Nuclear ribosomal spacer regions in plant phylogenetics: problems and prospects

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Abstract The nuclear ribosomal locus coding for the large subunit is represented in tandem arrays in the plant genome. These consecutive gene blocks, consisting of several regions, are widely applied in plant phylogenetics. The regions coding for the subunits of the rRNA have the lowest rate of evolution. Also the spacer regions like the internal transcribed spacers (ITS) and external transcribed spacers (ETS) are widely utilized in phylogenetics. The fact, that these regions are present in many copies in the plant genome is an advantage for laboratory practice but might be problem for phylogenetic analysis. Beside routine usage, the rDNA regions provide the great potential to study complex evolutionary mechanisms, such as reticulate events or array duplications. The understanding of these processes is based on the observation that the multiple copies of rDNA regions are homogenized through concerted evolution. This phenomenon results to paralogous copies, which can be misleading when incorporated in phylogenetic analyses. The fact that non-functional copies or pseudogenes can coexist with orthologues in a single individual certainly makes also the analysis difficult. This article summarizes the information about the structure and utility of the phylogenetically informative spacer regions of the rDNA, namely internal- and external transcribed spacer regions as well as the intergenic spacer (IGS).

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Introduction

The ribosomal RNA (rRNA) genes and their spacer regions have become widely used as a source of phylogenetic information across the entire breadth of life [1]. The popularity of the rDNA locus for phylogenetics might be attributed to the phenomena that they serve the same function in all free-living organisms. They have the same or almost the same structure within a wide range of taxa. The coding regions, like the small- and large subunit gene, represent some of the most conservative sequences in eukaryotes [2, 3], which is a result of a strong selection against any loss-of-function mutation in components of the ribosome subunits [4]. The most conservative part appears to be the 3′ end of the 26S rDNA representing the α-sarcin/ricin (S/R) loop [5]. The information provided by the rDNA locus in phylogenetic research is significant, and it can be used at different taxonomic levels, since the specific regions of the rDNA loci are conserved differentially. The spacer regions of the rDNA locus possess information useful for plant systematics from species to generic level. They have also been used on studies of speciation and biogeography, due to the high sequence variability and divergence. There are three notable spacer regions: the external- and internal transcribed spacers (ETS, ITS) and the intergenic spacer (IGS). The general properties of these rDNA spacer regions will be reviewed in a phylogenetic context. Besides the general description, organization and

structure of each spacer, the recent advances made in the utilitization of each unit will also be discussed. Some of these are well summarized in other studies (like for ITS), while for ETS and IGS the relevant new findings have not been adequately reviewed. Thus, the aim of this study is to summarize the features of all rDNA spacer regions suitable for phylogenetic research.

The internal transcribed spacer as a phylogenetic marker

The internal transcribed spacer (ITS) is intercalated in the 16S-5.8S-26S region separating the elements of the rDNA locus (Fig. 1). The ITS region consists of three parts: the ITS1 and ITS2 and the highly conserved 5.8S rDNA exon located in between [6]. The total length of this region varies between 500 and 750 bp in angiosperms [7] while in other seed plants it can be much longer, up to 1,500–3,500 bp [8, 9]. Both spacers are incorporated into the mature ribosome, but undergo a specific cleavage during the maturation of the ribosomal RNAs [10–12]. It is now certain that ITS2 is sufficient for the formation of the large subunit (LSU) rRNA during the ribosome biogenesis [13]. The correct higher order structure of both spacers is important to direct endonucleolytic enzymes to proper cut sites [14]. Although, the sequence length of the ITS2 is highly variable between different organisms, Hadjiolova et al. [15] identified structurally homologous domains within mammals and *Saccharomyces cerevisiae*. In contrast to the

coding regions, spacers evolve more quickly, like the internal transcribed spacer (ITS) region, which is extensively used as a marker for phylogenetic reconstruction at different levels. Since its first application by Porter and Collins [16] it has become widely used for phylogeny reconstruction. As a part of the transcriptional unit of rDNA, the ITS is present in virtually all organisms [11]. The advantages of this region are: (1) biparental inheritance, in comparison to the maternally inherited chloroplast and mitochondrial markers; (2) easy PCR amplification, with several universal primers available for a various kind of organisms; (3) multicopy structure; (4) moderate size allowing easy sequencing; and (5) based on published studies it shows variation at the level that makes it suitable for evolutionary studies at the species or generic level [7–9]. Álvarez and Wendel [17] and Baldwin et al. [7] summarize that this variability is due to frequently occurring nucleotide polymorphisms or to common insertions/deletions in the sequence. This high rate of divergence is also an important source to study population differentiation or phylogeography [18–21]. It has been widely utilized across the whole tree of life, including fungi [22–31], animals [32–36], different groups of 'algae' [14, 37–39] lichens, and bryophytes [40, 41]. In addition it is often used in the other two major domains of the tree of life Archaea and Bacteria [42–46] where RISSC, a novel database for ribosomal 16S–23S RNA genes and spacer regions is developed to provide easy access to information [47].

