, 1980). Approximately 500 ng of total DNA per sample was digested with *Eco*RI and *Mse*I and ligated with *Eco*RI adaptor and *Mse*I adaptor (Applied Biosystems). The ligated DNA was preamplified using preselective primers for *Eco*RI and *Mse*I (Applied Biosystems) with rTaq DNA polymerase (TOYOBO). The conditions for preselective PCR were 1 cycle of 72°C, 2min; 20 cycles of 94°C, 20 sec; 56°C, 30 sec; 72°C, 2 min; and finally 1 cycle of 60°C, 30 min. The product of preamplification was amplified with 12 different combinations of selective primers (Applied Biosystems). The primer combinations used in this study were E-ACA and M-CAAC; E-AGC and M-CTAT; E-ACC and M-CAAG; E-AAG and M-CAAC; E-ACG and M-CACT; E-ACG and M-CAAG; E-AGC and M-CAAC; E-AGC and M-CACT; E-AAC and M-CACA; E-ACG and M-CGAC; E-AGG and M-CACG; and E-ACA and M-CACT. The conditions for selective PCR were 1 cycle of 94°C, 2min; 10 cycles of 94°C, 20 sec; 66°C, 30 sec; 72°C, 2 min, with a decrease of the temperature in the second step by 1°C for each cycle; 20 cycles of 94°C, 20 sec; 56°C, 30 sec; 72°C, 2 min; and finally 1 cycle of 60°C, 30 min. The products of selective amplification were electrophoresed in a 6% acrylamide gel for 3 hours at 50 W and detected using a DNA Silver Staining System (Promega).

Data analysis The bands were scored manually within the range of 100 bp to 400 bp. To evaluate the genetic diversity, the average value of 1 minus the proportion of shared bands for each pair of samples (1-psb) was calculated. Based on the value of 1-psb, the nucleotide diversity (π or d), i.e., the average number of nucleotide substitutions per nucleotide site, was estimated following the method of Innan et al. (1999). π and d are estimated essentially identically but when they are used for intra- and inter-specific comparison they are usually called π and d , respectively. The maximum parsimonious phylogenetic tree was constructed using the computer software PAUP 3.1.1 (Swofford, 1993) and PHYLIP 3.57c (Felsenstein, 1995).

Table 1. Plant materials used in the present study

Species	2n	Accession	Original code	Country	Collection site	Year of collection
<i>E. tsukushiensis</i>	6x	AG98-1		Japan	Fukuoka	1998
		AG98-2		Japan	Fukuoka	1998
		AG98-3		Japan	Fukuoka	1998
		EB18	JP08-97	Japan	unknown	
		EB22	JP18-97	Japan	unknown	
<i>E. humidus</i>	6x	AG98-4		Japan	Fukuoka	1998
		AG98-5		Japan	Fukuoka	1998
		AG98-6		Japan	Fukuoka	1998
		AG98-7		Japan	Fukuoka	1998
		AG98-8		Japan	Fukuoka	1998
		AG98-9		Japan	Fukuoka	1998
		AG98-10		Japan	Fukuoka	1998
		AG98-11		Japan	Fukuoka	1998
		AG98-12		Japan	Fukuoka	1998
		AG98-13		Japan	Fukuoka	1998
		AG98-14		Japan	Fukuoka	1998
		EB12	3279S	Japan	unknown	
		EB13	5163S	Japan	unknown	
<i>E. dahuricus</i>	6x	98E7	H4007	Pakistan	Gilgit	1983
		98E9	H4077	Pakistan	Gilgit	1983
		98E10	H4083	Pakistan	Gilgit	1983
		98E12	H7066	China	Qinghai	1986
		98E13	H7099	China	Sichuan	1986
		98E15	H7253	China	Sichuan	1986
		98E16	H7283	China	Gansu	1986
		98E17	H7501	China	Xinjiang	1986
		98E18	H7597	China	Xinjiang	1986
		98E21	H10007	Russia	Altai	1990
		98E23	H10012	Russia	Altai	1990
		98E25	H10026	Russia	Altai	1990
		98E28	H10035	Russia	Altai	1990
		98E32	H10048	Russia	Altai	1990
		98E35	H10075	Russia	Altai	1990
<i>E. yezoensis</i>	4x	EB35	JP13-97	Japan	unknown	

