Syntenic Relationships between the U and M Genomes of *Aegilops*, Wheat and the Model Species *Brachypodium* and Rice as Revealed by COS Markers

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Abstract

Diploid *Aegilops umbellulata* and *Ae. comosa* and their natural allotetraploid hybrids *Ae. biuncialis* and *Ae. geniculata* are important wild gene sources for wheat. With the aim of assisting in alien gene transfer, this study provides gene-based conserved orthologous set (COS) markers for the U and M genome chromosomes. Out of the 140 markers tested on a series of wheat-*Aegilops* chromosome introgression lines and flow-sorted subgenomic chromosome fractions, 100 were assigned to *Aegilops* chromosomes and six and seven duplications were identified in the U and M genomes, respectively. The markerspecific EST sequences were BLAST-ed to *Brachypodium* and rice genomic sequences to investigate macrosyntenic relationships between the U and M genomes of *Aegilops*, wheat and the model species. Five syntenic regions of *Brachypodium* identified genome rearrangements differentiating the U genome from the M genome and from the D genome of wheat. All of them seem to have evolved at the diploid level and to have been modified differentially in the polyploid species *Ae. biuncialis* and *Ae. geniculata*. A certain level of wheat-*Aegilops* homology was detected for group 1, 2, 3 and 5 chromosomes, while a clearly rearranged structure was showed for the group 4, 6 and 7 *Aegilops* chromosomes relative to wheat. The conserved orthologous set markers assigned to *Aegilops* chromosomes promise to accelerate gene introgression by facilitating the identification of alien chromatin. The syntenic relationships between the *Aegilops* species, wheat and model species will facilitate the targeted development of new markers specific for U and M genomic regions and will contribute to the understanding of molecular processes related to allopolyploidization.

Citation: Molnár I, Šimbková H, Leverington-Waite M, Goram R, Cseh A, et al. (2013) Syntenic Relationships between the U and M Genomes of *Aegilops*, Wheat and the Model Species *Brachypodium* and Rice as Revealed by COS Markers. PLoS ONE 8(8): e70844. doi:10.1371/journal.pone.0070844

Editor: Samuel P. Hazen, University of Massachusetts Amherst, United States of America

Received April 2, 2013; Accepted June 23, 2013; Published August 5, 2013

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Funding: This work was funded by the Hungarian National Research Fund (PD83444), by TÁMOP-4.2.2.A-11-1-KONV-2012-0008, by a János Bolyai Research Scholarship from the Hungarian Academy of Sciences (for MI and CSA), by the Agrisafe Programme (EU-FP7-REGPOT-2007-1, No. 203288), by the Czech Science Foundation (grant award P501/12/G090), by the Ministry of Education, Youth and Sports of the Czech Republic and the European Regional Development Fund (Operational Programme Research and Development for Innovations No. ED0007/01/01) and by the United Kingdom Biotechnology and Biological Sciences Research Council. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

The genus *Aegilops* is closely related to *Triticum* and contains 23 species, 12 of them with U and/or M genomes [1]. The allotetraploid species *Ae. biuncialis* Vis. (2n = 4x = 28, U³U³M⁴M⁴) and *Ae. geniculata* Roth. (2n = 4x = 28, U³U³M⁴M⁴), which originated from the natural hybridization of the diploids *Ae. comosa* Sm. in Sibth. & Sm. (2n = 2x = 14, MM) and *Ae. umbellulata* Zhuk. (2n = 2x = 14, UU), have the greatest ecological adaptability [1]. These species represent an outstanding reservoir of useful genes and alleles responsible for tolerance to pests and diseases, including the stem rust strain UG99, and abiotic stresses such as salt, drought, frost and heat stress [2-9]. These species have also been reported to carry alleles affecting the nutritional and bread making quality of wheat [10,11]. These traits are attractive candidates for transfer into bread wheat by interspecific hybridization. Over the past decades, extensive research efforts have been made to introgress *Aegilops* chromatin into wheat, resulting in a range of addition, substitution and translocation lines between bread wheat and *Ae. comosa*, *Ae. umbellulata*, *Ae. geniculata* and *Ae. biuncialis* [2,3]. As a result of the introgression process, several genes for resistance to rusts and powdery mildew (*Lr9, Lr57, Sr34, Tr6, Tr40, Pm 29*) have been transferred into hexaploid wheat from the U and M genomes of *Aegilops* [2,3].

The identification of alien chromatin in the wheat genome is an essential part of the pre-breeding process and determines the efficiency of gene transfer efforts. The cytogenetic methods used most commonly to detect *Aegilops* chromatin in the wheat genetic background [2,12,13] are powerful techniques, but they tend to be less efficient in identifying small introgressions when the goal is to screen a large population. At the same time, only a small number of cost-effective molecular markers specific for the U and M genomes are available [14–17], a fact that limits the highthroughput marker-assisted selection of *T. aestivum - Aegilops* introgression lines. The shortage of suitable DNA markers also slows the development of high density genetic and physical maps,
the mapping of favourable agronomic traits and the map-based positional cloning of genes.

Until now, only a few wheat-Aegilops translocations have been used in breeding programmes and the introgression of favourable agronomic traits from wild relatives to cultivated wheat remains difficult due to undesirable linkage drag and yield reduction [19,22]. The utilisation of interspecific translocations in the breeding process is only successful if the introgressed alien chromosome segment compensates for the loss of the wheat chromatin [24]. Compensating wheat-alien translocations are likely to be developed from wheat and alien chromosomes having a strong homoeologous relationship due to the similar gene order along the introgressed chromosome. The collinearity between the homoeologous wheat and Aegilops chromosomes could be interrupted by genome rearrangements occurring independently in wheat and Aegilops after their evolutionary divergence [19,20]. For example, Zhang et al. [21] identified at least eleven rearrangements that differentiate the D genome of wheat from that of Ae. umbellulata. Therefore, it is extremely important to establish syntenic relationships between the wheat and Aegilops chromosomes and to map the breakpoints of genome rearrangements in the U and M genomes relative to wheat.

