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

Pritam Chattopadhyay[1] • Nirmalya Banerjee[1] • Bhupendra Chaudhary[1,2*]

[1] Department of Botany, Visva-Bharati, Santiniketan 731 235 WB India
[2] School of Biotechnology, Gautam Buddha University, Greater Noida 201 308 UP India

Corresponding author: * **bhupendra@gbu.ac.in**

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 among all species studied. Seed, embryo and testa cell length and width were six major quantitative variables 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 asymbiotic orchid seed germination to be effective, many conditions must be addressed such as photoperiod, temperature, and mineral nutrition (Kauth *et al*. 2008). At the generic level seeds vary considerably in size ranging from 150 to 6000 mm and the shape differs from filiform to fusiform, clavate to ellipsoidal and sometimes prominently winged (Molvray and Kores 1995). In cases, seeds were observed to be covered by a hard seed coat; however, in most the seed coat is papery in texture and loosely encapsulates the embryo (Molvray and Kores 1995).

The seeds are wind dispersed and exhibit a great deal of varied morphological characters and structure mostly studied with the scanning electron microscopy (SEM) (Arditti *et al.* 1979). The morphometric characters of seeds are ever challenging to the taxonomic and phylogenetic studies that would be a great help both in understanding the orchid seed biology as well as in applied ventures (Rani *et al.* 1993).

The various morphological characters in orchid seeds have their significant importance in orchid classification (Clifford and Smith 1969). These morphological properties could serve as taxonomic markers and for understanding the orchid seed biology (Barthlott and Ziegler 1981). These characters were successfully harnessed to distinguish closely related species (Barthlott and Ziegler 1981; Haas 1977a, 1977b; Healey *et al.* 1980); as well as to identify their contribution in hybrid genotypes (Arditti *et al.* 1979). Therefore, seed morphology could serves as a good source of systematic character to circumscribe subgeneric groups or relationships among species within a complex genus like *Dendrobium* (Mathews and Levins 1986; Ness 1989; Larry 1995) to assist in deducing phylogenetic relationship as well (Barthlott and Ziegler 1981).

Although many studies on orchid systematics are based on morphological characters, however, significant seed 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; Shipunov *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-

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

Dendrobium spp.	Section*	Ecological status**	Indian distribution**												rITS 2 sequence derived from
			India N-W	India N-E								India central	India peninsula	India Andaman -Nicobar Island	
				Skm	ArP	Asm	Meg	Ngl	Mnp	Mzr	Trp				
D. chrysanthum	Eugenanthe II		+	+	+			+	+	+					gi/14993592
D. chrysotoxum	Callista	R, Th			+			+	+	+					gi/14993593
D. crepidatum	Eugenanthe II		+	+	+	+		+	+	+					gi/14993599
D. densiflorum	Callista			+	+			+	+	+				+	gi/14993595
D. fermerei	Callista			+											--
D. fimbriatum	Eugenanthe I		+	+	+	+	+	+	+	+		+	+	+	gi/38906434
D. formsum	Nigrohirsutae		+	+	+	+	+	+	+			+	+		gi/33088269
D. heterocarpum	Eugenanthe II		+	+	+			+	+	+			+		gi/38906483
D. hookerianum	Eugenanthe II			+	+	+		+		+					--
D. infundibulum	Nigrohirsutae						+								--
D. macrostachyum	Eugenanthe II	R							+	+					--
D. moschatum	Eugenanthe I		+		+							+			gi/38906185
D. nobile	Eugenanthe II			+	+			+	+	+		+			gi/211907896
D. ochreatum	Eugenanthe II					+		+	+						--
D. parishii	Eugenanthe II	R, Th			+			+	+	+					gi/157101713
D. primulinum	Eugenanthe II		+		+		+		+	+			+	+	gi/15420569
D. transparence	Eugenanthe II		+	+	+	+		+	+	+	+	+	+		--
D. willumsonii	Nigrohirsutae	R				+	+		+						gi/14993595

