Precise Seed Micromorphometric Markers as a Tool for Comparative Phylogeny of Dendrobium (Orchidaceae)

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ABSTRACT

Orchid seeds are microscopic in nature and have been widely used in defining phylogenetic relationship among different genera. The genus Dendrobium is the second largest in the family Orchidaceae. The present study aims at evaluation of seed micromorphology of Dendrobium in the light of recent phylogenetic studies and to test whether the seed micromorphology strictly reflects the phylogenetic relationships at the inter-specific level. The seeds from 18 different Dendrobium species collected from throughout the Indian continent representing different sections were directly measured for six scorable micromorphometric traits (variables) through multivariate analysis and investigated for deducing their phylogenetic relationship. Concurrently seed sculpturing was also investigated from different populations of a particular species to normalize potential intra-specific variations using scanning electron microscopy. A reference phylogenetic tree from the ribosomal ITS 2 sequences was also constructed as a standard reference reflecting a DNA-based phylogenetic relationship of a particular species to normalize potential intra-specific variations using scanning electron microscopy. A reference phylogenetic tree in the light of recent phylogenetic studies and to test whether the seed micromorphology strictly reflects the phylogenetic relationships among all species studied. Seed, embryo and testa cell length and width were six major quantitative traits observed to be important in deducing inter-specific phylogeny exhibiting congruence with ITS2-based phylogeny. However, seed coat sculpturing has not been observed in congruence with phylogeny; rather, they may be involved in ecology of the particular species.

Keywords: multivariate analysis, rITS 2 sequence, SEM

INTRODUCTION

Orchidaceae is the largest family among angiosperms, consisting of about 850 genera with 25000 species (Dressler 1993). Dendrobium is the third largest genus of Orchidaceae comprising about 1184 species (Leitch et al. 2009). To date, more than 103 species of the genus Dendrobium have been reported from Indian continent (Singh et al. 2001). Dendrobium species are characterized by a broad geographical distribution, a tremendous diversity in growth habit and the ability to produce a large number of interspecific hybrids with different morphology. The systematics of the genus Dendrobium was extensively studied on the basis of morphological key characters (Hooker 1890; Pradhan 1979; Dressler 1993), and on the basis of molecular markers (Xu et al. 2001; Boonsrangsom et al. 2008; Begum et al. 2009) and chloroplast DNA sequences (Yukawa et al. 1996). However, the classification of many Dendrobium species remains ambiguous (Clements 2003) and seed micromorphology may help in this context.

Orchid seeds are the smallest among all seeds produced by flowering plants and vary considerably in outer morphology and in advance structures. For both symbiotic and chlorophyllous plants and vary considerably in outer morphology and in advance structures. For both symbiotic and mineral nutrition in the orchid seed biology (Barthlott and Ziegler 1981). These morphological characters have been least exploited for taxonomic division of orchid species. Though orchid seeds were always considered important for micropropagation (Kauth et al. 2008), only few of the orchids from India have been classified based on seed morphological variables (Rao and Avadhani 1964; Goh 1976; Mitra et al. 1976; Vij et al. 1981, 1992; Jeeja and Ansari 1994; John and Jack 1998). Comparative study of molecular and morphological methods for describing phylogeny was tried in different group of plants including orchid (Roldan-Ruiz et al. 2001; Rodriguez et al. 2003; Shifunov et al. 2004; Zhang et al. 2005; Bhargava et al. 2007), but there were fewer reports of comparative study of molecular and micromorphological methods (Lumga et al. 2006). However a comprehensive study on seed micromorphology and morphometry of Dendrobium from India would rein-
force significantly in orchid taxonomy and phylogeny. *Dendrobium* is a taxonomically complex genus and occupies second largest position in the family Orchidaceae. Considering the importance of seed characters in taxonomic and phylogenetic relationships, an attempt has been made in the present study to investigate the importance of seed micromorphology in respect to phylogeny and/or ecology in *Dendrobium* species. The present investigation deals with a unique approach to consider both quantitative, such as seed length and seed width, and qualitative micromorphological characteristics such as seed coat sculpturing, for the first time in 18 *Dendrobium* species of Indian origin. The present study will confirm the role of seed micromorphometric markers as tools for comparative phylogeny among Indian species of *Dendrobium*.

