# The intergenic spacer region of the rDNA in *Haplopappus gracilis* (Nutt.) Gray

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**Abstract**

In this paper, we provide further information on the genome organisation of *Haplopappus gracilis*, one of the six angiosperms showing the lowest chromosome number, i.e. 2n=4, by determining the nucleotide sequence of the intergenic spacer region of the ribosomal RNA genes and its cytological localization on metaphase chromosomes. DNA sequence analysis reveals the occurring of a product of 4,382 bp in length, characterised by the presence of four blocks of different repeated sequences. Our analysis also evidenced putative promoter regions with three transcription initiation sites for polymerase I, as previously reported in *Artemisia absinthium*, belonging to the same Asteraceae family. A fluorescent *in situ* hybridization with the intergenic spacer probe indicates the presence of rDNA genes only in the satellited chromosomes of *H. gracilis*; besides, differences in the signal intensity between homologous chromosomes were frequently observed, thus suggesting for these chromosome sites the presence of a variable number of rDNA gene copies, even if a divergent chromatin organisation in corresponding regions cannot be ruled out.

**Keywords** *Haplopappus gracilis*, Ribosomal DNA, Intergenic spacer, *In situ* hybridization, Homologous

chromosomes

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## Introduction

*Haplopappus gracilis* (Nutt.) Gray (Asteraceae), also known as *Machaeranthera gracilis* (Nutt.) Shinners or *Xanthisma gracile* (Nuttal) D.R. Morgan & R.L. Hartman, is one of the six angiosperms showing the lowest chromosome number, i.e. 2n=4, as also occur in *Zingeria biebersteiniana* (Claus) P. Smirnov (Poaceae), *Colpodium versicolor* (Stev.) Schmalh (Poaceae), *Brachycome dichromosomatica* C.R. Carter (Asteraceae), *Ornithogalum tenuifolium* (Hyacinthaceae) and *Rhynchospora tenuis* Link (Cyperaceae) (for a recent review, see Ruffini Castiglione and Cremonini 2012). This species has a DNA content of 2C=4.10 pg (Bennett 1972) and the chromosome complement is composed of a pair of V-shaped chromosomes (I) and a pair of J-shaped chromosomes (II), i.e the nucleolar chromosomes (Jackson 1957, 1959), thus representing an interesting model for studying genome organisation. In a previous paper, we carried out a cytological investigation of the chromosome complement of *H. gracilis* (Ruffini Castiglione et al. 2008a). Karyomorphometric data, generated by an automated image analysis system, enabled an accurate determination of the karyomorphological indices (Huziwara 1962; Greilhuber and Speta 1976), which are directly related with the evolution of the karyotype. Moreover, the DNA methylation pattern by using a monoclonal antibody against 5-methylcytosine (5mC), the fluorochrome banding by chromomycin A3 and 4′,6-diamidino-2-phenylindole (DAPI) and the effects of DNase I treatment on both interphase and metaphase chromatin were determined. In the present report, we provide further information on the genome organisation of *H. gracilis* by determining the nucleotide sequence of the intergenic pacer region (IGS) of the ribosomal genes and its cytological localization. The nucleolus organiser (NOR) is a complex genetic locus consisting of numerous tandemly arranged copies of ribosomal RNA genes at one or more chromosomal locations (Rogers and Bendich 1987). One repeating unit consists of the 18S, 5.8S and 25S ribosomal RNA (rRNA) coding regions, the corresponding internal transcribed spacers (ITS1 and ITS2) and an IGS, which includes non transcribed spacer and transcribed spacer (ETS) regions. The IGS plays an important role in cellular processes as rDNA transcription regulatory sequences and pre-rRNA processing signals are located within (Hemleben and Zentgraf 1994; Ruffini Castiglione et al.1998; Fernandez et al. 2000; and references therein). A general feature of the IGS is the presence of several types of repeated elements, also referred as subrepeats (s.r.); for some of these elements, a function of enhancer of transcription has been suggested (Flavell et al. 1986). Complete nucleotide sequence and internal structural organisation of IGS have been reported for many angiosperm dicotyledonous species. However, to our knowledge, no data of IGS sequence structure for Asteraceae has been published, except for Artemisia species (Garcia et al. 2009, 2010). Therefore, studies on the molecular organisation of the IGS are important and comparative analyses in different species can give information on the evolutionary changes of conserved and variable sequences.

