Short Communication

Physical localization and probable transcriptional activity of 18S–5.8S–26S rRNA gene loci in some Asiatic Cymbidiums (Orchidaceae) from north-east India

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ABSTRACT

Fluorescence in situ hybridization based physical localization of 45S ribosomal DNA in eight horticulturally important species of Cymbidium (Orchidaceae) from north-east India (South-East Asia) has been carried for the first time. Observations revealed only one pair of chromosomes had NOR loci. Three, out of eight Cymbidiums showed decondensed, dispersed, extended form of hybridization signals of rDNA as dots of fluorescence (transcriptionally active), where as the rest of the Cymbidiums revealed condensed (non-active) forms, hence demonstrated the heteromorphism in size, intensities and their appurtenance which may be under epigenetic control. Except for the ribosomal genes, no other active genes have been reported to reside within the nucleoli. Such observations provide useful chromosome landmarks and provide valuable evidence about the genome evolution, speciation and ploidy both at molecular and chromosomal levels which is more or less highly ambiguous in family Orchidaceae.

1. Introduction

India is endowed with rich treasure of phyto-diversity of agricultural, horticultural, and medicinal plants besides several unique flora that are endemic to the region. The northeastern region of India is regarded as one of the mega biodiversity hotspots (Myers et al., 2000) for a number of plant species including various orchids (Sharma et al., 2010a). The family Orchidaceae is considered as threatened and most of the genera are endangered in their natural habitat. Cymbidium, or boat orchid, is a genus comprising of 52 evergreen species, of which about 20 species are reported from India and found mostly in Arunachal Pradesh, Sikkim and Meghalaya provinces. It belongs to subtribe Cyrtopodiinae, tribe Cymbidieae and family Orchidaceae (Dressler, 1993). Chromosome variations in orchids are rampant and as a whole, this is quite intriguing since many of the genera exhibit higher ploidy levels with variable base numbers (Ehrendorfer, 1980; Goldblatt, 1980). Raven (1975) opined that it is premature to suggest a distinct base number for Orchidaceae. Cytogenetical studies in orchids are by and large lacking and still fewer reports are available for Indian orchids (Sharma and Chetterji, 1966; Singh, 1984; Sharma et al., 2010a). Most of the Cymbidium plants are epiphytic and hence, root tip mitosis and karyotype analysis is relatively difficult in Cymbidiums. Recently, Sharma et al. (2010a) reported details of karyotypes in three species of Asiatic Cymbidium viz. C. eburneum, C. hookerianum and C. mastersii. However, unequivocal differentiations between species are hampered by almost identical chromosome numbers (2n = 40) and only few differences with regard to chromosome morphology, presence of low heteromorphism with no clear indications for distinct satellite chromosomes (Sharma et al., 2010a). The basic chromosome number of several genera belonging to this family is still unclear leading to difficulties in determining accurate ploidy level and to understand the pattern of speciation and evolution vis-à-vis chromosomes in the family Orchidaceae (Sharma et al., 2010a).

Recent studies in Orchidaceae have utilized DNA sequence data to resolve phylogenetic relationships and infrageneric classification (Cox et al., 1997; Pridgeon et al., 1997; Ryan et al., 2000; Van den Berg et al., 2002, 2005). A similar approach has been employed to resolve phylogenetic and classification ambiguities in the genus Cymbidium using repeat unit length variation and internal transcribed spacer (ITS) sequences of nuclear ribosomal DNA coupled with plastid matK gene which basically revealed low level of variation and possible South-East Asian origin of the genus Cymbidium (Van den Berg et al., 2005). Genes coding for ribosomal RNA occur universally in all organisms. In eukaryotes, they consist of tandemly repeated rDNA units composed of transcribed regions, coding for

