Phylogeny of two tetraploid *Hordeum* species, *H. secalinum* and *H. capense* inferred from physical mapping of 5S and 18S-25S rDNA

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We previously showed that *H. secalinum* and *H. capense* are allotetraploids carrying the Xa genome of *H. marinum* and the I genome of an unidentified diploid species. In this study, intraspecific variation in each tetraploid species was investigated with regard to intergenomic translocations and chromosomal distribution of rDNA sites. Genomic *in situ* hybridization revealed that three *H. secalinum* accessions examined did not carry intergenomic translocations, but that two of three *H. capsense* accessions analyzed carried a pair of intergenomic translocations. Multicolour fluorescence *in situ* hybridization showed that *H. secalinum* included two types of rDNA pattern differing in the presence or absence of an extra 5S rDNA site in a submetacentric chromosome pair of the Xa-genome origin. The extra 5S rDNA site was found in all *H. capense* accessions examined. This 5S rDNA site is characteristic of *H. marinum* ssp. *gussoneanum*, but is absent in ssp. *marinum*. Polymorphisms in the 5S rDNA site infer that *H. secalinum* included two types, one having ssp. *gussoneanum* 2x and the other having ssp. *marinum*, as the Xa-genome donor. We conclude that *H. capense* originated from a limited number of *H. secalinum* accessions introduced probably through migrating birds to South Africa.

Key Words: chromosome, FISH, GISH, nucleolus organizer region, translocation, wild barley.

Introduction

The genus *Hordeum* (Triticeae) is classified into 31 species (in total 45 taxa), and 51 cytotypes exist at the diploid, tetraploid, and hexaploid levels with a basic chromosome number of $x=7$ (Baden and Bothmer 1994, Bothmer *et al.* 1995). Based on the metaphase I chromosome pairing in hybrids (Bothmer *et al.* 1986, 1995), the genus has been divided into four genome groups (H, I, Xa, and Xu). *H. vulgare* and *H. bulbosum* both carry the H genome, *H. marinum* carries the Xa genome, *H. murinum* has the Xu genome, and the remaining species share variants of the I genome (genome designation follows Linde-Laursen *et al.* (1997)). Taketa *et al.* (1999b) revealed by genomic *in situ* hybridization (GISH) that three polyploid species/cytotypes, *H. secalinum*, *H. capense* and the hexaploid cytotype of *H. brachyantherum*, were allopolyploids having a combination of the Xa and I genomes. These species are considered to originate as a result of natural hybridization between Xa- and I-genome species. A close relationship between Xa-genome and I-genome (designated as H-genome in Komatsuda *et al.* (1999)) species was revealed from a molecular phylogenetic study based on a nuclear gene (Komatsuda *et al.* 1999).

The genus *Hordeum* shows a wide distribution ranging from Eurasia to the North and South American Continents. One species, *H. capense*, is distributed in South Africa, distantly isolated from other species (Bothmer *et al.* 1995). There has been much debate about the origin of *H. capense* (Petersen and Seberg 2004). One possibility is that *H. capense* represents an ancient relict originated independently in South Africa, and the other is that *H. capense* is a recent human introduction of *H. secalinum* (a closely related species distributed mainly in Europe) to South Africa followed by diversification. Although GISH has shown that *H. capense* and *H. secalinum* share the same genomic constitution IXa (Taketa *et al.* 1999b), their progenitor species/subspecies and origins are still unclear. To address these questions, GISH and fluorescence *in situ* hybridization (FISH) techniques were applied in the present study.

Materials and Methods

Three accessions each of *H. secalinum* and *H. capense* were analyzed by GISH and FISH. *H. secalinum* accessions are from different countries of Europe. *H. capense* accessions are from different locations of South Africa. For comparison, several accessions of the *H. marinum* complex and one accession of *H. brachyantherum* ssp. *californicum* 2x were included. The *H. marinum* complex includes two subspecies ssp. *gussoneanum* ($2n = 2x = 14$ and $2n = 4x = 28$), and

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Table 1. Materials used in this study

Species	Accession	Collection site
<i>H. capense</i>	H334	Prov. Ventersted, Oviston, South Africa
	H335	Lesotho, Dogna, Mountain Road, South Africa
	H3923	Cape province, R406 towards South Africa
<i>H. secalinum</i>	H296	14kmE Huelva, Spain
	H231	Skåne, Saxån, at Landskrona, Sweden
	H3121	Cambridgeshire, between Hardwick, United Kingdom
<i>H. marinum</i> ssp. <i>gussoneanum</i> 2x		
	H28	distr. Szolnok, Hungary
	H826	CI, Vil Aydin, NE Soke, Turkey
<i>H. marinum</i> ssp. <i>marinum</i> 2x		
	H155	Dodecanisos, Greece
	H759	Crete E, S Ag. Nikolos, Greece
<i>H. marinum</i> ssp. <i>gussoneanum</i> 4x		
	H64	W Dushambe, Tadizhikistan
	H2303	California, Marin, co, 0.5 kmW San Quentin, USA
<i>H. brachyantherum</i> ssp. <i>californicum</i> 2x		
	H3317	California, Ventura c., USA

ssp. *marinum* ($2n = 2x = 14$), and these two subspecies are morphologically distinct (Bothmer *et al.* 1995). Materials are listed in Table 1.