RESULTS

Band scoring An AFLP profile of the 34 accessions of *Elymus* is shown in Fig. 1. The number of bands scored is summarized in Table 2. On average, 135.3 bands were scored per accession. Of the total 329 bands scored, 299 (90.9%) were found to be polymorphic. Of the 299 polymorphic bands, 45 (15.1%) were unique for the outgroup taxon. Excluding the outgroup taxon, the total number

of bands was found to be 302, of which 254 (84.1%) bands were polymorphic. For each species, 155–200 bands were scored, among which 30–52% were polymorphic within that species. Despite such a high proportion of polymorphic bands, 10 accessions of *E. humidus* (AG98-4, AG98-5, AG98-6, AG98-8, AG98-9, AG98-10, AG98-11, AG98-12, AG98-13 and EB13) had identical band patterns. Also, the band patterns of three accessions of *E. tsukushiensis* (AG98-1, AG98-2 and AG98-3) were

Fig. 1. AFLP profile of the 34 accessions of *Elymus* using the primer combination E-ACG and M-CGAC. Species names are indicated at the top. Approximate fragment size is indicated on the left. The order of the accessions from top to bottom in Table 1 corresponds to that from left to right in this figure.

identical to each other. Considering these results and the fact that those accessions were collected in very small area in Fukuoka prefecture, we used only one accession as a representative of the accessions showing the identical band patterns in the following genetic variation anal-

ysis.

Each species showed a species-specific band pattern. However, there was one exception in *E. humidus*: accession AG98-7 showed numerous bands different from those of the other accessions. If AG98-7 is excluded, the pro-

Table 2. Number of bands scored

Species	N ^a	Average number of bands per accession ^b	Number of bands	Number of polymorphic bands	Percentage of polymorphic band
<i>E. tsukushiensis</i>	5	129.2 ± 2.7	155	53	34.2
<i>E. humidus</i>	13	138.1 ± 1.0	163	49	30.1
<i>E. humidus</i> excl. AG98-7	12	138.1 ± 1.1	145	13	9.0
<i>E. dahuricus</i>	15	136.2 ± 4.1	200	103	51.5
Total	34	135.3 ± 5.7	329	299	90.9
Total excl. <i>E. yezoensis</i>	33	136.0 ± 4.1	302	254	84.1

^a: Number of accessions.

^b: Standard deviation is noted.

Table 3. Genetic diversity within each germplasm group

Species	N ^a	1-psb (× 100) ^b	Nucleotide diversity (π × 1000) ^b
<i>E. tsukushiensis</i>	3	13.8 ± 6.6	7.5 ± 3.8
<i>E. humidus</i>	4	9.1 ± 6.7	4.9 ± 3.7
<i>E. humidus</i> excl. AG98-7	3	3.1 ± 2.1	1.6 ± 1.1
<i>E. dahuricus</i>	15	17.2 ± 12.2	10.3 ± 9.0
within <i>E. dahuricus</i>			
Pakistan	3	5.3 ± 4.0	2.8 ± 2.1
China	6	12.4 ± 1.4	6.7 ± 0.8
Russia	6	5.2 ± 1.3	2.7 ± 0.7

^a: Number of accessions.

^b: Standard deviation is noted.

