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Original article

Chromosomal localization of a novel repetitive sequence in the *Chenopodium quinoa* genome

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Abstract. In this study, a novel repetitive sequence pTaq10 was isolated from the *Taq I* digest of the genomic DNA of the pseudocereal *Chenopodium quinoa*. Sequence analysis indicated that this 286-bp monomer is not homologous to any known retroelement sequence. FISH and Southern blot analysis showed that this sequence is characterized by an interspersed genomic organization. After FISH, hybridization signals were observed as small dots spread throughout all of the chromosomes. pTaq hybridization signals were excluded from 45S rRNA gene loci, but they partly overlapped with 5S rDNA loci. pTaq10 is not a species-specific sequence, as it was also detected in *C. berlandieri*.

Keywords: *Chenopodium*, chromosomes, FISH, repetitive sequence.

Introduction

Highly repetitive DNA is a major component of higher plant genomes. It represents at least 20% and sometimes more than 90% of the genome. Two main types of repetitive DNA are distinguishable by their genomic organization. One group includes various types of satellite DNAs, the rDNA, and telomeric repeats. They are organized into tandem repeating units, where individual copies are adjacent, forming tandem arrays of monomeric units (Sharma and Raina 2005). The second group, dispersed repetitive DNA, is a heterogeneous class of sequences whose repeats are interspersed throughout the genome, scattered in all or many chromosomes of the complement (Kumar et al. 1998; Bennetzen 1999). Many of these repeats are derived from retrotransposons, such as *Ty1-copia*-like or *Ty3-gypsy*-like elements (Kumar and Bennetzen 1999; Vitte and Panaud 2005). However, there are also dispersed sequences that are not related to retrotransposon-like sequences, such as VfB from *Vicia faba* (Frediani et al. 1999). The repetitive DNA families may be widely distributed in various taxonomic groups, or may be specific to an individual species, genome or even a chromosome. These features have formed the basis for the intensive use of repetitive sequences for taxonomic and phylogenetic studies (Schmidt and Heslop-Harrison 1996; Zhang et al. 2002; Dechyeva et al. 2003; Weiss-Schneeweiss et al. 2003). Tandemly repetitive sequences are also excellent cytological markers for karyotyping, especially in species with numerous small chromosomes (Hasterok et al. 2001; Weiss-Schneeweiss et al. 2003)

The genus *Chenopodium* comprises several anciently domesticated species, such as *C. quinoa*, *C. berlandieri* or *C. pallidicaule.* Among them, the most important is *C. quinoa* (2n=4x=36). This plant is utilized for human consumption as a grain crop, and the whole plant is used for animal feed. It is a grain crop native to South America and has been introduced in the United States and Europe (Popenoe et al. 1989). The genome structure of *C. quinoa* remains largely uninvestigated, al-

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though there is some evidence that indicates its disomic behaviour and therefore a likely allotetraploid origin (Wilson 1990; Ward 2000; Maughan et al. 2004;). Very little information is available on the repetitive DNA sequence organization in the *C. quinoa* genome. Until now only rDNA sequences have been the subject of molecular and cytogenetic analysis. rRNA genes are localized in the *C. quinoa* genome in very few loci. *C. quinoa* has two pairs of rDNA loci and only one pair of 45S rDNA loci, suggesting a reduction in the number of 45S rDNA loci during the evolution of the species. Molecular analysis of IGS confirmed a very close relationship between *C. quinoa* and tetraploid *C. berlandieri*. A characterization of a 5S rDNA spacer region has revealed two different NTS sequence classes that presumably originated from the two subgenomes of allopolyploid *C. quinoa.* One of these is very similar in sequence to the NTS present in *C. berlandieri*. This finding suggested that these two allotetraploid species have at least one common diploid ancestor (Kolano et al. 2001; Maughan et al. 2006). In this paper, we report the chromosomal organization of a novel repetitive sequence from the *C. quinoa* genome.