Genomic *in situ* hybridization followed the methods of Taketa *et al.* (1999b) using *H. marinum* ssp. *gussoneanum* 2x (accession H826) total DNA as a probe and *H. brachyantherum* ssp. *californicum* 2x (accession H3317) total DNA as a blocking DNA. Double-target *in situ* hybridization using two ribosomal probes, pTa794 and pTa71, followed the methods of Taketa *et al.* (1999a). Clone pTa794 is a *Bam*HI fragment of the 5S rDNA, having a 120-bp coding sequence for the 5S rRNA gene and the intergenic spacers isolated from common wheat, *Triticum aestivum* L. (Gerlach and Dyer 1980). Clone pTa71, the 18S-25S rDNA is a 9-kb *Eco*RI fragment from common wheat, containing the coding sequences for the 18S, 5.8S, and 25S rRNA genes and the intergenic spacer sequences (Gerlach and Bedbrook 1979). For quadruple multicolor FISH, two DNA clones (pTa794 and pTa71), and total DNA of *H. marinum* ssp. *gussoneanum* 2x (Xa genome) were used as probes. Clone pTa794 was labeled with digoxigenin-11-dUTP by PCR. Clone pTa71 was labeled with biotin-11-dUTP by nick translation. Total DNA of Xa genome (accession H826) was labeled with Cy5-AP3-dUTP by nick translation. The *in situ* hybridization procedure described by Taketa *et al.* (1999a) was adopted. Slides were examined with an Olympus AX70 FL-1 epifluorescence microscope with appropriate filter sets (U-MWU for UV, U-MWIB for FITC, U-DM-Cy3 for Cy3, and U-DM-Cy5 for Cy5). Images were captured with a cooled CCD camera ORCAII C4742 (Hamamatsu Photonics) and analyzed with a software Image Pro Plus.

Results

Presence or absence of intergenomic translocations

H. capense and *H. secalinum* were analyzed by GISH. By using *H. marinum* ssp. *gussoneanum* 2x DNA (Xa genome) as a probe and *H. brachyantherum* 2x DNA (I genome) as a blocking DNA, the two genomes were clearly discriminated. Identical results were obtained when *H. marinum* ssp. *marinum* DNA was used as a probe for GISH (data not shown). In *H. capense*, two accessions (H334 and H335) carried a pair of intergenomic translocations (Fig. 1A), but the accession H3923 did not carry similar structures (Fig. 1B). Thus, *H. capense* was polymorphic in the presence or absence of intergenomic translocation. On the other hand, in *H. secalinum*, none of three accessions studied carried intergenomic translocations.

rDNA mapping

In *Hordeum*, ribosomal DNA sites are excellent chromosome markers to study phylogenetic relationships of species (Bustos *et al.* 1996, Taketa *et al.* 1999a, 2001, 2005). Both classes of rDNA sites are known to evolve dynamically, and our interpretation assumes that all rDNA sites are generally conserved in these species. Because *H. marinum* is shown to be a genome donor to *H. secalinum* and *H. capense* (Taketa *et al.* 1999b), rDNA variation of *H. marinum* was studied at first. In *H. marinum*, two subspecies are polymorphic in a 5S rDNA site; namely the diploid cytotype of ssp. *gussoneanum* carries an extra 5S site in the distal region of the long arm of a submetacentric chromosome pair (Fig. 1C). This extra 5S rDNA site is absent in ssp. *marinum* (Fig. 1D). However, other rDNA sites are commonly present in both diploid cytotypes (Fig. 2). These results generally confirm the previous reports of rDNA patterns in diploid cytotypes of *H. marinum* (Bustos *et al.* 1996, Taketa *et al.* 1999a). The tetraploid cytotype of ssp. *gussoneanum* was previously examined only by using 18S-25S rDNA probe (Linde-Laursen *et al.* 1992). In the present study, FISH analysis of ssp. *gussoneanum* 4x by using both 5S and 18S-25S rDNA probes, detected rDNA sites on three pairs of chromosomes (Fig. 1E); two of them are similar to those found in ssp. *gussoneanum* 2x, and the rest is similar to the submetacentric pair of ssp. *marinum* (Fig. 2). This result suggests that the tetraploid cytotype of ssp. *gussoneanum* is not a simple autotetraploid of its diploid cytotype.