Rice has been considered as a model system for the Triticeae species because of its small genome size (1C = 389 Mb) and the availability of the genome sequence [22,23]. The comparative mapping of cereal genomes has provided evidence of a high level of conservation of gene order across regions spanning many megabases (i.e. macrocollinearity) [22]. However, the collinearity between rice and Triticeae species frequently breaks down at micro level due to translocations, deletions and duplications [24,25] leading to increased interest in the genome of the wild grass, Brachypodium distachyon. This was proposed as a better model organism for structural and functional genomics in cereals because of its biological features (such as self-fertility, inbreeding annual life cycle of less than 4 months, small size, undemanding growth requirements, high capacity for plant regeneration via somatic embryogenesis and resistance to several cereal-adapted pests and diseases), small genome size (1C = 272 Mb) and its closer phylogenetic position to the tribe Triticeae [26-29]. The genomic sequence of Brachypodium distachyon has recently become available [30], allowing a deeper comparison of syntenic regions between crop species and Brachypodium as a reference.

Comparative genomic and phylogenetic studies between the Triticeae/Aegilops taxa and the model systems rice and Brachypodium identified a set of genes conserved throughout evolution in both sequence and copy number. This set of >1000 conserved genes, referred to as conserved orthologous set (COS) markers, was identified by the in silico comparison of the rice, wheat and Brachypodium EST databases [31]. The COS markers were designed over the exon-intron boundaries of genes conserved between the model and target species. The markers are potentially highly polymorphic, as they span the introns, which have an increased frequency of polymorphisms relative to the exons (6.07 SNP/kb versus 3.00 SNP/kb in introns and exons, respectively, in rice) [32]. These markers define orthologous regions, thus enabling the comparison of regions on the chromosomes of related species. It was shown that COS markers are highly transferable between species such as rice, wheat, maize, sorghum and barley [33]. Wheat-specific COS markers are also transferable to Aegilops, as demonstrated by Howard et al. [34], who mapped a major QTL controlling the content of B-type starch granules on chromosome 4S in Ae. peregrina. Burt and Nicholson [35] used COS markers to map the eyespot resistance gene Pch1 originating from Ae. ventricosa in hexaploid wheat. Therefore, the COS markers have potential for the identification of alien chromatin introgressed from various species of Aegilops into hexaploid wheat, and also to identify the chromosomal locations of orthologous regions in the U and M genomes relative to wheat using rice and Brachypodium as references.

The aim of the present study was to assign COS markers to U and M genome chromosomes with the help of a series of wheat-Aegilops disomic addition, substitution and translocation lines and using subgenomic DNA samples obtained by flow cytometric sorting of well-defined groups of U and/or M genome chromosomes [17]. A further aim was to compare the Aegilops genomes with wheat by identifying orthologous chromosomal regions in the U and M genomes relative to wheat (D genome) using rice and Brachypodium as references.

**Materials and Methods**

**Plant Materials**

The assignment/identification of the COS markers on the U and M genome chromosomes of diploid and allotetraploid Aegilops species (Ae. umbellulata, Ae. comosa, Ae. biuncialis and Ae. geniculata) was carried out on wheat-Aegilops introgression lines and on flow-sorted subgenomic DNA fractions with well-defined chromosomal content.

The parental wheat (Tritium aestivum L.) genotypes (Chinese Spring, Mv9kr1) of the wheat-Aegilops introgression lines and the wheat genotype Mv25, which were used for the first backcross during the production of wheat-Ae. biuncialis additions, were used as control. The parental Aegilops genotypes of the introgression lines (Ae. umbellulata JIC2010001, Ae. comosa JIC2110001, Ae. biuncialis MvGB642 and Ae. geniculata TA2899) and the genotypes used for the production of flow-sorted subgenomic DNA fractions in previous work [17] (Ae. umbellulata MvGB470, Ae. comosa MvGB1039, Ae. biuncialis MvGB382 and Ae. geniculata AE1311/00) were also included in the present study.

The wheat (Chinese Spring/Ae. umbellulata JIC2010001) addition lines 1U, 2U, 4U, 5U, 6U and 7U, the wheat (Chinese Spring/Ae. comosa JIC2110001) addition lines 2M, 3M, 4M, 5M, 6M and 7M, and the substitution 6M(6A) were supplied from the John Innes Centre germplasm collection, Norwich, UK by Dr. Steve Reader. The partial set of wheat (Mv9kr1/Ae. biuncialis MvGB642) addition lines 1U, 1U6U, 3U, 4M, 2M, 3M and 7M [12], and the substitution 3M(4B) and the centric fusion 3M(4BS), both obtained from a cross between Mv9kr1/Ae. biuncialis (MvGB642) 3Mb addition × Chinese Spring ph1b mutant [36], were produced in Martonvásár. The wheat (Chinese Spring/Ae. geniculata TA2899) addition lines 1U, 2U, 3U, 4U, 5U, 6U, 7U, 1M, 2M, 3M, 5M, 6M and 7M [37] were provided by Dr. Bernd Friebe (Kansas State University, Manhattan, Kansas).

**Chromosome Sorting and Amplification of Subgenomic DNA Samples**

Flow cytometric chromosome sorting from individual peaks (I–IV) on flow karyotypes of Ae. umbellulata (MvGB470), Ae. comosa (MvGB1039), Ae. biuncialis (MvGB382) and Ae. geniculata (AE1311/00) and the determination of the chromosome content of flow-sorted fractions by FISH were carried out as described by Mohr et al. [17]. The assignment of chromosomes to peaks on flow-karyotypes of Aegilops species is summarized in Table 1. In order to prepare template DNA for PCR with COS markers, chromosomes were sorted in batches of 25–50,000 (equivalent to...
COS Marker Analysis
DNA preparation and genotyping was carried out as described by Howard et al. [34] using the following templates; wheat-\textit{Aegilops} genetic stocks, parental wheat (Chinese Spring, Mv9kr1, Mv25) and \textit{Aegilops}/\textit{Ae. umbellulata} JIC20110001, \textit{Ae. comosa} JIC21110001, \textit{Ae. biuncialis} MvGB492, \textit{Ae. geniculata} TA2099 genotypes and the \textit{Aegilops} genotypes used for the flow cytometric analysis (\textit{Ae. umbellulata} MvGB470, \textit{Ae. comosa} MvGB1039, \textit{Ae. biuncialis} MvGB382 and \textit{Ae. geniculata} AE1311/00).

