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RESEARCH ARTICLE

Characterization of the nuclear ribosomal DNA unit in *Oxalis tuberosa* (Oxalidacea) and related species

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Abbreviations: IGS: intergenic spacer
ITS: internal transcribed spacers
PAES: polyanethole sulfonic acid
SDS: sodium dodecylsulfate

Oxalis tuberosa* is an octoploid Andean tuber crop called "oca" that belongs to the worldwide distributed genus *Oxalis*. The genus is very heterogeneous and its systematics is still problematic. It has been proposed that *O. tuberosa* evolved by polyploidization of a still not defined ancestor that belongs to an alliance of species sharing the same basic chromosome number (x = 8). Nuclear rDNA units of *O. tuberosa* and a selected group of four related diploid species were characterised by RFLP using different restriction endonucleases and southern hybridization probes to produce a restriction map for EcoRI and BamHI. The major rDNA unit length in *O. tuberosa* was estimated at 10.7 kbp. As expected, restriction site variation was observed mainly in the intergenic spacer (IGS), but was also detected in coding regions. Restriction site mapping organization of the transcribed rDNA unit of *O. tuberosa* is very similar to *O. oblongiformis*. Nucleotide sequencing of a region of *O. peduncularis* IGS generated a complex organization pattern of repeats and subrepeats. Diploid species *O. peduncularis*, *O. tabaconasensis* and *O.* aff. *villosula* exhibited a ladder pattern that is a consequence of a 170 bp subrepeat unit indicating that these species share organization similarity and sequence homology. The variation pattern provided information to compare among diploid species, although it did not help to clarify taxonomic relationships between *O. tuberosa* and the putative diploid ancestors analysed in this study. Nonetheless, the RFLP pattern exhibited by *O. tuberosa* for the IGS region was quite unique and will be a useful tool to prospect in other related species.

The genus *Oxalis* is distributed worldwide and is represented by at least 800 species, most of them in the southern hemisphere and mainly in America and South Africa (Lourteig, 1983). Cytogenetic studies revealed a great deal of variability in the basic number of chromosomes (from x = 5 to x = 12), as well as variation in the ploidy degree, ranging from 2x to 8x (Marks, 1956; Cronquist, 1981; Naranjo et al. 1982; de Azkue and

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Martínez, 1983; de Azkue and Martínez, 1984; de Azkue and Martínez, 1988; de Azkue and Martínez, 1990). Within the genus, a group of species shares the same basic number of chromosomes (x = 8) (de Azkue and Martínez, 1990). This group has particular economic importance because it includes the octoploid *Oxalis tuberosa* Mol., an Andean crop commonly known as "oca", that is unique in this alliance due to its domestication by pre-Columbian civilizations. This crop is still widely cultivated in the Andean regions from Venezuela to Argentina (Hodge, 1951; Cárdenas, 1969; King, 1987). Out of this region, *O. tuberosa* is commercially cultivated in Mexico and New Zealand (New Zealand yam) (León, 1964; Vietmeyer, 1986).

Due to the importance of this crop in 1990 de Azkue and Martínez (de Azkue and Martínez, 1990) proposed the name "*Oxalis tuberosa* alliance" to this group of x = 8 species. However, these species are separated in different sections according to Knuth's taxonomical classification of the genus (Knuth, 1930; Knuth, 1935; Knuth, 1936). This grouping is also not concordant with the recent revision of Alicia Lourteig (Lourteig, 2000) who has introduced some new names, has reduced others to synonymy and has reassigned species to different sections.

The basic organization of ribosomal DNA has been maintained in most eukaryotic systems. It is organized in large arrays or blocks of tandemly repeated units, which occur at one or more chromosome locations; each of them considered as an individual genetic locus. In plants, their copy number can vary from few hundred to several thousand. One repeating unit consists of the conserved 18S, 5.8S and 25S rDNA coding regions and the corresponding internal transcribed spacers (ITS) as well as intergenic spacers (IGS) (Rogers and Bendich, 1987). The rDNA units can vary in length, in base sequence (restriction site variation) and in the number of repeats per species, these types of variation has been described for many plant species (Appels and Dvorak, 1982; May and Appels, 1987; Zimmer et al. 1988; Baldwin et al. 1995; Volkov et al. 1996; Pillay, 1996; Polanco and Pérez de la Vega, 1997; Warpeha et al. 1998; Chou et al. 1999; Volkov et al. 1999a; Fernández et al. 2000; Bhagwat et al. 2001; Gupta et al. 2002; Hsieh et al. 2004; Hsieh et al. 2005; Pillay and Kenny, 2006). ITS regions are more variable than the rRNA genes but are more conserved than the IGS regions. The coding region is highly conserved, whereas the IGS region is variable in sequence and length and show inter- and intra- specific variation (Rogers and Benedich, 1987).

Intraspecific IGS length variability is generally associated with different numbers motifs referred to as subrepeats that range from 40 to 400 bp in length in plants (*e.g.* 40 bp in *Sisymbrium irio*, Grellet et al. 1989; 115 bp in barley Saghai-Maroof et al. 1984; Gupta et al. 2002; 130 bp in wheat, Appels and Dvorak, 1982; May and Appels, 1987; 340 bp in alfalfa Cluster et al. 1996; 460 bp in carrot Taira et al. 1988). The length and sequence of subrepeats within most individual species are quite conserved. However, the number of subrepeats within a rDNA repeat is often quite variable at different NOR loci within species and also at the same NOR locus in different individuals at the same species. The variation in the number of subrepeats alters the length of the entire IGS region leading to the occurrence of spacer length variants that can be detected by restriction

Table 1. Chromosome number and collection locality of the Oxalis species.

Species	Locality	2n	Herb No/ Acc. No
O. oblongiformis Knuthc	Perú, Amazonas, Chachapoyas, near Balsas	16	2917[a]
O. peduncularis H.B.K.	Perú, Cajamarca, Cenedin, Quilimbash	16	2908[a]
O. tabaconanensis Knuth[c]	Perú, Amazonas, Bongara, near Pomacocha	16	2872[a]
O. aff. villosula Knuth	Perú, Amazonas, Bongara, Churruja	16	2901[a]
O. tuberosa MOL	Argentina, Salta, Santa Victoria	64	1074[b]
O. tuberosa MOL	Argentina, Jujuy, Tumbaya	64	1018[b]
O. tuberosa MOL	Argentina, Jujuy, Yavi	64	1051[b]

[a] BACP (Cefaprin, Buenos Aires).
[b] CIP Germplasm and INTA- Cerrillos Germplasm.
[c] In the last monography involving the taxonomic treatment of genus *Oxalis* published by Lourteig (2000), she considered *O. oblongiformis* synonymous to *O. tabaconasensis*. Despite her observation, we decided to use both names in this work, since the analysis of rDNA sequence organization of the "two species" showed that they differed considerably.

enzyme digestion coupled whit Southern hybridization (Appels et al. 1980; Appels and Dvorack, 1982; Grellet et al. 1989; Sano and Sano, 1990; Clegg, 1990; Maggini et al. 1992; Lakshmikumaran et al. 1994; Cluster et al. 1996; Jeandroz et al. 1996; Gupta et al. 2002 and reference there are in; Skalická et al. 2003; Lim et al. 2004; Kovarik et al. 2005).