18S, 5.8S and 26S rRNA, and non-transcribed regions. Nuclear ribosomal DNA units have been studied in species, populations and even individuals (or inbred lines) as in wheat (Flavell et al., 1986), maize (McMullen et al., 1986), *Vicia* (Raina and Ogihara, 1995), *Arachis* (Singh et al., 2002), *Nymphaea* (Dkhar et al., 2010) and many others. Repetitive sequence families are major components of plant genomes (Heslop-Harrison, 2000). These genes are organized into two multigene families in eukaryotes. One of these families contains the major rDNA (45S) that codes for 18S, 5.8S and 28S rRNAs, and the smaller, that codes for the minor rDNA (5S). The nucleolar organizing regions (NORs) that contain the 45S rDNA can be easily identified in chromosomes by using certain fluorochrome based *in situ* hybridization techniques (Leitch and Heslop-Harrison, 1992). This technique permits more exact chromosome identification and mapping, demonstrates the relatedness of individual species, and elucidates their phylogenetic relationships with valuable chromosomal landmarks (Heslop-Harrison, 2000). Few reports are available on physical localization of ribosomal DNA in orchids (Demerico et al., 2001; Clements, 2003; Cheng et al., 2004; Tsai et al., 2004; Cabral et al., 2006; Begum et al., 2009). Only a single report by Nagl (1977) showed the localization of amplified DNA in nuclei of *Cymbidium* by *in situ* hybridization and revealed the fact that the nuclear DNA of the orchid genus *Cymbidium* is unique among monocots indicating the location of the highly amplified AT-rich DNA fraction.

From the perusal of literature it is amply clear that besides chromosome number reports, the information about the 45S rDNA locus particularly in the genus *Cymbidium* is lacking and various Asiatic *Cymbidium* species did not attract the attention of scientists for such observations as yet. Therefore, the present investigations were carried out for physical localization of 45S ribosomal DNA in eight species of *Cymbidium* from India (South-East Asia).

2. Materials and methods

2.1. Plant materials and chromosome preparation

Eight species belonging to the genus *Cymbidium* were collected mainly from Arunachal Pradesh, Meghalaya and Sikkim provinces of northeastern region of India. Plant samples of *C. cyperifolium* Wall ex Lindl., and *C. tracyanum* L. Castle., were obtained from Dr. U. C. Pradhan, Chairman, Orchid Specialist Group, Government of India whereas *C. hookerianum* Rchb.f., *C. iridioides* D. Don, and *C. tigrinum* Parish ex Hook. f. were obtained from the Orchid Research Centre, Government of Arunachal Pradesh, Tipi, Arunachal Pradesh. The plant samples of other species were obtained from authentic nurseries viz. Green Light nursery, Upper Shillong, Meghalaya and International nursery, Kalimpong, West Bengal. The plants were grown in the greenhouse of the Plant Biotechnology Laboratory, Department of Botany as well as Department of Biotechnology and Bioinformatics of North-Eastern Hill University, Shillong. For each species, a minimum of five individuals and more than one population were studied. Actively growing root tips from the potted plants were fixed in Carnoy's fluid followed by pre-treatment with saturated solution of ρ-dichlorobenzene for 3 h at room temperature. Root tips were appropriately hydrolyzed with a mixture of 1 N HCl:45% acetic acid (2:1) for 10 min at room temperature and squashed after staining in 1% acetocarmine.
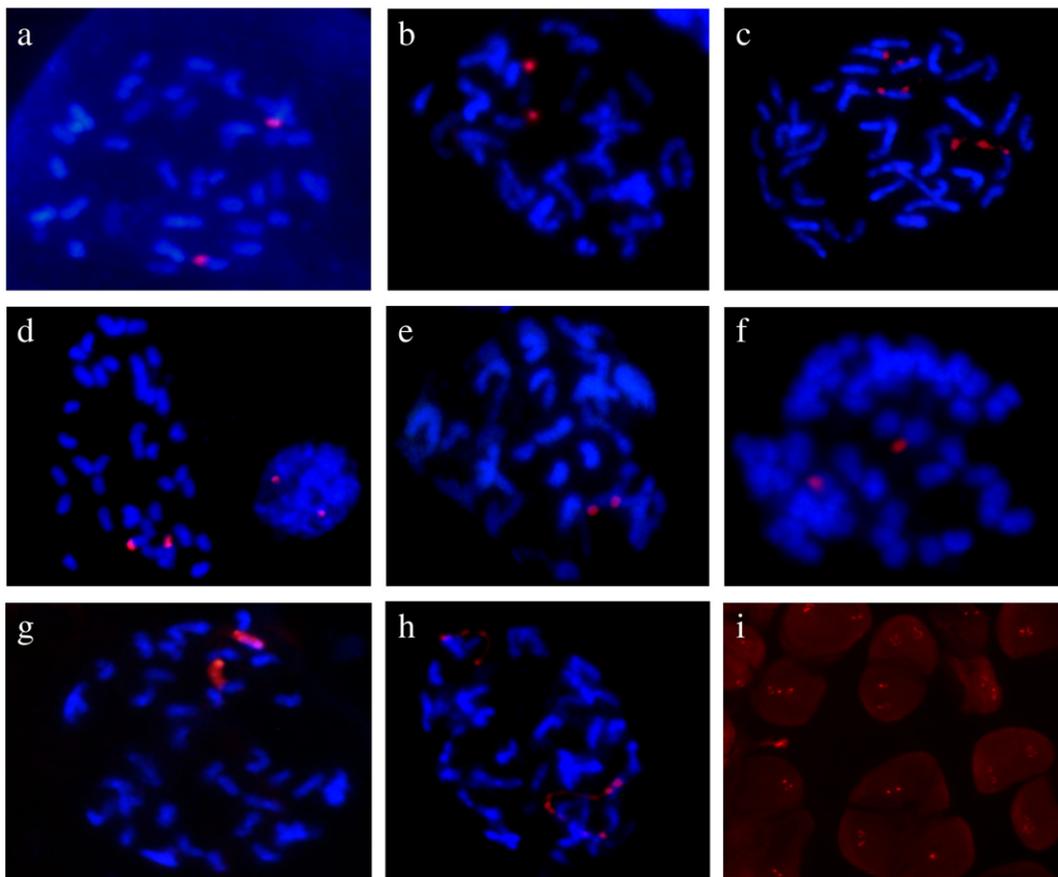

