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CELLULAR AND MOLECULAR CYTOGENETICAL ANALYSIS OF ORCHIDS FROM NORTHEAST INDIA A STUDY IN CYMBIDIUMS

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Abstract

The paper assesses genetic variations at chromosomal level in 10 species of *Cymbidium* (all with 2 n = 40 chromosomes), using both the cellular and molecular techniques. Karyotypic analysis revealed intraspecific morphological uniformity in 9 of the 20 chromosome pairs; only moderate to marked changes are seen in morphology of the remaining 11 pairs. Such a uniformity of chromosome number (2n=40) and overall karyotypic symmetry suggests that species diversification in the genus as not accompanied major changes in the chromosome complements. Endomitosis in the tapetal cells during male meiosis and persistence of their nucleoli all through the endomitotic cycle seems intersting. *In situ* hybridization studies using the 45S rDNA clone indicates two sites on metaphase chromosomes without any variation in their location/position and suggested that the genomic distribution pattern of 45S rDNA is uniform in all the tested species. 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 interphases and metaphases of *C. aloifolium, C. tigrinum* and *C. tracyanum*, evidently represent the transcriptional activity of ribosomal genes. The results indicating a little discrepancy among the species at chromosomal level, suggest that an analysis of DNA-methylation in the genome may help in interpreting molecular phylogenies.

Introduction

NORTHEAST INDIA with its unique floristic diversity is considered as one of the mega hotspots for a number of plant species, especially the orchids and is on the priority lists of leading conservation agencies of the world (Myers 2000). Incidentally, India is a major centre for orchid speciation with nearly 700 species growing in North-Eastern region along. These include several with floriculturally significant traits. Many Indian orchids are threatened of survival due mainly to their habitat specificity, poor natural regeneration, and requirement for specific fungi and pollinators for continued reproduction in nature. Unregulated commercial collection and habitat destruction pressures further at woes to their natural populations. They need to be conserved.

In order to typify the taxa and prioritize their conservation needs, it is important to analyze genetic variation existing in natural populations using appropriate cellular and molecular marker approaches. Cytogenetical information, including information of chromosome numbers, structure, behavior etc. can be of great help in this direction. However, cytogenetical studies are difficult in orchids owing to very thick and velamen covered roots; difficulty in penetration of pre-treatment fluid; occurrence of variable base numbers in the family; and high ploidy levels in most of the genera. Taxonomic and genetic treatment of the familly is open difficult due to following trends in chromosome complements: i) all species in the genus show the same chromosome number, eg. *Ophrys*; ii) species in a genus differ in having an additional chromosome number, eg. *Dendrobium*; iii) species in a genus forming an aneuploid series, eg. *Paphiopediulm*; and iv) species in a genus forming an euploid series, e.g. *Dactylorchis*. Such a generalization of the chromosome number variation would have been same in all the tribes. A general survey of the chromosome numbers so far known, shows that this is not the case that, and each tribe has its own pattern of evolution (Vij *et al.*, 1986).

Cymbidium, a genus of boat orchids, comprises 44 evergreen species, of which about 20 species are reported from India found mostly in Arunachal Pradesh, Sikkim and Meghalaya provinces. It belongs to subtribe Cyrtopodiinae, tribe Cymbidieae (Dressler, 1993). The somatic chromosome number in various species of *Cymbidium* was reported as 2n= 40. A few exceptions of triploid or tetraploid named cultivars of e.g., *C. insigne* 'Bieri' (2n = 60), *C. floribundum* 'Geshohen' (2n = 80) and *C. floribundum* 'Yoshina' (2n = 60) are also reported. The data on chromosome number and comparative karyo-morphology is very significant to comprehend the genome structure, its organization and evolution within the genus at inter- and intra-specific levels. The differences and similarities in the karyotype are regarded as basis of genetic variation, as well as distance or relatedness among diverse genomes. Chromosome studies are regarded as important cytogenetical tool for elucidating taxonomic and phylogenetic relationships as well as in breeding for better horticulture types.

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.*, 2010). The basic chromosome number of several genera belonging to

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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.*, 2010). Therefore, the present investigations were carried out for cellular and molecular cytogenetical analysis of orchids from North-East India with special reference to cymbidiums.

Material and Methods

Ten species belonging to the genus *Cymbidium* were collected mainly from Arunachal Pradesh, Meghalaya and Sikkim provinces of NorthEastern region of India. 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. The standard protocols as detailed by Sharma *et al.*, (2010) were followed for preparation of mitotic complements as well as karyotypes. Physical localization of 45S rDNA and species relationships based on nuclear ribosomal internal transcribed spacer (nrITS) region was also carried out in eight species and the details of protocols can be accessed through (Sharma *et al.*, 2012a,b).