A total of 140 markers (whose primer sequences and PCR conditions were summarised in Table S1) potentially covering wheat homoeologous groups I–VII were chosen from two publicly available COS marker collections, the Wheat Genetic Improvement Network (WGIN) (http://www.wgin.org.uk/resources/Markers/TAmarkers.php) and Tools and Resources (TR) collections (http://www.modelcrop.org/cos_markers). When the chromosomal locations of the markers were not available in the D genome of hexaploid wheat, the source EST sequences of the COS markers were searched from the GrainGenes database (http://wheat.pw.usda.gov/cgi-bin/westsql/map_locus.cgi?t = estacc&q = ).

Reverse PCR primers were directly labelled with a fluorescent dye (6-FAM) and the following programmes performed on an MJ Research Tetrad PTC-225 Thermal Cycler (Waltham, Massachusetts) were used to amplify PCR products from 10 ng of genomic DNA in 10 μl reactions. WG1N: 95°C (1.5 min), 39 cycles of (95°C (0.5 min), 58°C (0.5 min), 72°C (0.5 min)), hold at 72°C (5 min) then at 10°C. TR: 94°C (10 min), 16 cycles of (95°C (0.5 min), 58°C (1 min), decreasing by 0.5°C per cycle to 30°C, 72°C (1 min)), 25 cycles of (94°C (0.5 min), 50°C (1 min), 72°C (1 min)), hold at 15°C. The fragment analysis of PCR products was carried out on a POP7 column attached to a 3730xl DNA Analyzer (Applied Biosystems, USA). The results were analysed using GeneMapper v4.0.

Sequence Analysis
To compare the orthologous regions defined by the COS markers between D genome of \textit{T. aestivum}, U and M genomes of \textit{Aegilops} species and rice and \textit{Brachypodium}, a physical map was constructed showing the physical positions of the COS markers on the chromosomes of rice and \textit{Brachypodium} as reference. To identify the physical positions of the markers, the EST source sequences of the COS markers (shown as Accession No. in Tables S2, S3 and S4) were downloaded from The Institute of Genomic Research (TIGR) database (http://plantta.jcvi.org/index.shtml) and used as queries in BLASTn searches to identify homologues in the assembled genomic sequences of \textit{Brachypodium} distachyon and \textit{Oryza sativa} (\textit{Brachypodium} distachyon v1.0 [30]; \textit{Oryza} sativa Japonica Group, The IRGSP pseudomolecules, Build 4.0, GenBank Assembly ID: CA_000005425.2) using the EnsemblPlants Database (http://plants.ensembl.org/). As the best hits were considered the hits with the highest score value and characterized by their BLAST parameters E-value, % of Identity and Alignment length (Tables S2, S3 and S4). Throughout the study, BLAST hits with E-values smaller than 2.8e-06, Identity % >58.44 and Alignment length >100 bp were considered as significant (Tables S2, 3 and 4).

The start genomic positions of the best hits in \textit{Brachypodium} and rice were used to construct physical maps of the COS markers. The lengths (in bp) of \textit{Brachypodium} and rice chromosomes as well as the start genomic positions of the best hits of the ESTs were converted to pixels and the physical maps of the COS markers were designed.

Results
Assignment of COS Markers to U and M Chromosomes
A set of COS markers specific to different ESTs was mapped to \textit{Aegilops} chromosomes using wheat-\textit{Aegilops} introgression lines carrying the U- and M-genome chromosomes of diploid \textit{Aegilops} species (\textit{Ae. umbellulata} and \textit{Ae. comosa}) and their allotetraploid hybrids (\textit{Ae. biuncialis} and \textit{Ae. geniculata}). Subgenomic DNA samples amplified from each of the flow-karyotype peaks of the four goatgrass species and representing individual chromosomes or groups of chromosomes were also used (Table 1).

Of the 140 COS markers, 133 showed PCR products in the wheat genotypes (Chinese Spring, Mv9kr1 and Mv25) or in at least one of the eight \textit{Aegilops} genotypes, while seven markers did not amplify any product. The 133 markers resulted in 822 PCR products (range: 1–5 PCR products/marker/ genotype, mean: 2.04 PCR products; Table S5) with different sizes on the eight genomic regions of the four \textit{Aegilops} species, 492 (59.85%) of which showed size polymorphism relative to wheat, while 330 (40.15%) were non-polymorphic. Out of the 492 polymorphic PCR products, 295 (59.95%) products of 89 COS markers could not be unambiguously assigned to \textit{Aegilops} chromosomes because a relevant wheat-\textit{Aegilops} addition line bearing the polymorphic locus was not available and the locus was located in a subgenomic DNA

Table 1. Chromosome content of subgenomic DNA samples prepared from chromosomes flow-sorted from peaks on flow karyotypes of \textit{Ae. umbellulata} (MvGB470), \textit{Ae. comosa} (MvGB1039), \textit{Ae. biuncialis} (MvGB382) and \textit{Ae. geniculata} (AE1311/00).

| Subgenomic DNA samples | Chromosome content* |
|-------------------------|---------------------|
| **Species**             | **Flow karyotype peak** |
| **Ae. umbellulata**     | I 1U, II 6U, III 3U, IV 2U, 4U, 5U, 7U |
| **Ae. comosa**          | I 1M, 4M, II 2M, 6M, III 2M, 5M, IV 3M, 7M |
| **Ae. biuncialis**      | I 1U, II 3U, 4U, 5U, 7U, 7U, 7M, 1M, 3M, 5M, IV 7M |
| **Ae. geniculata**      | I 1U, 6M, II 3U, 4U, 6U, III 2U, 5U, 7U, 7M, 7U, 7M, IV 3M, 5M, 7M |