The high copy numbers allow for highly reproducible amplification and sequencing results, as well the potential

Fig. 1 Schematic presentation of the universal structure of the rDNA region in plants. (a) The chromosomal location of the rDNA regions. (b) Tandem arrays of the consecutive gene blocks (18S-5.8S-26S). In the tandem arrays each gene block is separated by an intergenic spacer (IGS) consisting of a 5′ end and 3′end external transcribed spacer (ETS). The two ETS regions are separated by a non-transcribed region (NTS). The transcription start site (TIS) labels the start position of the 5′ETS. The small subunit (18S) and large subunit genes (5.8S and 26S) are separated by the internal transcribed spacer 1 (ITS1) and internal transcribed spacer 2 (ITS2)

to study concerted and reticulate evolution. The number of studies utilizing ITS in phylogenetic studies is increasing, publicly available ITS sequences has tripled since 2003 [11]. The plant families most intensively studied are Asteraceae, Fabaceae, Orchideaceae, Poaceae, Brassicaceae, and Apiaceae. At the genus level there are for example more than 1,000 sequences available for different species of *Carex* (NCBI GenBank, nucleotide search preformed in 15.02.2009).

Besides several advantages there are many drawbacks for use of rDNA ITS data in evolutionary studies. There are hundreds or thousands of ITS copies in a typical plant genome [17]. Inferring phylogeny from multigene families like ITS can lead to erroneous results, because there is variation among the different repeats present in a single eukaryote genome [48]. Evidence now suggests that this variation among ITS sequences of an organism is found only within organisms that are hybrids or polyploids [49].

Multiple rDNA arrays and paralogy

Several ribosomal loci, both transcriptionally active and inactive, are usually present in plant genomes [50]. As ribosomes are the workhorses of the protein biosynthesis, translating mRNA to build polypeptide chains, they are extremely important structures in the cell. For this reason many copies are required to tend to the needs of an organism for this important process. These copies as well as their number and distribution in the plant genome are highly variable [51–56]. As both ITS regions are part of the cytoplasmic ribosome genes playing a role in the formation of the mature ribosome, there are hundreds, or in some cases thousands of tandem copies [57, 58]. Because of the high copy number this region is recognized as a multi-copy gene family, which provides easy amplification via PCR. This is an advantage, but on the other hand it can be a problem in phylogenetic analyses, if paralogous sequences are present. However, the general assumption for phylogenetic studies is that all ribosomal copies present within the genome have fairly identical sequences due to functional constraints. Orthologous genes and gene products found in different species are the basic requirement of phylogenetic inferences concerning common ancestry among species [59]. Unidentified paralogous relationships and infrequent recombination between paralogues can result in erroneous species phylogenies [60]. Paralogous sequences can occur at many levels: within an individual, among individuals within a species, and among species. To determine intra-individual paralogues among sequences of an individual and to find which are maintained and shared with other species is a potential problem in phylogenetic analysis. Another problem is PCR amplification, because

the ITS sequence amplified is a consensus of many targets sharing the same priming sites in one or several loci usually located in separate chromosomes. This consensus sequence used as a row of data in phylogenetic analysis is a molecular phenotype from which the genotype of the organism cannot always be inferred [50]. It is also impossible to determine the zygosity of the marker. There are two types of alternative copies which can be detected with PCR. First there are sequences having the same size as the others from different loci, but there are SNPs (single nucleotide polymorphisms) in different positions within their sequence. Sequences can differ also in size, because of permanent insertion/deletion events. Both types occur when different ITS repeats are merged within a single genome via hybridization (including allopolyploidy) or introgression. These processes are very common in plants; recent estimates suggest that 70% of all angiosperms have experienced one or more episodes of polyploidization [61].

Concerted evolution

In plants the ribosomal genes are present in several copies. For example in *Arabidopsis thaliana* more than 1,400 genes encode rRNAs, and occur on different chromosomes, with specific polymorphic alleles largely homogeneous in each rDNA array [62]. All copies within and among ribosomal loci are expected to be homogenized through genomic mechanism of turnover like gene conversion, the non-reciprocal transfer of genetic information between similar sequences, and unequal crossing over [63]. This phenomenon was first reported by Schlötterer and Tautz [64] and later by Polanco et al. [65] studying polymorphisms within the ITS in populations of *Drosophila*. They found that individual rDNA arrays are homogenized for different polymorphic alleles, which indicate that intrachromosomal recombination events occur at rates much higher than those for recombination between homologous chromosomes at the rDNA locus. The intra-genomic rDNA diversity is generally low, and this low diversity results from concerted evolution within and between ribosomal loci [66]. The mechanism of concerted evolution completely, or almost completely, reduces the level of inter-repeat sequence variation between the multiple arrays of rDNA in every organism [17].

The fact that the ribosomal multigene family evolves through the process called concerted evolution certainly makes phylogenetic analysis much more difficult. It is important to recognize that concerted evolution is a complex process. According to various authors there are special stages during the process of concerted evolution, which lead to different classes in the plant genome [49, 67–69]. These stages can be important features in