Skm (Sikkim), ArP (Arunachal Pradesh), Asm (Assam), Meg (Meghalaya), Ngl (Nagaland), Mnp (Monipur), Mzr (Mizorum), Trp (Tripura)
R = rare, Th = threatened
* According to Pradhan 1979
** According to Singh et al. 2001

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. chrysotoxum*, and *D. densiflorum* (Callista); *D. fimbriatum*, and *D. moschatum* (Eugenanthee I); *D. aqueum*, *D. chrysanthum*, *D. crepidatum*, *D. heterocarpum*, *D. hookerianum*, *D. macrostachyum*, *D. nobile*, *D. ochreatum*, *D. parishii*, *D. primulinum*, and *D. transparence* (Eugenanthee II), *D. formosum*, *D. nutans*, and *D. williumsonii* (Nigrohirsutae). The mature seed-capsules were freshly collected from the wild, National Botanical Gardens of India, commercial growers and also from the departmental orchidarium during 2006 to 2009 throughout India (**Table 1**). To cover intra-specific variations, 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 $CaCl_2$ 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 epiilumination (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 JSMCT-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 ver-sion 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

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

Species	Seed shape	Seed colour	Testa cell shape
D. chrysanthum	Fusiform	Yellow	Fusiform
D. chrysotoxum	Fusiform	Orange yellow	Fusiform
D. crepidatum	Fusiform	Yellow	Fusiform
D. densiflorum	Fusiform	Orange yellow	Fusiform
D. fermerei	Fusiform	Yellow	Fusiform
D. fimbriatum	Fusiform	Yellow	Fusiform
D. formosum	Oval and twisted	Orange yellow	Fusiform
D. heterocarpum	Fusiform	Yellow	Fusiform
D. hookerianum	Fusiform	Brownish yellow	Fusiform
D. infundibulum	Fusiform	Yellow	Fusiform
D. macrostachyum	Spindle-shaped	Yellow	Fusiform
D. moschatum	Fusiform	Orange yellow	Fusiform
D. nobile	Fusiform	Yellow	Fusiform
D. ochreatum	Fusiform	Yellow	Fusiform
D. parishii	Fusiform	Yellow	Fusiform
D. primulinum	Elliptic	Golden yellow	Fusiform
D. transparens	Fusiform	Yellow	Fusiform
D. williumsonii	Elliptic	Orange yellow	Fusiform

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 2[(W/2)2(1/2L)(1.047)], where, 1.047= π/3; W = width; L = seed length and embryo volume may be calculated by using the formula: 4/3πab2, where a=1/2 its length and b=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. williumsonii* (**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