Despite the demonstrated analytical power of this comparative information for systematic clarification, the study of rDNA organization (and its variation) in *Oxalis* is still lacking. In this context, previous studies of rDNA using the ITS support the *O. tuberosa* alliance hypothesis (Tosto and Hopp, 1996; Emshwiller and Doyle, 1998). The naturalness of the group has been also supported by other studies based on the chloroplast- expressed glutamine synthetase (Emshwiller and Doyle, 1999; Emshwiller and Doyle, 2002) and AFLPs (Tosto and Hopp, 2000).

In the present work, we analyse the restriction fragment length polymorphism of rDNA units as a first step to characterise the rDNA variation in *O. tuberosa* and in five related species that will help to provide a clue to clarify the species relationships.

MATERIALS AND METHODS

Plant material

The species of the genus *Oxalis* used in this study were: *O. peduncularis, O. oblongiformis, O. tabaconasensis, O.* aff. *villosula* and *O. tuberosa.* The different species were chosen as representatives of three of the four different sections (according to Knuth, 1930; Knuth,1935; Knuth, 1936, classification) that are present in the alliance. The only exception, section *Herrerea,* is not represented because *O. herrerae,* the only species that belongs to this section, was not available for this work. From section *Ortgieseae* we have chosen two species (apart from *O. tuberosa)* because it is the section where *O. tuberosa* belongs to.

The collection locality of the plants, their herbarium accession and chromosome number are detailed in Table 1. Voucher specimens of the diploid species examined are kept in the herbarium of CEFAPRIN (BACP). Clones of analyzed plants of *O. tuberosa* are kept at the germplasm bank of the International Potato Center (CIP) at Quito, Ecuador and at the germplasm bank of the INTA- Cerrillos, Salta, Argentina.

DNA isolation, digestion and hybridization

Leaf material was harvested and stored at -70ºC until DNA extraction. Total DNA was isolated from 4 gr of frozen leaf material from individual plants following the Dellaporta procedure (Dellaporta, 1983) with slight modifications. Routinely 3-5 µg samples of each DNA were digested for 4 hrs at 37ºC with 10 IU of restriction endonucleases according to the supplier's recommendations and using the supplied buffers. DNA restriction fragments were separated by electrophoresis in 1% agarose gels in 0.04 M Tris-acetate 2 mM EDTA (pH 7) buffer at 40 V/cm for 15 hrs. As size marker, the 1 kb ladder of Life Technologies (USA) was included in the electrophoresis as well as the HindIII-digested fragments from lambda phage DNA. Ethidium bromide was used to stain gels for documentation purposes. Southern blotting was performed using Hybond N nylon membranes (Amersham Pharmacia, UK) according supplier's recommendations. DNA probes (approximately 25 ng) were labeled using the Random Primed DNA labelling kit of Boehringer Mannheim in the presence of 20 µCi α^{32}P-dCTP (3000 Ci/ nmol). Nylon menbranes were prehybridized and hybridized at 65ºC for 16 hrs in the presence of polyanethole sulfonic acid (PAES) 0.1%, sodium pyrophosphate 0.1%, SSPE 2.5% and sodium dodecylsulfate (SDS) 1%. Final wash was for 15 min at 65ºC in 0.2 x SSC (SSC is 0.15M NaCl, 0.015M Sodium citrate pH7), 0.1% SDS buffer. DNA fragment sizes were estimated after transforming their relative mobility into kbp using the local reciprocal method (Elder and Southern, 1983). After each hybridization, filters were reused with the successive probes to ensure optimal comparison between bands revealed in each case. To do so, they were stripped after hybridization by washing with boiling low-salt buffer (0.01x SSC) before re-probing.

DNA probes

a.- The 8.95 kbp Eco RI fragment of wheat rDNA cloned in pTA71 (Gerlach and Bedbrook, 1979) as heterologous probe.

b.- The internal transcribed spacers ITS1, ITS2 and the 5.8S rDNA (ITS) were obtained from *O. oblongiformis* and *O. tuberosa* DNA by PCR using the following primers: 5' TCC TCC GCT TAT TGA TATGC 3' (ITS4) and 5' GGA AGG AGA AGT CGT AAC AAG G 3'(ITS5).

c.- An EcoRI- BamHI fragment (1000 bp) corresponding to the 3' end of the 25S fragment of wheat rDNA (May and Appels, 1987).

d.- The IGS region was obtained from *O.* aff. *villosula* DNA by PCR using the following primers: 28ii 5' ggc tct tcc tat cat tgt gaa gca gaa ttc ac3', 18i 5' ttt ctc agg ctc cct ctc cgg aat cga acc ct 3' and 18k 5' ccc gtg ttg agt caa att aag ccg cag gc 3' (Hillis and Dixon, 1991). A diagram with the location of the probes is shown in Figure 1.

PCR reactions were carried out in a Perkin Elmer DNA Thermo Cycler (Pec 9600). In the case of the ITS region and the IGS region, PCR amplifications were performed in 50 µl of 1x PCR buffer (Life Technologies, USA), 200 µM of each deoxyribonucleotide triphosphate, 300 nM of each primer ITS4 and ITS5 or 300nM of each primer IGS1 and IGS2, and 2.5 units of *Taq* polymerase (Life Technologies, USA). The IGS region was amplified using the Taq polimerasa "Expand Long Template PCR System" (Roche

Applied Science, Germany) according to manufacturer's instructions. For the ITS region, the reaction was performed for 40 cycles. Each cycle lasted for 30 sec at 94ºC, 1 min at 50ºC and 2 min at 72ºC. In the case of the IGS region the reaction was perfomed for 35 cycles. Each cycle lasted for 1 min at 94º C, 2 min at 60ºC and 5 min at 72ºC. In both cases, denaturation of the template was performed for 5 min at 94ºC. A final extension step was carried out for 10 min at 72ºC.

Extracted DNAs from different species were amplified using primers and PCR procedures described above. PCR amplification showed bands with different lengths between 2 and 4. PCR products were cloned into the pGEM-T easy plasmid (Promega Biotech, EUA) according to manufracturer´s instructions. A PCR product of 2,4 kbp from *O. peduncularis* (AY 356350) was sequenced in the DNA Sequencing Facility at the BioResources Center of Cornell University, Ithaca, NY.