Materials and methods

Plant material and slide preparation

Seeds of *C. quinoa* were obtained from the Botanic Garden in Nancy, France (accession no. 262). Accessions of cultivated forms of *C. berlandieri* subsp. *nuttalliae* (Huauzontle and Quelite) came from Future Foods Company (UK) and the wild form *C. berlandieri* subsp. berlandieri (PI 612858) from the North Central Regional Plant Introduction Station, Iowa State University (USA). The accession of *C. album* came from the Berlin-Dahlem Botanical Garden, Germany (accession no. 518). For cytogenetic analysis young leaves were pretreated with 2 mM 8-hydroxyquinoline for 4 h at room temperature, fixed in methanol-glacial acetic acid (3:1) and stored at –20°C until use. Metaphase spreads were prepared as described by Maughan et al. (2006). The fixed material was washed in a citrate buffer and digested in an enzymatic mixture of 4% cellulase "Onozuka R-10" (Serva) and 20% pectinase (Sigma) for 4 h at 37°C. The protoplast suspension was pelleted and resuspended in distilled water. After pelleting, the protoplasts were washed three times with 45% acetic acid. The pellet was resuspended in 20–30 μL of 45% acetic acid and incubated for 1 min at 70°C then 5 μL of the suspension was applied to a slide. After freezing, the coverslips were removed and the preparations were immediately postfixed in prechilled ethanol- glacial acetic acid (3:1), dehydrated in absolute ethanol and air-dried.

Extended DNA fibres were prepared according to Fransz et al. (1996), with some modification. After spotting onto microscope slides and air-drying, nuclei were disrupted in 10 μL of STE (0.5% sodium dodecyl sulphate, 50 mM EDTA, 100 mM Tris-HCl pH 7.0). After 60 s of incubation at room temperature, the released chromatin was stretched by tilting the slide. DNA fibres were fixed in ethanol-acetic acid (3:1) and air-dried.

For chloroplast observation, young leaves were fixed in 4% paraformaldehyde. Then the material was squashed on slides in a drop of PBS (phosphate buffered saline). After removing the coverslip, the slides were dried and stained with DAPI (4'-6-diamidino-2-phenylindole).

DNA extraction, molecular cloning, and sequence analysis

Total DNA was extracted from young leaves as described by Doyle and Doyle (1987) and digested with various restriction enzymes (*EcoR I, Hind III, Taq I, Sal I, Xba I, Xho II*). After polyacrylamide gel electrophoresis and ethidium bromide staining, only DNA digested with *Taq I* showed a band of about 200–300 bp, which revealed the presence of repetitive elements. This band was isolated and purified from polyacrylamide using the 'crush and soak' technique and the fragments were ligated into the *Cla* site of pBluescript vector. Transformed *E. coli* cells (NM522) were selected by blue-white selection and twenty eight recombinant clones were isolated. Recombinant clones were transferred onto positively charged nylon membranes (Roche). The clones were screened using the total genomic DNA from *C. quinoa* labelled with digoxigenin as a probe. Four clones were visualized by strong hybridization signals. The clones were sequenced on an automated sequencer LI-COR IR^2, following a modified dideoxynucleotide chain-terminator method, using SequiTherm EXCRL^{TM}II DNA Sequencing Kit-LC (Epicentre). For analysis of DNA sequences, the software programs E-seq and AlignIR were used. Homology searches employed public domain sequence databases (GenBank).

DNA probes and labelling

The 2.3 kb sub-clone of the 25S rDNA coding region of *Arabidopsis thaliana* (Unfriend and Gruendler 1990), labelled with digoxigenin-11-dUTP by nick translation, was used for the detection of 18S-5.8S-25S rRNA genes. The 5S rDNA 410 bp clone isolated from *Triticum aestivum* (Gerlach and Dyer 1980) and the newly isolated DNA clones (pTaq7, pTaq8 and pTaq10) were labelled with rhodamine-4-dUTP or digoxigenin-11-dUTP by PCR using universal M13 primers: forward (5'-CAG GGT TTT CCC AGT CAC GA-3') and reverse (5'-CGG ATA ACA ATT TCA CAC BAG GA-3'). The thermal cycling program was as follows: 94°C for 1 min, 35 cycles of 94°C for 40 s, 55C for 40 s, and 72°C for 1 min, and 1 cycle of 72°C for 5 min.