phylogenetics, leading to questions: Are the several copies homogenized properly? Are there any heterogenic sequences? Which copy is the dominant sequence in the genome? Are there any variations between the sequences of an individual? However, concerted evolution does not act immediately after organismal processes such as hybridization or polyploidization, or after genomic changes like gene and chromosome segment duplication, and various forms of homologous and non-homologous recombination [11]. Thus, divergent rDNA copies could be present throughout the genome, disturbing phylogenetic analysis and sequencing. Because paralogous copies occur due to polyploidy or hybridization they can be utilized to study these processes. The presence of parental rDNA repeat types in a hybrid is determined by many forces affecting their molecular evolution [70]. The detection of these alternative copies depends on their number. If hybrids are recent, both parental types are almost always present [71]. Such hybridization can be easily reveled by direct sequencing, where an additive pattern of sequence variation is present. In such cases, the sites differing between species yield signals from two different nucleotides. According to Rauscher et al. [70] it is unclear how common a repeat type must be, relative to the other parental type. In the case of *Gossypium* spp. the homogenization process was complete, leaving no easily traceable evidence in the ITS region to track polyploidy [69]. However, in the *Glycine tomentella* complex the ITS region was successfully used to evaluate parental relationships and hybrid speciation [70]. In this study repeat-specific and exclusion PCR primers were designed to detect rare parental ITS types. In another study Koch et al. [72] clarified the multiple hybrid origin of natural populations of *Arabis divaricarpa*, the putative hybrid of *A. holboellii* and *A. drummondii*. They detected multiple intraindividual ITS copies in several *A. divaricarpa* accessions which were also present in the parental species. But concerted evolution in this case also resulted in different ITS types, in the hybrid *A. divaricarpa* and in the parental taxa, respectively. In other groups like *Potamogeton* [73], *Bromus* [74], *Nymphaea* [75], *Armeria* [76] and *Cardamine* [77] ITS was a valuable source to reveal complex reticulate events between putative hybrids [78–81]. Concerted evolution is sometimes incomplete and some copies of the tandem arrays became non-functional pseudogenes [50]. Mayol and Rosselló [82] reanalyzed datasets by two different and independent laboratory teams [83, 84] generated for the study of systematics of the genus *Quercus*. Their surprising result was that the divergent ITS alleles reported by one of the teams were non-functional paralogous copies (pseudogenes). It was also concluded that the incorporation of these ITS paralogues in evolutionary studies can lead to

erroneous hypotheses about phylogeny. Standard definitions of pseudogenes are hard to be apply to rDNA pseudogenes. In the context of phylogenetic reconstruction Bailey et al. [85] determined rDNA pseudogenes as sequences with nucleotide divergence pattern that has not been constrained by function irrespective of expression patterns.

Secondary structure modeling of the ITS region

The construction of the secondary structure model of the ITS RNA transcript was proposed as a novel tool for phylogenetics. These new methods have also made analyses easier in a user-friendly interface (e.g., online databases and programs). The importance of this recent advance enable inference of phylogenies not only based on sequence information, but also based on predicted secondary structures. The phenomena that rRNA single-stranded chains form a secondary structure which contain stemmed regions and different loops correlating with base pairing opened a new field to infer phylogenies. During phylogenetic analysis it is hard to determine whether a pseudogene or a paralogous sequence has interfered the results. ITS2 is a well suited marker with a broad use in low level phylogenetic analyses, as its sequence evolves quite fast. This feature, which made the region useful for analyses at generic and infrageneric level, is a 'hindrance' for the application of this marker for more general phylogenetic analyses [86]. The possibility to predict the folding structure has enhanced the role of ITS in phylogenetic studies, since this will enhance sequence alignment which can be based on secondary structures [87]. When comparing the structure of the ITS2 RNA transcript, it turned out that a conserved core is found in different species. Many methods have been applied to infer the secondary structure of the ITS2, like electron microscopy [88], chemical and structure probing [89], and site-directed mutagenesis [90, 91] Also different softwares have been developed for this purpose [86]. The surprising result of these studies has been that the examined eukaryote groups share the same general ITS2 secondary structure [92]. It was concluded that the secondary structure for the ITS2 consists of four helixes. Among plants, nucleotide sequence evolves most rapidly in region IV followed by helix I [48]. It was also described that 'helix II is more stabile, and characteristically has a pyrimidine-pyrimidine bulge while helix III contains on the 5′ side the single most conserved primary sequence, a region of approx. 20 bp encompassing the TGGT' [48]. The fundamental role of the helicoidal ring of ITS2 during the pre-RNA processes is to trigger the maturation of the 26S rRNA, because it was observed that the lack of its structure blocks the productions of the mature large

subunit, when specific structures of the ring model can not be formed [93–95]. These features led Schultz et al. [86] to construct an ITS2 database, a web-based tool for phylogenetic analysis. This new server is open for structure prediction and provides a way for utilizing more information from sequences. Recent studies have used a parsimony approach to predict the appropriate structure for ITS2 according to free energy measurements. The method developed by Schultz et al. [86] to predict ITS2 structures is based on the Needleman–Wunsch algorithm [96], but applies a BLAST search with the newly predicted structure in the database to compare it with others [86, 97, 98]. The phylogenetic estimates made from secondary structure characters are based on newly generated information that differs from the sequence level data.

When comparing the secondary structures of the ITS2 it turned out that it is also useful to distinguish species based on compensatory base changes (CBCs). CBCs occur in a paired region of a primary RNA transcript when both nucleotides of a paired site mutate, while the pairing itself is maintained [99]. In other words following the definition of Kimura [100] compensatory mutations are a pair of mutations at different loci (or nucleotide sites) that are individually deleterious but are neutral in appropriate combinations. Basically the RNA secondary structures comprises single-stranded and double-stranded regions, where the double-stranded stems are formed by Watson–Crick (WC) pairing of complementary bases.