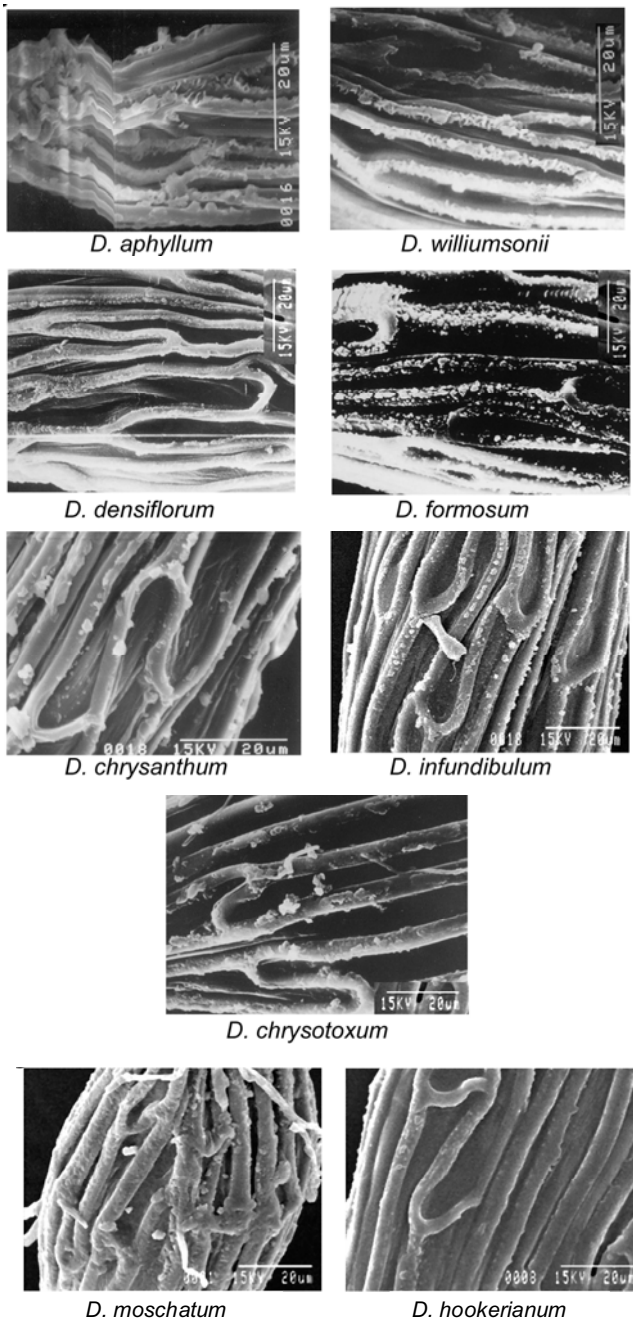

Fig. 1 Scanning electron micrographs of *Dendrobium* seeds showing different ornamentational patterns.

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 obliterates 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 II), *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 I), *D. formosum* (section Nigrohirsutae) (**Fig. 1**).

