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Dispersion of rDNA loci and its implications on intragenomic variability and phylogenetic studies in *Camellia*

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ABSTRACT

Intragenomic variability is known to be present in multi-copy gene sequences of several plant species and causes severe problems on molecular phylogenetic analysis. Initial investigation revealed extensive presence of intragenomic variability in *nrITS* of *Camellia*. Detailed investigation using 20–30 sequences from each leaf sample of seven species of *Camellia* revealed considerable sequence variability in terms of Kimura pair-wise distance, nucleotide diversity and Tamura's *D* parameters. Fluorescence in situ hybridization (FISH) revealed the dispersion of rDNA loci into multiple chromosomes and a small percentage of species in *Camellia* were allotetraploids. The distribution of rDNA loci into nonhomologous chromosomes along with long generation period and incidence of the interspecific hybridization are considered the major reasons for the failure of complete homogenization of the *nrITS* repeats by concerted evolution in *Camellia*. Nevertheless, in the majority diploid species or in the dominant sequence types of the allotetraploids, *nrITS* sequence variability was not high enough to obliterate the phylogenetic signals. Thus, utilization of *nrITS* in the phylogeny of *Camellia* is still possible, if adequate care is taken.

1. Introduction

Camellia (Theaceae) is one of the economically important trees being grown in south-east Asian countries. Out of the 240 species reported (Chang, 1998), *Camellia sinensis* and *Camellia assamica* are extensively used commercially for tea industry and some other species like *Camellia japonica*, *Camellia sasanqua* along with a group of species called "golden camellia" with yellow flowers are used for floriculture purposes. The interspecific relationship of *Camellia* was a matter of great dispute. Hence, Vijayan et al. (2009) employed internal transcribed spacer (ITS) of ribosomal DNA to develop a molecular phylogenetic frame work for *Camellia*. Interestingly, this study revealed considerable intragenomic variations in more than 70% of the species. It is a known fact that the nuclear ribosomal DNA (*rDNA*) exists as tandemly repeated units of 18S-ITS1-5.8S-ITS2-26S regions. However, in most of the species, high homogeneity exists among the copies. Therefore, ITS1-5.8S-ITS2 region has been used extensively for phylogenetic analyses in plants and animals (Alvarez and Wendel, 2003; Baldwin et al., 1995). The high sequence homogeneity among the copies of rDNA was brought about by a process called 'concerted evolution' (Zimmer et al., 1980). Two mechanisms such as unequal crossing over and gene conversion have been attributed to concerted evolution (Dover, 1982; Arnheim, 1983; Gonzalez and Sylvester, 2001; Eickbush and Eickbush, 2007). However, failure of concerted evolution in complete homogenization of the multiple copies was noticed in a number tree species (Bayly and Ladiges, 2007; Campbell et al., 1997; Ritland et al., 1993; Vijayan et al., 2009). Several reasons have been postulated to explain this anomaly. High rate of mutations, less number of generations due to long life cycle, interspecific hybridization and introgression, lineage sorting, and pseudogenization of cistrons due to nuclear dominance are a few among them (Alvarez and Wendel, 2003; Appels and Honeycutt, 1986; Baldwin et al., 1995; Bailey et al., 2003; Buckler et al., 1997; Feliner and Rossello, 2007; Hillis and Dixon, 1991; Muir et al., 2001; Rogers and Bendich, 1987; Vollmer and Palumbi, 2004). Although, intragenomic variability was observed in the ITS1-5.8S-ITS2 regions of *Camellia* (Vijayan et al., 2009), it has not been investigated in details to understand the magnitude and causes of such variation along with their impact on molecular phylogenic inferences. The present study aimed at investigating these aspects using both diploid and tetraploid species of the genus *Camellia*.

Physical mapping of ribosomal DNA loci using fluorescence in situ hybridization (FISH) has been used to find out the dispersal pattern of rDNA in the genome (Kovarik et al., 2004). The merits and demerits along with technical demands of FISH in plant genome analyses have been reviewed by Jiang and Gill (2006).

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Plant tissue generally exhibits autofluorescence which impairs signal detection. Similarly, dyes frequently adhere to cell walls; this necessitates optimization of hybridization conditions and of labeling and detection systems to obtain a higher signal-to-noise ratio. In *Camellia*, Gu and Xiao (2003) used FISH to locate rDNA. This study revealed dispersion of *rDNA* loci into four chromosomes in diploids and existence of some species of *Camellia* in different ploidy levels. For instance, *Camellia reticulata* exists in diploid, tetraploid and hexaploid forms. Further, it demonstrated that most of the *rDNA* signals were located at the terminal region of the chromosomes. In the light of these findings and the report on intragenomic diversity of the *nrDNA* in *Camellia*, present study was undertaken to study the intragenomic variability in the rDNA sequences of *Camellia* in detail so as to decide whether ITS can be used for phylogenetic assessment or not.

2. Materials and methods

2.1. Plant species

For analyzing the intragenomic variability, nrITS sequences were generated from seven species of *Camellia* which were *C. japonica*, *C. oleifera*, *C. reticulata*, *C. saluensis*, *C. sinensis*, *C. tenuiflora* and *C. yunnanensis* (Table 1). For FISH analyses, only four species, i.e., *C. japonica*, *C. sinensis*, *C. oleifera* and *C. tenuiflora*, were used for the present study and for the remaining three species such as *C. reticulata*, *C. saluensis*, and *C. yunnanensis* the FISH information generated by Gu and Xiao (2003) was used for further assessment.