*Chromosomes were assigned to peaks in which they occurred at the highest frequency (Molnár et al. 2011b).

doi:10.1371/journal.pone.0070844.t001
Some markers produced similar sized amplicons both in the diploid progenitors and in the tetraploids \textit{Ae. biuncialis} and \textit{Aegilops geniculata}, but the chromosomal location of the locus could not be identified due to the fact that the sets of addition lines were incomplete. When the chromosomal location of a locus could be determined unambiguously in at least one species (in the diploid progenitor, or in \textit{Ae. biuncialis} or \textit{Aegilops geniculata}) and the highest product yield in the other two species was detected in the subgenomic DNA sample containing the same chromosome, it was concluded that the locus was located on the same chromosome in all three \textit{Aegilops} species. For example the X2N marker produced a 558 bp PCR fragment in \textit{Ae. umbellulata} (AE740/035), \textit{Ae. biuncialis} (McGB982) and \textit{Aegilops geniculata} (TA2899 and AE1311/000), which was also found in the 2U\textsuperscript{b} wheat-\textit{Ae. geniculata} disomic addition line and on the subgenomic samples specific for peaks IV and III of the flow-karyotype containing the 2U and 2Ub chromosomes of \textit{Ae. umbellulata} and \textit{Ae. biuncialis}, respectively. As a consequence, it was suggested that the X2N marker detects loci on chromosomes 2U and 2Ub of \textit{Ae. umbellulata} and \textit{Ae. biuncialis} as well as in \textit{Aegilops geniculata}.

Some PCR products could be detected on more than one \textit{Aegilops} chromosome, so 156 loci were assigned unambiguously to the U genome chromosomes. 30 loci (19.29\%) were located on group 1 chromosomes, 8 (5.12\%) on group 2, 44 (28.20\%) on group 3, 21 (13.46\%) on group 4, 10 (6.41\%) on group 5, 28 (17.94\%) on group 6, and 15 (9.61\%) on group 7. Out of the 132 loci assigned to the M genome chromosomes, 4 loci (3.03\%) were located on group 1 chromosomes, 27 (20.45\%) on group 2, 47 (35.60\%) on group 3, 3 (2.27\%) on group 4, 8 (6.06\%) on group 5, 19 (14.39\%) on group 6 and 24 loci (18.18\%) on group 7 chromosomes. Some markers assigned to \textit{Aegilops} chromosomes showed different chromosomal location in the allopolyploid species relative to the diploid ancestors. The proportion of these markers was significantly higher (27.9\%) in the M genome (12 out of 43 markers assigned to chromosomes in \textit{Ae. comosa} and one of the allopolyploid \textit{Aegilops} sp.) than in the U genome (6 of the 74 assigned markers, 8.1\%).

\textit{Aegilops} chromosome-specific markers with a significant level (≥2 bp) of length polymorphism between the parental wheat and \textit{Aegilops} genotypes were considered to be suitable for the marker-assisted selection of new wheat-\textit{Aegilops} introgression lines in prebreeding programmes (Table 2). In this study, 169 polymorphic loci of 51 markers covering all 7 homoeologous groups of the U and M genomes were found to be suitable for the high-throughput detection of diploid and allotetraploid \textit{Aegilops} chromosomes.

### Duplications in the U and M Genome of Diploid and Polyploid \textit{Aegilops}

The chromosomal location of COS markers revealed several intragenomic duplications in the diploid and polyploid \textit{Aegilops}.
Table 2. COS markers showing polymorphic (≥2 bp) PCR amplicons between wheat and Aegilops species are considered as suitable for the marker-assisted introgression into hexaploid wheat of the U and M genome chromosomes from Ae. umbellulata (UU) and Ae. comosa (MM) and from Ae. biuncialis and Ae. geniculata.

| diploid progenitors | Ae. Biuncialis | Ae. geniculata |
|---------------------|---------------|---------------|
| 1U                  | X1B(224), X2B(162) | X1B(226), X2B(162), X tr248(208) |
| 2U                  | X2N*(558), X2P*(292), Xtr146(303), Xtr451(192) | X2N*(558), X146*(203), Xtr451*(262) |
| 3U                  | X3(205), Xtr63(180), Xtr63(545), Xtr80(429), Xtr83(360) | X3(205), Xtr63(180), Xtr63(545), Xtr77(364), Xtr80(429), Xtr83(360) |
| 4U                  | X4*(236), Xtr72(179), Xtr76(179), Xtr72(231), Xtr102(218), Xtr103(270) | X4*(236), Xtr72(179), Xtr76(179), Xtr72(231), Xtr102(218), Xtr103(270) |
| 5U                  | X5*(270), XSQ*(311), Xtr128(214), Xtr131(470), Xtr48*208 | X5*(270), XSQ*(311), Xtr128(214), Xtr131*(470) |
| 6U                  | X6*2(262), X4C(385), X4G(239), X6A(250), Xtr77(363), Xtr90(290), Xtr91(287), Xtr100(127) | X6*2(262), X4C(385), X4G(239), X6A(250), Xtr77(363), Xtr90(290), Xtr91(287), Xtr100(127) |
| 7U                  | X7B*(234), X7C*(327), X7I(248), Xtr4(266) | X7B*(234), X7C*(327), X7I*(248), Xtr4*(266) |
| 1M                  | X1B*(163) | X1B*(163) |
| 2M                  | X1J*(228), Xtr146(381) | X1J*(228), X2R*(267), Xtr72(168), Xtr76(168), Xtr131(356), Xtr134(250), Xtr248(208) |
| 3M                  | Xtr62(178), Xtr63(444), Xtr67(351), Xtr73(473), Xtr80(487), Xtr83(356), Xtr85*(226) | Xtr62*(178), Xtr63*(444), Xtr73*(473), Xtr80*(487), Xtr83*(356), Xtr85*(226), Xtr146(381) |
| 4M                  | Xtr88(407) | Xtr88*(407), Xtr88*(407) |
| 5M                  | XSQ*(311), Xtr128(212), Xtr76(209), Xtr76(214) | XSQ*(311), Xtr471*(209), Xtr76*(214) |
| 6M                  | X6*2(262), Xtr93(477), Xtr103(261), Xtr104(406), Xtr112(390) | X6*2(262), Xtr93(477), Xtr103(261), Xtr104(406), Xtr112(390) |
| 7M                  | X7C*(328), X7I(249, 312) | X7C*(328), X7I(249, 312) |

The size (in bp) of the chromosome-specific loci is shown in brackets. Asterisks indicate the loci with predicted chromosomal location when the PCR amplicon was detected separately in Ae. umbelulata and Ae. comosa, or when the highest PCR product yield in the other two species was detected in the subgenomic DNA sample containing the same chromosome.