Results and Discussion

Karyomorphological Studies

All the species had shown the occurrence of 2n= 40 chromosomes in root tip cells which were clearly resolved into 20 pairs forming a series from the longest to shortest pair within the complements (Fig. 1). Variation was recorded with respect to number of metacentric and submetacentric chromosomes, presence or absence of heteromorphic pairs and sub-telocentric chromosomes in the chromosome complements. Karyotype asymmetry indices were recorded as 2B in most of the species however 3B was recorded in *C. giganteum* and 2C in *C. tracyanum*. Sub-telocentric/telocentric chromosome pairs which were recorded only one in *C. eburneum* and *C. tracyanum* while two in *C. mastersii*. X = 10 is the most acceptable true basic number for the genus *Cymbidium*.

In general, nine pairs out of twenty viz. I-II, IX-X, XIV-XVI and XVIII to XIX, showed uniformity with respect to the chromosome morphology at inter-specific level. Moderate to greater degree of variation was recorded in the remaining eleven pairs of the chromosome complements pattern. The ratio of longest and shortest chromosome was ranged between lowest in *C. devonianum* (2.03) and highest in *C. tracyanum* (4.38). Such observations indicate certain level of genetic variation in the genus. The absence of any nucleolar organizers chromosome, deviant chromosome numbers and overall symmetry suggests that the diversification at inter-specific level has occurred without any significant numerical changes. The observation related to heteromorphic pairs recorded in *C. aloifoium*, *C. devonianum*, *C. eburneum*, *C. elegans*, *C. lowianum*, *C. mastersii*, *C. tigrinum*, and *C. tracyanum* is indicative of the fact that these pairs in the chromosome complements comparatively exhibit less genome integrity and thereby helping the species to attempt in structural alterations as means of speciation.

Tapetal cell endomitosis

Some of the reasons like different and environment dependent flowering, insect dependent pollination, more importantly a less number of flowers especially in cymbidiums make them not a fine material for male meiotic studies. However, meiotic analysis in three species viz. *C. aloifoilum*, *C. devonianum* and *C. tigrinum* was carried out but unfortunately most of the cells were at tetrad stages and thus indicated the very short period of meiotic divisions. Tapetal cell endomitosis was found to be more interesting and informative in three species of *Cymbidium* (Fig. 2). Therefore, much attention has been focused on endomitosis in tapetal cells rather than male meiotic analyses of cymbidiums. Such investigation adds one more example of occurrence of 'inhibited mitoses' in plant species and for the first time in orchids (Sharma *et al.*, 2012c).

Molecular Cytogenetic Analysis using Fluorescence In situ Hybridization (FISH)

FISH analysis was indicating that only one pair of chromosomes carried NOR loci in this horticulturally important orchid. According to the hybridization signal intensities, they could be resolved as either condensed or dispersed/ extended forms. Out of eight, five species viz. *Cymbidium cyperifolium*, *C. elegans*, *C. gigantium*, *C. hookerianum*, and *C. mastersii* did show one pair of condensed hybridization signals with more or less moderate intensities. Such condensed pattern of hybridization signals indicated the presence of inactive rDNA in the five cymbidiums. Alternatively, three species viz. *C. aloifolium*, *C. tigrinum*, and *C. tracyanum* revealed less condensed, highly dispersed/extended hybridization signals as dots of fluorescence at pro-metaphase. 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 (Fig. 3). 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