Fig. 1. Fluorescent *in situ* hybridization of 45S rDNA to root-tips cells showing two major sites in eight *Cymbidium* species (a) *C. elegans* (b) *C. cyperifolium* (c) *C. aloifolium* (d) *C. iridioides* (e) *C. hookerianum* (f) *C. mastersii* (g) *C. tracyanum* and (h) *C. tigrinum* (i) hybridization pattern at interphase of *C. mastersii*. (c, g and h) show the decondensed dispersed extended rDNA signals as dots of fluorescence (transcriptionally active) at pro-metaphases. (a, b, d, e, and f) show the condensed hybridization signals (non-active) at metaphase.

2.2. Fluorescence in situ hybridization (FISH)

A Clone pTa71, a 9-kb *Eco*R1 fragment from common wheat, containing the coding sequences from the 18S, 5.8S and 26S rRNA genes and the intergenic spacer sequence, was utilized as FISH probe. The clone pTa71 was labeled with rhodamine-6 dUTP using nick translation system (Roche Applied Science, Indianapolis, Ind.). Hybridization conditions, post hybridization washes and imaging were as described by Zhang et al. (2001).

3. Results

In situ hybridization analysis using the rRNA gene as probe indicated FISH sites on one pair of somatic chromosomes in all the species studied during the present study (Fig. 1). The chromosome analysis confirmed the chromosome number of 2n = 40 for the eight species of *Cymbidium* analyzed. FISH analysis indicated that only one pair of chromosomes carried NOR loci in this horticulturally important orchid. Notably, not a single species, out of eight presently analyzed, did show the presence of secondary constriction or satellite which are usually associated with NOR chromosome(s). The intensity of hybridization signals were observed in all the species. According to the hybridization signal intensities, they could be resolved either as condensed or dispersed extended forms. Out of eight, five species viz. *Cymbidium elegans*, *C. cyperifolium*, *C. iridioides*, *C. hookerianum* and *C. mastersii* did show one pair of condensed hybridization signals with more or less moderate intensities (Figs. 1a, b, d, e, and f). Such condensed pattern of hybridization signals indicated the presence of inactive rDNA in the five Cymbidiums. Similar condensed hybridization signals could be seen during interphase and subsequent metaphases of these species that indicated the presence of probably transcriptionally inactive rDNA (Fig. 1i). Alternatively, three species viz. *C. aloifolium*, *C. tigrinum* and *C. tracyanum* revealed less condensed, highly dispersed and extended hybridization signals as dots of fluorescence at prometaphase (Figs. 1c, g, and h). This relative distribution of rDNA in the form of some fluorescence dots can be seen more clearly in the interphase nucleoli of the same species where the middle part of the rDNA is dispersed (Figs. S1 and S2) and may be associated with a nucleolus. However, the chromosome complements belonging to the rest of the species revealed condensed hybridization signals, hence showed heteromorphism in size and intensity. When ribosomal genes are active at interphase they can appear more decondensed and visualized as satellites or secondary constriction at pro-metaphase and subsequently become condensed at metaphase. Interestingly, both kinds of hybridization signals, either in condensed or dispersed (extended) form were present at the distal end of the short arms of the chromosome in case of each Cymbidiums. The tendency of fluorescence to expand beyond the region marked by the probe did not distinguish between terminal and sub-terminal rDNA sites (Fig. 1g) and such results were also noted earlier by Mukai et al. (1991). However, in case of some Cymbidiums i.e. *C. aloifolium*, *C. tigrinum* and *C. tracyanum*, the 45S rDNA sites were located at the terminal position possibly since these sites were larger, variable in size as fluorescence dots compared to the sites observed in the rest of the species.