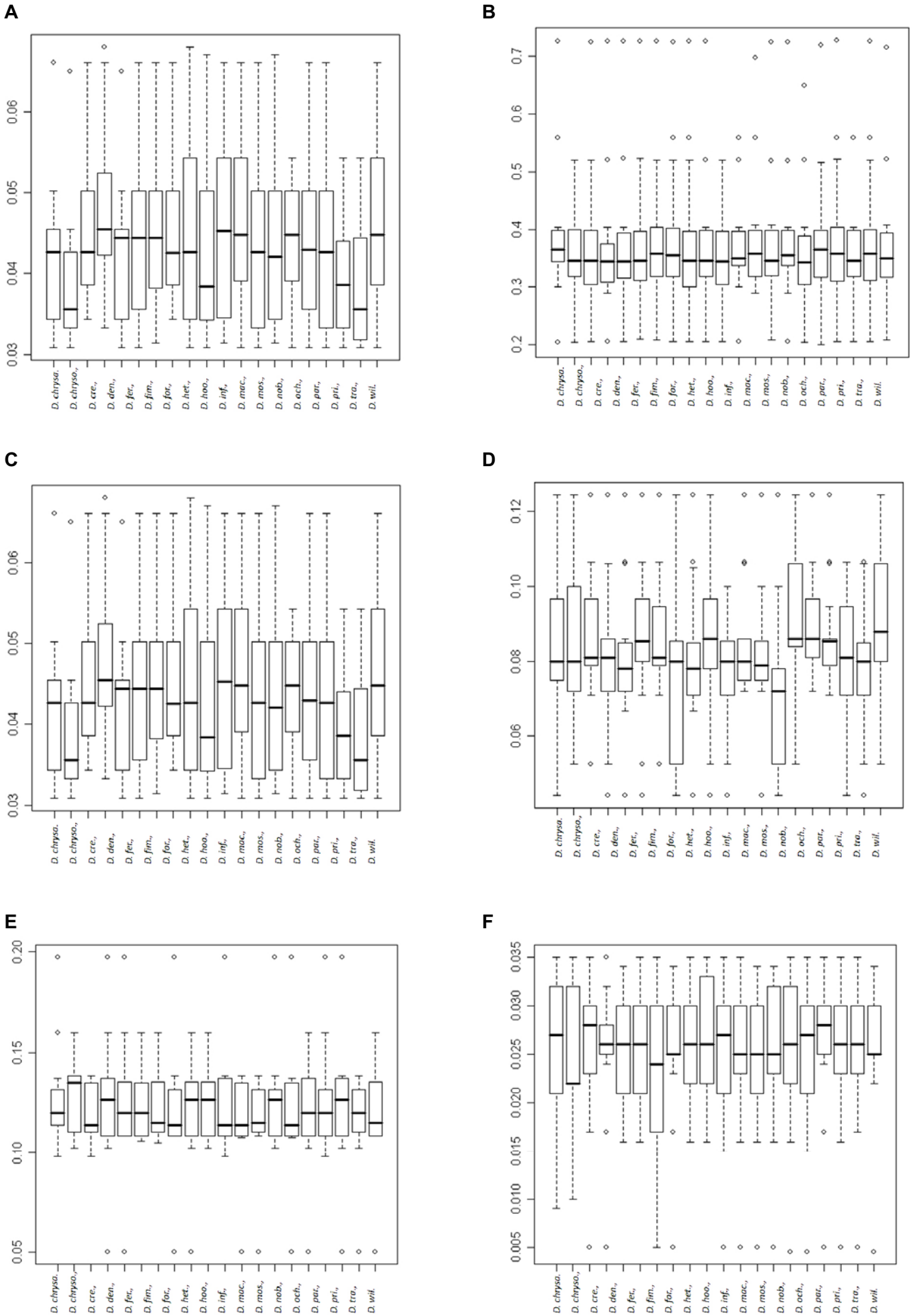

Fig. 2 Box plots of six seed micromorphomtric variables (length and width of seed, embryo and testa cells) for the 18 different species of *Dendrobium*.

Type C: Bead-like structures were present at the central position of testa walls, for example, *D. crepidatum* (section Eugenanthee 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 Eugenanthee subsection I), *D. hookerianum* (section Eugenanthee 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. chrysanthum*, 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. primulianum*, 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 Eugenanthee 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. chrysanthum*, *D. hookerianum*, and *D. nobile* (members of the section Eugenanthee subsection II);

Group IV: represented by *D. moschatum* and *D. fimbriatum* (members of the section Eugenanthee subsection I);

Group V: represented by *D. macrostachyum*, *D. parishii*, and *D. primulinum* (members of the section Eugenanthee 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 Eugenanthae subsection I);

Cluster IV: represented by group III and group V of PCA (members of the section Eugenanthae subsection II);

Cluster V: represented by group I of PCA (members of the section Eugenanthae subsection II).

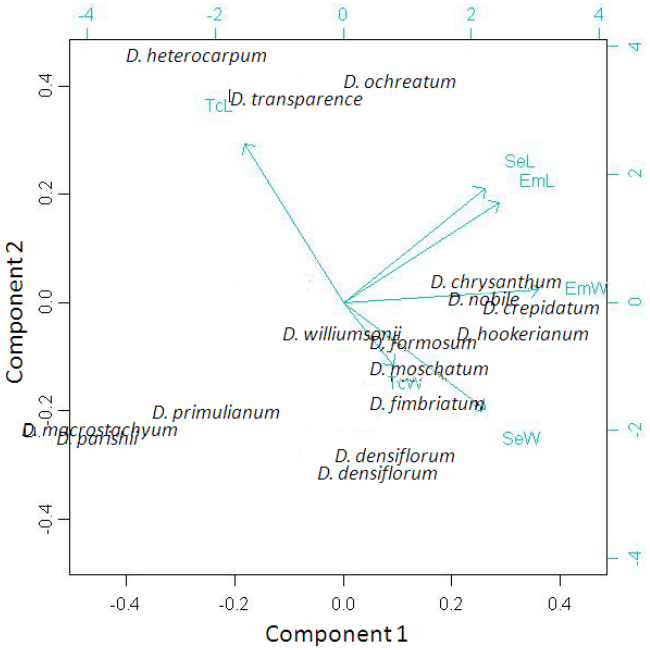

Fig. 3 Plot of first two principal components segregating 18 *Dendrobium* species into different groups.