*pLoci with predicted chromosomal location.

doi:10.1371/journal.pone.0070844.t002

species (Table 3). In the case of the U genome, six duplications were detected. Three (1U/3U, 4U/7U, 6U/7U) were found in the diploid progenitor Ae. umbelulata and in one tetraploid Aegilops. One duplication (3U/4U) was detected separately in Ae. umbelulata and Ae. geniculata by markers Xr76 and X7T, respectively, while two species-specific duplications (1U*/2U*/7U*, 4U*/5U*) were found in Ae. geniculata.

In the M genome, seven different duplications were detected in the diploid (Ae. comosa) and the allotetraploids Ae. biuncialis and Ae. geniculata (Table 3). Some species-specific markers, like the 1M*/2M* duplication for Ae. geniculata, the 4M*/7M* duplication for Ae. comosa and the massive 2M*/3M* duplication detected by 11 COS markers for Ae. biuncialis. Two duplications were detected in more than one species, such as the 2M*/7M* duplication in Ae. biuncialis and Ae. geniculata and the 7M*/7M* duplication in all the three M genome species.

Relationship of U and M Genomes Relative to Rice, Brachypodium and Wheat

The source EST sequences of the 100 COS markers identified (Table S1) were related mainly to R1 and Br2, whereas the Ae1 chromosomes (1U) showed homology to R3 and R10 and to Br2 and Br3. (Figure 1A).
Syntonic Relationship of U and M Genomes Relative to Wheat

The markers whose EST sequences could be located on wheat allowed the direct investigation of syntenic relationships between wheat and the U and M genomes of *Aegilops*. Table 5 summarises the conserved genomic regions while Table S7 shows the syntenic relationship established based on COS marker positions (Figure 2) between the U and M genomes of diploid and tetraploid *Aegilops* species relative to wheat.

**Discussion**

Assignment of COS Markers to U and M Chromosomes

In the present study 94.3% of the COS markers produced amplicons in at least one *Aegilops* species, indicating the high transferability of the conserved orthologous set markers between the related species. The good transferability of COS markers was also reported for *Ae. peregrina* and *Ae. ventricosa* by Howard et al. [34] and Burt and Nicholson [35], respectively. The present results also indicate that the transferability of COS markers is better than other types of molecular markers such as SSRs, where transferability of wheat-specific markers was 89.3% (for *Ae. geniculata*), 79.62% (for *Ae. biuncialis*) and 54.1% for one of the species, *Ae. umbellulata*, *Ae. comosa*, *Ae. biuncialis* and *Ae. geniculata* [40,16,17]. High transferability between the species could be explained by the sequence conservation of the primer target sites of COS markers, which could be less variable than those of genomic simple sequence repeat markers (SSR).

Wheat SSR markers have been used widely for the molecular characterization of various *Aegilops* species, including *Ae. biuncialis* and *Ae. geniculata* [14,16,40–42]. Previous studies assigned 33 SSR and 37 sequence-specific amplified polymorphism (S-SAP) markers to U and M chromosomes [15–17,40]. This work significantly increased the number of U and M genome-specific markers by identifying the *Aegilops*-specific chromosomal location for 100 COS markers. One hundred and sixty nine loci of 51 markers covering all 7 chromosomes of the U and M genomes resulted in polymorphic amplicons relative to wheat, so they are potentially useful markers for detecting *Aegilops* chromosomes in bread wheat.

The results also confirmed previous observations on the suitability of MDA-amplified chromosomal DNA for molecular marker analysis [38,17] and indicate that flow-sorted chromosomes can be used for the physical mapping of molecular markers, especially when a complete set of cytogenetic stocks representing the whole chromosome complements is not available. Furthermore, the possibility of purifying chromosomes in *Aegilops* species [17] opens a way for next-generation survey sequencing to identify low-copy and generic sequences for the development of new markers, including SSR, ISBP, COS and SNP, for genotyping by sequencing of different accessions and for the high-resolution analysis of synteny and the characterization of structural chromosome differences between wheat and its progenitors and relatives [43,44].

Relationships between the Genomes of Diploid and Tetraploid *Aegilops* Species

The theory of pivotal-differential evolutionary patterns in *Aegilops* species suggested by Zohary and Feldman [45] states that the pivotal U genomes remain essentially unchanged during allopolyploid speciation, while the differential M genomes have accumulated substantial modifications as compared with the parental genome [45–47]. Consistently with this theory, the inactivation of major NORs on the 1M and 6M chromosomes, the redistribution of 3S rDNA sites, and the loss of minor 18S–26S rDNA loci were observed in *Ae. geniculata* and *Ae. biuncialis* relative to *Ae. comosa*

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**Table 3.** Duplications in the U and M genomes identified in diploid progenitors (*Ae. umbellulata* and *Ae. comosa*) and in their tetraploid hybrids *Ae. biuncialis* and *Ae. geniculata* by COS markers assigned to *Aegilops* chromosomes.