In all eukaryote groups where a broad array of species has been compared for ITS2 sequence secondary structure and tested for any vestige of species sexual compatibility, an interesting correlation has been found: 'when sufficient evolutionary distance has accumulated to produce even one CBC in relatively conserved pairing positions of the ITS2 transcript secondary structure, taxa differing by the CBC are observed experimentally to be totally incapable intercrossing' [101]. Using the ITS2 database now consisting of 65,000 ITS2 sequences Müller et al. [99] concluded that CBCs in an ITS2 secondary structure are sufficient indicators to distinguish even closely related species. Secondary structures can be determined by an alignment against sequences with already known structures to depict common base-pairing patterns [102]. Secondary structures are particularly useful because they include information not found in the primary sequence [4]. The modeled structures are of importance in phylogenetic analysis, because they can be used to enhance alignments obtained by different methods [102, 103].

The secondary structure of ITS1

Structural prediction of the ITS2 is more common in phylogenetic studies than the rarely used ITS1, but several models exist also for ITS1. The successful prediction of the secondary structures of ITS1 and to compare these sequences among genera or families is more difficult than in the case of ITS2. ITS1 seems to evolve faster, and has less conserved sites than ITS2. As discussed above the prediction for ITS2 is easier since there are conserved motifs in the structure across a wide range of divergent lineages, like the four helixes or the 5′ UGGU motif and many others [86]. This is unfortunately not the case in ITS1. Liu and Schardl [104] identified a 20 bp region in the inferred secondary structure of ITS1, which is highly conserved among angiosperms, and this was also confirmed by Goertzen et al. [105] but there are no reports of other conserved motifs within the structure of ITS1. However, there are some studies that have utilized ITS1 structure prediction in phylogenetic analyses [106–108]. The reconstruction of the ITS1 RNA transcript secondary structure model is not always successful between different plant genera. The failure in some cases might be related to the features of ITS1 which are different from ITS2. The ITS1 is more variable than the ITS2. The two spacers evolve with different substitution rates, and the rates seem to vary from species to species [109]. Thus, a universal rate could not be applied to plants; it must be edited and determined for each genera. This difference between the two spacers is due to their function and role in the ribosome biogenesis. The sequence of the ITS2 is more conserved because it is more important in the formation of the mature ribosome than the ITS1. Thus, the higher order and secondary structure is more important for the appropriate ribosome formation, which is reflected in its sequence. It must be recognized that there is more conformational similarity between the aligned and predicted structures of ITS2 than in the ITS1. Baldwin et al. [7] also concluded that in contrast to ITS1, ITS2 displays more sequence similarity across plant families in the central region of the spacer. The conformational similarities in the higher order in the predicted structures of the RNA transcript might be attributed to stronger functional constrains of the ITS2. Baldwin et al. [7] also tried to predict the structure of ITS1 for five plant families, but the sequences showed insufficient retention of similarity during the alignment. The final conclusion was that the ITS1 lacks similarity in the structure across plant families, but there were minor differences in the free energy between the most parsimonious structures. It would be highly interesting to predict the ITS1 structures with the novel approach of Schultz et al. [86]. A tabulation based on the available sequences in the Genbank across all ITS1 sequences would give new insights to the theoretical and practical application of the secondary structure of the ITS1. With a proper algorithm it would be possible to predict the potential secondary structure models from the available sequences. Comparing them with each

other might elucidate the utility of ITS1 secondary structure models in phylogenetic analyses. In summary a more extensive study on the ITS1 structure should provide new data and information for evolutionary studies.

The intergenic spacer region

The IGS region, separating the rDNA tandem arrays (Fig. 1), consists of regulatory elements like promoters, enhancers and terminators, and the NTS (Non-transcribed spacer) as well as the external transcribed regions (discussed in the next section). For the last several years this region have been used for evolutionary biology and genetics rather than studies of phylogeny. The major findings of recent studies are the exact and descriptive structure of the rDNA IGS for many species. This region contains several kinds of repeating elements, also refered as subrepeats, various types of enchancers, and promoter regions which are often distributed throughout the whole region in duplicated forms. There are elements which form conserved secondary structures. The full detailed exposition of the structural elements of the IGS region is beyond the phylogenetic context of this review. For further details in this context see Weider et al. [110] and Gorokhova et al. [111]. In general it has to be noticed that these elements play an important and essential role in the control of the rRNA transcription and also in the processing of the transcript during the replication of the unit. Thus, IGS is an important functional region, because it contains the nucleotide sequences that trigger and/or terminate transcription [112–116]. The phylogenetic interpretation and utility of this region is naturally constrained by these features. Before the full detailed discussion of the IGS region some nomenclatural clarification must be made in order to refer precisely to each region. There are two non-transcribed (NTS) regions in plants. The 5S repeats in plants are located in different locus or loci than the nucleolar organizer region (NOR, 18S-5.8S-26S rDNA) locus or loci, although the 5S and NOR can also be located in the same chromosome [117]. This organization is unique in plants in contrast to other eukaryotes, where the 5S region repeats are intercalated in the IGS region. The 5S units of the rDNA are also present in repetitive tandem arrays and are separated by simple NTS regions frequently referred as the 5S NTS which are not equal to the regions found between the separately organized NOR arrays. Predominantly they have the same features and structure, but the term NTS in this review will attribute only to the spacer region found between the NOR tracts of rDNA locus, as the 5S NTS will not be discussed here.