Southern hybridization

Genomic DNA (10 μg) was restricted to completion with Taq I restriction enzymes and separated in 1.5% agarose gels. For Southern hybridization, agarose gels were transferred onto positively charged nylon membranes (Roche) according to the standard protocols. The DNA probe was labelled with digoxigenin by PCR. Hybridization was performed according to the manufacturer's protocol using the DIG High Prime DNA Labelling and Detection Starter Kit II (Roche). High-stringency washes were performed to allow hybridization at the level of high sequence similarity (2 × 5 min in 2 × SSC and 0.1% SDS at room temperature, and 2 × 15 min in 0.1 × SSC and SDS at 65°C). Signals were detected by luminescence on Kodak BIOMAX MR Film.

Fluorescent in situ hybridization (FISH)

Methods of *in situ* hybridization were adapted with some modification from Schwarzacher and Heslop-Harrison (2000). In short, slides were pretreated with RNase (100 μg mL^{-1}) for 1 h at 37°C, washed in 2 × SSC for 15 min, dehydrated in an ethanol dilution series, and then air-dried. The hybridization mixture consisting of 100 ng of labelled DNA probe, 50% formamide, 10% dextran sulphate, 0.1% SDS (sodium dodecyl sulphate), and 10 μg of sheared salmon sperm DNA, was denatured for 10 min at 85°C, then chilled for 5 min on ice, and applied to the chromosome preparation. The slides and hybridization mixture were denatured together at 72°C for 5 min in an *in situ* Thermal Cycler (Hybaid) and then allowed to hybridize overnight in a humid chamber at 37°C. Stringent washes (20% formamide in 0.1 × SSC at 42°C) were followed by immunodetection of digoxigenin-labelled DNA probe using FITC-conjugated primary anti- digoxigenin antibodies and signal amplification with FITC-conjugated anti-sheep secondary antibodies. The preparations were mounted in Vectashield containing 2 μg mL^{-1} DAPI. Images were acquired with a Hamamatsu CCD camera attached to an Olympus Provis epifluorescence microscope, then processed uniformly, and superimposed using Micrografx Picture Publisher software. After taking images, the slides were washed with 4 × SSC-Tween20 and a new FISH experiment was performed with 25S rDNA and 5S rDNA as probes.

Results and discussion

The genomic DNA of *C. quinoa* partially digested with *Taq I* showed a band at 250–300 bp, indicating the presence of the elements in the *C. quinoa* genome in a high copy number. The DNA fragments cloned into the plasmid vector resulted in twenty eight clones named: pTaq1, pTaq2, etc. Among the obtained clones four (pTaq4, pTaq7, pTaq8, pTaq10) showed positive results in dot-blot hybridization with a total genomic DNA of *C. quinoa* and these clones were sequenced and selected for analysis. The clones pTaq4 and pTaq8 had the same nucleotide sequence, so for further analysis only pTaq8 was chosen. Nucleotide sequences of the three cloned DNA fragments are presented in Figure 1. The clones pTaq7, pTaq8 and pTaq10 were respectively 255 bp, 225 bp and 286 bp long, with an AT content of 43% for pTaq7 and 46% for pTaq8 and pTaq10. The sequences pTaq7 and pTaq8 showed homology to chloroplast DNA sequences isolated from a number of dicotyledonous species. Sequence similarity, especially to fragments derived from *Spinacia oleracea* chloroplast DNA, was very high and reached 94%. The clone pTaq10 showed no homology to any known mobile element sequences, but it revealed a significant similarity to a *C. quinoa* BAC clone sequence containing the salt overly sensitive 1 (*SOS1*) gene (GenBank Acc. No. EU024570). However, the homologous sequence within the BAC clone is located about 2750 bp upstream of the *SOS1* coding sequence. The level of similarity between pTaq10 and the corresponding BAC sequence is 90% (259 nucleotides identical out of 286) without any gaps. Inser-