The search of tandem repeat motifs was performed with the Tandem Finder Repeat program (Benson, 1999). Multiple sequence alignment was carried out with CLUSTALX version 1.8 (Thompson et al. 1997).

RESULTS

Variation in rDNA organization in the genomes of selected species of *Oxalis* (*O. oblongiformis*, *O. peduncularis, O. tabaconasensis*, *O.* aff. *villosula* and *O. tuberosa*) was analyzed by RFLP using different combinations of restriction endonucleases and probes. They were cleaved using the restriction endonucleases EcoRI, BamHI, EcoRI-BamHI (double digestion) and EcoRV. Southern blots were probed with: a) the insert present in plasmid pTA71 (comprising the entire wheat rDNA repetition unit); b) the so-called "ITS probe" (a region comprising the internal transcribed spacers ITS1 and ITS2 and the 5.8S rRNA gene); c) a fragment of the 25S rRNA gene and d) the IGS sequence from *O.* aff. *villosula*.

Restriction endonuclease mapping of Oxalis rDNA repetition unit

Hybridization experiments with wheat rDNA contained in plasmid pTA71 allowed the proper estimation of the repeat unit size. After digestion with EcoRV a singleband pattern of approximately 11 kbp was obtained in *O. tuberosa* (Figure 2a). This estimation was supported by the pattern of bands generated after BamHI digestion. Two bands summing up 10.7 kbp (6.8 kbp and 3.9 kbp) were detected (Figure 2b). Since the 6.8 kbp band also hybridized with the 25S probe and the 3.9 kbp one to the ITS probe (see below) it was possible to ascribe the sequences present in these bands (25S coding segment and ITS and their respective flanking regions, Figure 6).

The 3.9 kbp band resulting from BamHI digestion (Figure 3) was monomorphic for all the species analyzed, in contrast, digestion with EcoRI evidenced polymorphism: a fragment of 3.5 kbp hybridizing to the ITS probe was characteristic of the genomes of *O. tuberosa*, *O. tabaconasensis* and *O. oblongiformis* while two bands (of 3.9 kbp and 3.5 kbp) were detected in *O. peduncularis* and *O.* aff. *villosula* genomes. After BamHI- EcoRI double digestion, a hybridization band of 2.5 kbp was detected in *O. oblongiformis* and *O. tuberosa* while a 2.9 kbp band was detected in *O. peduncularis* and *O.* aff. *villosula* and two bands (of 2.9 kbp and 2.5 kbp) were present in *O. tabaconasensis*.

When BamHI digested genomic DNA was hybridized with the 3' region of the 25S rDNA probe, different patterns of bands were revealed in the analyzed species perhaps reflecting length variation in the IGS (Figure 4): a band of 6.8 kbp in *O. tuberosa*, a 7.4 kbp band in *O. oblongiformis* and a smeared group of bands showing predominant intensity at about 6.9 kpb in *O.* aff. *villosula*, two bands of 6.9 kbp and 7.4 kbp in *O. tabaconasensis* and a band of 7.4 kbp in *O. peduncularis*. While the pattern of hybridization with this probe (3' end of 25S rDNA) after EcoRI digestion was the same as the one obtained after the hybridization with the ITS, the double digestion with EcoRI and BamHI resulted in the occurrence of 2.5 kbp and 1 kbp in *O. tuberosa*, *O. tabaconasensis* and *O. oblongiformis*. In *O.* aff. *villosula* three bands were detected (3.7 kbp, 3.2 kbp and 2.7 kbp) while *O. peduncularis* showed five hybridization bands (of 3.8 kbp, 3.4 kbp, 2.8 kbp, 2.6 kbp and 2.5 kbp and the same bands at 1 kpb).

An interesting observation of these experiments is that EcoRI digested genomic DNA of *O. peduncularis* and *O.* aff. *villosula,* probed either with the ITS region or with the 3´ end of the 25S resulted in bands of highermolecular weight than expected (Figure 3 and Figure 4). This is possibly due to the lack of an EcoRI site caused by methylation as reported in *Solanum* (Borisjuk et al. 1994) and in *Lycopersicom esculentum* (Kiss et al. 1989) or due to a restriction site mutation. In this context, the two fragment pattern of *O. peduncularis* can be explained as a product of the coexistence of these two possible forms. In *O. peduncularis* both forms are more or less equally represented, while in *O.* aff. *villosula* the hypothetically methylated or mutated band preponderated.

This complexity of patterns that result from a combination of polymorphism, heterogeneity and, in the case of *O. tuberosa*, polyploidy makes it difficult to precisely elucidate the variation of rDNA organization. The interpretation of the described restriction patterns led to the *O. tuberosa* restriction map proposed in Figure 6.

Restriction endonuclease mapping of the IGS

After hybridization of BamHI digested DNA with the IGS probe, banding patterns were coherent with the ones detected after hybridization with the 3' 25S probe. These results suggest the absence of BamHI sites in the IGS

Table 2. IGS tandem repeat characterization.

Indices[a]	Period Size[b]	Copy Number[c]	Consensus Size[d]	Percent Matches[e]	Percent Indels[f]	A	C	G	T	Entropy[g] (0-2)
1000--1130	62	2.1	62	85	5	31	28	27	12	1.93
1301--1725	62	6.9	61	80	7	27	27	29	15	1.96
1490--1659	31	5.5	30	67	17	28	27	29	14	1.95

[a] Indices of the repeat relative to the start of the sequence.
[b] Period size of the repeat.
[c]Number of copies aligned with the consensus pattern.
[d] Size of consensus pattern.
[e]Percent of matches between adjacent copies overall.
[f] Percent of indels between adjacent copies overall.
[g]Entropy measure based on percent composition.

region (Figure 5) of the different species with the exception of *O. oblongiformis*, where three faint bands could be detected, indicating the specific presence of up to three BamHI sites in some rDNA units in this species. When genomic DNA was digested with EcoRI and hybridized with the same probe, *O. tuberosa* showed a group of 3 bands of around 4 kbp (4070 bp, 3890 bp and 3710 bp) and another group of 3 bands of around 2 kbp (2240 bp, 2060 bp and 1880 bp). In agreement with these results, the two groups of bands were conserved after double digestion with BamHI and EcoRI. Compared to EcoRI patterns, the 2 kbp group remained unchanged while the 4 kbp group was reduced to 3 kbp due to the BamHI site (see B1 in Figure 6 map) present in the 18S (Figure 5).

Taken together, the two results indicate that at least 3 variants of rDNA units coexist in *O. tuberosa* genome differing in their presence/absence of EcoRI sites or/and number of subrepeats in the IGS.