**MATERIALS AND METHODS**

**Collection of plant material**

Detailed studies were carried out with seeds of 18 species (Table 1) of *Dendrobium* representing different sections: *D. chrysotum*, and *D. densiflorum* (Callista); *D. fimbriatum, and D. moschatum* (Eugenanthe I); *D. aqueum, D. chrysanthum, D. crepidatum, D. heterocarpum, D. hookerianum, D. macrostachyum, D. nobile, D. ochreatum, D. parishii, D. primulinum, D. transparence*, and *D. willumsonii* (Nigrohirsutae). The mature seed-capsules were freshly collected and subsequently split open and biological replicates of capsules from 2-6 individuals per species were sampled. Capsules were subsequently split open and the seeds were collected and stored in small-cap vials (Tarson) over CaCl₂ at 4°C in desiccators.

**Taxa classification, seed biology and nomenclature**

The name of different taxa and classification of the genus *Dendrobium* follows those of Hooker (1890), Pradhan (1979), and Dressler (1993). Plants, from which capsules were collected, were identified at the Botanical Survey of India, Shillong. Terminology and formulae regarding seed biology and seed micromorphology were adopted from Arditti et al. (2000). Seed nomenclature follows that of the ‘Glossary of seed and seed terminology’ (http://www.bio.uu.nl/~seed/glossary/glos-int.htm).

**Light microscopic (LM) study**

Twenty seeds from each capsule with three replications were observed under a Leica DM 2500 microscope and measurements were recorded digitally at 40X magnification using Leica Queen-8 software. Measurements were recorded for seed, embryo and testa cell length and width. Testa cell measurements were recorded only for the median testa cells of the longest axis. The color and shape of the seeds were observed and described in subjective terms with the help of a stereoscopic microscope (Hund WETZLAR 1021471) with epilumination (Hund WETZLAR FLQ 150).

**Scanning electron microscopic (SEM) study**

The seeds were mounted on aluminum copper stubs using double adhesive tape. The samples were then sputter-coated with gold palladium alloy for five minutes and photographed on a JEOL-35 SEMCT-SEM at an accelerating Voltage of 15 KV. Detailed seed coat (testa cells) surface studies were conducted under SEM. The parameters considered were seed coat sculpturing and wall thickening of two adjacent testa cells.

**Construction of reference phylogenetic tree**

From the database partial rDNA sequences were retrieved in FASTA version 3.4 (Pearson and Lipman 1988) and trimmed for ITS 2 among ITS 1, 5s rDNA, ITS 2, and 12s rDNA using BioEdit version 7.0.9.0 (Hall 1999). Original source of these sequences is provided in Table 2. Using CLUSTAL W (Thompson et al. 1994) multiple alignments of sequences was executed and to find a consensus neighbour-joining tree (Saitou and Nei 1987) out of 1000 phylogenetic trees produced through MEGA version 4 (Tamura et al. 2007). Bootstrap values (1000 replicates) were calculated to validate the reproducibility of the branching pattern (Felsenstein 1985).

**Measurements and data analysis**

In this study we considered those variables that could be measured directly with respect to a constant unit. Therefore, we could consider only 6 variables (the length and width of seed, embryo, and testa cells) measured up to the 4th decimal point in mm. These are

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**Table 1** Present status of the experimental species in Indian context.