High sequence variability

As some parts of the IGS are also variable or more variable than the widely utilized ITS, they have also been used as phylogenetic markers. The utility of IGS for phylogenetic studies is criticized by its major features, which are: (1) high sequence variability; (2) subrepeat tracks present in the region; and (3) the length of these subreapeats are highly variable disrupting the sequence alignment between taxa. It must be realized, that the IGS is a rapidly evolving region of the rDNA with several internal subrepeats present in its sequence, which evolve rapidly both in size and structure making comparative anlayses difficult in some cases. The length polymorphisms have been observed between populations, species or even in individuals [116]. The region seems to be extremely dynamic [118, 119]. The primer design for this region can be problematic too, because the rDNA IGS is known for gradual decrease in sequence conservation upstream from the 18S gene to the center of the rDNA IGS which consists of repetitive elements [120, 121]. These troublesome properties of the rDNA IGS region cause serious obstacles to the development of primers and in the alignability apart from the ETS, even at low taxonomic levels [11]. Most plant species show length variation in the IGS [116, 117]. IGS has not proven itself as a useful tool for phylogenies of species that are not very closely related, not only because the IGS has a large number of related subrepeats but also because the subrepeat length and primary sequences are too dissimilar to be aligned [120]. Several length variants of the IGS can be present in the same genome [122]. Species of the same genus often differ significantly in the length of the IGS, which can range from 1.7 to 6.4 kb in the case of *Trillium* [123]. Even in the case of individuals of the same population, the length of the IGS can differ, but there are examples for low variability or even uniformity among species of the same genus [124]. The upstream of the TIS accumulates base substitution with a high rate: obvious sequence similarity can be found here only among members of the same genus or closely related genera [5]. As summarized above there are several factors which affect the use of IGS as a potential phylogenetic marker. Amplification of subrepeats in the IGS have occurred several times during evolution, as it is known from the examples of the genus *Nicotiana*, where original duplication of the ancestral A-subrepeat sequence in the ETS took place before the divergence of the subgenera, and produced two subvariants, A1 and A2 [125]. Longer stretches of the A1/A2-subrepeats are formed independently during the later speciation of *Nicotiana*, while similarly several rounds of amplification/delations generated C-subrepeats in the upstream of the TIS [126, 127]. As it is seen in the example of *Nicotiana* this "chaotic-mix" of subrepeats

prohibits the routine use of easy amplification with PCR and the development of universal primers across plant families.

The phylogenetic utility of IGS subrepeats

Despite of the fact that there are many reiterated subrepeats present within the sequence of the IGS it has been successfully used to infer phylogenies. These studies were mainly carried out among closely related species. When closely related species are compared with each other, the possibility that the IGS region could be aligned properly greatly increases. Maughan et al. [128] analyzed the IGS region of different cultivars of Andean grain crop quinoa (*Chenopodium quinoa*) and a related ancestor *C. berlandieri* subsp. *zschackei*. During this analysis it turned out that the IGS regions of quinoa and its wild relative have strikingly similar subrepeat sequences which differ in their number and in the presence of species-specific motifs toward the 3′ end of the IGS. Sequence comparison indicated that the two allotetraploid species descended from at least one common diploid ancestor. In another study Fernández et al. [119] compared the IGS sequence of *Lens culinaris* with other species of Vicieae and Phaseoleae. The amplified spacer was also composed of nonrepetitive sequences and four tandem arrays of repeated sequences. According to the number and length of subrepeats, different repeat types were identified named A to D. Among the sequences conserved motifs were also found, which were attributed as functional sequences. The most noticeable result of this study is that despite of the rapid evolution of the IGS sequences within and between the two legume tribes, some motifs have been conserved in their sequence and relative position. Some of these motifs were found in other phylogenetically distant taxa. Although, there are some exceptions where the IGS can be successfully applied as a phylogenetic marker this can not done universally to all plant species. Additionally, IGS sequences could only be employed as phylogenetic markers when closely related species are the subjects of interest [118, 129–132]. Subrepeats appear in the IGS region of all plant species and in eukaryotes, except for one unambiguous case of *Caenorabditis elegans*, which has a simple organized structure for an unknown reason. Because of these subrepeats present in the well characterized region of IGS in plants, the length of this region varies dramatically. But is there any consequence in the duplication of these regions?

The repeats can be grouped into different classes in *Oryza sativa* [133], *Vicia faba* [134], *Triticum aestivum* [135] and several other species. Later, this has been confirmed also for *Olea europea* [136] and *Quercus* [137]. If we apply the widely accepted model of Dover and Tautz [138] to subrepeats; that is DNA sequences evolve through successive cycles of tandem duplications and perhaps, the divergence of an ancestral sequence, interesting conclusions can be made. Ryu et al. [139] presented that the evolution of the IGS can be thought to include duplications/delations through divergence processes, resulting in a dynamic change in the subrepeat composition. These duplications, often called as homopolymeric runs or mononucleotide microsatellites can be abbreviated with the term poly (N). Ryu et al. [139] concluded that if the repeats within the IGS are interpreted as poly (N) runs the nature of this fraction could be better understood. They characterized the IGS region of several species and developed an alignment algorithm, which can take into account the differences induced by the poly (N) runs and recover the underlying phylogenetic signals from the IGS subrepeat comparisons. The method is called dropout alignment. Thus, prior to the alignment of the sequences all consecutive bases in each poly (N) run are deleted (dropped out) except one base, because many difficulties arose from the differences in the expansion of the poly (N) repeat types. This new method allowed the proper alignment of different types of subrepeats within the same taxa, and enabled alignment and comparison of the subrepeats between different species. The method of Ryu et al. [139] suggests that most of the variation found within the IGS is manifested by the occurrence of poly (N) runs. The new method could be a useful tool for the further evolutionary investigation of the IGS and opens the opportunity for the better exploitation of IGS sequences for phylogenetic purposes.