The patterns observed in the diploid species (*O. peduncularis*, *O. tabaconasensis* and *O.* aff. *villosula*) after digestion with EcoRI showed the presence of the same group of bands of around 4 kbp and an additional group of fragments giving rise to a typical ladder ranging from 2700 bp to 200 bp (Figure 5), suggesting the existence of seven subrepeats of approximately 170 bp each, some of which could remain linked after digestion when the Eco RI site was modified. Double digestion of DNA showed the same EcoRI pattern except for the 4 kbp bands which were replaced by 3 kbp bands, as happened in *O. tuberosa*. This IGS subrepeat organization and the putative polymorphism in the number of subrepeats could explain the smeared pattern of bands observed in these particular species after BamHI digestion. Figure 7 shows an interpretation model for the resulting ladder. Further localization of the subrepeats within the IGS was achieved by mapping the ladder using a more limited probe, just comprising the 3' end of the 25S gene and 200bp of the 5' IGS end. Hybridization with this probe revealed a shorter ladder ranging from 2.7 to 1.4 kbp which was compatible with both, the number and the length of the proposed sub repeats (data not shown).Thus, the putative position of the array was at approximately 1 kpb from the 5' IGS end.

An exception to the general IGS organization of diploid species was the case of *O. oblongiformis,* which showed a completely different banding pattern, since BamHI and EcoRI, as well as the double digestion rendered a single high molecular weight band with high intensity and three bands with less intensity after BamHI digestion. These results indicated the lack of EcoRI sites in the IGS region of *O. oblongiformis* and the presence of three BamHI sites in some of the copies, as already described above (Figure 5).

IGS nucleotide sequence

A fragment of 1725 nucleotides of *O. peduncularis* IGS was sequenced (GenBank accession: AY 356350) because it includes the region that revealed the characteristic ladder pattern using probe d (Figure 1) in Southern blot experiments in *O. peduncularis,* in *O. tabaconasensis* and in *O.* aff. *villosula* species (Figure 5). Comparison of this sequence with other similar sequences deposited in Genbank showed that although there is very low holomogy with the IGS sequences of other plants, some short conserved motifs could be identified. These motifs (CTTTT, CCCTCCC, TGAAAAT and its variant GAAAAT) probably represent regulatory elements required for transcription initiation and termination processes (Jeandroz et al. 1996; Volkov et al. 1996; Volkov et al. 1999b; Fernández et al. 2000). Interestingly, comparative sequence analysis of the 5' and 3' IGS extremes of this and the rest of the *Oxalis* species studied here revealed a relatively high degree of conservation since sequence

homology ranged from 90 to 92.5% (not shown). Thus, as previously reported for other species, the highly polymorphic characteristic of this region was mainly based on the variable numbers of tandem repeats. Three kinds of tandem repeat elements could be defined as characteristic of *Oxalis peduncularis*: the A repeat type, a 62 bp element present two times between the 1000 and 1130 positions of the cloned fragment; the B repeat type, a 62 bp element repeated seven times between positions 1301 and 1725 and the C repeat, which was a subrepeat of 31 bp contained within the B repeat type (Table 2 and Figure 8). As happens in other systems, these repeated motifs were not always identical. In this context, the most interesting one was the motif of B type repeats which showed Eco RI restriction endonuclease site polymorphism. The alignment of B repeats is shown in Figure 8 IV. Presence of an EcoRI site in one of the repeats is marked and its deficiency due to one nucleotide mutation is pointed out in the other repeats.

DISCUSSION

RFLP analysis of general rDNA organization in *Oxalis* (*O. oblongiformis*, *O. peduncularis*, *O. tabaconasensis*, *O.* aff. *villosula* and *O. tuberosa*) shows that they share the same general organization found in many plants species, particularly the location of BamHI, EcoRI and EcoRV restriction endonuclease sites found here are essentially as those found in other species (see Doyle and Beachy, 1985; Rogers and Bendich, 1987; Zimmer, 1988; Crisci et al. 1990; Lakshmikumaran et al. 1994; Warpeha et al. 1998; Bhagwat et al. 2001; Pillay and Kenny, 2006, for some examples).

Restriction site mapping organization of the transcribed rDNA unit of *O. tuberosa* is very similar to *O. oblongiformis* and both species differ from the others particularly when mapping EcoRI restriction endonuclease sites within the 3' region of the 25S ribosomal gene. The precise analysis was difficult due to heterogeneity in rDNA repetition unit composition due to variation of EcoRI sites in *O. peduncularis, O.* aff. *villosula* and *O. tabaconanensis* (Figure 2 and Figure 3). This reminds similar observations reported in *Solanum* (Borisjuk et al. 1994) and in *Lycopersicom esculentum* (Kiss et al. 1989) where it was proposed the occurrence of methylation. Methylation is a matter of discussion for some authors (Cluster et al., 1996) and other explanations were proposed, *i.e.* in *Solanum* Harding (1991) proposed a deletion/insertion of a 100/150 bp near the 3' end of the 25S. From the data shown in *Oxalis*, it is not possible to decide between these two hypotheses, but having in mind that gene conversion is one of the mechanisms proposed for the homogenization process and that it depends on the methylation degree (Lim, 2000), our results would probably agree with the methylation hypothesis. In any case, these results suggest that gene conversion has not homogenised repeat length in *Oxalis peduncularis, O.* aff. *villosula* and *O. tabaconanensis*.

Despite this general organization similarity to other plant species, specificities are also clear, particularly when mapping EcoRI restriction endonuclease sites within the IGS region. Interspecies homology comparison between spacer regions located between rDNA coding regions show extensive sequence divergence compared to the conserved coding regions. The presence of tandem subrepeats in IGS organization is a common feature in different plant species. However the length, the sequence and the structure vary between species (Appels and Dvorak, 1982; May and Appels, 1987; Rocheford et al. 1990; Jeandroz et al. 1996; Warpeha et al. 1998; Fernández et al. 2000; Gupta et al. 2002, for some examples). RFLP analysis after IGS region hybridization after EcoRI digestion revealed a ladder pattern in the diploid species and poliploid species (except in *O. oblongiformis*). While in the diploid species the ladder consists in multiples of about 170 bp (160-180 bp), in the octoploid, the bands differ in 200 bp multiples (190-210 bp), suggesting a different evolution process of the IGS sequences. Although *O. oblongiformis* failed to show the ladder pattern after EcoRI digestion, cross hybridization experiments using the 5' end of IGS as probe revealed identical Southern banding patterns. Thus, in spite of the fact that *O. oblongiformis* lacks EcoRI sites in the IGS, its sequence ought to be very similar to the rest of the diploid species. In conclusion, the absence of EcoRI sites does not mean the absence of the subrepeats.