(a)
GCCTGATTATCCCTAAACCCAATGTGAGTTTTTCTATGTTGACTTGCCCCCCCGCCGTGA 60
TTGAATGAGAATGAATAAGAGGCTCGTGGGATTGACGTGAGGGGGTAGGGATGGCTATAT 120
TTCTGGGAGCGAACTCCAGGCGAATATGAAGCGCATGGATACAAGTTAGGCCTTGGAATG 180
AAAGACAATTCCGAATCCGCTTTGTCTACGAACAAGGAAGCTATAAGTAATGCAACTATG 240
AATCTCATGGAGAGT 255

(b)
TCACTCTGACTGCAAGCTTTTTCTGTCCGTGAGGATCCCACCAGAGCGCCTTCTACTTCT 60
AATAGGCCATGAACTAGATAAGAATCATTCTCAACGAGTCCATAAGAAGTGATCCCGTTT 120
TTTTCATCGGGTCCAGGTAGAGACCAAAGATCTTGAGCGACCGATCCGGCAGAACAACTC 180
AAAAGATAAAGAAGTATCGTTAATTTCTTCATGCTCGTTCCAAGT 225

(c)
GGCTTTCGCCCACAGGAACTACCTCAGCCGGACCCCTAAGGCAGGCGGTACCGGCTGGTA 60
TCAATTGACGAGCCACAGGGGCTACCTCATGACGAGAGGTAAAGCAAACAAATGGAAGGA 120
CTGGCGGAGCACCTTCCTTATCATTGAGAGTTCAGACACAGAGCTGAACAAGCGCTTCAA 180
CAGGTGGAACAAGAATCCGCCCCTTCTTGCAAGCGGCCGCAGCATGCCGCCTGTTCCCCC 240
CAAGGCTAAGAAAGAGATCCTAGATAAAGGACTCTTCAAAGTAGAG 286

Figure 1. DNA sequences of (a) pTaq7, (b) pTaq8, and (c) pTaq10

tion of a repetitive sequence without homology to mobile elements into the noncoding region of a gene was earlier reported for a sea urchin histone gene (Yager et al. 1987). However, a detailed analysis of the BAC sequence reveals two regions homologous to polyprotein GAG-POL of *Ty3-gypsy*-like retrotransposon (about 50% of amino acid identity), located 3 kbp and 46 kbp upstream of the pTaq10 sequence, respectively. Thus we hypothesize that the closely located *Ty3-gypsy*-like retrotransposon could be responsible for the distribution of pTaq10-like sequences within the *C. quinoa* genome. On the other hand, the hypothetical link between the transposable element and pTaq10 sequence is not clear, and their colocalization in the analysed BAC can be also just accidental.

Fluorescent *in situ* hybridization was used to investigate the physical distribution of the newly isolated sequences along the chromosomes. DNA sequences with homology to a chloroplast genome were also used in these experiments, because as Shahmuradov et al. (2003) had indicated earlier, plastid DNA can be inserted into the nuclear genome. After FISH with pTaq7 and pTaq8 no signals were seen on the chromosomes but about 6–11 signals were observed in small round structures stained with DAPI (Figure 2a). The microscopic analysis of the preparations fixed in paraformaldehyde and stained with DAPI shows that the structures colocalized with the red autofluorescence of chlorophyll (Figure 2b, c). This observation revealed that the structures are chloroplasts. This confirmed the results obtained from the sequence similarity analysis, indicating that clones pTaq7 and pTaq8 contain sequences that belong to the chloroplast genome. A lack of hybridization signals on chromosomes and interphase nuclei suggested that the sequences are not present or are present in a very low copy number in the nuclear genome of quinoa.

FISH showed that pTaq10 had spread all over the 36 chromosomes (Figure 2d). Weak but discrete hybridization signals were observed in the pericentromeric, interstitial or terminal localization on chromosome arms. The obtained results showed that the pTaq10 sequence is characterized by disperse organization in the *C. quinoa* genome. In interphase nuclei, hybridization signals of pTaq10 clone were present, particularly in heterochromatic regions (Figure 2g, h). Clone pTaq10 hybridized to all chromosomes of allotetraploid *C. quinoa*. This may indicate that this repetitive sequence was present in both putative ancestral genomes. It is also possible that pTaq10 existed only in one ancestral genome and has spread to a new genome since polyploidization. Such intergenomic concerted evolution following polyploidization was revealed in polyploid *Gossipium* species, where some dispersed repetitive sequences specific to an A-subgenome spread to a new localization in the D-subgenome (Hanson et al. 1998; Zhao et al. 1998).