## Materials and methods

*Germination of seeds*

Seeds of *H. gracilis* (Nutt.) Gray (accession number HAPLO1), kindly provided by the IPK, Gatersleben (Federal Republic of Germany), were germinated on a moist filter paper in Petri dishes at 23 °C, after gentle stirring for 6 h in 0.1 % Tween 20 (Sigma).

DNA extraction, polymerase chain reaction, DNA cloning and sequencing Genomic DNA was extracted from seedlings of *H. gracilis* with MasterPure Plant Leaf DNA Purification Kit (Epicentre, USA). The IGS region was amplified by a standard polymerase chain reaction using AccuPrime (Invitrogen, USA) and primers drawn from conserved regions of 25S rDNA and 18S rDNA, as deduced from the comparison of nucleotide sequences of ribosomal genes present in the EMBL database. The primers were 25Sdir (GACGACTTAAATACGCGACGGG) and 18Scom GACTACTGGCAGGATCAACC).

Amplification was carried out using the following parameters: 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 68 °C for 5 min and finally 7 min at 68 °C. One PCR-amplified DNA fragment of about 4,500 bp was present when analysed on 1 % agarose gel. Single band was recovered and purified with GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, UK) and cloned in pGEM-T Easy Vector System (Promega, USA). For the sequencing reactions, several primers were used: 25Sdir, 18Scom, Ast8 and Ast6 (Markos and Baldwin 2001) in combination with the primers 512dir (GGATTTCCCCAAGAGAGGTTCCC) and 781dir (CTCGTCACAATCCTTCAAAG), drawn from the sequence itself. The presence of a highly repeated region lacking of nucleotide sequences suitable for drawing specific primers required a different strategy to complete the sequencing.

Indeed, the fragment NheI-NheI (1,879 bp) (cf. Fig. 1) was deleted and the resulting recombinant plasmid (3,015 bp +2,503 bp of the insert) was sequenced with 512dir, in order to obtain the sequence of the residual portion of the IGS. The obtained nucleotide sequence was analysed with DNAMAN (Lynnon Biosoft, Canada) and Bioedit (Hall 1999).

*Probe preparation and in situ hybridization*

The region of the IGS spanning from position 2,045 to position 3,838 (Fig. 1) was amplified and labelled by PCR in the presence of digoxygenin-dUTP (Roche, Germany), using AccuPrime (Invitrogen, USA) and the primers Ast6 and Ast8 (Markos and Baldwin 2001). Amplifications were carried out using the following parameters: 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 68 °C for 5 min and finally 7 min at 68 °C. *In situ* hybridization was carried out as previously described (Ruffini Castiglione et al. 2009) with some modifications. In brief, mitotic preparations were treated with RNase A and pepsin. After post-fixation in 4 % neutral formaldehyde, the slides were processed for heat DNA denaturation, dehydrated in cold increasing ethanol series and airdried.

The hybridization mix contained 40 ng of labelled IGS probe, 10%dextran sulphate, 0.125%SDS, 160 ng/µl salmon sperm and 50 % formamide in 2× SSC. Heat-denatured hybridization mixture was applied on slides, covered with plastic cover slips. After hybridization at 37 °C for 18 h, slides were washed and treated with anti-digoxigenin–fluorescein antibodies (Roche, Germany) for fluorescent detection of hybridization sites and counterstained with DAPI (Sigma, USA). Slides were visualised using a Zeiss Axio Observer.Z1 fluorescent microscope (Zeiss, Germany). Images were registered by a CCD camera AxioCam MRc5 (Zeiss, Germany) and processed with the leased imaging software.