As previously reported for other species, the sequence of part of the IGS region in *O. peduncularis* confirmed that the highly polymorphic characteristic of this region could be mainly based on the variable numbers of tandem repeats. Three kinds of tandem repeat elements ranging from 31 to 62 in length could be defined as characteristic of *Oxalis*. In agreement with published data from other plant species, the length of IGS sub-repeats, within most individual species, does not seem to differ by more than a few base pairs and their sequences show a high degree of similarity. Among these differences, one of the sub-repeat types showed a variable EcoRI recognition sequence; similarly to the previously described BamHI site found in *Sisymbrium irio* (Grellet et al. 1989). Although not thoroughly analyzed here, it is likely that rDNA unit heterogeneity and polymorphism is due to variation in the number of sub-repeats within an rDNA repeat, as happens at different NOR loci between and within individual species and sometimes at the same NOR locus in different individuals of the same species.

Estimated length sub repeat from the southern blot experiments was 170 bp (160-180 bp), although this length does not strictly matches with tandem sub-repeats of 62 bp found in the sequenced clone, these subrepeats and the EcoRI sites (both complete and deficient) suggest how could the IGS be structured. It is possible that the 170 bp variants could be generated from a 62 bp (or 31 bp) ancestral motif that could have been submitted to repetition steps leading to a 170 bp block and that subsequent amplification steps have given rise to the present structure,

a similar situation was observed in the crucifer *Sisymbrium irio*. IGS region presents some short motifs which are highly conserved in some plants, these sequences were proposed to be involved in signaling RNA transcription and processing. This could be interpreted as an indirect evidence supporting that these sub-repeats may have functions acting as enhancers of transcription (Jeandroz et al. 1996).

Hence, IGS may have evolved in different ways in *Oxalis*: by varying its sub-repeat lengths and by losing or acquiring the EcoRI sites. A similar situation occurs in *Nicotiana tomentosiformis* and *N. sylvestris* (two close diploid relatives of the allotetraploid *N. tabacum*), where the sub-repeats have very similar in sequence (90%), but they differ for the presence of a Cla I site (Volkov et al. 1996).

EcoRI ladder patterns observed in the IGS (present in O. *peduncularis*, *O. tabaconasensis* and *O.* aff. *villosula*) revealed the presence of multiple rDNA families and incomplete homogenization. Variation in the intensity of the bands (depending on the species analyzed) was detected, thus indicating different degrees of the homogenization.

Particularly, *O. tabaconasensis* showed higher relative intensity in some of the IGS bands representing a relatively abundant rDNA family as happen in *Nicotiana* (Lim et al. 2004). This differential degree of homogenization observed in the IGS of *O. tabaconanensis* is consistent with EcoRI restriction site patterns in the transcribed region (ITS + 25S) which also shows homogenization of the number of bands respect to *O. peduncularis* and *O*. aff. *villosula*.

The IGS can go through different evolutionary processes in the poliploid species: in some cases, as in the allotetraploids *Tragopogon mirus* and *T. miscellus,* IGS could be a usefull tool to identify the different genomes that contribute in the poliploid formation (Kovarik et al. 2005). In other cases the rDNA of one parent is eliminated from the genome as in *Nicotiana tabacum* (Volkov et al. 1999a) or the case of *Nicotiana rustica*, an allotetraploid, where the 80% of its IGS sequences are of an *N. undulata* type, one of the diploids progenitor (Matyasek et al. 2003). The other possibility is the case where parental IGS variants are not represented in the interspecific hybrid or autopoliploid, as in the genera *Nicotiana* (Kovarik et al. 1996), *Oryza* (Cordesse et al. 1990) and *Medicago* (Cluster et al. 1996). The studies by Skalická et al. (2003) in a synthetic tobacco allotetraploid line are interesting as well; he observed that unidirectional gene conversion of rDNA untis may occur and also that newly amplified rDNA units may be identified.

The fact that none of the diploid species share the IGS pattern of the octoploid (*O. tuberosa*) does not rule out the possibility that some of these species took part in the evolution of *O. tuberosa* genome. Polyploidization of *O. tuberosa* may resemble the case where parental IGS variants are not represented in the interspecific hybrid or autopoliploid (Kovarik et al. 1996; Cordesse et al. 1990; Cluster et al. 1996; Skalická et al. 2003). Other possibility could be that the diploid species included in this study were not involved in the octoploid origin, in this case, the IGS pattern revealed for *O. tuberosa* was unique enough to be a useful tool for prospection in other species that belong to the *O. tuberosa* chromosome alliance.

The comparison of restriction site conservation located in more conserved regions of the cistron in *O. tuberosa* and *O. oblongiformis* showed remarkably identical restriction patterns for the transcribed rDNA regions while the other diploid species showed some differences, indicating that rDNA organization of these two species is closely related, despite of the IGS data. This observation is in better agreement with the traditional taxonomy since both species are the only ones among those studied here that were classified in the same section by Knuth (in *Ortgiesae*) and by Lourteig (in *Lotoideae*).

In spite of the apparent failure of IGS to clarify phylogenetic relationships between the octoploid and the diploid species included in this study, it is interesting to notice that three diploid species (*O. peduncularis*, *O. tabaconasensis* and *O*. aff. *villosula*) shared the same IGS pattern, thus suggesting that they could be closely related. However, this conclusion is in conflict with morphologically based taxonomy since these three species were classified by Knuth (1930) in three different sections, while Lourteig separated them into two different sections. Furthermore, the analysis of the IGS variation does not support the proposed sinonimy of *O. oblongiformis* and *O. tabaconanensis* (Lourteig, 2000).

Differences in rDNA organization described in this work showed that the evolution of this part of the genome was very dynamic in this group of species. The molecular mechanisms underlying this evolution seem to combine epigenetic modifications or dynamic mutation of individual tandemly repeated segments with incomplete homogenization. The observed differences could be very helpful to complement other taxonomic criteria.

