Evolutionary dynamics and chromosomal distribution of repetitive sequences on chromosomes of *Aegilops speltoides* revealed by genomic *in situ* hybridization

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Simultaneous genomic *in situ* hybridization (GISH) with probe preannealing was used to detect the relationship between chromosomal position and sequence conservation on *Aegilops speltoides* var. *aucheri* chromosomes. DNA of *Secale sylvestre*, *Hordeum spontaneum*, *Festuca pratensis*, *Semiarundinaria fastuosa*, *Arundo donax* and *Zea mays* that represent several main groups of Poaceae were used as probes. Different GISH-banding patterns that characterize diverse evolutionary trajectories in the repetitive DNA fraction and correlate with evolutionary distance between tested species were observed. Fast-evolving sequences were detected in subterminal telomeric and subtelomeric heterochromatic regions, whereas sequences in pericentromeric regions showed high levels of conservation. GISH experiments revealed extensive conservation in NOR regions on chromosomes 1 and 6 which, in fact, appears to be a complicated mix of rDNA clusters and heterochromatin blocks of different nucleotide composition.

Keywords: *Aegilops speltoides*, genomic *in situ* hybridization, Poaceae, repetitive DNA sequences.

Introduction

The method of genomic *in situ* hybridization (GISH), with total genomic DNA as a probe in hybridization experiments, provides unique information about similarities between repetitive DNAs from related species, as well as the physical location of conserved sequences on chromosomes (Fuchs *et al.*, 1996; Heslop-Harrison & Schwarzacher, 1996). Simultaneous GISH with probe preannealing permits differences at the chromosome level to be displayed between chromatin fractions of a model species (Belyayev & Raskina, 1998). This type of data could shed light on the function and evolutionary dynamics of the noncoding DNA fraction. It is known that different chromosomal segments have different levels of sequence conservation. There are several chromosome structures that can be detected at the cytogenetical level such as rDNA loci or terminal telomeric sequences that share much in common with those from animals and fungi, hence there are some minor changes over a billion years (Appels & Honeycutt, 1986; Kipling, 1995). On the other hand, some chromosome regions show rapid changes in comparat-

ively short time periods and some differences can be detected even at the intraspecies level (Kipling, 1995; Belyayev & Raskina, 1998).

In the present study we aimed to identify evolutionary dynamics and chromosomal distribution of repetitive DNA by hybridizing *Aegilops speltoides* Tausch chromosomes with DNA of species representing several groups of Poaceae using the GISH technique. *Ae. speltoides* belongs to the section *Sitopsis* (Triticeae) and is most similar to the donor of the B-genome of bread wheat (Maestra & Naranjo, 2000).

Specifically, we investigated two questions. (1) In what order are chromosomal segments involved in the process of evolutionary divergence, i.e. what is the relationship between chromosomal position and sequence conservation? (2) At what evolutionary distance is it still possible to find cytologically visible similarity in repetitive noncoding DNA sequences between Poaceae species?

Materials and methods

Aegilops speltoides var. *aucheri* ($2n = 2x = 14$) from Latakia Province, Syria, original germplasm source, USDA PI 487235, was used for preparations of chromosome spreads and DNA isolation. DNA was

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also obtained from the following species: *Secale sylvestre* Host., Russia, Komarov Botanical Institute collection; *Hordeum spontaneum* C. Koch, from Mt. Hermon, Israel, Institute of Evolution collection; *Festuca pratensis* Huds., Russia, Komarov Botanical Institute collection; *Semiarundinaria fastuosa* (Mitf.) Makino ex Nakai, Russia, Komarov Botanical Institute collection; *Arundo donax* L., from nature, near Nes-Tsiona, Israel; *Zea mays* L., Weizmann Institute collection; *Lycopersicon esculentum* L, Weizmann Institute collection. These species (except *L. esculentum*) represent several evolutionary branches of the Poaceae (Fig. 1) *S. sylvestre* is closely related to *Ae. speltoides* and belongs to the same subtribe Triticinae, whereas *Z. mays* and *A. donax* are distantly related. The dicotyledon *L. esculentum* was selected to discriminate highly conserved chromosome regions.