## Results

From the sequencing data, the IGS of H. gracilis is 4,382 bp in length. The general organisation of this intergenic region is reported in Fig. 1. The IGS presents a 47.8 % guanine–cytosine (GC) content, and the beginning is characterised by a pyrimidine-rich motif (CCCTCCCCCC) of common occurrence in plants (Hemleben and Zentgraf 1994). The region spanning from base 1,430 to base 2,042 is characterised by the presence of the putative promoter regions with the transcription initiation sites (TIS) for polymerase I (Fig. 2). In *H. gracilis*, three sequences (TATATATAGGGGGG) are found that fit the reported TIS of plants (Gernster et al. 1988; Kato et al. 1990; Ueki et al. 1992), at the positions 1,492, 1,691 and 2,029 after the end of 25S. Every putative TIS is preceded by a sequence of about 60 bp in length, highly conserved (88.6 % of homology) showing a 72 % AT content (Hap. 1–3) (Fig. 2).
Moreover, both after the first and the second putative TIS, there is a highly conserved sequence of 69 bp (repeated elements type 1), followed by another sequence of about 40 bp in length (repeated elements type 2) reiterated once after the first putative TIS and four times after the second one; these repeats are absent after the third putative TIS (Fig. 2). The comparison of the region containing the putative promoter sequences of *H. gracilis* with analogous regions of *Artemisia absinthium* (Asteraceae) (from Garcia et al. 2009) shows significant homologies (Fig. 3). Indeed, in both the cases, three sequences homologous to the putative TIS are present and every putative TIS, both in *H. gracilis* and in *A. absinthium*, is preceded by a highly conserved AT-rich sequence (named Hap. 1–3 and Art. 1–3, respectively). From the comparison of these repeated sequences, a homology of about 81 % between *H. gracilis* and *A. absinthium* can be derived (Fig. 3).
The IGS of H. gracilis is characterised by the presence of four different blocks of repeated sequences localised both upstream and downstream the region containing the putative TIS (Fig. 1). The first and second blocks are located upstream: the first block (s.r. A) is localised in the region spanning from base 57, from the end of 25S, to base 489 and contains three related tandem elements of about 100 bp in addition to two incomplete s.r. of 44 and 89 bp, respectively (77.5 % of homology). The second repeated region (s.r. B) (604-bp long, from base 777 to base 1,380) includes four related s.r., 139–159 bp in length (74.5 % of homology). The repeated sequences belonging to group A and B show no significant homology with sequences present in the EMBL database.
The other repeated sequences are located downstream the region containing the TIS; in particular, the third block (s.r. C), spanning from base 2,323 to base 2,466, is composed by four related tandem elements, 36 bp in length with 90.5 % of homology, followed by another family of repeated sequences (s.r. D), from base 2,476 to base 3,688, composed from 11 s.r. about 110 bp in length showing 91.94 of homology. Moreover, it is noteworthy that the region containing the sequences of types C and D, spanning from base 2,323 to base 3,688, is surrounded at its ends by an identical sequence of 102 bp. The region adjacent to the 5′ end of the 18S rRNA genes, including the s.r. D, exhibits a significant homology with analogous sequences of Asteraceae (Markos and Baldwin 2002; Garcia et al. 2009), but no intriguing aspects rise from the comparison of these ETS regions. The s.r. A, B, C and D are related with none of the repeated sequences enclosed in the putative promoter regions.
The results of *in situ* hybridization experiments of digoxigenin-labelled IGS probe, detected with green fluorescence, are shown in Fig. 4a, b. The probe hybridised with only the J-shaped chromosomes, the nucleolar chromosomes, while no signal was detectable in the V-shaped chromosomes. The location of the IGS following hybridization involved the whole NOR, but prominently, the secondary constriction; besides, differences in the signal intensity between homologous chromosomes were frequently observed (Fig. 4a, b).