The slides were rehybridized with the rDNA sequences. In *C. quinoa* the genes for 25S rRNA were mapped at the terminal position in one chromosome pair. The 5S rRNA genes were localized in two chromosome pairs in terminal and interstitial positions (Figure 2e). A lack of colocalization was observed between pTaq10 and 25S rDNA loci, but hybridization signals for 5S rDNA and pTaq10 partly overlapped, suggesting that pTaq10 and 5S rDNA sequences could be colocalized in

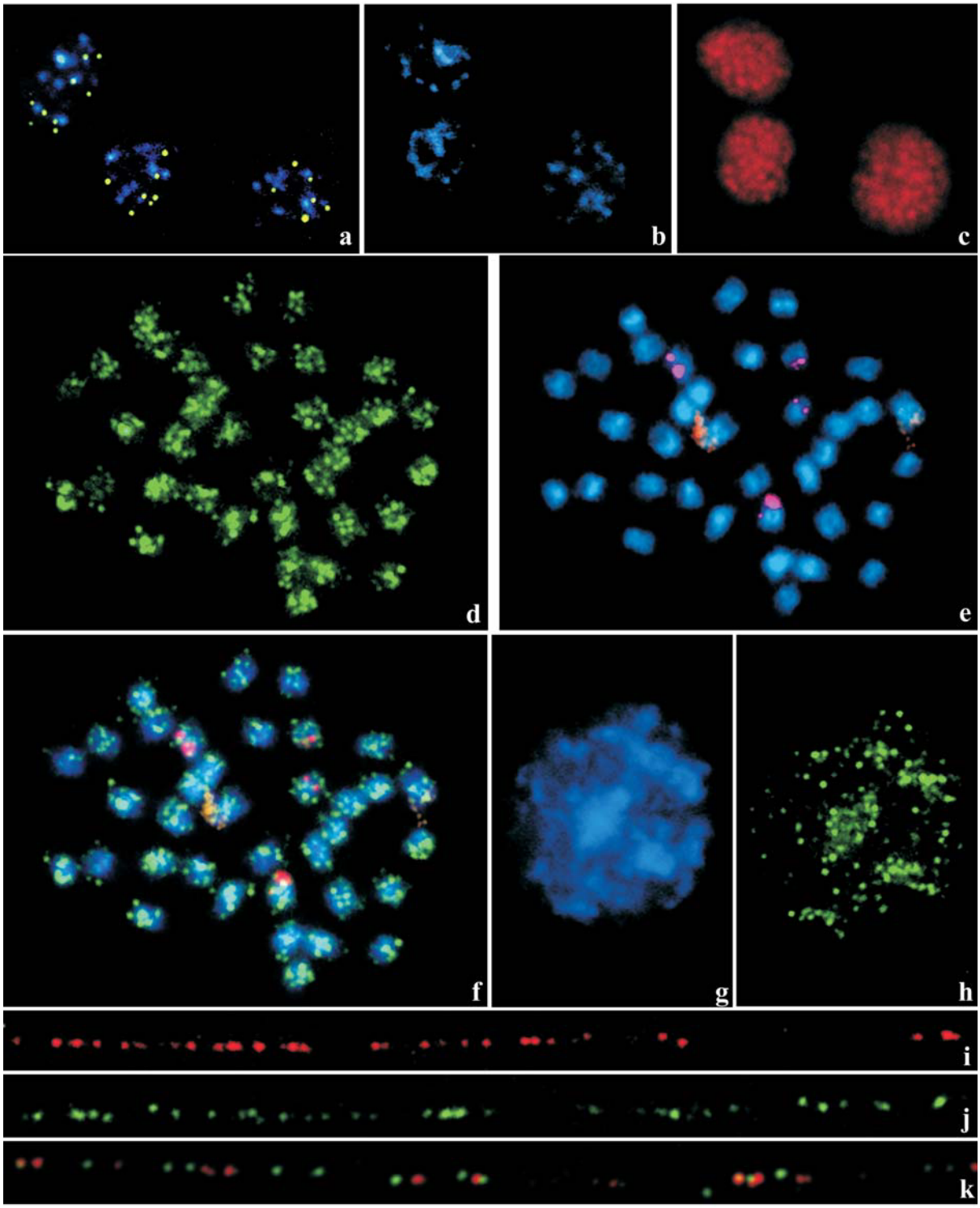

Figure 2. Physical localization of repetitive sequences in the *C. quinoa* genome. (a) *In situ* hybridization of pTaq7 (green) to chloroplast DNA counterstained with DAPI. (b) Chloroplast DNA stained with DAPI and (c) red autofluorescence of chlorophyll. (d) Metaphase chromosomes after *in situ* hybridization with pTaq10. (e) Rehybridization of the same metaphase with 25S rDNA (yellow) and 5S rDNA (red) probes, counterstained with DAPI (blue). (f) Overlaid image of d and e. (g) Blue fluorescence of interphase nuclei after DAPI staining. (h) The same interphase nuclei after FISH with pTaq10. (i-k) FISH to extended DNA fibres. (i) Hybridization signals of 5S rDNA sequences and (j) pTaq10 clone. (k) Double FISH revealed interspersion of pTaq10 (green) and 5S rDNA sequences (red).

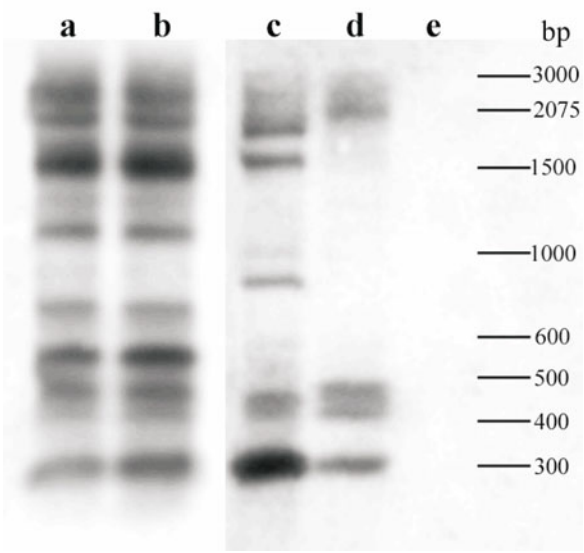

Figure 3. Southern hybridization of pTaq10 clone to *Taq I*-digested *Chenopodium* DNA: (a) *C. berlandieri* subsp. *nuttalliae* Huauzontle, (b) *C. berlandieri* subsp. *nuttalliae* Quelite, (c) *C. quinoa,* (d) *C. berlandieri* subsp. *berlandieri,* (e) *C. album*