GISH procedure was described previously in detail (Belyayev & Raskina, 1998; Belyayev *et al.*, 2000). Sonicated genomic DNA from *Aegilops speltoides* was labelled with biotin and preannealed with digoxigenin-labelled sonicated total genomic DNA from each of the other species at 37°C overnight. The probe mixture was hybridized to mitotic chromosomes of *Ae. speltoides*. The detection of biotin with fluorescein isothiocyanate (FITC)-conjugated avidin (green fluorescence) and digoxigenin with rhodamine-conjugated sheep antidigoxigenin Fab fragment (red fluorescence) was made simultaneously. As a result, chromosome regions of preferential hybridization of self-DNA were visualized as green, whereas regions of combined hybridization showed orange-yellow fluorescence.
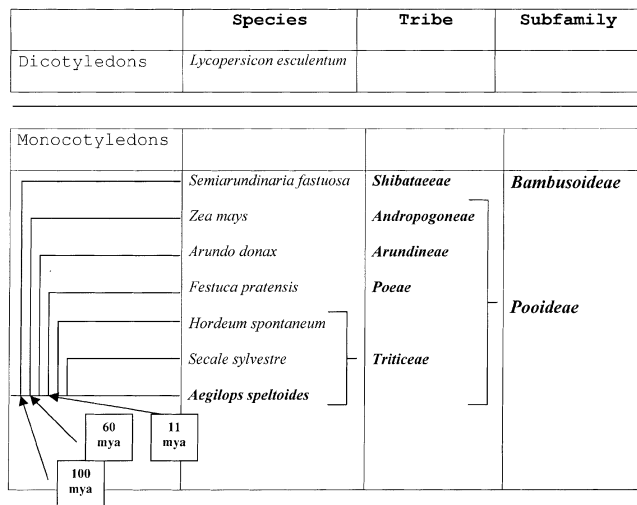
	Species	Tribe	Subfamily
Dicotyledons	*Lycopersicon esculentum*		

Fig. 1 Scheme of evolutionary separation between investigated species. Dendrogram is according to Tsvelev (1976). In the text boxes approximate time of taxa divergence million years ago (mya) are shown.

We established several steps for standardization of experimental procedures to be sure that data were comparable and reproducible. Several preliminary experiments to optimize the GISH protocol with regard to optimal concentrations, temperatures and other variables were made. Only the optimized GISH protocol was used for experiments. Previously (Belyayev & Raskina, 1998), we carried out a control experiment in which equal amounts of DNA from *Ae. speltoides* were separately labelled with biotin and digoxigenin, mixed in a 1:1 ratio and, following overnight preannealing, were hybridized to *Ae. speltoides* chromosomes. No green bands were detected on the yellow-orange chromosomes, hence both probes hybridized evenly along the chromosomes.

We repeated each experiment three times (two slides in each hybridization). In each slide we analysed a minimum of five complete metaphase plates. Chromosomes were identified according to Friebe & Gill (1996) as described in Belyayev & Raskina (1998).

Results

1 GISH using DNA from *Ae. speltoides* and *Secale sylvestre* on the chromosomes of *Ae. speltoides*. We obtained an orange-yellow combined signal that resulted from hybridization of both *Ae. speltoides* (detected in green) and *S. sylvestre* (detected in red) DNA in pericentromeric and intercalary chromosome regions. Green bands resulted from preferential hybridization of *Ae. speltoides* DNA to subterminal telomeric (all chromosomes) and subtelomeric (long arms of chromosomes 2, 3, 4, 5 and 7) heterochromatic regions (Figs 2A and 3).

2 GISH using DNA from *Ae. speltoides* and *Hordeum spontaneum* on the chromosomes of *Ae. speltoides*. DNA of *H. spontaneum* hybridized mainly to pericentromeric regions and in the regions of secondary constrictions (Figs 2B and 3). On chromosomes 1, 4, 5 and 6 the hybridization sites correspond to the pericentromeric heterochromatin regions revealed by Hoechst-33258 staining. On chromosomes 2, 3 and 7 the size of the hybridization sites exceeded that of the heterochromatic regions. In euchromatic regions a dispersed red signal of low intensity was detected. Telomeric and subtelomeric heterochromatin regions showed green fluorescence of high intensity. These data are similar to those obtained previously (Belyayev & Raskina, 1998).