## Discussion

The IGS of *H. gracilis* is 4,382 bp in length and is characterised y the presence of putative promoter regions with three sequences that fit the reported TIS for RNA polymerase I in plants (Gernster et al. 1988; Kato et al. 1990; Ueki et al. 1992), in addition to the four different blocks of repeated sequences, indicated as s.r. A, B, C and D (Fig. 1).

The IGS regions of rRNA genes in animals and plants often contain one or more Pol I promoters (Coens and Dover 1982; Gernster et al. 1988; Gruendler et al. 1991; Hemleben and Zentgraf 1994; Suzuki et al. 1996; Bauer et al. 2009; Garcia et al. 2009). The significance of the duplication of some portions/or of the entire promoter in plants is unclear, even if in analogy to animal rDNA (Moss 1980; De Winter and Moss 1986), it was reported that one of the promoter sequences is the gene promoter while the others are designated as spacer promoters and may act as regulatory sequences (Suzuki et al. 1996; Mayer et al. 2006). As a rule, the gene promoter is a relatively large sequence of about 150–160 bp, preceded by a long AT-rich stretch and overlapping the transcribed gene by several base pairs (Gernster et al. 1988; Hemleben and Zentgraf 1994). The significance of a similar AT-rich region has been discussed; indeed, Borisjuk et al. (2000) and Volkov et al. (2004) suggested that these sequences, not the subrepeats, may act as enhancers of transcription and that the length of the AT-rich region upstream the TIS modulates differential transcription of rDNA.

As a rule, the spacer promoters are similarly organised as the gene promoter (Fernandez et al. 2000; Komarova et al. 2004 and references therein). It is noteworthy that in *Arabidopsis*, the nucleotide sequences of the gene promoter and spacer promoter were highly conserved, and the transcripts from both the promoters were clearly detected; but the putative gene promoter had a longer AT-rich sequence in its upstream region (Doelling et al. 1993). On the contrary, in *H. gracilis*, every putative TIS is preceded by a AT-rich stretch of about 60 bp in length, highly conserved as far as the sequence length and composition; an analogous situation has detectable in *A. absinthium*, too, where three TIS are present, preceded by motifs AT-rich, highly conserved (from Garcia et al. 2009). A different situation characterises *Olea europaea*, where a single sequence is found that fits the TIS of plants, preceded by an extremely AT-rich sequence; but the striking aspect of the region containing the promoter in *Olea* is the occurrence, after an initial AT-rich stretch, of a subregion showing 51.9 % of homology with the gene promoter but lacking of any sequence homologous to the TIS (Maggini et al. 2008). Therefore, in our opinion, it is very difficult to draw a general picture on the significance of these duplications, if present, in plants.

Another point of interest arising from our study is represented by the high level of homology between the AT-rich sequences preceding the TIS of *H. gracilis* (Hap. 1–3) and *A. absinthium* (Art. 1–3) (Fig. 3), since the conservation of otifs in the promoter regions of the otherwise highly variable IGS sequences could point to the relevance of these motifs as functional sequences, at least in the Asteraceae.

Moreover, by studying the distribution of 5S rRNA genes in the chromosome complement of several species belonging to the family of Asteraceae, Garcia et al. (2009, 2010) evidenced he presence of 5S units embedded in the 26-18S rDNA spacer in species belonging to the tribes Anthemideae, Gnaphalieae
and to the “Heliantheae alliance” of subfamily Asteroideae (Panero and Funck 2008), but not in the remaining five tribes of the Asteroideae. From the comparison of the sequencing data, we can exclude the presence of 5S rRNA genes in the IGS of *H. gracilis* (data not reported), suggesting a conventional unlinked arrangement of these genes in this species, belonging to the tribe Astereae of the subfamily Asteroideae, thus confirming the results of Garcia et al. (2009, 2010).

As far as the location of the IGS probe following hybridization is concerned, it involves the whole NOR, but specially the secondary constriction (Fig. 4a, b). No signal is detectable in other regions of the chromosome complement, as observed, on the contrary, in *C. versicolor* and in *Z. biebersteiniana* where additional minor ribosomal sites were evidenced (Bennett et al. 1995; Kim et al. 2009).