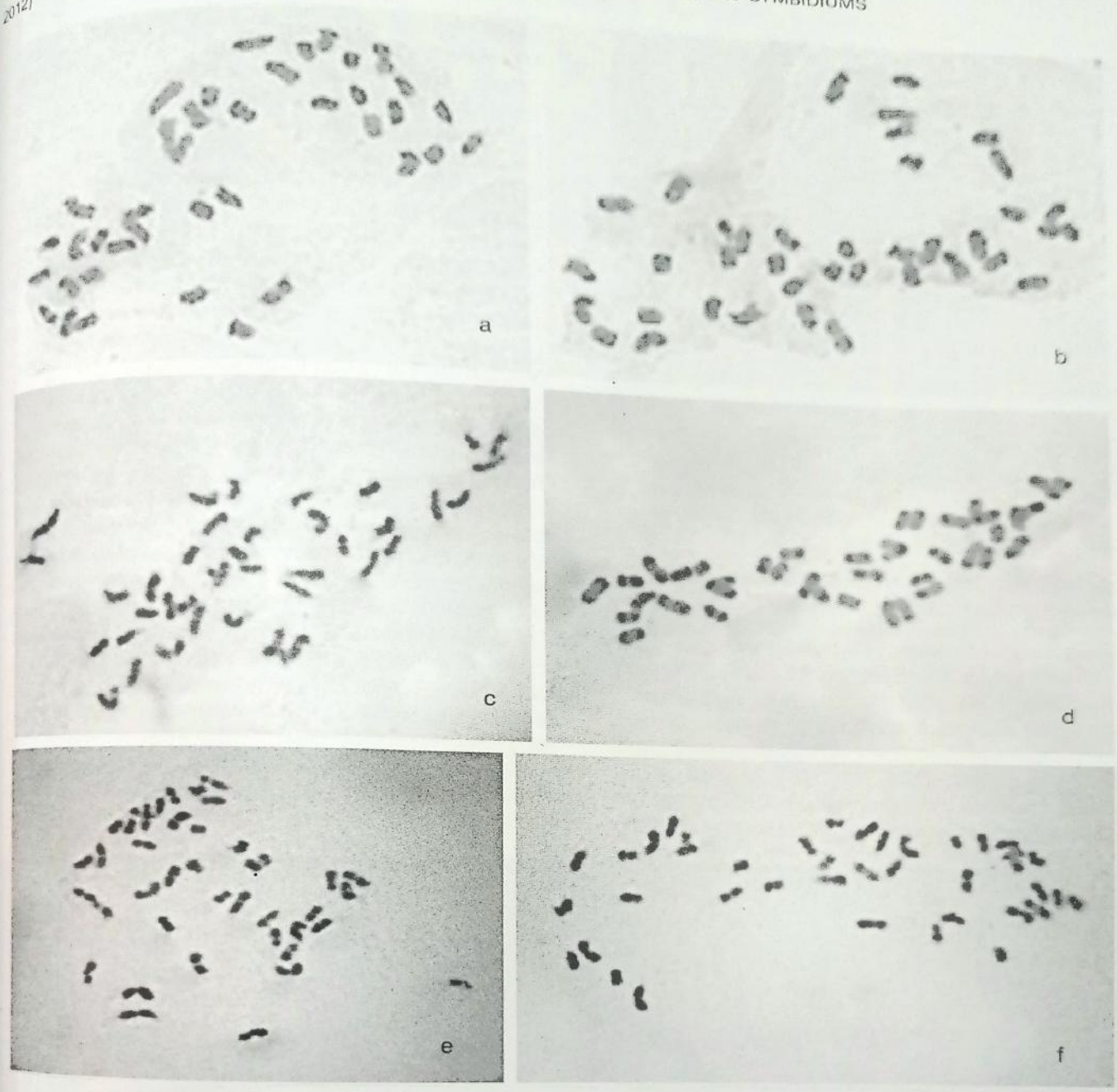

Fig. 1a-f. Chromosome complements of several cymbidiums showing somatic chromosome number 2n = 40

chromosome in case of each cymbidiums. Lim *et al.* (2000) reported the same pattern of hybridization signal in *Nicotiana tabacum* and opined that the number of dots represents the number of trancriptionally active genes with high copy number. The extended hybridization signals in *C. aloifolium*, *C. tigrinum*, and *C. tracyanum*, evidently represents the transcriptional activity of ribosomal genes which can be under epigenetic control (Lim *et al.*, 2000). DNA methylation and histone acetylation are the best-known epigenetic factors influencing chromatin packaging and gene activity in higher eukaryotes (Raska *et al.*, 2004). Inactive genes occur in chromatins that are highly methylated and more condensed than the chromatin of active genes. More importantly, except for the ribosomal genes, no other active genes have been reported to reside within nucleoli, which have been also reported in many other plant species (Lim *et al.*, 2000; Muravenko *et al.*, 2003; Vanzela *et al.*, 2002). The intact number and location of 45S rRNA gene is indicative of the high degree of gene stability in the genus at inter-specific level. It also indicates lack of chromosome structural rearrangements during speciation in *Cymbidium*.