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APPENDIX
FIGURES

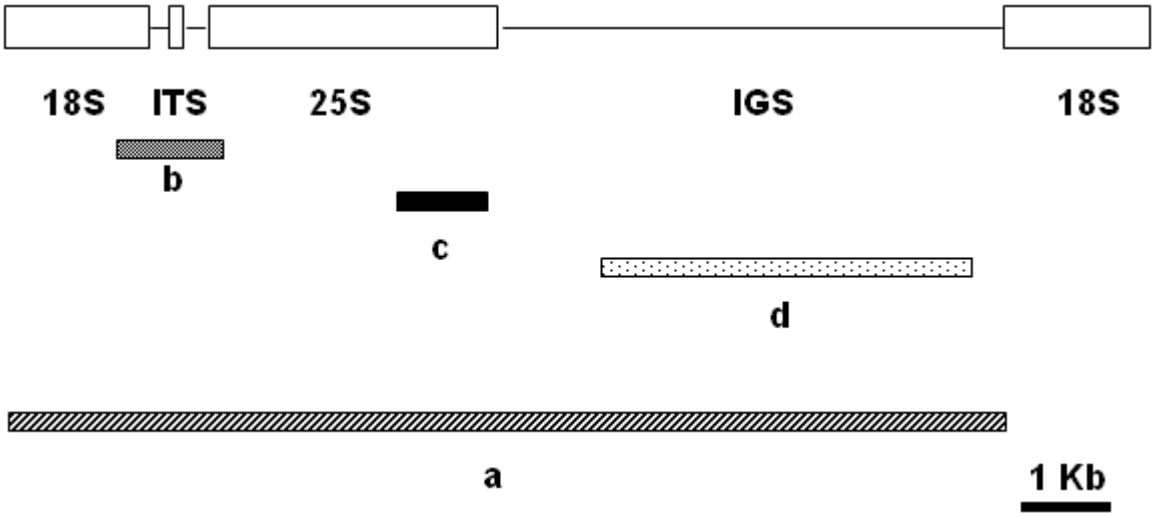

Figure 1. Schematic diagram of plant ribosomal DNA organization and the probes used in this study. Abbreviations on the figure denote the following: 18S: 18S rDNA; ITS: internal transcribed spacer; 25S: 25S rRNA coding region; IGS: intergenic spacer. The position of the probes used for sequential probing is shown below the repeat unit. (a) The 8.95 kbp Eco RI fragment of wheat rDNA cloned in pTA71 (Gerlach and Bedbrook, 1979) as heterologous probe, (b) The internal transcribed spacers ITS1, 5.8S and ITS2 rDNA (ITS), (c) An EcoRI- BamHI fragment (1000 bp) corresponding to the 3' end of the 25S fragment of wheat rDNA (May and Appels, 1987), (d) The intergenic spacerIGS region.

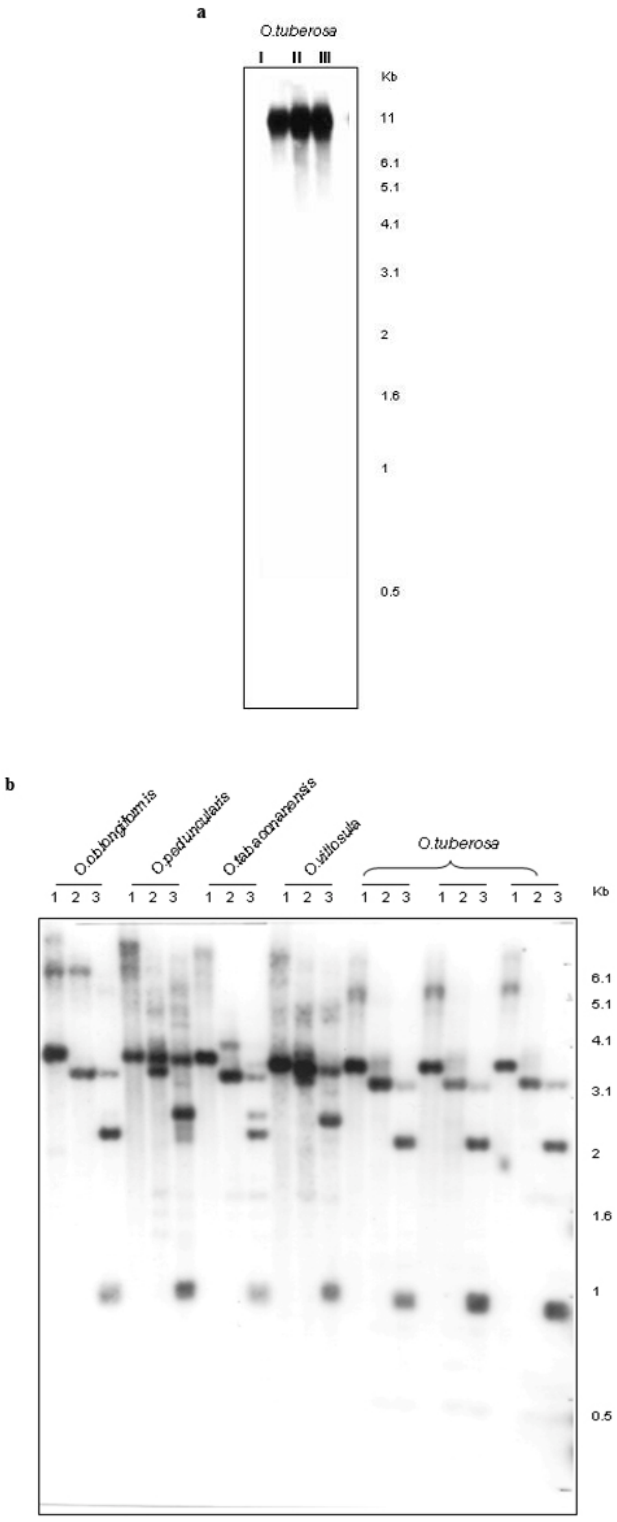

Figure 2. Hybridizaton patterns of *Oxalis* nuclear DNA RFLPs hybridized with the pTA71 probe (wheat heterologous probe of the entire repeat).

(a) EcoRV digested DNA from *O. tuberosa*, lanes: I, II, III, three different accessions of *O. tuberosa* (No 1074, 1148 and 1051, respectively).

(b)Total digested DNA from Oxalis species, lanes 1: digested by BamHI, lanes 2: digested by EcoRI and lanes 3: the double digestion BamHI- EcoRI. Numbers in top denote the different plants, 1: *O. oblongiformis*, 2: *O. peduncularis*, 3: *O. tabaconasensis*, 4: *O. aff. villosula*, 5, 6, 7: O. *tuberosa* (acc. No 1074, 1051 and 1018 respectively).