<table>
<thead>
<tr>
<th><em>Dendrobium</em> spp.</th>
<th>Section*</th>
<th>Ecological status**</th>
<th>India N-W</th>
<th>India N-E</th>
<th>India central</th>
<th>India peninsula</th>
<th>India Andaman -Nicobar Island</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. chrysanthum</em></td>
<td>Eugenanthe II</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>gi/14993592</td>
</tr>
<tr>
<td><em>D. chrysotum</em></td>
<td>Callista</td>
<td>R, Th</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>gi/14993593</td>
</tr>
<tr>
<td><em>D. crepidatum</em></td>
<td>Eugenanthe II</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>gi/14993599</td>
</tr>
<tr>
<td><em>D. densiflorum</em></td>
<td>Callista</td>
<td>R, Th</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>gi/14993595</td>
</tr>
<tr>
<td><em>D. fermeirei</em></td>
<td>Callista</td>
<td>R, Th</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>gi/14993594</td>
</tr>
<tr>
<td><em>D. fimbriatum</em></td>
<td>Eugenanthe I</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>gi/38906434</td>
</tr>
<tr>
<td><em>D. formosum</em></td>
<td>Eugenanthe I</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>gi/33808269</td>
</tr>
<tr>
<td><em>D. heterocarpum</em></td>
<td>Eugenanthe II</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>gi/38906483</td>
</tr>
<tr>
<td><em>D. hookerianum</em></td>
<td>Eugenanthe II</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>gi/38906480</td>
</tr>
<tr>
<td><em>D. infundibulum</em></td>
<td>Eugenanthe I</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>gi/38906481</td>
</tr>
<tr>
<td><em>D. macrostachyum</em></td>
<td>Eugenanthe II</td>
<td>R R</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>gi/38906185</td>
</tr>
<tr>
<td><em>D. moschatum</em></td>
<td>Eugenanthe I</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>gi/211907896</td>
</tr>
<tr>
<td><em>D. nobile</em></td>
<td>Eugenanthe II</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>gi/15710171</td>
</tr>
<tr>
<td><em>D. ochreatum</em></td>
<td>Eugenanthe II</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>gi/15420569</td>
</tr>
<tr>
<td><em>D. parishii</em></td>
<td>Eugenanthe II</td>
<td>R, Th</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>gi/15420568</td>
</tr>
<tr>
<td><em>D. primulinum</em></td>
<td>Eugenanthe II</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>gi/15420567</td>
</tr>
<tr>
<td><em>D. transparence</em></td>
<td>Eugenanthe II</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>gi/15420566</td>
</tr>
<tr>
<td><em>D. willumsonii</em></td>
<td>Eugenanthe I</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>gi/14993595</td>
</tr>
</tbody>
</table>

* Skm (Sikkim), ArP (Arunachal Pradesh), Asm (Assam), Meg (Meghalaya), Ngl (Nagaland), Mnp (Monipur), Mzr (Mizorum), Trp (Tripura)
* R = rare, Th = threatened
* According to Singh et al. 2001
considered as the basic data set as measurements like seed volume, embryo volume, air space percentages are measured with the help of those six variables only. For examples, seed volume may be calculated using the formula: \( V = \frac{4}{3}\pi a^2 b \), where \( a = \frac{1}{2} \) its length, \( b = \frac{1}{2} \) its width (Arditti et al. 1980). Here we solely used the primary data set for further analysis.

To represent the variability of each species, box plots were prepared against all six variables and then used to perform multivariate analyses using R-programme package version 2.4.0 (R Development Core Team 2006; http://www.R-project.org). Principal component analyses (PCA) was carried out using the matrix of basic data set (Hatcher and Stepanski 1994). Based on seed micromorphometric variables higherarchial cluster analysis was also performed in order to get a cluster dendrogram (Sneath and Sokal 1973) to compare with the phylogenetic tree derived from ITS 2 sequences.

**RESULTS**

**Light microscopic study**

Among the qualitative traits studied under LM, the colour of seeds exhibited a range of different shades from yellow to brownish-yellow (Fig. 1). Yellow was the most common colour among all seeds from different species and thus was not considered as a distinguishing character. The shapes of the seeds of different species also exhibited little variation where majority of the seeds were fusiform (Fig. 1). Therefore, this trait was also found to be unsuitable as a species delimiting factor.

**Scanning electron microscopic study**

The SEM study revealed that in majority of the species testa cells arrangement were simple with cells attached in a straight longitudinal head to tail fashion; anticlinal cell walls were strongly raised and bordered than the periclinal cells, cell lumens were almost obliterated due to extensive development of the cell wall thickenings. The cells varied in length and orientation and the micropylar and the chalazal end cells were much shorter and stouter than the medial cells which enveloped into the embryo in all cases. The medial cells were also more elongated in length and encased the embryos of the seeds. Examples are **D. aphyllum**, **D. williamsonii** (Fig. 1). However, in **D. densiflorum** and **D. fimbriatum**, the arrangement was observed to be slightly spiral fashioned (Fig. 1), whereas in **D. ochreatum** and **D. chrysanthum** a bead-like deposition material was uniformly present on the cell lumens (Fig. 1). With these analyses it is quite clear that there is a similarity in broad aspects of the seed ultrastructural features like arrangement of testa cells, thickening of anticlinal cell wall of testa cells, and obliterate testa cell lumens.