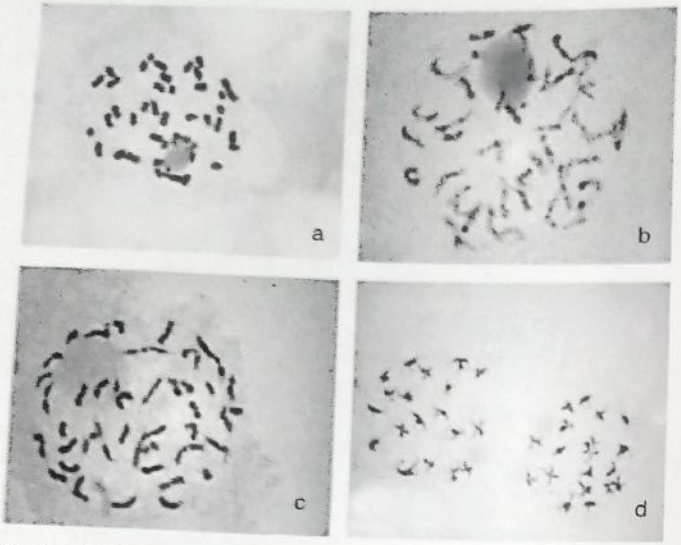

Fig. 2 a-d. Stages of endomitosis in *Cymbidium*: a, *C. devonianum*; b, *C. tigrinum*; c, *C. aloifolium*; D, Endo-anaphase (40:40)

Phylogenetic Analysis using Nuclear Ribosomal Internal Transcribed Spacer (ITS) Sequences in Cymbidium

Sequence data obtained through ITS region revealed that the position of *C. mastersii* (subgenus *Cyperorchis*, section Eburnea) and *C. elegans* (subgenus *Cyperorchis*, section *Cyperorchis*) is the hallmark features of the investigation which are almost every time clustered together (Fig. 4). Species of subg. *Cyperorchis*; section Iridorchis revealed that the pattern for *C. hookerianum* and *C. giganteum* followed the general pattern of congruence within section. The other member of section Eburnea *i.e. C. eburneum* showed position differences as earlier observed by Van den Berg *et al.*, (2002). It is apparent that forcing this taxon to be sister to *C. mastersii* (section Eburnea) requires several additional steps. *C. tigrinum* (subg. *Cyperorchis*, section Parishiella) placed alone without any affinity to any of the members of subg. *Cyperorchis*, which may be due to its peculiar morphological and climatic characters. Such observations were also recorded by Van den Berg *et al.*, (2002) for *C. dayanum* (section Himantophyllum) which is also a morphologically abnormal *Cymbidium*. Such apparent clustering of *C. tigrinum* was also observed while studying the genetic variation using three SPARs. The position of *C. devonianum* poses problems and tends to close with subg. *Cyperorchis*. The apparent base of the trees formed by *C. aloifolium* may be due to its several unique features. It is a medicinal, cultivated *Cymbidium* and a biological indicator of tropical environment with very thick, rigid leaves, which reminds one of Aloe. The ITS region of nrDNA appears to be adequate for resolving infrageneric relationships in this group. This study establishes the ITS rDNA region as a reliable indicator of phylogenetic relationships especially the ITS 2 as probable DNA barcode at higher levels within and among orchid genera. Such other coding DNA regions viz matK, rbcL, rpoB, rpoC1, and 3 noncoding spacers viz atpF-atpH, trnH-psbA, psbK-psbI may be coupled with ITS data and may serve as unique DNA barcode for orchids.