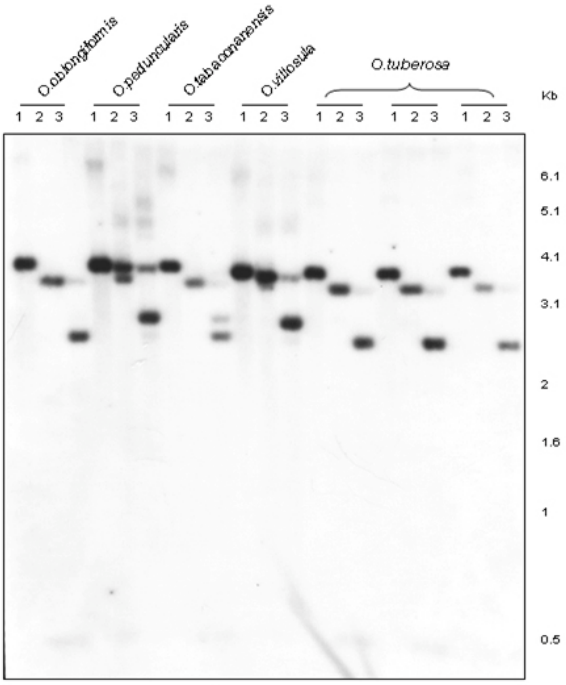

Figure 3. Hybridization patterns of *Oxalis* nuclear DNA RFLPs hybridized with the ITS probe. Lanes 1: BamHI digestion, lanes 2: EcoRI digestion, lanes 3: double digestion BamHI- EcoRI. Numbers in top in denote the different plants, 1: *O. oblongiformis*, 2: *O. peduncularis*, 3: *O. tabaconasensis*, 4: *O. aff. villosula*, 5, 6, 7: O. *tuberosa* (acc. No 1074, 1051 and 1018 respectively).

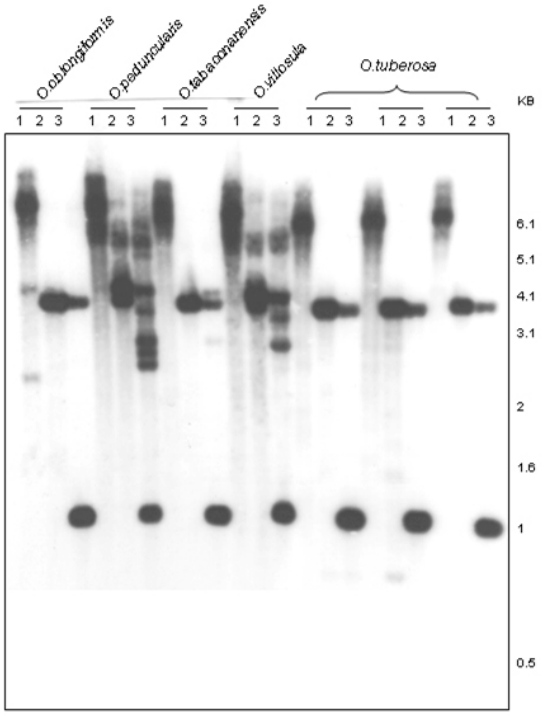

Figure 4. Hybridization patterns of *Oxalis* nuclear DNA RFLPs hybridized with the 25S probe. Lanes 1: BamHI digestion, lanes 2: EcoRI digestion, lanes 3: double digestion BamHI- EcoRI. Numbers in top in denote the different plants, 1: *O. oblongiformis*, 2: *O. peduncularis*, 3: *O. tabaconasensis*, 4: *O. aff. villosula*, 5, 6, 7: O. *tuberosa* (acc. No 1074, 1051 and 1018 respectively).

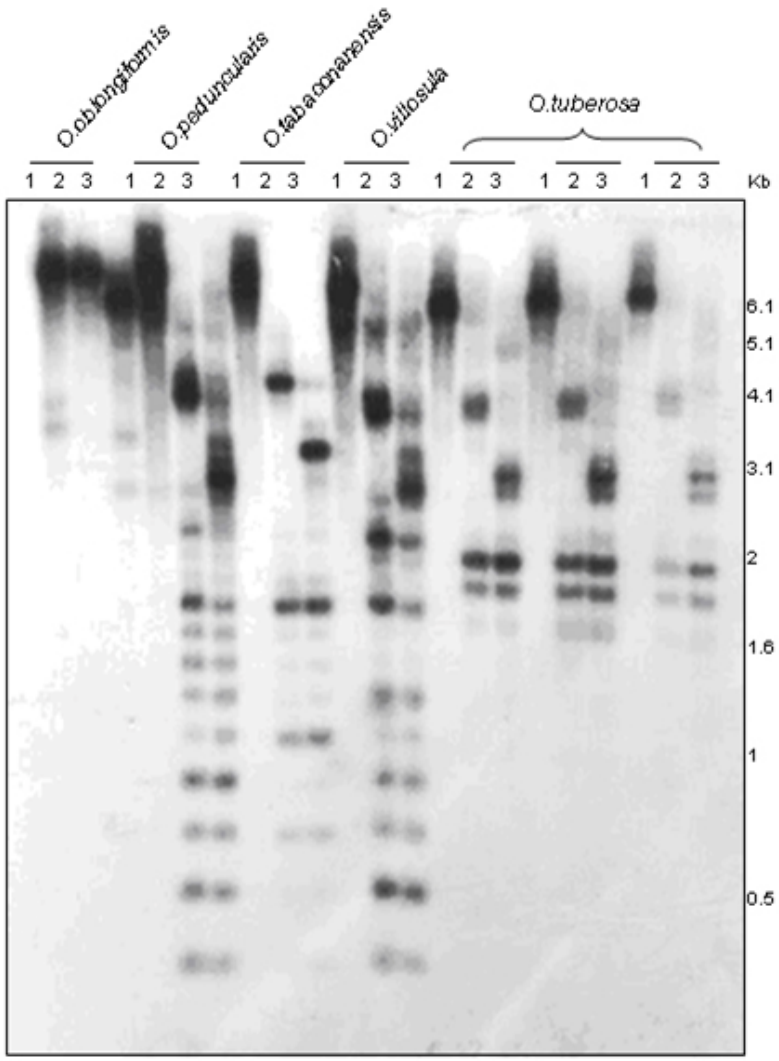

Figure 5. Hybridization patterns of *Oxalis* nuclear DNA RFLPs hybridized with the IGS probe. Lanes 1: BamHI digestion, lanes 2: EcoRI digestion, lanes 3: double digestion BamHI- EcoRI. Numbers in top in denote the different plants, 1: *O. oblongiformis*, 2: *O. peduncularis*, 3: *O. tabaconasensis*, 4: *O. aff. villosula*, 5, 6, 7: O. *tuberosa* (acc. No 1074, 1051 and 1018 respectively).