However, a detailed study of ultrastructure, including testa cell wall deposition, varies greatly within the recognized sub-generic groups. These studies also revealed that according to the deposition pattern on testa cells, **Dendrobium** seeds may be categorized into 5 types:

**Type A**: Testa cell walls were covered with cottony white substances in a reticulate fashion, for example, **D. aphyllum** (section Eugenanthee, subsection I), **D. williamsonii** (section Nigrohirsutae) (Fig. 1).

**Type B**: Slightly rough surfaces due to the deposition of a bead-like structure in 2-3 rows on the testa walls, for example, **D. densiflorum** (Section Callista), **D. fimbriatum** (Eugenanthee subsection II), **D. formosum** (section Nigrohirsutae) (Fig. 1).

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**Table 2** Study of qualitative micromorphometric characters from **Dendrobium** seeds.

<table>
<thead>
<tr>
<th>Species</th>
<th>Seed shape</th>
<th>Seed colour</th>
<th>Testa cell shape</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. chrysanthum</em></td>
<td>Fusiform</td>
<td>Yellow</td>
<td>Fusiform</td>
</tr>
<tr>
<td><em>D. chrysotoxum</em></td>
<td>Fusiform</td>
<td>Orange yellow</td>
<td>Fusiform</td>
</tr>
<tr>
<td><em>D. crepidatum</em></td>
<td>Fusiform</td>
<td>Yellow</td>
<td>Fusiform</td>
</tr>
<tr>
<td><em>D. densiflorum</em></td>
<td>Fusiform</td>
<td>Orange yellow</td>
<td>Fusiform</td>
</tr>
<tr>
<td><em>D. fermerei</em></td>
<td>Fusiform</td>
<td>Yellow</td>
<td>Fusiform</td>
</tr>
<tr>
<td><em>D. fimbriatum</em></td>
<td>Fusiform</td>
<td>Yellow</td>
<td>Fusiform</td>
</tr>
<tr>
<td><em>D. formosum</em></td>
<td>Oval and twisted</td>
<td>Orange yellow</td>
<td>Fusiform</td>
</tr>
<tr>
<td><em>D. heterocarpum</em></td>
<td>Fusiform</td>
<td>Yellow</td>
<td>Fusiform</td>
</tr>
<tr>
<td><em>D. hookerianum</em></td>
<td>Fusiform</td>
<td>Brownish yellow</td>
<td>Fusiform</td>
</tr>
<tr>
<td><em>D. infundibulum</em></td>
<td>Fusiform</td>
<td>Yellow</td>
<td>Fusiform</td>
</tr>
<tr>
<td><em>D. macrostachyum</em></td>
<td>Spindle-shaped</td>
<td>Yellow</td>
<td>Fusiform</td>
</tr>
<tr>
<td><em>D. moschatum</em></td>
<td>Fusiform</td>
<td>Orange yellow</td>
<td>Fusiform</td>
</tr>
<tr>
<td><em>D. nobile</em></td>
<td>Fusiform</td>
<td>Yellow</td>
<td>Fusiform</td>
</tr>
<tr>
<td><em>D. ochreatum</em></td>
<td>Fusiform</td>
<td>Yellow</td>
<td>Fusiform</td>
</tr>
<tr>
<td><em>D. parishii</em></td>
<td>Fusiform</td>
<td>Yellow</td>
<td>Fusiform</td>
</tr>
<tr>
<td><em>D. primulinum</em></td>
<td>Fusiform</td>
<td>Golden yellow</td>
<td>Fusiform</td>
</tr>
<tr>
<td><em>D. transparens</em></td>
<td>Fusiform</td>
<td>Yellow</td>
<td>Fusiform</td>
</tr>
<tr>
<td><em>D. williamsonii</em></td>
<td>Elliptic</td>
<td>Orange yellow</td>
<td>Fusiform</td>
</tr>
</tbody>
</table>

---

Fig. 1 Scanning electron micrographs of **Dendrobium** seeds showing different ornamental patterns.
Fig. 2 Box plots of six seed micromorphometric variables (length and width of seed, embryo and testa cells) for the 18 different species of *Dendrobium*.
**Type C:** Bead-like structures were present at the central position of testa walls, for example, *D. crepidatum* (section Eugenanthe subsection II), *D. infundibulum* (section Nigrohirsutae) (Fig. 1).