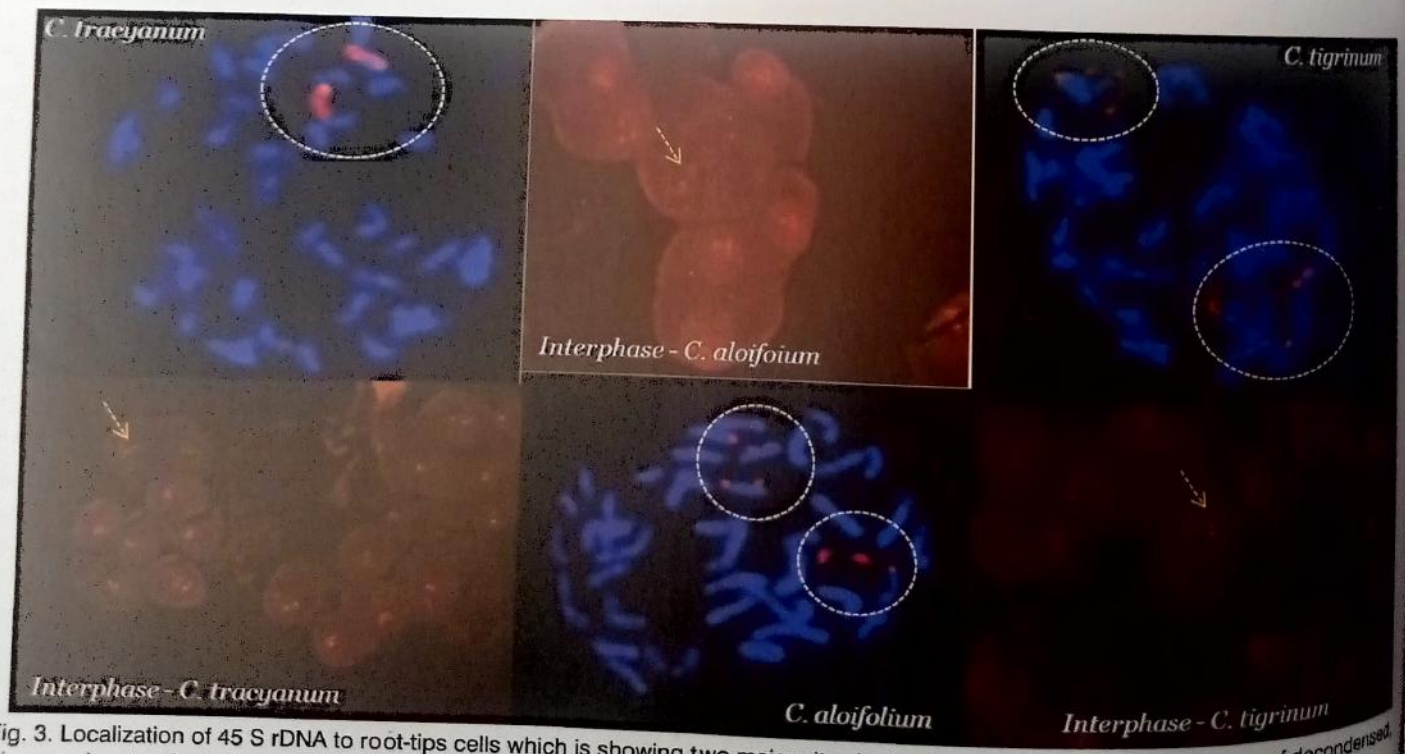

Fig. 3. Localization of 45 S rDNA to root-tips cells which is showing two major sites in three *Cymbidium* species in the form of decondensed dispersed, extended rDNA signals as dots of fluorescence at pro-metaphases

2012)

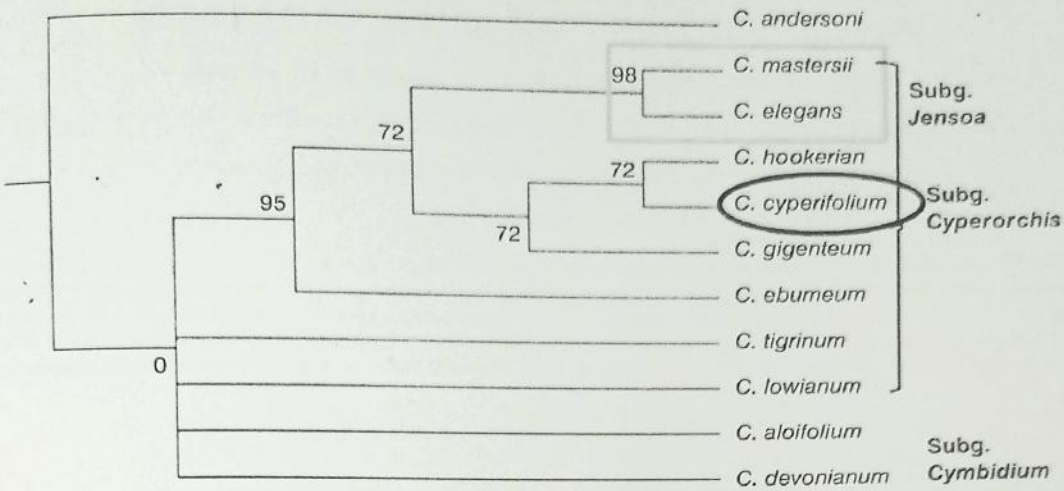

Fig. 4. Phylogenetic analysis using nrITS sequence data.

Conclusion

Most of the genera in the family due to high economic value are threatened in their natural habitat and falling in the category endangered so it is very important to make some strategy for their conservation. According to the New York Natural Heritage Program, when rare plants are protected, distinctive populations of species are preserved along with their genetic variation within their natural habitat. Orchids are the most evolved of all flowering plants, they are very site-specific and need optimum conditions to thrive in a given ecosystem.

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Appeal

Orchids are good indicators of a healthy and well functioning ecosystem so

SAVE ORCHIDS - SAVE NATURE