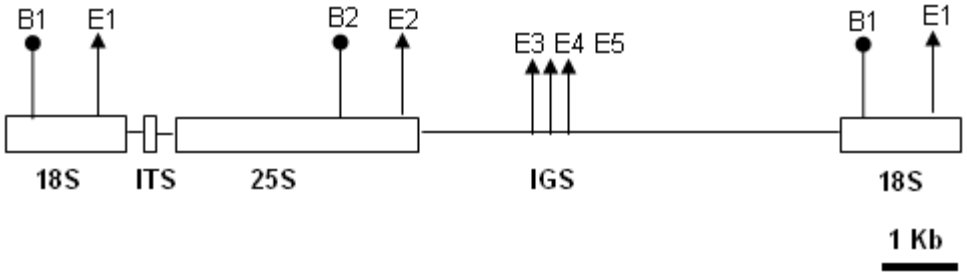

Figure 6. Deduced restriction sites location in nuclear *O. tuberosa* rDNA unit. Diagram is based on the results showed in Figure 2a and in lines corresponding to *O. tuberosa* on Figure 2b, Figure 3, Figure 4 and Figure 5. Abbreviations on the figure denote the following: 18S: 18S rDNA; ITS: internal transcribed spacer; 25S: 25S rRNA coding region; IGS: intergenic spacer. EcoRI (▲), BamHI (●) sites.

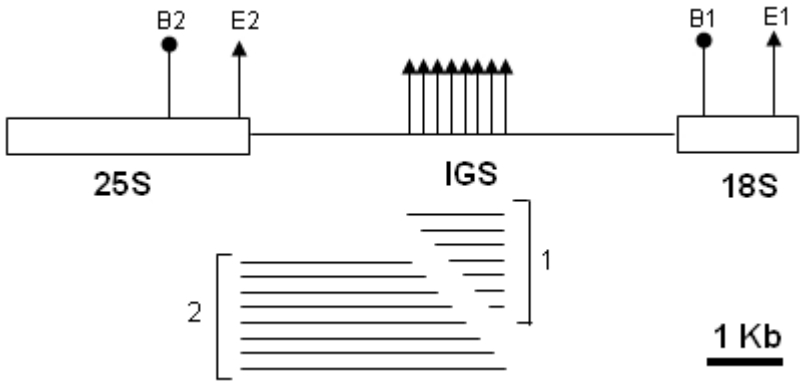

Figure 7. Schematic interpretation of EcoRI restriction patterns of most of diploid species with the IGS probe. Intergenic spacer is shown with its flanking 25S and 18S rRNA domains and the putative EcoRI sites are marked (▲). Bellow, (1) and (2) represent the different EcoRI fragments that could be present. This pattern was observed in the following diploid species: *O. peduncularis*, *O. tabaconasensis* and *O*. aff. *Villosula.*

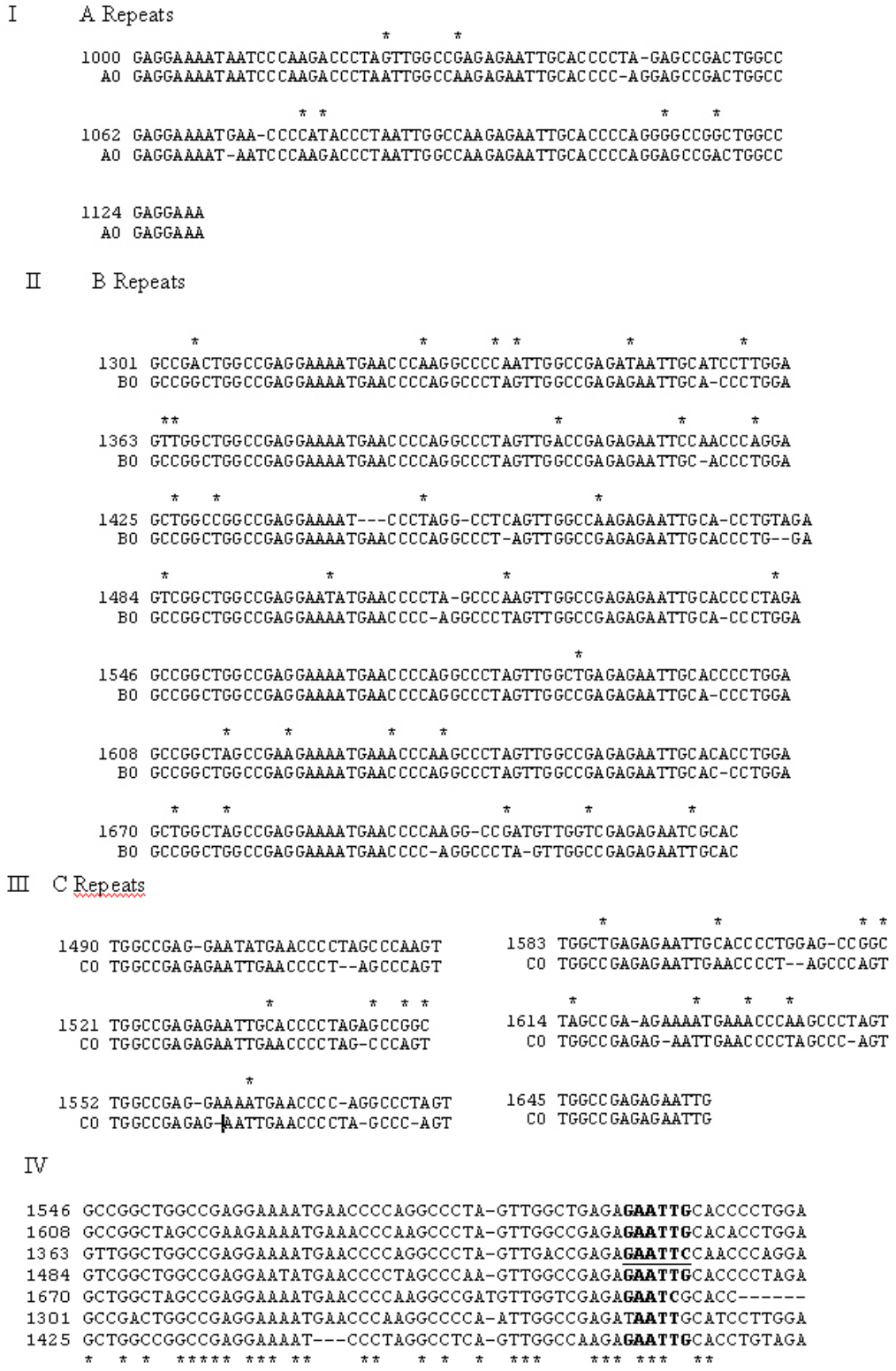

Figure 8. Repeat motives of nucleotide sequence of the rDNA intergenic spacer (IGS) fragment of *Oxalis peduncularis* (AY 356350). I Alignment of consensus sequence of the type A repeats (A0) with the sequences of each A repeats in *O. peduncularis* performed with the Tandem Finder Program. **II** Idem for B repeats. **III** Idem for C repeats. Symbol - indicates an insertion or deletion. Symbol * indicates a mismatch. **IV** Sequence alignment of B repeats (Clustal X). Perfect (underlined) and mutated EcoRI restriction site recognition sequences are emphasized in bold. Symbol - indicates an insertion or deletion. Symbol * indicates a match.