| Marker | Diploid progenitor | *Ae. biuncialis* | *Ae. geniculata* |
|--------|-------------------|-----------------|-----------------|
| X6N    | 1U/3U             | 1U/3U<sup>b</sup>| 1U/2U/7U<sup>b</sup>| |
| X76    | 3U/4U             | –               | 3U/4U           | |
| X7T    | –                 | –               | 3U/4U           | |
| X61    | 4U/7U             | –               | 4U/7U           | |
| X6A    | 6U/7U             | 6U/7U<sup>b</sup>| –              | |
| X5M    | –                 | –               | 4U/5U           | |
| X1J    | –                 | –               | 1M/2M<sup>b</sup>| |
| X6N    | –                 | 2M<sup>b</sup>/7M<sup>b</sup>| 1M/2M/6M<sup>b</sup>| |
| Xtr150 | –                 | 2M<sup>b</sup>/7M<sup>b</sup>| 2M<sup>b</sup>/7M<sup>b</sup>| |
| X7L    | 4M/7M             | –               | –              | |
| X7I    | 7M/7M             | 7M<sup>b</sup>/7M<sup>b</sup>| 7M<sup>b</sup>/7M<sup>b</sup>| |
| X4E    | –                 | 2M<sup>b</sup>/3M<sup>b</sup>| –              | |
| X4G    | –                 | 2M<sup>b</sup>/3M<sup>b</sup>| –              | |
| X4I    | –                 | 2M<sup>b</sup>/3M<sup>b</sup>| –              | |
| X4Q    | –                 | 2M<sup>b</sup>/3M<sup>b</sup>| –              | |
| X4S    | –                 | 2M<sup>b</sup>/3M<sup>b</sup>| –              | |
| X4O    | –                 | 2M<sup>b</sup>/3M<sup>b</sup>| –              | |
| X4I    | –                 | 2M<sup>b</sup>/3M<sup>b</sup>| –              | |
| X72    | –                 | 2M<sup>b</sup>/3M<sup>b</sup>| –              | |
| X76    | –                 | 2M<sup>b</sup>/3M<sup>b</sup>| –              | |
| X29    | –                 | 2M<sup>b</sup>/3M<sup>b</sup>| –              | |
| X131   | –                 | 2M<sup>b</sup>/3M<sup>b</sup>| –              | |
| X134   | –                 | 2M<sup>b</sup>/3M<sup>b</sup>| –              | |

Five large-scale chromosomal rearrangements (I–V) identified by more than one marker were detected on the *Aegilops* genomes relative to the wheat D genome and also on the U genome relative to the M genome (Figure 2). The first of these (Rearrangement I), the region spanning from *X4G* to *X1C* on Br1 (or R3) and related to W4, was located on 6U and on various chromosomes of the M genome (6M, 2M<sup>b</sup>/3M<sup>b</sup>, 1M<sup>b</sup> in *Ae. comosa*, *Ae. biuncialis* and *Ae. geniculata*, respectively). Another Br1 (or R6 and R7) region from X2C to Xtr372 (Rearrangement II), related to W2 (X2C) and W7 (X7T, Xtr383, Xtr372), was identified on the group 6 chromosomes of the U genome and also on group 6 (X7T) and 7 (Xtr383–Xtr372) of the M genome. A further region on Br1 (or R3 and R7), defined by the markers *Xtr400* and *X4K* (Rearrangement III) which are related to W2 and W4, respectively, was also found on the 6U chromosomes of *Ae. umbellulata* and in some cases on the polyploid *Aegilops* species.

The *Xtr85–X3B* region on the distal part of the short arm of Br2 (or R1) (Rearrangement IV), which is related to the group 3 chromosomes of wheat and to the M genomes, was homoeologous with the 7U chromosome in the diploid and polyploid *Aegilops*. The massive region spanning from *Xtr100* to *Xtr103* on the long arm of Br3 (or R2) (Rearrangement V) was homologous with the group 6 chromosomes of wheat and the M genome (in diploid and polyploid *Aegilops* species), whereas it was related to the 4U chromosomes of the three *Aegilops* species. Additional genome rearrangements detected by single markers were also found in the U and M genomes relative to each other and to wheat.
Figure 2. Brachypodium–wheat–Aegilops orthologous relationships from the genomic perspective of Brachypodium distachyon. The physical positions of the source ESTs of the COS markers are indicated on the Brachypodium chromosomes (Left). Each marker assigned to chromosomes of the wheat D genome or to the chromosomes of Ae. umbellulata (U), Ae. camosa (M), Ae. biuncialis (U^b, M^b) and Ae. geniculata (U^g).
Table 4. Syntenic genome relationships between the chromosomes of U and M genomes in the diploid progenitors Ae. umbellulata and Ae. comosa and their allotetraploid hybrids Ae. biuncialis and Ae. geniculata, and the chromosomes of rice (R) and Brachypodium (Br).

| diploid progenitors | Ae. biuncialis | Ae. geniculata |
|---------------------|----------------|---------------|
|                     | Rice           | Brachy        | Rice           | Brachy        |
| U                   | R2*, R3*, R5, R7*, R9*, R10, R11* | Br1, Br2, Br3, Br4* | Br1, Br2, Br3, Br5* | Br1, Br3, Br5* |
| 2U                  | R4, R7, R8*    | Br1, Br3, Br5 | R4, R7, R8*    | Br1, Br3, Br5 |
| 3U                  | R1, R2*, R6*   | Br1*, Br2, Br3* | R1, R2*, R6*  | Br1*, Br2, Br3* |
| 4U                  | R1, R2, R3*   | Br1*, Br2, Br3 | R1, R2, R3*   | Br1*, Br2, Br3 |
| 5U                  | R3, R4*, R12* | Br1, Br2, Br4*, Br5* R3, R9*, R12* | Br1, Br2, Br4* | R3, R9, R12* |
| 6U                  | R1, R2, R3, R4*, R6, R7, R9*, R11 | Br1, Br2, Br3, Br4, Br5* | R2, R3, R4*, R6, R7, R11* | Br1, Br3, Br4, Br5* |
| 7U                  | R1, R2*, R6, R7*, R8*, R10* | Br1, Br2, Br3 | R1, R2*, R6, R7*, R8*, R10* | Br1, Br2, Br3 |
| 1M                  | R3*, R5*, R7*  | Br1, Br2*     | R5*, R7*       | Br1*, Br2*    |
| 2M                  | R3*, R4, R8*, R10*, R11* | Br1, Br3*, Br4*, Br5* | R1, R2*, R3, R4, R8*, R11* | Br1, Br2, Br3 |
| 3M                  | R1          | Br2            | R1, R2, R3, R8*, R11* | Br1, Br2, Br3, Br4* |
| 4M                  | R2*, R8*     | Br3            | R2*            | Br3*          |
| 5M                  | R3, R8*, R9*  | Br1*, Br2*, Br3*, Br4* R3* | Br1*         | R3, R9        |
| 6M                  | R1*, R2, R3*, R6*, R11* | Br1, Br2*, Br3, Br4* | R1*, R2, R3*, R6*, R11* | Br1*, Br2*, Br3* |
| 7M                  | R2, R6*, R8    | Br1, Br3       | R2, R3*, R4*, R6, R8, R10* | Br1, Br3, Br5* |