Table 4. Genetic diversity between germplasm groups

Comparison	N ^a	1-psb (× 100) ^b	Nucleotide diversity (d × 1000) ^b
<i>E. tsukushiensis</i> vs. <i>E. humidus</i>	12	28.2 ± 5.3	16.9 ± 3.6
<i>E. tsukushiensis</i> vs. <i>E. humidus</i> excl. AG98-7	9	30.7 ± 2.0	18.7 ± 1.5
AG98-7 vs. <i>E. tsukushiensis</i>	3	20.6 ± 4.3	11.7 ± 2.7
AG98-7 vs. <i>E. humidus</i> excl. AG98-7	3	15.1 ± 0.5	8.3 ± 0.3
<i>E. dahuricus</i> vs. <i>E. tsukushiensis</i>	45	49.6 ± 1.9	35.3 ± 2.0
<i>E. dahuricus</i> vs. <i>E. humidus</i>	60	51.2 ± 1.1	37.2 ± 1.2
<i>E. dahuricus</i> vs. <i>E. humidus</i> excl. AG98-7	45	51.4 ± 1.0	37.4 ± 1.1
within <i>E. dahuricus</i>			
Pakistan vs. China	18	11.8 ± 1.8	6.3 ± 1.0
Pakistan vs. Russia	18	16.6 ± 1.1	9.2 ± 0.7
China vs. Russia	36	15.5 ± 2.5	8.5 ± 1.5

^a: Number of pairs of accessions compared.

^b: Standard deviation is noted.

Fig. 2. One of the maximum parsimony trees constructed with a heuristic search using PAUP 3.1.1 (Swofford, 1993). *E. yezoensis* accession EB35 was used as the outgroup. The total branch length was 488. The consistency index (CI), retention index (RI) and rescaled consistency index (RC) were 0.613, 0.910 and 0.558, respectively. The scale is shown below the tree. Numbers on the branches indicate the probability for 100 bootstrap replications. Only probabilities of more than 50% are shown.

portion of polymorphic bands in *E. humidus* (= 30.1%) drops to 9.0%.

Genetic variation within a species The intraspecific genetic variation of the three *Elymus* species was estimated using two parameters, 1-psb and π (Table 3). As explained above, the number of accessions was reduced to three and four for *E. tsukushiensis* and *E. humidus*, respectively. For both parameters, *E. dahuricus* showed the highest level of variation. The other two species had relatively lower levels of intraspecific variation. For example, the values of 1-psb were 0.172, 0.138 and 0.091 for *E. dahuricus*, *E. tsukushiensis* and *E. humidus*, respectively. For *E. dahuricus*, the genetic variation within a region (country) was also estimated. Among the three regions, China had the highest level of variation, followed by Pakistan and Russia. The values of 1-psb were 0.124, 0.053 and 0.052 for China, Pakistan and Russia, respectively. This result was probably due to the wide range of collection sites of Chinese accessions. The level of intraspecific variation of two of the indigenous Japanese *Elymus* species was higher than or at least equal to that of the intraregional variation of *E. dahuricus*. However, when the AG98-7 accession is excluded, the level of intraspecific variation for *E. humidus* decreases to less than that of the intraregional variation of *E. dahuricus*.

Genetic divergence between species Each of the three hexaploid *Elymus* species showed a species-specific band pattern (Fig. 1). Several bands were diagnostic for each species. Twenty-two, 12 and four bands were diagnostic for *E. dahuricus*, *E. humidus* and *E. tsukushiensis*, respectively. These results emphasize the distinct genetic divergence of the three *Elymus* species. However, the band pattern of *E. humidus* (accession AG98-7, as mentioned above) did not conform to this generality, which suggests the occurrence of interspecific introgression. AG98-7 had several bands present in *E. tsukushiensis* but absent in *E. humidus*. The number of bands segregating *E. tsukushiensis* and *E. humidus* was 24, while that segregating *E. tsukushiensis* including AG98-7 and *E. humidus* excluding AG98-7 was 29. Thus, AG98-7 showed a band pattern intermediate between those of *E. humidus* and *E. tsukushiensis*. This result implies the possibility that accession AG98-7 originated via an interspecific hybridization between *E. humidus* and *E. tsukushiensis*.