To know which of the two subspecies of *H. marinum* is the donor of the Xa genome, rDNA patterns of *H. capense* and *H. secalinum* were studied, with particular attention to the presence or absence of the diagnostic 5S rDNA site. Genomic origin of chromosomes with rDNA sites was determined using multicolor FISH. In the accessions H334 and H335 of *H. capense*, a pair of intergenomic translocations was observed, but the translocated chromosomes are independent of the rDNA sites (Fig. 1F). The Xa-genome donor to these accessions is likely to be *H. marinum* ssp. *gussoneanum* 2x, due to the presence of the diagnostic

Fig. 1. Multicolor FISH on metaphase plates of various species and accessions. GISH using Xa genome as a probe (yellow) and I genome as a blocking DNA (orange) (A–B). Arrows indicate translocated chromosomes. (A) *H. capense* H335, (B) *H. capense* H3923. Double-target *in situ* hybridization using 5S rDNA (green) and 18S-25S rDNA (red) (C–E). Arrowheads indicate 5S rDNA sites unique to *H. marinum* ssp. *gussoneanum*. (C) *H. marinum* ssp. *gussoneanum* 2x H28, (D) *H. marinum* ssp. *marinum* 2x H155, (E) *H. marinum* ssp. *gussoneanum* 4x H2303. Multicolor FISH using 5S rDNA (green), 18S-25S rDNA (red) and total Xa genome DNA (white) (F–H). Arrows and arrowheads indicate translocated chromosomes and 5S rDNA sites unique to *H. marinum* ssp. *gussoneanum*, respectively. (F) *H. capense* H334, (G) *H. capense* H3923, (H) *H. secalinum* H231. The number of strong 18S-25S rDNA sites is two in (F), four in (G) and two in (H).

5S rDNA site (Fig. 1F and Fig. 2). In *H. capense* accession H3923, three pairs of rDNA-carrying chromosomes are assigned to the Xa genome. In accession H3923, *H. marinum* ssp. *gussoneanum* 2x has probably donated the Xa genome because of the presence of the diagnostic 5S rDNA site

(Fig. 1G). This accession additionally carried a submetacentric chromosome pair of Xa-genome origin having a strong 18S-25S rDNA signal on its short arm. Moreover, this accession also carried a metacentric chromosome pair of I-genome origin with a minor 18S-25S rDNA site (Fig. 2).

Fig. 2. Idiograms of the morphology and the chromosome pairs carrying 5S and 18S-25S rDNA in *H. marinum*, *H. secalinum* and *H. capense* accessions investigated here. Open circles indicate 5S rDNAs sites and filled circles indicate 18S-25S rDNA sites. In *H. secalinum* and *H. capense*, chromosomes boxed in dotted lines are those of Xa-genome origin. Asterisks indicate 5S rDNA sites unique to *H. marinum* ssp. *gussoneanum*.

In *H. secalinum* accession H296, the Xa genome seems to be derived from ssp. *gussoneanum* 2x because of the presence of the diagnostic 5S rDNA site (Fig. 2). In *H. secalinum* accessions H231 and H3121, however, the Xa genome appears to be derived from *H. marinum* ssp. *marinum* because of the absence of the diagnostic 5S rDNA site (Fig. 1H). Accession H3121 additionally carried a metacentric chromosome pair of I-genome origin with a minor 18S-25S rDNA site in the middle of the short arm (Fig. 2).

Discussion

Intergenomic translocation

All three *H. secalinum* accessions studied lacked intergenomic translocations, whereas *H. capense* was polymorphic in intergenomic translocations. Translocated chromosomes in two *H. capense* accessions (H334 and H335) are morpholog-

ically similar and lacking rDNA sites. These accessions are thus likely to carry identical translocations. Bustos *et al.* (1996) studied a *H. secalinum* accession from Spain using the repetitive sequence pHch950 isolated from *H. chilense* (Ferrer *et al.* 1995). Bustos *et al.* (1996) also reported that two chromosome sets displayed a small region with interchange response when hybridized with pHch950. The Spanish *H. secalinum* accession needs to be examined by our GISH and FISH protocols in order to reveal if it contains true intergenomic translocations. Therefore, more accessions need to be studied to conclude whether or not intergenomic translocations are unique to *H. capense*.

Phylogeny of *H. capense* and *H. secalinum* based on rDNA mapping

Polymorphisms of the diagnostic 5S rDNA site on the Xa-genome chromosomes suggest that *H. secalinum* has a

polyphyletic origin. *H. secalinum* includes two different types; one of them obviously has ssp. *gussoneanum* 2x and the other has ssp. *marinum*, as the Xa-genome donor. *H. capense* includes only one type, which apparently has ssp. *gussoneanum* as the Xa-genome donor. Although the 4x cytotype of ssp. *gussoneanum* could be a potential donor of the Xa genome, a lack of rDNA markers unique to the 4x cytotype prevented us to test the possibilities of its involvement in the origin of *H. secalinum* and *H. capense*.