3 GISH using DNA from *Ae. speltoides* and *Festuca pratensis* on the chromosomes of *Ae. speltoides*. Compared with the previous experiment, there was a further decrease of total red signal (Figs 2C and 3). DNA of *F. pratensis* also hybridized mainly in pericentromeric regions, but the sizes of these bands were reduced.
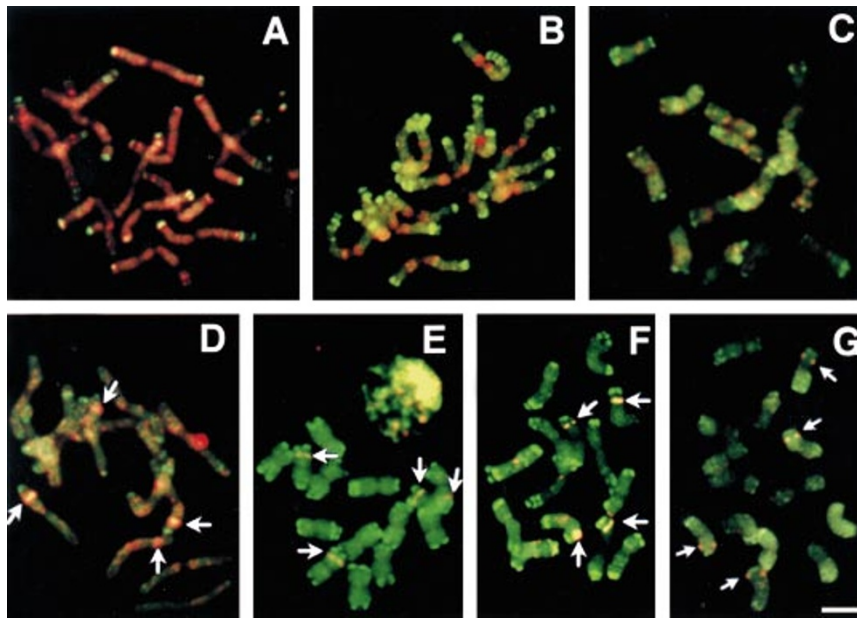
Fig. 2 GISH on the chromosomes of *Aegilops speltoides*. A. Probe: DNA of *Ae. speltoides* and *Secale sylvestre*. B. Probe: DNA of *Ae. speltoides* and *Hordeum spontaneum*. C. Probe: DNA of *Ae. speltoides* and *Festuca pratensis*. D. Probe: DNA of *Ae. speltoides* and *Semiarundinaria fastuosa*. Pro-meta-phase plate. E. Probe: DNA of *Ae. speltoides* and *Semiarundinaria fastuosa*. Metaphase plate. F. DNA of *Ae. speltoides* and *Zea mays*. G. Probe: DNA of *Ae. speltoides* and *Lycopersicon esculentum* (Solanaceae). Bar 10 μm. Signals from rRNA gene locations on *Ae. speltoides* chromosomes 1 and 6 are arrowed in D–G.
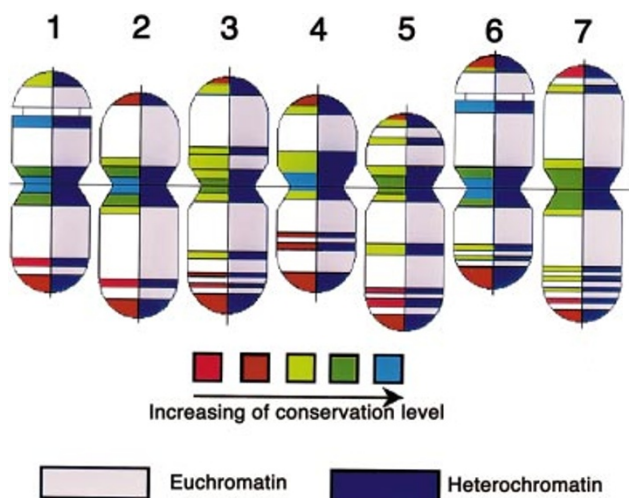

Fig. 3 Summarized GISH-banding karyotype of *Ae. speltoides* (left part of chromosome) versus C-banding (right part of chromosome). Heterochromatin discriminated according to its conservation. Sequences of these regions are different between *Ae. speltoides* and: red — *S. sylvestre*; dark red — *H. sponta-neum*; green — *F. pratensis*; dark green — *A. donax*. Highly conserved regions that are common for all investigated species including *Z. mays* and *S. fastuosa* are shown in blue.

Hybridization also revealed orange bands in the NOR regions.

4 GISH using DNA from *Ae. speltoides*, *Arundo donax*, *Zea mays* and *Semiarundinaria fastuosa* on the chromosomes of *Ae. speltoides*. Most distinct signals from hybridization of DNA of these species appeared in the NOR regions on chromosomes 1 and 6. An orange signal of low intensity was also observed in some pericentromeric regions (Figs 2D, E, F and 3).

5 GISH using DNA from *Ae. speltoides* and *Lycopersicon esculentum* on the chromosomes of *Ae. speltoides*. In this experiment, orange signal of low intensity from the DNA of *L. esculentum* could be observed only in NOR regions (Fig. 2G).