The genetic variation between species is summarized in Table 4. As expected, the level of genetic variation between species was much higher than that within species. The level of interspecific variation between *E. tsukushiensis* and *E. humidus* was two or three times higher than that of the intraspecific variation for each species, and was also almost two times higher than that

of the interregional variation of *E. dahuricus*. For example, the value of 1-psb between *E. tsukushiensis* and *E. humidus* was 0.282, whereas those within *E. tsukushiensis*, within *E. humidus*, and between Pakistan and Russia in *E. dahuricus* were 0.138, 0.091 and 0.166, respectively. When the possible hybrid accession AG98-7 is excluded, the value of 1-psb between *E. tsukushiensis* and *E. humidus* increases to 0.307. The level of divergence between *E. dahuricus* and the two indigenous Japanese species was higher than that between *E. tsukushiensis* and *E. humidus*, suggesting that the divergence of the indigenous Japanese *Elymus* from *E. dahuricus* was an early evolutionary event. With respect to the possible hybrid accession AG98-7, the level of divergence between AG98-7 and *E. tsukushiensis* was higher than that between AG98-7 and *E. humidus*, but the two values were not markedly different. The value of 1-psb between AG98-7 and *E. tsukushiensis* was 0.0206 and that between AG98-7 and *E. humidus* was 0.0151.

Phylogenetic tree In the maximum parsimony tree, the three species were clearly separated (Fig. 2). *E. tsukushiensis* and *E. humidus* indicated the close genetic relationship to each other. Accession AG98-7 joined with the *E. humidus* cluster. With respect to intraspecific variation in *E. dahuricus*, three accessions from Pakistan and six accessions from Russia formed each clusters whereas six accessions from China did not form a clear cluster.

DISCUSSION

In this study, the divergence of *E. humidus* from *E. tsukushiensis* was clearly indicated by AFLP markers. The level of genetic variation between *E. humidus* and *E. tsukushiensis* was more than two times higher than that of the variation within each of these species, and also two times higher than that of the interregional variation of *E. dahuricus*. Based on these results, we concluded that *E. humidus* is genetically separated from *E. tsukushiensis* at the species level. Until now, the taxonomical identification of *E. humidus* from *E. tsukushiensis* was based on its morphological characters and growth habitat (Ohwi and Sakamoto, 1964). However, as stated by Sakamoto (1978), it is difficult to distinguish the two species after they are collected because they have quite similar morphology after they have been dried as specimens. Moreover, the two species appear to have identical chromosomes and can produce F_1 hybrids in nature, although they are highly sterile (Sakamoto and Muramatsu, 1966; Sakamoto, 1966). The present study is the first report evaluating the degree of divergence between *E. humidus* and *E. tsukushiensis* at the molecular level. Because the AFLP technique simultaneously surveys numerous loci throughout the genome, the results of this study indicate

that the differentiation of the two species was caused not only by changes in a few key character genes but also by genetic differentiation throughout the whole genome. Concerning the classification of *E. humidus*, however, it is still possible that *E. humidus* is included in the large intraspecific variation of *E. tsukushiensis*, because *E. tsukushiensis* is known to have wide morphological (and probably genetic) variation. The materials of *E. humidus* and *E. tsukushiensis* used in the present study were collected from very limited areas in Japan. If materials collected from more diverse areas were used, the level of intraspecific variation of both the Japanese *Elymus* species might increase. In that case, we would need to consider the possibility that *E. humidus* is one of the genetically differentiated varieties of *E. tsukushiensis*. However, we still suppose that *E. humidus* should be classified as a different species from *E. tsukushiensis* for the following reasons: 1) the level of variation between *E. humidus* and *E. tsukushiensis* is much higher than that of the intraspecific variation of *E. dahuricus*, which is known to have high level of intraspecific variation, 2) the level of variation between *E. humidus* and *E. tsukushiensis* detected in this study is much higher than that of the intraspecific variation of other Triticeae species previously reported (Sasanuma et al., 2002, see also the last paragraph of this section). Thus, we suppose that even if *E. tsukushiensis* has the highest level of intraspecific variation in Triticeae species, this level should be less than that between *E. humidus* and *E. tsukushiensis*. To confirm this supposition, it will be necessary to collect and survey more accessions of *E. humidus* and *E. tsukushiensis* from wider geographical areas.