**Type D:** Deposition materials were clumped together randomly on the sides of the wall, for example, *D. chrysotoxum* (Section Callista) (Fig. 1).

**Type E:** Testa cell walls were smooth with only occasional deposition, for example, *D. moschatum* (section Eugenanthe subsection I), *D. hookerianum* (section Eugenanthe subsection II) (Fig. 1).

Although these ultra-structural features might be useful to classify seed types they were unable to establish any phylogenetic relationship, as each type is represented by different sections at a time (**Table 3**).

**Box plot analyses**

To represent the variability across species, box plots against all 6 variables were analyzed (Fig. 2). It was evident that the variability is more significant in the length of any particular variable than its counterpart width. Among all studied micromorphometric traits, seed length was observed to show maximum variability with considerable overlapping among species (Fig. 2A), whereas testa cell width exhibited minimum variation (Fig. 2F).

From the box plot analyses maximum and minimum seed lengths among the experimental species were observed in *D. chrysanthum* and *D. macrostachyum*, respectively. Maximum and minimum seed width and embryo width was observed in *D. aqueum* whereas maximum and minimum embryo length was observed in *D. formosum* and *D. chrysanthemum*, respectively. Testa cell length was maximum and minimum in *D. heterocarpum* and *D. fimbriatum*, respectively while maximum and minimum testa cell width was observed in *D. crepidatum* and *D. primulinum*, respectively (Fig. 2).

**Principal component analyses**

In PCA, the first three principal components (PC) show 76.50, 90.3 and 97.10%, respectively of total variability. Interestingly, with 6 micromorphometric variables, 18 experimental species were clustered into six groups when first two PCs were plotted (Fig. 3):

- **Group I:** represented by *D. aqueum*, *D. heterocarpum*, and *D. transparence* (members of the section Eugenanthe subsection II);
- **Group II:** represented by *D. formosum*, *D. nutans*, and *D. williamsonii* (members of the section Nigrohirsutae);
- **Group III:** represented by *D. crepidatum*, *D. chrysanthemum*, *D. hookerianum*, and *D. nobile* (members of the section Eugenanthe subsection II);
- **Group IV:** represented by *D. moschatum* and *D. fimbriatum* (members of the section Eugenanthe subsection I);
- **Group V:** represented by *D. macrostachyum*, *D. parishii*, and *D. primulinum* (members of the section Eugenanthe subsection II);
- **Group VI:** represented by *D. chrysotoxum*, and *D. densiflorum* (members of the section Callista).

**Cluster analysis**

Hierarchical cluster analysis resulted into a cluster dendrogram representing five distinguishing clusters (Fig. 4):

- Cluster I: represented by group II of PCA (members of the section Nigrohirsutae);
- Cluster II: represented by group VI of PCA (members of the section Callista);
- Cluster III: represented by group IV of PCA (members of the section Eugenanthe subsection I);
- Cluster IV: represented by group III and group V of PCA (members of the section Eugenanthe subsection II);
- Cluster V: represented by group I of PCA (members of the section Eugenanthe subsection II).

**Reference phylogenetic tree from ITS 2**

Analysis of the available rDNA ITS 2 sequence data results into a phylogenetic tree that could be treated as a reference tree for the present study (Fig. 5). In the reference phylo-
genetic tree five considerable groups were observed much similar to that of cluster dendrogram (Fig. 4). *D. Williamsonii* and *D. formosum* grouped together (members of section Nigrohirsutae) with close proximity to *D. densiflorum* and *D. chrysotoxum* (members of section Callista). However, *D. parishii*, *D. primulinum*, *D. chrysanthum*, and *D. crepidatum* were grouped together (members of sub-section Eugenanthae II). Interestingly, they all have a common root of origin. Moreover, *D. nobile* and *D. heterocarpum* grouped together (other members of sub-section Eugenanthae II) but showed a different root of origin. *D. moschatum* and *D. fimbriatum* grouped together (members of sub-section Eugenanthae I) possessing an intermediate position in the tree.
DISCUSSION

Qualitative seed micromorphology is not a delimiting character within Dendrobium

The color of the mature seed coat has been used as a distinguishing character to identify species by earlier workers (Barthlott and Zeiger 1981; Zeiger 1981). However, in the present study seed coat color could not be assessed as a distinguishing character because most of the studied species from different sections showed similarity in colour (Fig. 1).