H. secalinum and *H. capense* were both polymorphic in the rDNA sites. Bustos *et al.* (1996) also analyzed 5S and 18S-25S rDNA in a Spanish *H. secalinum* accession, and the rDNA pattern reported is similar to that of our accession H3121 from United Kingdom. There is a typical rDNA pattern common to the two tetraploid species (Fig. 2). Such a typical pattern was observed in *H. secalinum* accession H296 and *H. capense* accessions H334 and H335. Two chromosome pairs with rDNA sites were here observed for each of the I and Xa genomes, and only one strong 18S-25S rDNA site of I-genome origin is the major nucleolus organizer region (NOR) (Fig. 1F and Fig. 2). This agrees with the reports by Linde-Laursen *et al.* (1986, 1995) that the karyotypes of *H. capense* and *H. secalinum* comprise only one satellited chromosome pair as a result of nucleolar dominance (Cermeno and Lacadena 1985). Our results show that in the typical rDNA pattern of the two tetraploid species, the 18S-25S rDNA site of the Xa-genome origin is silenced. However, *H. capense* accession H3923 had two pairs of chromosomes with a strong 18S-25S rDNA site, one each for the I and the Xa genomes, respectively (Fig. 1G). In this accession, a new major NOR appeared on a submetacentric chromosome pair of the Xa-genome origin. It is currently not possible to state the origin of this new major NOR site.

In the typical rDNA pattern of *H. capense* and *H. secalinum*, the I-genome chromosome pairs with rDNA sites did not match the rDNA patterns of any Central Asian wild *Hordeum* species (*H. brevisubulatum*, *H. roshevitzii*, or *H. bogdanii*), but were rather similar to those of the “chilense type” species that are most frequently found in the American Continents (Taketa *et al.* 2001). However, in accessions with a non-typical rDNA pattern, such as *H. secalinum* accession H3121 and *H. capense* H3923, rDNA patterns similar to that of *H. roshevitzii* were found among I-genome chromosomes (Fig. 2). Taking geographical distribution into consideration, the latter connection is more likely. The typical rDNA pattern of the two tetraploid species may have resulted from a loss of a metacentric pair with a minor 18S-25S rDNA site. If this was the case, then *H. roshevitzii* could be the I-genome donor to *H. secalinum* and *H. capense*.

Molecular phylogenetic studies have been conducted to clarify the origin of *H. secalinum* and *H. capense*. Nishikawa *et al.* (2002), based on analysis of three chloroplast DNA sequences (*matK*, *atpB-rbcL* and *trnL-trnF*), showed that a close relationship between *H. marinum* ssp. *marinum* and *H. secalinum*, and concluded that *H. secalinum* was probably formed from a cross involving *H. marinum* ssp. *marinum* as

the maternal parent. They also revealed that *H. secalinum* and *H. capense* are closely related, and that *H. capense* was developed from *H. secalinum*. On the other hand, Petersen and Seberg (2004) studied two single copy nuclear genes (DMC1 and EF-G) and one plastid gene (*rbcL*). They concluded that these two tetraploid species have a common hybrid origin involving diploid *H. marinum* ssp. *gussoneanum* and *H. brevisubulatum*. Their data from the plastid gene showed that *H. brevisubulatum* was the maternal parent. Thus, the two molecular phylogenetic studies contradict about the maternal parent of *H. capense* and *H. secalinum*, and which subspecies of *H. marinum* contributed the Xa genome to these tetraploid species. In both studies, only one accession each of *H. capense* and *H. secalinum* were studied, therefore differences in accessions may be responsible for the discrepancies.

Blattner (2004, 2006) analyzed three nuclear genes (DCM1, EF-G and ITS) and confirmed that *H. marinum* and an I-genome species were involved in the evolution of both *H. capense* and *H. secalinum*. Moreover, he estimated the dating of the split between *H. capense* and *H. secalinum* at 0.4 million years ago. Petersen and Seberg (2004) concluded that *H. capense* is an ancient relict, not a recent introduction of *H. secalinum*, because they observed as many as five nucleotide substitutions in *rbcL* gene between the two tetraploid species. In the present physical mapping of rDNA sites, *H. capense* showed less variation in 5S rDNA sites on the Xa-genome chromosomes compared to *H. secalinum*. The present molecular cytogenetic data suggest that *H. capense* originated from a limited number of *H. secalinum* accessions introduced probably through long distance bird migration to South Africa as also indicated by Blattner (2004, 2006).

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