In this study we found an accession of a possible natural hybrid between *E. humidus* and *E. tsukushiensis*. As mentioned above, natural hybrids between these two species are sometimes observed. In nature, these hybrids have a distinguishable morphology from their parents and are sometimes recognized as a different species called "Ootachikamoji" as the Japanese vernacular name (Osada, 1993). However, in this study the possible hybrid accession AG98-7 showed no obvious morphological difference from the other accessions of *E. humidus*. Sakamoto (1966) observed the occurrence of backcrosses to F_1 hybrids of the two Japanese *Elymus* species in nature. He reported that the morphology of backcrossed hybrid plants was quite similar to one of their parental species. He also indicated that the backcrossed hybrid was seed fertile. Considering these previous and our present findings, we propose that accession AG98-7 is the progeny of a natural hybrid between *E. tsukushiensis* and *E. humidus* with backcrossing from *E. humidus*. Thus, our present results indicate the usefulness of molecular analysis for the detection of hybrids that have no obvious morphological differences from their parental species. Because the *Elymus* genus is one of the most complex tax-

onomic groups in Triticeae, the utilization of molecular techniques such as those used in this study could be a strong help for the identification and reclassification of species.

Our molecular data not only confirmed the genetic relevance of the previous morphological classification, but also provided new insights into *Elymus* genetics. One of the new insights was the estimation of the time of divergence. According to Nei (1987), the time of divergence of two species (T) can be estimated using the formula: $d = 2\lambda T$, if the rate of nucleotide substitutions per site per year (λ) and nucleotide diversity between the two species (d) are available. Based on this formula, we estimated the time of divergence of *E. humidus* from *E. tsukushiensis*. Here, we used the net distance between *E. humidus* and *E. tsukushiensis* as d in the formula. The net distance between species A and B is estimated by the formula: $\text{net (A and B)} = d_{AB} - (\pi_A + \pi_B)/2$ (Nei, 1987). Then, the net distance between *E. humidus* and *E. tsukushiensis* is 14.2×10^{-3} (if accession AG98-7 is excluded). The average synonymous substitution rate in plant nuclear genes is $5.8\text{--}8.1 \times 10^{-9}$ (Wolfe et al., 1987). When it is used as λ in the formula, the time of divergence between *E. humidus* and *E. tsukushiensis* is estimated to be $0.9\text{--}1.2 \times 10^6$ years ago. This estimate indicates that the two species diverged approximately one million years ago (MYA). This is slightly shorter than that for the time of interspecific divergence of other Triticeae species (1.2–7.4 MYA, Sasanuma et al., 1996; Allaby et al., 1999) but not markedly shorter. Thus, we consider that *E. humidus* is not a species newly formed by rapid adaptation to a humid climate but rather a species that diverged genetically from *E. tsukushiensis* long ago (at least 1 MYA). This indicates that the divergence of *E. humidus* from *E. tsukushiensis* occurred during the glacial era. Based on this fact, we suppose that *E. humidus* was subjected to several drastic climate changes such as glacial and interglacial periods, and gradually gained the adaptation to the present humid climate in Japan. Hence, *E. humidus* can be regarded as a promising potential source of resistance to humidity and humidity-induced diseases in Triticeae cereal breeding. In addition, the estimate implies the possibility that *E. humidus* was or even is distributed in other East Asian countries, because the estimated time of divergence of *E. humidus* precedes the separation of the Japanese islands from the Eurasian Continent. To address this point, more field surveys in East Asian countries will be necessary.