It is noteworthy that the size and intensity of the hybridization signals frequently differed between homologous
chromosomes of *H. gracilis*, (Fig. 4a, b). Also, in *Quercus* species, Zoldos et al. (1999) evidenced after *in situ* hybridization with rDNA probes that the size and strength of the hybridization signals differed between homologous site of NOR-1 and Liu et al. (2006), studying the physical location of 45S rDNA, founded differences between homologues sites in several species of *Sophora, Robinia* and *Amorpha*.
The heteromorphism of homologuous NORs may suggest the presence of a variable number of ribosomal genes that can be related to the amplification, deletion or unequal crossing over of genes. In this connection, it is worth mentioning that FISH is considered a semi-quantitative technique, since the size and intensity of hybridization signals can reflect the number of the gene copies indirectly (Maluszynska and Heslop-Harrison 1993).
As far as the chromosome complement of *H. gracilis* is concerned, differences between corresponding regions of homologous chromosomes, including the NOR, have been also evidenced after anti-5mC binding (Ruffini Castiglione et al. 2008a). Indeed, while some chromosome regions nearly always showed identical labelling, other regions frequently showed anti-5mC binding in only one chromosome of the pair.
Differences between the homologues were also evident when the DNase I treatment was taken into account, as evidenced by the different absorbance values, even when referred to the whole chromosome (Ruffini Castiglione et al. 2008a).
Nowadays, few data are available in the literature about the behaviour of homologous chromosomes and possible divergences. Apart from the case of the two X chromosomes in female, in which differences in the methylation pattern between homologous loci in the active versus inactive chromosome have been found (Lyon 1988; Široky et al. 1998), differences between homologues after anti-5mC binding have been also observed in *Allium cepa* (Ruffini Castiglione et al. 1995), *Vicia faba* (Frediani et al. 1996), triticale (Castilho et al. 1999), *Z. biebersteiniana* (Cremonini et al. 2003), more recently in barley reconstructed karyotypes (Ruffini Castiglione et al. 2008b, 2010) and in *C. versicolor* (Ruffini Castiglione et al. 2009).
Moreover, differences between homologues have been revealed by asymmetric puffing in polytene chromosomes (Pavan and Perondini 1967; Cionini et al. 1982), by banding techniques (Sumner 1990) and after cytological hybridization with specific DNA sequences both in plants (Durante et al. 1977; Loiero et al. 1982) and in animals (Hennen et al. 1975; Nardi et al. 1977; London-Vallejo et al. 2001). More recently, next-generation sequencing-based assays have demonstrated that, in humans, each cell contains pairs of homologous chromosomes whose chromatin structure and expression status are not necessarily identical (Birney et al. 2010).
In conclusion, also in our system, even if it is not possible to rule out completely the possibility that differences observed between homologues are the result of variation in the accessibility to two DNAs; however, in this case, too, a divergent chromatin organisation in corresponding regions would be hypothesised. Nevertheless, the observed heteromorphism in homologous NORs could be part of short-term dynamics of rDNA loci that, in spite of the variable amount of rDNA between homologous, not necessarily involve changes in the number of transcribing ribosomal genes but may represent a driving force in evolutionary terms.

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**Figure Legend**

Fig. 1 Restriction map of the IGS of *H. gracilis*. The positions of subrepeats A (vertical stripes), subrepeats B (white), subrepeats C (black), subrepeats D (grey) and multiple putative promoter regions (horizontal stripes) are also reported

Fig. 2 Nucleotide sequence of the IGS region, spanning from position 1,430 to position 2,042, containing the putative promoter sequences with the TIS. Bold = AT-rich repeated elements preceding the TIS (Hap. 1-3); bold and boxed = TIS (TATATATAGGGGGG); underlined = repeated elements type 1; italic = repeated elements type 2