2.2. DNA extraction, PCR amplification and cloning of nrITS

Procedures of DNA extraction, PCR amplification of the ITS1-5.8S-ITS2 region with ITSleu1 and ITS4 primer pairs, cloning of the PCR products and sequencing were followed exactly as reported (Vijayan and Tsou, 2008). For detailed examination, the number of sequences generated for each leaf sample of the species was increased to 22–30.

2.3. Assessment of intragenomic variability

By aligning both forward and reverse sequences using PILEUP program available in the GCG version 8.1 (Genetic Computer Group, 1994), the complete sequence was constructed for each clone. Nucleotide compositions and sequence length were estimated with BioEdit version 5.0.9 (Hall, 1999). Mean number of nucleotide differences per site between pairs of sequences were estimated using Kimura's two-parameter model available in MEGA 4.1Beta (Kumar et al., 2008). Standard errors of the sequence divergence were estimated by applying 1000 bootstrap replications. In order to find the presence of any paralogous sequences in the form of pseudogenes, indels and substitution patterns in the 5.8S region along with the number of CpG and CpNpG methylation sites, secondary structure stability and sequence diversity in the whole ITS1-5.8S-ITS2 regions were assessed (Buckler et al., 1997). The minimum free energy secondary structures were generated at 37 °C for RNA using all default values present in MFOLD version 3.2 available at http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi (Zuker, 2003).

2.4. Phylogenetic analysis

The impact of the intragenomic variability upon the species discriminatory potential of the ITS sequence was studied by generating a gene tree with all the multiple sequences from each of the seven species using PAUP* 4.0b8 (Swofford, 2001). Heuristic searches were conducted using stepwise addition of random sequences with 100 replicates keeping tree bisection reconnection (TBR) branch swapping, multree, collapse and steepest descent options in effect. In order to utilize the phylogenetic information present in gaps without any exaggeration, the gaps were coded into a binary matrix (presence/absence) following the coding rules and methods of Lloyd and Clader (1991), Simmons and Ochoterena (2000) and used for analysis. To estimate branch support for clades, jackknife support (Farris et al., 1996) was calculated with 100 replicates of heuristic search with random additions of sequences. Trees were rooted using the sequences from the sister genus *Pyrenaria* as out group and were visualized using the software Tree view (Page, 1996).

2.5. FISH examinations

2.5.1. Preparation of mitotic chromosomes

Mitotic study was carried out in *C. japonica* by collecting actively growing root tips from mature plants by effecting air layering. Root tips were collected in the early morning, pretreated with a mixture of 0.1% colchicine and 0.002 mol 8-hydroxyquinoline for 3 h at 25 °C, washed thoroughly with water and subsequently fixed in Carnoy's fixative of 3:1 absolute ethyl alcohol to glacial acetic acid for 2 h. Root tips were stored at −20 °C until used. In order to digest with enzymes, the fixed root tips were first washed thoroughly with distilled water and kept in 0.3% (w/v) citrate buffer for 5 min at room temperature. The root tips were then trimmed and transferred to an enzyme mixture containing 0.3% pectolyase Y-23, 0.3% cellulase Onozuka, and 0.3% cytohelicase in the citrate buffer and incubated at 37 °C for 1 h. After incubation the root tips were washed for 5 min in citrate buffer and 20 min in water. The root tips were then transferred on to clean glass slides and smeared in one or two drops of the fixative by tapping the tissues with fine tipped forceps. The slides were finally flame dried, examined under phase contrast microscope and those slides having good metaphase plates were stored at −20 °C for further use.

2.5.2. Preparation of meiotic chromosomes

Meiotic chromosomes plates of *C. sinensis*, *C. oleifera* and *C. tenuiflora* were prepared from immature flower buds that were collected during the regular flowering periods (November–December) from mature plants growing in the natural habitats. Flower buds were fixed in Carnoy's fixative (95% ethanol:chloroform:glacial acetic acid [6:3:1]) between 8.00 am and 9.00 am. After 2 days, the flower buds were transferred to 70% ethanol and stored till further use. Anthers were dissected out from the flower buds into 45% acetic acid on acid cleaned glass slides and covered with cover slips. The anthers were smeared by gentle tapping. The cover slips were removed after freezing the slides in liquid nitrogen. The slides were then dried on a hot plate (60 °C).

2.5.3. Probe preparation and hybridization

The probe was prepared from the plasmid DNA containing 18S-ITS1-5.8S-ITS2-26S sequences by labeling with Digoxigenin-11-dUTP by nick translation according to the instruction of the manufacturers (Roche Applied Science, Indianapolis, USA). For in situ hybridization the slides were treated with 200 μl of 100 μg/ml DNAse-free RNAse at 37 °C for 1 h. The slides were then washed three times in 2× SSC (1× SSC = 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0) at 20 °C for 5 min. Chromosomes in each slide were denatured with 70% formamide in 2× SSC at 70 °C for 2 min. The slides were then dehydrated in a series of ethanol solutions and air dried. For in situ hybridization, 40 μl denatured hybridization mixture containing 100 ng of probe DNA, 2 mg sheared salmon sperm DNA, and 10% (w/v) dextran sulfate in 2× SSC was applied to each slide. The slides were covered with cover slips (alcohol cleaned) and kept in moist chamber for over night.