The external transcribed spacer

The external transcribed spacer (ETS) lies in the intergenic spacer region separating the repetitive 18S-5.8S-26S ribosomal gene blocks from each other. There are two ETS sites: the 3′- and 5′ prime parts which are bordering the 18S and 26S exons (Fig. 1). There is a substantial difference between the 5′- and 3′ prime ETS. In some studies the 5′ ETS is also referred as ETS1, while the 3′ external transcribed spacer as ETS2. But what are the 3′- and 5′ prime ETS regions? There is a considerable confusion around the nomenclature of the ETS region. In the literature both spacers are named differently and the denomination of 5′- and 3′ parts are sometimes confusing depending on what were the bases of the nomenclature. In the first model the 18S-5.8S-26S is treated as a separate unit and the bordering sequences in both ends are considered to be the external transcribed spacers. Thus, when the rDNA locus is treated as a separate unit, the external transcribed spacer from the 18S part is labled as 5′ETS and the 3′ spacer region of the 26S exon is called as 3′ ETS (e.g., Hershkovitz et al. [140]). In the second model the IGS is taken

as the base labeling the ETS regions. The 5′ prime part of
the IGS would then be the 5′ ETS and likewise the 3′ part
of the IGS should be named as 3′ ETS (e.g., Calonje et al.
[11]). In this review the terms 3′- and 5′ETS will be used to
refer exactly and directly to the two parts of the rDNA
region instead of ETS1 and ETS2. Because the tandem
repeat sections of the rDNA locus begin with the 18S gene
in order with the main direction of the DNA strand, the site
upstream from the 18S exon, should be unambiguously
labeled as the 5′ETS (5′end ETS or ETS1) and the site
downstream from the 26S exon (the 3′end of the 26S gene)
likewise 3′ETS (3′end ETS or ETS2). In this review
nomenclatural terms will be treated as stated above.
Another confusion surrounding the ETS is whether the
3′ETS exist or not? In some studies the 5′end part of the
IGS is referred to as the 3′ETS. Since the transcription of
this region was not always obvious, as well as the exact
position of the transcription termination site, the nomen-
clatural term 3′ETS is not applied by every study. In
summary it has been shown that the 5′end of the IGS
contains non-repetitive sequences which are highly similar
across different species [118, 130, 141, 142]. Recent
studies have shown that this region is transcribed and plays
a role in the ribosome transcription [12, 143, 144]. The
transcription termination site in the 3′ETS—as the tran-
scription initiation site in the 5′ETS—is highly variable in
plants. In the recent years a great progress was made sur-
rounding the external transcribed spacers, revealing inter-
esting new features about the region. As this mainly
concerns the 5′ETS; and much more data is available for
this region since it is more widely utilized in phylogenetic
studies, this review will mainly focus on the 5′ETS. Con-
sequently, in the following the major characteristics of this
region in context of the new findings will be discussed to
provide a conclusive description about their potential use in
evolutionary studies.

The phylogenetic signal of the ETS

Since its first application by Baldwin and Markos [145]
several analyses successfully adopted this marker as a
valuable phylogenetic tool. Sequence comparisons of the
rDNA external transcribed spacer (ETS) indicated that it
represents an even more valuable instrument for the phy-
logenetic analysis than ITS [130]. The ETS has been used
in phylogenetic analysis of families Asteraceae [145, 146],
Fabaceae [147] and Myrtaceae [148] only to mention some
examples. The 5′ETS is more frequently used in phyloge-
netic studies, than the 3′ part. The length of the 5′end ETS
range from 425 to 575 bp [149–152] making it easily
sequenced. There are less sequences available for ETS
compared to ITS. This might be attributed for the ambig-
uous amplification and primer design for this region.

Several studies utilized this marker prior to the prominent
article of Baldwin and Markos [145] and Bena et al. [153].
The information about the molecular features of the ETS
region has increased recently. According to a fast tabula-
tion among submitted sequences in GenBank (search pre-
formed in 04.06.2009 in NCBI, GenBank). A large portion
of sequences is available mainly for crop plants.