Discussion

In cereals, the amount of repetitive DNA families that serve as a base for genomic *in situ* hybridization reaches up to 85% of the total DNA (Flavell *et al.*, 1993; Heslop-Harrison & Schwarzcher, 1996). Using the GISH technique with probe preannealing (to increase probe specificity) we obtained several types of banding patterns on chromosomes of *Ae. speltoides*. The high temperature of hybridization allows high-precision annealing of chromosome/probe sequences (Bostock & Sumner, 1978), and at the same time it sharpens differences between reassociation kinetics of eu- and heterochromatin, revealing chromosome substructure. The hybridization mixture consists of self-DNA of *Ae. speltoides* detected in green and DNA of one of the tested species detected in red (probes overlapping). Thus, the green signal shows chromosome regions with preferential hybridization of *Ae. speltoides* DNA. The combined signal that is visualized as red–orange fluor-escence shows regions of hybridization of both DNAs, i.e. the chromosomal distribution of common sequences. The alignment of these signals shows correlation with evolutionary distance (Fig. 1) (Tsvelev, 1976). By comparison of the patterns obtained it is possible to

reconstruct the dynamics of the evolutionary processes in repetitive DNA families in the whole genome and in single chromosomes (Figs 2 and 3).

The green hybridization pattern that was obtained in the experiment with DNA of *S. sylvestre* from the same subtribe Triticinae (Tsvelev, 1976), shows the physical location on chromosomes of *Ae. speltoides* of rapidly evolving genus-specific repetitive sequences (Fig. 2A, red in Fig. 3). It corresponds to the subterminal telomeric and part of the subtelomeric heterochromatin regions (Belyayev & Raskina, 1998). Sequences in these regions are therefore the first to be involved in the process of evolutionary divergence in *Ae. speltoides*. This correlates with the known fact that in many species terminal repeat arrays and unique internal sequences are separated by repetitive sequences which are often species-specific, and many of which are found only at telomeres (Anamthawat-Jonsson & Heslop-Harrison, 1993; Kipling, 1995). The origin of the species-specific sequences is unclear. This can be due to accumulation of mutations caused by different factors, by activity of mobile elements, for instance (Belyayev *et al.*, 2001), and/or due to replacement of sequences by other classes of repeats.

Probing chromosomes of *Ae. speltoides* with DNA of *H. spontaneum* (subtribe Hordeinae) revealed a decrease of total red signal as compared with the previous experiment (Fig. 2B, dark red in Fig. 3). The red signal of low intensity can still be observed in intercalar hetero- and euchromatin regions, but the main red pattern corresponds to pericentromeric regions. This GISH experiment and hybridization with the DNA of a species that does not belong to the tribe Triticeae reveals pericentromeric heterochromatin as the location of slow-evolving family-specific sequences (Figs 2C, D and 3). It has long been established that the centromere suppresses the processes of recombination and unequal crossing over (Choo, 1997) that appear to be one of the main evolutionary mechanisms (Elder & Turner, 1995). Several repetitive DNA elements were found in centromeric regions through all cereals (Aragón-Alcaide *et al.*, 1996; Dong *et al.*, 1998). Most likely, this evolutionary conservation results from the centromere's functional importance.

Hybridization of *Ae. speltoides* chromosomes with DNA of species that do not belong to the subfamily Pooideae (Fig. 1) (Tsvelev, 1976) – *S. fastuosa* (Bambusoideae) (Fig. 2D, E, from dark green to blue on Fig. 3), *A. donax* (Arundinoideae) (data not shown) and *Z. mays* (Panicoideae) (Fig. 2F) gave very similar results. Red signal of low intensity was observed in pericentromeric regions, most distinctly on prometaphase chromosomes (Fig. 2D). The size of these regions does not exceed that revealed by Hoechst-33258 staining (Belyayev & Raskina, 1998). Bright orange bands of combined hybridization

of self and alien DNA shows sequence similarity in NOR regions on chromosomes 1 and 6 which, in fact, appears to be a complicated mix of rDNA clusters (Badaeva *et al.*, 1996) and heterochromatin blocks of different nucleotide composition (Schweizer, 1980; Belyayev & Raskina, 1998). The signal from those regions was also observed when DNA from the dicotyledon *L. esculentum* (Fig. 2F) was used.

Our data obtained from GISH experiments on *Ae. speltoides* chromosomes show that the first evolutionary changes that can be detected at the chromosome level in noncoding repetitive DNA sequences take place in subterminal telomeric heterochromatin. Cytologically visible similarity in noncoding repetitive DNA sequences is still detectable at pericentromeric regions of Poaceae species belonging to different tribes.

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