some chromosome regions (Figure 2f). A higher mapping resolution of pTaq10 and 5S rDNA sequences was achieved using extended DNA fibres. After double FISH, linear tracks of red (5S rDNA) and green (pTaq10) signals were observed (Figure 2i, j). 5S rDNA signals showed continuous dotted tracks with minor and major gaps, suggesting that tandem repeats of the monomers were interrupted by other sequences. Hybridization signals of pTaq10 repeats were observed as short continuous dotted tracks or they were arranged in a more dispersed way. Signals for 5S rDNA and pTaq10 were mainly present as separate tracks but colocalization of these two sequences was also observed (Figure 2k). It is likely that some interspersion of the 5S rRNA genes and pTaq10 occurred, and that they were not completely spatially separated. rRNA genes are organized in long arrays of tandem arranged repeats and usually other repetitive sequences are excluded from the rDNA loci (Galasso et al. 1997; Schmidt et al. 1998). In *C. quinoa* chromosomes, pTaq10 repeats are most often excluded from rDNA loci, except for part of the interstitial 5S rDNA site. This locus is joined, or even to some extent overlaps with pericentromeric heterochromatin, which suggested that at least part of the locus was not transcriptionally active. The correlation between heterochromatin and rDNA loci was reported for *Allium* species, where some of the hybridization signals of 5S and 45S rDNA overlapped with C-bands. Do et al. (2001) indicated that in the *Allium cepa* genome these rDNA loci that colocalized with constitutive heterochromatin also overlapped with the hybridization signals of repetitive sequences.