The shape of the orchid seed has been reported varying from filiform to fusiform, clavate to ellipsoidal, spindle shaped and sometimes permanently winged (Molvray and Kores 1995). Vij et al. (1992) observed that the seed shape is fusiform in Cyripedieae, fusiform and spathulate in Neottieae, fusiform and ovoid in Orchideae, fusiform, filamentous, ovoid, elliptical, and cylindrical in Epipendrea. In the present study testa cells in all Dendrobium species studied were fusiform shaped which is in accordance with Clifford and Smith (1969) with few exceptions like D. aqueum and D. primulinum (Elliptic), D. macrostachyum and D. nutans (spindle-shaped) (Fig. 1). However, fusiform shape has been revealed as to be a common feature among wide range of species (Arditti et al. 1979, 1980; Healey et al. 1980). The fusiform shape appears to be the basic in orchids and their subsequent evolution into other morphotypes may have been an adaptive strategy (Vij et al. 1992).

Thus it was concluded that seed shape was not a species delimiting factor among the species studied.

Seed ultra-structure also does not reveal any phylogenetic consequences

Based on ultra-structural features Zeiger (1981) characterized seed into three types: Orchis type, Goodyera type and Disa-Diuris type. However, all the experimental Dendrobium species exhibited a similar trait to Disa-Diuris type where the individual cells varied in shape which may be sub-quadrate, oblong, sub-elliptical or irregular in outline. Their size may be uniform or varies within the areas such as the median region and the chalazal end (Molvray and Kores 1995).

In the present study, the SEM images also revealed more simple form of testa cell arrangements in the epiphytic members such as Dendrobium spp. which showed incongruence with the arrangements suggested earlier being common in terrestrial taxa and spiral form in epiphytic species (Vij et al. 1992). The anticlinal walls may be sculptured and take the form of ridges, reticulations, perforations, or scattered varicosities, and can be fairly consistent within genera or sub-tribe (Molvray and Kores 1995).

The present study revealed that according to the ornamentation pattern of testa cells, Dendrobium seeds may be categorized into five types (Fig. 5). Though these ultrastructural features might be useful to classify the seed types but were unable to establish any phylogenetic relationship between species due to the non-specificity of each type to different sections (Fig. 5). These results support the view that the testa cells arrangement vary within genera and do not reflect as of any phylogenetic significance.

Quantitative seed micromorphology is best suited to elucidate phyogeny

Arditti et al. (1980) studied the patterns of seed growth and suggested that during maturation the seed elongated as a result of an increase in testa cell elongation and not numbers, this increases buoyancy of seed in air and in wind dispersion. It could be the reason that the variability was always more in the length than their counterpart width. It was more significant for the entire three basic components (seed, embryo, and testa cell) of seed micromorphology to be used for boxplot analyses (Fig. 2). Among the six variables studied it was observed that the seed length vary most and testa cell width vary list at intra specific level.

Results from the PCA were observed to be in accordance with the classification provided by the earlier workers, particularly by Pradhan (1979). According to Pradhan (1979), D. formosum, D. nutans, and D. williamsonii were representatives of the section Nigrohirsetae (=Formosae; as proposed by Hooker 1890), D. chrysanthum, and D. densiflorum were representatives of the section Callista; whereas D. basilliatum, and D. chrysanthum were members of the sub-section Eugenanthe I (Table 1). However, clustering from the PCA the members of sub-section Eugenanthe II was not prominent though there was a clear indication of two to four distinct groups (Fig. 3). One group was at the middle of upper two quadrates (D. heterocarpum, D. aqueum, D. transparence, D. ochreatum), another at the middle of right two quadrates (D. chrysanthum, D. nobile, D. crepidatum) whereas the third group was well separated at lower left quadrates which again may be subdivided into two (D. chrysotoxum, D. densiflorum in one and D. macrostachyum, D. parishii, D. primulinum in another). Interestingly, in the lower right quadrates a transition from Eugenanthe II to Callista through Eugenanthe I may be noted. The members of Callista and Nigrohirsetae were also within close proximity indicating a close relatedness. The results of PCA in cluster analysis were consistent with the PCA (Fig. 3).