4. Discussion

The most conserved ribosomal RNA genes in eukaryotes comprising 18S–5.8S–25S rRNA repeating units form long arrays. Comparative studies of plant repetitive sequences are useful to investigate the evolutionary relationships between plant species (Kamm et al., 1995). Begum et al. (2009) suggested that comparative study of

repetitive sequences would be useful for investigation of the relationships among orchid species.

The condensation patterns of rDNA in root-tip meristematic cells have been used to map their location on chromosomes in this study. They can be considered as ideal material for FISH analysis because rapidly growing root tips, with high metabolic activity, are likely to include cells with high levels of rDNA decondensation and minimal rRNA gene redundancy (Lim et al., 2000). *In situ* hybridization analysis using the 45S rDNA clone from wheat codes for 18S, 5.8S and 26S rRNA genes (Mukai et al., 1991) indicated two sites on metaphase chromosomes without any variation in their location/position and suggested that the genomic distribution pattern of 45S rDNA is completely the same in all the *Cymbidium* species. These similarities are indicative of the high degree of gene stability in the genus at inter-specific level indicating lack of chromosome structural rearrangements during speciation in *Cymbidium*. Such observations support our earlier views which indicated more or less stable genome of the genus *Cymbidium* at inter-specific levels. The absence of deviant chromosome numbers and overall symmetry also suggests that the diversification at inter-specific level has occurred without any significant numerical changes (Sharma et al., 2010a). Present investigation showed that 45S rDNA sites in all Cymbidiums are located mostly in short arms. Such observations were already depicted in the study of Lima-De-Faria and Jaworska (1972), who analyzed the nucleolus-organizing cistrons in over 700 plant species and reported that the nucleolus was invariably located on the short arm of the chromosomes in about 87% cases. Such striking conservatism in karyotype morphology suggests some molecular or physical constraints for chromosome arms to associate with the nucleolus (Lim et al., 2000).

Out of eight, five species did show minor condensed hybridization signals with more or less moderate intensities. Many *in situ* hybridization experiments have revealed the presence of minor condensed 45S rDNA sites in addition to the major 45S rDNA sites in secondary constriction (SCs) and satellites of various plant species (Lim et al., 2000). Such observations were also made by Sharma et al. (2010a) who reported the absence of any nucleolar organizer in the form of SCs in the three *Cymbidium* species, collected from north-east India. The hybridization signals in the minor sites were always smaller than those in SCs and many of them were without transcriptional activity. The origin of minor 45S rDNA sites is neither critically investigated nor properly understood (Taketa et al., 1999).

In the present investigation, the hybridization signals of 45S rDNA loci showed prominent differences in size and intensity. The extended form of hybridization signals as dots of fluorescence, both at interphase and metaphase of the three species viz. *C. aloifolium*, *C. tigrinum* and *C. tracyanum*, evidently represents the transcriptional activity of ribosomal genes which can be under epigenetic control (McStay and Grummt, 2008; Grummt, 2007). DNA methylation and histone acetylation are the best-known epigenetic factors influencing chromatin packaging and gene activity in higher eukaryotes (Chen and Pikaard, 1997). Hypomethylated DNA and histone H3K4 methylation are considered as an epigenetic marker for euchromatin and responsible for rDNA activation. On the other hand, inactivation of rDNA correspond to the hypermethylated DNA, histone H3K9 methylation and histone deacetylation, thus identified as interdependent and integral factor for rRNA gene silencing. Recently, Carchilan et al. (2007) reported transcriptionally active heterochromatin in Rye B chromosomes which had undergone decondensation during interphase, contrary to the heterochromatic regions of A chromosomes, and showed extended forms of hybridization signals. Present investigation did not reveal such occurrence of B chromosomes but showed extended hybridization signals as well. Inactive genes occur in chromatin that is highly methylated and more condensed than the chromatin of