In this study, genetic variation within each species was also evaluated. The estimate of π for *E. dahuricus* ($= 0.010$) was close to the value estimated based on AFLP for other plant species, e.g. $\pi = 0.008$ in soybean (Maughan et al., 1996; Innan et al., 1999), 0.006 in wild yam (Innan et al., 1999), 0.011 in *Arabidopsis thaliana* (Miyashita et al., 1999) and $0.010\text{--}0.012$ in wild wheat (Sasanuma et

al., 2002). In each of the two Japanese *Elymus* species, we also detected intraspecific variation despite the small range of collection sites. The value of π estimated for *E. tsukushiensis* ($= 0.008$) was in the same range as that for other plant species, although the accessions used in this study were derived from limited areas in Japan. This fact implies the possibility that *E. tsukushiensis* may have a high level of genetic diversity. With respect to *E. humidus*, the level of intraspecific variation ($\pi = 0.002$) was lower than the general level for other plant species. *E. humidus* is regarded as the most likely source of cereal crop improvements from the viewpoint of humidity adaptation. The present results indicate the fact that collection of germplasm of *E. humidus* from wide areas will be necessary to broaden the genetic diversity base of the available gene pool.

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REFERENCES

- Agafonov, A. V., Baum, B. R., Bailey, L. G., and Agafonova, O. V. (2001) Differentiation in the *Elymus dahuricus* complex (Poaceae): evidence from grain proteins, DNA, and crossability. *Hereditas* **135**, 277–289.
- Allaby, R. G., Banerjee, M., and Brown, T. A. (1999) Evolution of the high molecular weight glutenin loci of the A, B, D, and G genomes of wheat. *Genome* **42**, 296–307.
- Ban, T. (1997) Evaluation of resistance to *Fusarium* head blight in indigenous Japanese species of *Agropyron* (*Elymus*). *Euphytica* **97**, 39–44.
- Dewey, D. R. (1984) The genomic system of classification as a guide to intergeneric hybridization with the perennial Triticeae. In: Gene manipulation in plant improvement (ed.: J. P. Gustafson), pp. 209–279. Plenum Publishing Corporation, New York.
- Felsenstein, J. (1995) PHYLIP version 3.57c. Distributed by the author. Department of Genetics, University of Washington, Seattle, WA 98195, USA.
- Guthridge, K. M., Dupal, M. P., Kölliker, R., Jones, E. S., Smith, K. F., and Forster, J. W. (2001) AFLP analysis of genetic diversity within and between populations of perennial ryegrass (*Lolium perenne* L.). *Euphytica* **122**, 191–201.
- Heun, M., Schäfer-Pregl, R., Klawan, D., Castagna, R., Accerbi, M., Borghi, B., and Salamini, F. (1997) Site of einkorn wheat domestication identified by DNA fingerprinting. *Science* **278**, 1312–1314.
- Innan, H., Terauchi, R., Kahl, G., and Tajima, F. (1999) A method for estimating nucleotide diversity from AFLP data. *Genetics* **151**, 1157–1164.
- Mackill, D. J., Zhang, Z., Redona, E. D., and Colowit, P. M. (1996) Level of polymorphism and genetic mapping of AFLP markers in rice. *Genome* **39**, 969–977.
- Maughan, P. J., Saghai Maroof, M. A., Buss, G. R., and Huestis, G. M. (1996) Amplified fragment length polymorphism (AFLP) in soybean: species diversity, inheritance, and near-isogenic line analysis. *Theor. Appl. Genet.* **93**, 392–401.