On the other hand the rDNA sequences of plants are widely analyzed for evolution and anatomy studies of plants (Joshi et al. 2004). The region contains ITS 1, 5.8S, and ITS 2 sequences. The ITS region could be used to authenticate Herba Dendrobii (Xu et al. 2001; Ding et al. 2002). The average difference of the internal transcribed spacer 1 (ITS1) between Dendrobium and non-orchids is 34.62%, between Dendrobium and the orchids is 22.31% and the interspecific difference among the Dendrobium species is 13.14%, showing that ITS1 may also be used for differentiating the target Dendrobium species (Zhang et al. 2005). Whereas ITS2 of 16 Dendrobium species and showed that they differ from one another by an average of 12.4% and from non-orchids by 29.8% (Lau et al. 2001). The intraspecific variation observed among examined Dendrobium species was found to be only about 1%. Therefore, ITS2 regions could be appropriately adopted as a molecular marker system for differentiating Dendrobium species from one another and also from non-orchids (Zhang et al. 2005).

Both the micromorphology based cluster dendrogram and the ITS 2 sequence based phylogenetic tree could also be explained as per the classification of Pradhan (1979). The phylogenetic tree based on ITS 2 sequence showed significant congruence with the classification of Pradhan (1979) with the only exception in the section Eugenanthe II which is not monophyletic (Fig. 4). While comparing it with the cluster dendrogram derived through multivariate analysis from six basic variables of seed micromorphometry, it was observed to be significantly comparable. Members of Nigrohirsetae and Callista were grouped together as it was expected from the earlier classification with a very good bootstrap support value (>90). Though Pradhan (1997) has classified section Eugenanthe into two subsections, but the confusion remains same with the corresponding members of these subsections. Here (Fig. 1) the representatives of Eugenanthe subsection 1, D. fimbriatum and D. moschatum grouped together with a very poor bootstrap value (45) stating the ambiguity. Both in Figs. 1 and 4, three different clusters may well be recognized: D. parisiili, D. primulinum, D. chrysanthum, D. crepidatum were in one cluster, D. fimbriatum, D. moschatum were in another cluster, and D. ochreatum and D. transparente (Fig. 1), from the cluster dendrogram (Fig. 4), it was clear that they were also representing the third cluster. Interestingly both in Fig. 1 and Fig. 4, the members of Eugenanthe I clustered with the members of Eugenanthe II. Therefore, these findings showed significant accordance with...
DNA sequence based phylogenetic relationship among different species. The results show that polygenic traits such as seed length, seed width, embryo length, embryo width, testa cell length, and testa cell width can be used to discriminate between species to discriminate between populations, probably because they depict variation under natural selection.

CONCLUDING REMARKS

The present study implicates multivariate analyses of seed micromorphometric traits that led to the phylogenetic relationship between different species of Dendrobium, providing the foundation of evolutionary trend of an important flowering plant. We provide clues here into seed micromorphometric traits that may have been selected in nature, may be thousands of years ago, through the development of morphological, floral, and species. Notably, the majority of microscopic characters such as seed length, seed width, embryo length, embryo width, testa cell length, and testa cell width are diagnosed as having become significant during evolution, at both inter- and intra-specific levels. The suggestion for other seed traits with little significance such as coat sculpture was primarily concomitant with evolutionary trend, is now bolstered by the remarkable observation that independent parallel selection of different traits such as seed length, its width as well as embryo ultrastructures, taking place in different geographic regions in thousands of years under original selection pressure which ultimately led to parallel recruitment of these numeric traits in different Dendrobium species. This repeated observation accompanying seed micromorphology at inter-specific level would appear to be with precedent. An exciting prospect for future work will be to dissect this micromorphological tools into its responsible constituents, and to learn the trends in the phylogenies across taxon precluding a consensus on phylogeny of major lineages within the family.

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