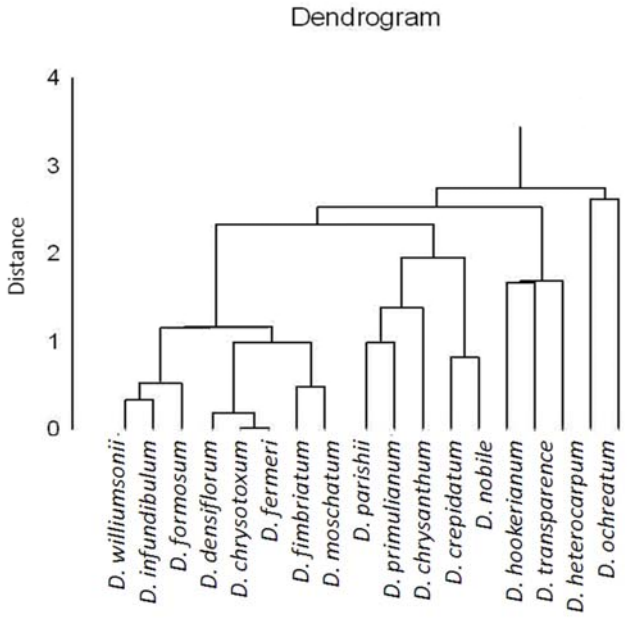

Fig. 4 Cluster analysis with 6 seed micromorphometric variables of 18 *Dendrobium* species.

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-

Fig. 5 Phylogenetic tree derived from rDNA ITS 2 sequences of *Dendrobium* along with the scanning electron micrographs of seeds of the corresponding species.

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 Zeigler 1981; Zeigler 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 Cypripedeae, fusiform and spathulate in Neottieae, fusiform and ovoid in Orchideae, fusiform, filamentous, ovoid, elliptical, and cylindrical in Epidendreae. 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 Zeigler (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, *Dendrobum* seeds may be catagorised 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

Arditii *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 variable 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 Nigrohirsutae (=Formosae; as proposed by Hooker 1890); *D. chrysanthum*, and *D. densiflorum* were representatives of the section Callista; whereas *D. fimbriatum*, and *D. moschatum* were representatives of the sub-section Eugenanthae I (**Table 1**). However, clustering from the PCA the members of sub-section Eugenanthaee 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 quadrate which again may be subdivided into two (*D. chrysotoxum*, *D. densiflorum* in one and *D. macrostachyum*, *D. parishii*, *D. primumulianum* in another). Interestingly, in the lower right quadrate a transition from Eugenanthae II to Callista through Eugenanthae I may be noted. The members of Callista and Nigrihirsutae were also within close proximity indicating a close relatedness. The results of PCA were also reflected in cluster analysis (**Fig. 4**).

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 Eugenanthae which is not monophyletic (**Fig. 4**). While comparing it with the cluster dendrogram derived through multivariate analysis from six basic variables of seed micromorphology, it was observed to be significantly comparable. Members of Nigrohirsutae and Callista were grouped together as it was expected from the earlier classification with a very good boot strap value (>90). Though Pradhan (1997) has classified section Eugenanthae into two subsections, but the confusion remains same with the corresponding members of these subsections. Here (**Fig. 1**) the representatives of Eugenanthae subsection I, *D. fimbriatum* and *D. moschatum* grouped together with a very poor boot strap value (45) stating the ambiguity. Both in **Figs. 1** and **4**, three different clusters may well be recognized: *D. parishii*, *D. primulinum*, *D. chrysanthum*, *D. crepidatum* were in one cluster, *D. fimbriatum*, *D. moschatum* were in another cluster, and *D. heterocarpum*, *D. nobile* were in another cluster. Though ITS 2 sequences were not available for the species like *D. aqueum*, *D. hookerianum*, *D. macrostachyum*, *D. nutans*, *D. ochreatum* and *D. transparence* (**Fig. 1**), from the cluster dendrogram (**Fig. 4**), it was clear that they were also representing the third cluster. Interestingly in both **Fig. 1** and **Fig. 4**, the members of Eugenanthae cluster I showed a close proximity with that of the members of Callista. 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 modern flowering 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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