The genomic organization of pTaq10 was analysed using Southern hybridization. A lack of periodicity of the hybridization bands showed the absence of large tandems of the sequence units indicating the dispersed character of pTaq10 in *C. quinoa* (Figure 3, line c). The series of bands ranged from 286 bp to 3000 bp, but the strongest hybridization signals corresponded to monomer length (286 bp). The genomic organization of pTaq10 was also analysed in *C. berlandieri* and *C. album* accessions (Figure 3). All these species (*C. quinoa, C. berlandieri* and *C. album*) belong to the same section *Chenopodium* of genus *Chenopodium.* Clone pTaq10 hybridized to the genomic digests of *C. berlandieri,* which is considered to be very closely related to quinoa. The pattern of hybridization obtained for *C. berlandieri* accessions were different from those observed for *C. quinoa*. Additionally, two subspecies of *C. berlandieri* exhibited different hybridization patterns. *C. berlandieri* subsp. *nuttalliae* accessions (Figure 3, lines a, b) revealed numerous strong signals between 286 bp and 3000 bp, whereas *C. berlandieri* subsp. *berlandieri* exhibits much fewer hybridization bands (Figure 3 line d). pTaq10 was not present in *C. album* (Figure 3, line e).

Dispersed repetitive sequences represent a large proportion of plant genomes. Most of them are considered to derive from or be the remains of transposable elements. pTaq10 has no homology to any known retroelements or remnants thereof, although the chromosomal distribution of the clone shows similarities to the physical distribution of retroelements in many plant species (Schmidt et al. 1995; Brandes et al. 1997). Dispersed repetitive sequences without homology to mobile elements were reported earlier for species such as *Beta procumbens*, *Avena sativa* or *Pisum sativum* (Neumann et al. 2001; Ananiev et al. 2002; Dechyeva et al. 2003). Repetitive DNA is subject to various rates of evolution. While some repetitive sequences are relatively conserved in distantly related species, others are subject to rapid diversification and evolution. After Southern hybridization with pTaq10 different band patterns were revealed by *C. quinoa* and C. *berlandieri* accessions. We assume that the ancestral sequence of pTaq10 was already present in a progenitor of the species, but after separation the amplification

of the ancestor took place in a different manner in each species. Interestingly, two subspecies of *C. berlandieri* differ in the copy number and genomic organization of the repeats. This is in agreement with the results obtained using molecular markers (Ruas et al. 1999). They indicated that the genomes of these two subspecies are quite divergent and support the idea that *C. berlandieri* should be divided into two species: *C. berlandieri* (wild plant) and *C. nuttalliae* (cultivated plant). pTaq10 did not hybridize with the DNA digest of *C. album*. That species, opposite to *C. quinoa* and *C. berlandieri*, originates from Eurasia (Wilson 1990). Geographical isolation made the genomes of New World and Old World species evolve separately for a long time. According to taxonomy *C. album* is placed in subsection *Leiosperma* (section *Chenopodium*) whereas *C. quinoa* and *C. berlandieri* in subsection *Cellulata* (section *Chenopodium*) Wilson (1990). Lack of hybridization of pTaq10 to the *C. album* DNA digest suggested that this repetitive sequence could be specific only for New World species belonging to subsection *Cellulata* and help to clarify phylogenetic relationships inside the section. It could be very useful in a search for diploid or tetraploid progenitors of cultivated chenopods of the New World. Our data suggests that the novel repeats have evolved quite recently and could be a very useful tool in phylogenetic analysis inside *Chenopodium*. genus

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