The protocol provided by Baldwin and Markos [145]
with the study of Bena et al. [153] for primer design
made it easy to exploit the ETS for phylogenetics. The
major principle of this method is the amplification of the
total IGS region with primers starting from the flanking
region of the 18S and 26S genes. The procedure requires
a long-PCR protocol; because the total length of the IGS
is classically longer then 4 kb [154] and can be up to
12 kb [58]. The next step is to design taxon specific
internal primers for the conserved regions of the amplified
intergenic spacer according to the nucleotide data of the
reverse sequenced product. Problems may arise when
duplications of promoter regions intercalate in the region.
An ideal point would be to design primers to the con-
served promoter motive flanking the RNA polymerase I
transcription initiation site, but the position of this region
in the 5′ETS is often variable in plants. Finding the
[TATA(G)TA] motif with reverse sequencing may require
additional time as compared to the routine use of the well
developed and widely applied ITS primers. In those cases
where additional work has been done in this context it is
easy to utilize the ETS region. In plant lineages where
such preliminary data is lacking it would require much
additional work in the primer design. But is this addi-
tional laboratory work warranted? Does the ETS region
contain enough information to be used in phylogenetic
studies? These questions could be answered according to
the several studies that have been published [155–159]
making primers available for the scientific community.
The major observation is that the ETS evolves at faster
rate compared to ITS. However, the restriction site studies
by Kim and Mabry [160] have found that variation within
ETS is comparable to ITS. The mean rate was estimated
as 2.86×10^{-9} subs/site/year according to the study of
Kay et al. [109] which was based on 29 independent ITS
substitution rates ranging from 0.38×10^{-9} (*Hamamelis*)
to 8.34×10^{-9} (*Soldanella*). These kinds of estimates are
not available for the ETS. According to Bena et al. [153]
the 5′ETS evolves 1.5× faster than the internal tran-
scribed spacer region. In another study Baldwin and
Markos [145] calculated a 1.3–2.4× higher rate of
sequence evolution, while Linder et al. [146] estimated
7× higher rate in Asteraceae and closely allied families.
5′ETS provided approximately 1.5× higher information
content in terms of parsimony informative sites. A pos-
sible explanation for this difference in the substitution

rates would be the reduction of ribosomal maturation processes in the case of ETS.

Divergent repeat types of ETS

The homogenization process of concerted evolution is the operating force to eliminate the different repeat types of ETS found within the genome of a single individual. However, concerted evolution is a well known and specific feature of multigene families—such as the rDNA locus— the rate and even its accuracy is not well known. In general the whole process of concerted evolution enhances the sequence similarity between multiply arrays of ITS and ETS. But does concerted evolution operate at different rates between each region? In a theoretical model ETS also evolves in concert and therefore its utility is affected by the same 'drawbacks' as ITS. The inclusion of paralogous sequences in the analysis would also be a problem as well as the appearance of pseudogenes in the datasets which can greatly disturb the reconstruction of phylogenies if they are accepted as orthologs. In addition, the putative repeat types of the ETS would also provide data to study complex evolutionary processes as well as to reveal hybridization no matter if they have arisen in recent or ancient hybridization. Another approach would be to combine the results of the alternative copies of ITS and ETS, thus further information would be available for tracking polyploidy. However, concerted evolution does not seem to operate at the same level between ITS and ETS. High level of intragenomic similarity has been found between 5′ETS sequences in *Helianthus* [146]. It was concluded that concerted evolution does not always eliminate all intragenomic variation in ETSs of all rDNA repeats, but it proceeds rapidly enough to not obscure specific relationships. Vander Stappen et al. [161] found no evidence for the presence of multiple ETS sequence types—or in a previous analysis for multiple ITS types—within individuals, indicating that concerted evolution acted affectively in both regions in the allotetraploid species of *Stylosanthes* [162]. Furthermore, it was also reported that the homogenization covers all parental rDNA repeat types. It seems that ETS homogeneity is maintained within rDNA clusters and throughout genomes [11]. However, it was reported that several subrepeat types exist within different taxonomic groups. These subrepeats, which are repeated regions in the ETS sequence, are found in *Solanum* sect. *Petota* and also in many other taxa like *Arnica mollis* and *Hemizonia perennis* [145]. Volkov et al. [163] reports A, B and C variants of 5′ETS subrepaets within *Solanum*. Their results showed that during the evolution of sect. *Petota* at least two large rearrangements of ETS occurred, resulting in B and C structural variants. These variants now succesfully has been used as sources of phylogenetic information for

potatoes and the major taxonomic groups can be separated based on this information. An interesting feature is that these repeat groups evolved through stepwise base substitutions allowing the additional discrimination of closely related species. The latter structural study of the 5′ETS also ambiguously supported the position of 'Lycopersicon' (tomato clade) in sect. *Petota* through the discovery of a new D subrepeat, among the members of the tomato clade [164]. Another interesting feature of these subrepeats is that variant D might have originated directly from the ancestral variant A found in ser. *Etuberosa*. Taken together, a high rate and accuracy of concerted evolution seem to operate in the 5′ETS region and the sequences of the ETS region can be successfully used in the reconstruction of phylogeny at different taxonomic levels and can be widely applied in plant phylogenetic research.

Combining ETS and ITS data

In several cases high genetic diversity have been reported in plants. This large diversity is attributed to reticulate evolution, hybrid speciation or polyploidy. Such events are common in angiosperms. To address changes or evolutionary patterns and/or complex events at the molecular level markers with robust phylogenetic signal are desirable for phylogenetic analyses. The ETS region can be used successfully in phylogenetic studies where ITS seems to have only a weak signal, such as in recently diverged lineages, because it shares the same favorable features of the ITS, and it is generally known to evolve faster and to contain more phylogenetically informative characters than the ITS in plants [145, 155, 161]. Since both ITS and ETS are part of the rDNA locus their application in a combined analysis seems obvious. Although, when the ITS and ETS regions are analyzed separately the results about the phylogenetic signal provided by each region is variable. This variability might depend on phylogenetic histories and might be displayed differently in various taxonomic levels. It has been found that the 5′ETS includes less parsimony informative sites than ITS in resolving higher level relationships. Oh and Potter [165] combined the data of ITS and 5′ETS in the study of tribe Neillieae (Rosaceae). They also report that 5′ETS region included less parsimony informative sites than the ITS. Markos and Baldwin [166] analyzed the ETS and ITS data separately and in combination to study the higher-level relationships and major lineages of *Lessingia*. In their analysis it turned out that the amplified ETS region possessed more variable sites and it was 1.4× more informative than the ITS site. In another case [167] the 5′ETS did not allow reliable alignment of the compared sequences. Tucci et al. [167] report that in *Cynara* and *Onopordon* the sequences showed only 27% similarity.