active genes (Keshet et al., 1986). Importantly, except for the ribosomal genes, no other active genes have been reported to reside within nucleoli (Raška et al., 2004), which have been also reported in many other plant species (Hanson et al., 1996; Lim et al., 2000; Vanzela et al., 2002; Muravenko et al., 2003). The gaps between the fluorescent dots are areas where probe hybridization is not detected either because of (i) lack of sequence homology of the probe to the IGS region and (ii) decondensed target sites of hybridization that are fall below the threshold limit for sensitivity of the method. Lim et al. (2000) reported the same pattern of hybridization signal and opined that the number of dots represents the number of active genes. Although *in situ* hybridization cannot show the precise number of gene copies, however, the size and intensity of hybridization signals may vaguely represent the copy number. Hence FISH technique is often considered as a semi-quantitative technique as well (Maluszynska and Heslop-Harrison, 1993). The variation in copy number of genes is related to the amplification, deletion, or unequal crossing over of genes, which may be the main cause for signal differences. According to the studies of Heliot et al. (1997) and Lim et al. (2000), rDNA in active NORs is approximately tenfold less condensed than the adjacent satellite DNA. Often, an axis of condensed AT-rich DNA is found within the SCs, which was observed earlier by Nagl (1977) and considered that the nuclear DNA of *Cymbidium* is unique among monocots indicating the location of the highly amplified AT-rich DNA fraction and opined that it might be repetitive sequences due to its location in heterochromatin.

Notably, following the SPAR approach (Sharma et al., 2010b) to analyze natural genetic variation, five *Cymbidium* species including *C. aloifolium* and *C. tigrinum* revealed lower genetic variation at inter-specific level (Sharma et al., 2011) and clustered independently of those of others. Phylogenetic analysis using nuclear ribosomal ITS sequence data (Sharma et al., 2012) of ten *Cymbidium* species identify ITS 1 for high sequence divergence and ITS 2 for high G + C content (>67%), however, clearly resolved the relationship among major clades as well as association between subgenera viz. *Cymbidium, Cyperorchis* and *Jensoa* (Sharma et al., 2012). Sequences with more than 50% G + C content presumed functional ITS paralogs, which are recently reported in *Cycas* by Xiao et al. (2010) who recorded 64.4% and 65.2% G + C content for ITS 1 and ITS 2, respectively. The levels of genetic variation found in *Cymbidium* are somewhat higher than other epiphytic orchids. Such genetic variations are manifested in the ITS phylogeny and SPAR approaches which collectively confirm the clustering of both the species at molecular level. *C. tigrinum* (subg. *Cyperorchis*; section Parishiella) placed alone without any affinity to any of the members of subg. *Cyperorchis*. Such observations were also recorded by Van den Berg et al. (2005) for *C. dayanum* (section Himantophyllum) which is also a morphologically abnormal *Cymbidium*. The clade of *Cymbidium* sect. *Cymbidium* comprising *C. aloifolium* was also quite distinct being the base of the trees in the ITS analysis (Sharma et al., 2012). The reason for such apparent clustering may be due to entirely different climatic conditions as well as morphological features, both the species exhibit. In this context, it is interesting to note that *C. aloifolium* (Section Cymbidium) is cultivated tropical *Cymbidium*, a biological indicator of tropical environment (Somashekarappa et al., 1996) with very thick, rigid leaves, which reminds one of *Aloe*. Section Cymbidium presents several typical anatomical characters, such as stomata within an elliptical cover and slit-shape pores and a complete layer of sub-epidermal schlerenchyma cells (Van den Berg et al., 2005). On the other hand, *C. tigrinum,* a rare and endangered temperate *Cymbidium*, exclusively grows in cooler and dry climate having wide leaves and small, round, clustered pseudo bulbs. It is the smallest member of the genus which does not look like a typical *Cymbidium* when not in flower. An alternative but not mutually exclusive explanation is that habitats as well as climatic conditions may simply permit a greater fixation rate of chromosomal as well as molecular variation following from founder effect and efficient inbreeding within small populations occupying novel ecological niches.

The 45S rDNA sites were useful chromosome landmarks and provide valuable evidence about genome evolution at both molecular and chromosomal levels (Taketa et al., 1999). They can reflect the degree of discrepancy among species or genera (Tsai et al., 2004). In the eight north-east Indian *Cymbidium* species, the number of 45S rDNA loci was exactly the same and their genomic distribution was identical. These results are indicative of little discrepancy among the species at chromosomal level and suggest that the analysis of DNA-methylation levels in the genome may help to interpret molecular phylogenies.

Supplementary materials related to this article can be found online at doi:10.1016/j.gene.2012.03.007.

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