Bold letters indicate rice or Brachypodium genomic regions represented by at least three markers.
*Genomic regions represented by one marker.

doi:10.1371/journal.pone.0070844.t004

M⁰ is colour-coded according to the homoeologous groups of *Triticum/Aegilops* chromosomes. Gaps between two markers assigned to the same *Triticum/Aegilops* chromosomes are filled in to show synteny (lighter colours). Blocks (designated I–V) indicate Brachypodium genomic regions related to the regions in the U genomes involved in evolutionary genome rearrangements relative to the wheat D genome or to M genomes. When a marker mapped to more than one wheat or *Aegilops* chromosome, other colour-coded locations are positioned adjacent to the first one. Asterisks indicate the predicted chromosomal location of a locus when the PCR amplicon was specific for the U or M genomes and could be determined unambiguously in at least one *Aegilops* species (in the diploid progenitor, or in *Ae. biuncialis* or *Ae. geniculata*) and when the highest PCR product yield in the other two species was detected in the subgenomic DNA sample containing the same chromosome.
in the U and M genome chromosomes is needed to explain the
distinct difference in the number of duplicated loci on the U and
M genomes and to obtain information on the molecular
mechanism of their selective alteration.

Relationships between Aegilops, Model Species and Wheat

Previously, wheat-A. umbellulata macrosynteny was investigated
by mapping wheat RFLP markers on the U genome chromosomes
of A. umbellulata [21,54,55]. The present work extended the
comparative analysis of wheat and Aegilops genomes to the M
genome of diploid A. comosa, allowing the U and M genomes in
polyploid A. biuncialis and A. geniculata to be investigated in
relation to wheat, Brachypodium and rice. The wheat-Brachypodium
and wheat-rice genome relationships obtained by the physical
location of marker-represented orthologue genes on Brachypodium
and rice were consistent with previous data reported on syntenic
relationships after the sequencing and assembly of the genomes of
Brachypodium and rice [30,23]. The physical maps allowed the
detection of macrosyntenic relationships between Aegilops and the
model species. In this respect, previous results indicated that rice
chromosome 10 (R10) was inserted into R5 to form Triticeae
chromosome 1, R7 was inserted into R4 to form Triticeae
chromosome 2, and R8 was inserted into R6 to form Triticeae
chromosome 7 [36,30]. These chromosomal rearrangements were
also detected in wheat and in ryegrass chromosomes 1 and 7
[50,57]. In the present study, the relationship between the R3-R5-R10 insertion and chromosome 1U was also detected, but it
was not confirmed for chromosome 1M, where mainly R5 and R7
regions were present (Table 4). In the case of group 2 Aegilops
chromosomes, the R1-R7-R4 insertion was indicated on 2U
chromosomes, but the 2M chromosomes were related mainly to
R4 and R8. Finally, the R6-R8-R6 insertion was detected for both
the 7U and 7M chromosomes. These results suggest that genome
rearrangements derived from common ancestors appear to
characterize the U genome of the Aegilops species, but were only
partly valid for the M genomes.

From an agronomic point of view, the macrosyntenic relation-
ships between Aegilops and the model species provide useful
background information for the targeted development of markers
specific for the Aegilops chromosome regions responsible for
important agronomic traits [35].

Physical maps of COS markers also allowed the investigation of
relationships between the U and M genomes and between wheat
and Aegilops species in the genomic perspective of Brachypodium and
rice. Besides the relatively close relationship between the U and M
genomes, the chromosomal location of Brachypodium and rice
syntenic regions in wheat and Aegilops genomes detected five
genome rearrangements differentiating the U and M genomes. All
of them seem to have evolved at the diploid level and to have been
modified differentially in the polyploid species A. biuncialis and A. geniculata. Three rearrangements (I, II and III) were connected to
cromosome 6U, one (IV) to 7U and one (V) to 4U. Interestingly,
in three of the five rearrangements (II, IV and V), the genomic
regions involved were located on chromosomes in the same
homoeologous group in wheat and in the M genomes of A. comosa,
A. biuncialis and A. geniculata, while they were located on different
homoeologous group chromosomes in the U genome of diploid
and polyploid Aegilops species. These results suggest that the M
genome is more closely related to the wheat D genome than the U
genome.

The relationship between the U genome of A. umbellulata and
the D genome of wheat was investigated by Zhang et al. [21], who
mapped 79 wheat RFLP markers on wheat cv. Chinese Spring-Ae.
related to W7, which is consistent with the results of Yang et al. [21]. Showed that 3U was closely related to W3 and also to 3M, was supported by the present work. Relationship of chromosome 1M to W1 and W2 was also detected, and chromosomes 2U and 2M were again found to be related to W2. 