Clearly there are differences in the diversity of ETS and ITS as reported by various studies. In some case the ITS is more informative at the higher taxonomic levels and the ETS can be less informative or vice versa. Possibly the ETS can accumulate great diversity or repetitive elements (like promoters) in its sequence and it cannot be aligned properly even in closely related genera. In studies where both ETS and ITS data have been used, no matter if the phylogenetic signal of the ITS or of the ETS was stronger, the combined analysis of the datasets have resulted in better resolved and more robust trees. Bena et al. [153] also reports that combining the two regions, greatly improves the resolution and increases the bootstrap support values for clades. It can be concluded that ETS data, besides the well utilized ITS, should be included to improve the estimation of phylogenies of different plant groups.

Concluding remarks

It can be summarized that the spacer regions of the rDNA locus are useful phylogenetic markers. They share the small size, high sequence level variability, conserved flanking regions, and rapid concerted evolution under similar functional constraints. In all eukaryote groups phylogenetic relationships can be inferred from rDNA markers, because different parts of this region evolve with different rates. However, rDNA regions coding for the large- and small subunits of the rRNA display relatively little variation, and thus they will remain the major targets of studies inferring phylogeny at the higher taxonomic levels. Other parts of these valuable loci can also be easily used in plant systematics. On the other hand doubts about the correct use of ITS and ETS data are considerable. These sequences can be difficult to handle if polyploidization or other events disturb the phylogenetic signal. The detailed nature of these regions should be taken into account. Paralogy, if overlooked and mistakenly incorporated in the in the phylogenetic data, can be a problem and speaks against the utilization of ITS. However, with considerable investment in amplification and analysis pseudogenes and paralogs can be isolated. If they include enough information, or they are the targets of interest they can be included in phylogenetic analyses.

As the nature of the ITS region is well understood new advances in its utilization (e.g., RNA transcript secondary structure prediction based reconstructions) are welcome additions to be used for phylogeny reconstruction. As demonstrated by several studies and summarized here, besides the routine use of ITS, other variable regions of rDNA could provide new information about phylogeny. Besides the nucleotide sequences of the ITS the recently developed database of the predicted secondary structures can be utilized.

Although, concerted evolution and the repetitive nature of the ITS could prevent its routine usage, it still might have great potential to study more complex evolutionary relationships. The process of concerted evolution is a fundamental phenomenon operating in all eukaryote organisms. The incorporation and application of new developed protocols and methods to study the divergence among and within repeat types of multigene families, such as the rDNA locus, is a developing area providing new data about phylogeny in both higher and lower level evolutionary studies. Besides the routine use of the nuclear ribosomal spacer regions (ITS, ETS or IGS) searching for alternative repeat type sequences which have escaped the homogenization process of concerted evolution is a field that deserves much further attention in future.

The internal transcribed spacer regions are widely employed markers and more attention should be focused on external transcribed spacers. Based on previous studies these regions seem to include enough information to warrant their use as phylogenetic markers. The fact that these regions are more variable in length or in their sequence composition makes them underutilized, because the routine amplification with available universal PCR primers is not always successful. ETS sequences can be used instead of ITS sequences or in combination when the ITS provides relatively weak phylogenetic signal. In the case of recently evolved lineages it is recommended to use ITS and ETS data in combination, because they share the same features and it has been shown repeatedly [168–171] that combining datasets in simultaneous analysis provides robust information. As pointed out by Wheeler et al. [172] simultaneous analyses hold the key for the use of 'problematic' areas in phylogenetic analyses. If a true, divergent phylogeny exists the signal should get stronger when more characters and terminals are added to the analysis. On the other hand if clear pattern does *not* emerge this might indicate a genuine lack of divergent lineages and thus need for other approaches such as the use of methods capable to handle reticulations [173–176].

Álvarez and Wendel [17] urge the routine utilization of single-copy nuclear genes as an alternative to the use of ITS sequences, because these single-copy sequences become more easily accessible via whole genome databases. Nowadays they can be routinely applied to the same purposes as rDNA markers. Single-copy genes do not suffer from the 'drawbacks' of concerted evolution, paralogy and homoplasy. The modeling of reticulate evolution or horizontal gene transfer is a developing area in evolutionary genetics. In order to visualize these scenarios several new algorithms are being developed, e.g., phylogenetic networks [177–180]. As reticulating events are

common in plants there is a great need of nuclear gene markers capable to reveal these events. The rDNA spacer regions could have potential to be used because of their universality and simplicity. The complex nature of the rDNA locus in plants could also be a useful feature. Consequently, plant phylogenetic studies should supplement the routine use of these markers and combine this data with recently developed new methodologies available for these regions. Combining the data of the ITS and ETS with the appropriately predicted secondary structures by utilizing newly developed algorithms in large plant phylogenetic super-networks will in many cases most likely provide robust phylogenetic signal.

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