- Miyashita, N. T., Kawabe, A., and Innan, H. (1999) DNA variation in the wild plant *Arabidopsis thaliana* revealed by amplified fragment length polymorphism analysis. *Genetics* **152**, 1723–1731.
- Muramatsu, M., Kaneta, S., Ikeda, R., Uetsuki, T., and Takahashi, K. (1983) Hybridization of Japanese indigenous *Agropyron* (*Roegneria*) species with hexaploid wheat and cytogenetics of some of the F_1 , BF_1 and amphiploid plants. In: Proc. 6th Intl. Wheat Genet. Symp., Kyoto, Japan (ed.: S. Sakamoto), pp. 1041–1048. Maruzen Co. Ltd., Kyoto.
- Murray, M. G., and Thompson, W. F. (1980) Rapid isolation of high molecular weight plant DNA. *Nucleic Acid. Res.* **8**, 4321–4325.
- Nei, M. (1987) Molecular evolutionary genetics. Columbia University Press, New York.
- Ohwi, J., and Sakamoto, S. (1964) Taxonomy, ecology and distribution of *Agropyron humidum* Ohwi et Sakamoto. *Journ. Jap. Bot.* **39**, 109–114.
- Osada, M. (1993) Illustrated grasses of Japan enlarged edition. Heibonsha Ltd. Publishers, Tokyo (in Japanese).
- Pakniyat, H., Powell, W., Baird, E., Handley, L. L., Robinson, D., Scrimgeour, C. M., Nevo, E., Hackett, C. A., Caligari, P. D. S., and Forster, B. P. (1997) AFLP variation in wild barley (*Hordeum spontaneum* C. Koch) with reference to salt tolerance and associated ecogeography. *Genome* **40**, 332–341.
- Sakamoto, S. (1966) Cytogenetic studies in the tribe Triticeae. IV. Natural hybridization among Japanese *Agropyron* species. *Japan. J. Genetics* **41**, 189–201.
- Sakamoto, S. (1978) Adaptation of two Japanese *Agropyron* species to the winter fallow paddy fields. *Weed Res. Japan* **23**, 101–108 (in Japanese).
- Sakamoto, S. (1982) Cytogenetical studies on artificial hybrids among *Elymus sibiricus*, *E. dahuricus* and *Agropyron tsukushiense* in the tribe Triticeae, Gramineae. *Bot. Mag. Tokyo* **95**, 375–383.
- Sakamoto, S., and Muramatsu, M. (1966) Cytogenetic studies in the tribe Triticeae. II. Tetraploid and hexaploid hybrids of *Agropyron*. *Japan. J. Genetics* **41**, 155–168.
- Sasanuma, T., Miyashita, N. T., and Tsunewaki, K. (1996) Wheat phylogeny determined by RFLP analysis of nuclear DNA. 3. Intra- and Interspecific variations of five *Aegilops* Sitopsis species. *Theor. Appl. Genet.* **92**, 928–934.
- Sasanuma, T., Chabane, K., Endo, T. R., and Valkoun, J. (2002) Genetic diversity of wheat wild relatives in the Near East detected by AFLP. *Euphytica*, **127**, 81–93.
- Swofford, D. L. (1993) PAUP (Phylogenetic Analysis Using Parsimony) version 3.1.1. Computer program distributed by the Illinois Natural History Survey, Champaign, Ill.
- Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., and Zabeau, M. (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acid. Res.* **23**, 4407–4414.
- Weng, Y., and Liu, D. (1989) Morphology, scab resistance and cytogenetics of intergeneric hybrids of *Triticum aestivum* L. with *Roegneria* C. Koch (*Agropyron*) species. *Scientia Agric. Sinica* **22**, 1–7 (in Chinese with English summary).
- Weng, Y. Q., Wu, L. F., Chen, P. D., and Liu, D. J. (1993) Devel-

opment of alien addition lines of wheat scab resistance from *Roegneria kamoji* C. Koch. In: Proc. 8th Intl. Wheat Genet. Symp., Beijing, China, (eds.: Z. S. Li and Z. Y. Xin), pp. 365–368. China Agric. Sci. Press, Beijing.

Wolfe, K. H., Li, W. H., and Sharp, P. M. (1987) Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast, and nuclear DNAs. Proc. Natl. Acad. Sci. USA **84**, 9054–9058.