Zhang et al. [21] showed that 3U was closely related to W3 and suggested the presence of a fragment related to W7 but no experimental evidence was presented. The close relationship of W3 to 3U, and also to 3M, was supported by the present work. Moreover, one marker indicated the presence of a fragment related to W7, which is consistent with the results of Yang et al. [58], who suggested that a translocation may exist between Ae. umbellulata chromosomes 3U and 7U. Due to the low number of markers, relationships could only be detected between 4U and W6 and between 5U/5M and W5. The present results confirmed the highly rearranged structure of chromosome 6U and indicated a rearranged structure for the 6M chromosomes for the first time. At the diploid and tetraploid levels, fragments related to W2, W4 and W7 were detected on 6U, as also reported by Zhang et al. [21]. Two markers also indicated the homology of Ae. umbellulata 6U with W5, but due to the low number of markers, no experimental evidence could be found for the presence of a fragment related to W6. The present study indicated homology between chromosome 6M and W6 and W7 in diploid and tetraploid Aegilops species and a relationship with W1 and W4 in *Ae. comosa*. Chromosome 7U contains regions syntenic with W3 and W7, consistently with the previous results [21], while the 7M chromosomes were related mainly to W6 and W7 in diploid and tetraploid *Aegilops*.

Evolutionary genome rearrangements are considered to be a common phenomenon in most plant taxa, including the *Triticaceae*, and to be one of the most important evolutionary driving forces for the formation of new species. Genome shuffling can be triggered by polyploidization events and is the main reason for syntenic breakage in grasses since their divergence from a common ancestor. Such mosaic synteny blocks were found in rye and ryegrass when their genomes were compared with wheat [59,57] and they were also formed during the evolution of barley and hexaploid wheat [60,61]. Based on the comparison of orthologous regions from rice, maize, sorghum and *Brachypodium*, Murat et al. [62] proposed that chromosome shuffling events were driven by non-random centric double-strand break repair processes. The centromeric/telomeric illegitimate recombination between non-homologous chromosomes results in nested chromosome fusions, followed by additional structural changes (inversions and repeat invasions) and the formation of syntenic break points [62,63]. By investigating the hardness locus in diploid and polyploid wheat species, Chantret et al. [64] detected various genome rearrangements and suggested that illegitimate DNA recombination is one of the major evolutionary mechanisms leading to various genomic rearrangements. Recently, it became clear that retrotransposons have a definitive role in these processes [65,66]. Genome rearrangements are also thought to induce gene duplications which lead to the pseudogenization (functionless paralogues), concerted evolution (conservation of function for paralogues), subfunctionalization (complementary function of paralogues) and neofunctionalization (novel function of paralogues) of new alleles [62]. As the investigated *Aegilops* species are closely related to *Triticum*, it can be concluded that similar mechanisms took part in the evolution of the U and M genomes. Genome rearrangements in *Aegilops*, which were also formed frequently after allopolyploid speciation [20], could have been triggered by gene duplication events, as detected in this study (Table 3). After functional divergence, these duplicated loci may serve as raw material for evolution and represent potentially useful alleles for increasing the genetic diversity of bread wheat. It should be noted that the present comparisons of *Aegilops* genomes with wheat and model species were based on 100 orthologous genes. *Ae. tauschii*, whose D genome is of similar size (5.1 pg DNA/1C) to *Ae. umbellulata* (5.05 pg DNA/1C) and *Ae. comosa* (6.18 pg DNA/1C), has approximately 36,000 genes [67,68], so the coverage in the present work cannot be more than 0.0027 \( \times \), allowing only macro level comparison. In the near future, the shotgun sequencing of individual U and M genome chromosomes isolated by flow sorting [17] will result in a much deeper comparative genome analysis of *Aegilops* (http://www.wheatgenome.org/Projects/Complimentary-Projects/Wild-Relatives) and will provide more detailed information about evolutionary rearrangements and polyploidization-related processes in *Aegilops* U and M genomes.

Conclusions

Major efforts are underway to improve wheat yield and quality under stress conditions by increasing genetic diversity in breeding materials. Various *Aegilops* species have already been used as sources of new alleles for wheat breeding through interspecific hybridization. The conserved orthologous set markers assigned here to *Aegilops* chromosomes promise to accelerate gene introgression by facilitating the identification of alien chromatin. The analysis of complex polygenic traits such as earliness, abiotic stress tolerance and nutritional quality will also be accelerated, contributing to sustainable increases in wheat yields. Finally, the syntenic relationships between the *Aegilops* species, wheat and model species established in this work will facilitate the targeted development of new markers specific for U and M genomic regions and will contribute to the understanding of allopolyploidization-related molecular processes.

Supporting information

Figure S1 Rice–wheat–*Aegilops* orthologous relationships from the genomic perspective of *Oryza sativa*. The physical positions of the source ESTs of the COS markers are indicated on the rice chromosomes (Left). Each marker assigned to chromosomes of the wheat D genome or to chromosomes of *Ae. umbellulata* (U), *Ae. comosa* (M), *Ae. biculialis* (Ub,Mb) and *Ae. geniculata* (Ug,Mg) is colour-coded according to the homoeologous relation-(TIF)

Table S1 Primer sequences and anealing temperatures of the COS markers used in the present study. (DOC)

Table S2 Genomic positions of the non-polymorphic COS markers in rice and *Brachypodium* which were not assigned to *Aegilops* chromosomes. (DOC)
Table S3 Results of BLASTn search of source ESTs of COS markers assigned to Aegilops chromosomes in the rice genomic database.

(DOC)

Table S4 Results of BLASTn search of source ESTs of COS markers assigned to Aegilops chromosomes in the Brachypodium genomic database.

(DOC)

Table S5 PCR products of the COS markers in the genotypes of wheat and Aegilops species.

(DOC)

Table S6 Assignment of COS markers to the chromosomes or to the peaks on flow karyotypes in Aegilops umbellulata, Ae. comosa, Ae. biuncialis and Ae. geniculata.

(DOC)

Table S7 Syntenic relationship of U and M genomes relative to wheat.

(DOC)

Acknowledgments

The authors are grateful to Dr. Jarmila Čiháliková, Be. Romana Sperkova, and Zdenka Dubská for their assistance with chromosome sorting and DNA amplification.

Author Contributions

Conceived and designed the experiments: IM SG MM-L JD. Performed the experiments: IM AF AC HŠ RG JV. Analyzed the data: IM ML-W RG. Contributed reagents/materials/analysis tools: SG JD HŠ JV. Wrote the paper: IM MM-L SG HŠ JD.

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