Fig. 3 Comparison of the nucleotide sequences of the AT-rich stretch preceding the three TIS in *H. gracilis* (Hap. 1 from 1,430 to 1,491 bp; Hap. 2 from 1,626 to 1,690 bp; Hap. 3 from 1,965 to 2,028 bp) and *A. absinthium* (Art. 1 from 1,711 to 1,773 bp; Art. 2 from 1,960 to 2,023; Art. 3 from 2,432 to 2,495 bp) (from Garcia et al. 2009, accession number EU649668). The asterisk indicates identity

Fig. 4 a, b Metaphase chromosomes and interphase nuclei of *H. gracilis* following fluorescence *in situ* hybridization with IGS probe. Note the different labelling intensity between homologues in b

Figure 1

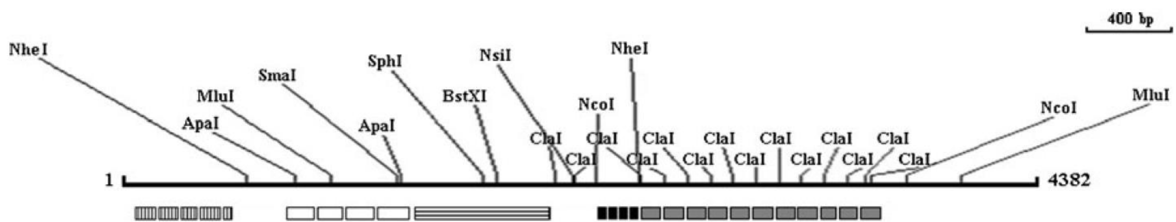


Figure 2

**CAAAATTATG ATTATTCAAA AAATCAATGT CTGTTCCTGT CACAAAGGAA AATTGTAATA TGTATATATA**
**GGGGGG**AGAG GTTGTTGAGC CGCGCACACC CCAGATCGTT TCCGAGCAAA AATGCCCCCA CCCCGCGCAC
AGCCCAGGTC GTTT*CCGAGT AAAAATGCCC CGACGCCGCA CACGGCCTTG GTCGCC***CAAA TATTATGATT**
**ATTCAAGGGA TTCAATGTCT GTTCCTGTCA CAAAGGAAAT TTGTAAATAT GTATATATAG GGGGG**AGTAG
GAGGTTGTTG AGCCGCGCAT GCCCAGATCG TTTCCTAGCA AAAAAGCCCC GACCCCGCGC ACAGACCCCC
CCATTTCCTT GGTCGCTA*TC GAGCAAAGTG CCCCGACCCC GTGCACGCCC AGATCGTTTC CGAGTAAAAA*
*TGCCCCGACC CCGTGCACGC CCAGATCGTT TCCGAGTAAA AATGCCCCGA CGCCGCACAC GCCCAGATCG*
*TTTCCGAGTA AAAATGCCCC GACGCCGCAC ACAGCC*TTGG TCACC**CAAAA ATTATGATTA TTCAAAAAAT**
**CAATGTCTGT TCCTGTCACT TTGGAAAATT GTAAATATGT ATATATAGGG GGG**

Figure 3

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Art. 1  AAAAAG-TATGATTCCTCAGAAATT-CAATGTCTGTTCCTGTCACTTAAGTTTTT-GTCCAATAAG
Hap. 1  CAAAAT-TATGATTATTCAAAAAAT-CAATGTCTGTTCCTGTCACAAAGGAAAATTGT- AATATG
Art. 2  AAAAAAGTATGATTCCTCAAAAAAT-CAATGTCTGTTCCTGTCACTTAAGTTTTT-GTCCAATAAG
Hap. 2  CAAATATTATGATTATTCAAGGGATTCAATGTCTGTTCCTGTCACAAAGGAAATTTGT-AAATATG
Art. 3  AAAAAAGTATGATTCCTCAAAAAAT-CAATGTCTGTTCCTGTCACTTAAGTTTTT-GTCCAATAAG
Hap. 3  CAAAAATTATGATTATTCAAAAAAT-CAATGTCTGTTCCTGTCACTTTGGAAAATTGT-AAATATG
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Figure 4