The Embryology, Seed Coat, and Conservation of some Kenyan species of the Orchidaceae

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ABSTRACT

Aspects of embryology, seed morphological features and symbiotic seed germination of the Kenyan orchid species of the subfamily Epidendroideae were examined critically in order to elucidate their development and importance in conservation. The embryology of *ca*. 70 species was studied by clearing-squash techniques and differential-interference-contrast optics and seeds of *ca*. 50 species were studied by scanning electron microscopy to determine their variation. Germination of seeds of 12 orchid species *in vitro* utilizing 12 mycorrhizal fungi, was conducted to explore its potential in *ex situ* conservation.

The study taxa were collected from many habitats across Kenya including open forests, bushed woodland, grassland, dense forests, swamps, rocky habitats, montane and dry areas. Kenya straddles the equator, and varies in topology from sea level to over 5 000 m, so that there is a wide range of habitats.

Embryological characters were markedly variable among the different lineages within the study taxa but did not vary within the genera. Megasporogenesis starts with formation of nucellar filament of seven cells in species of *Liparis*, *Oberonia*, *Cribbia*, *Diaphananthe*, *Chamaeangis*, *Angraecopsis*, and *Ansellia*; nine cells in *Acampe*, *Aërangis*, *Angraecum*, *Bulbophyllum*, *Calanthe*, *Cyrtorchis*, *Epidendrum*, *Eulophia*, *Microcoelia*, *Ypsilopus*, *Oeceoclades*, *Polystachya*, *Rangaeris*, *Tridactyle*; or 13 cells in *Vanilla* of which the terminal one forms the megaspore mother cell. The study questions strongly the previously assumed formation of archesporial cell during sporogenesis. Megasporogenesis is a rapid process, in many species taking place in the flower bud or soon after anthesis. Due to lack of suitable young ovaries during sampling, difficulty was experienced in establishing embryo sac types. However, in those cases where the

condition was observed the embryo sac was either eight-nucleate, as in *Epidendrum, Aërangis, Eulophia, Polystachya* or six-nucleate in *Bulbophyllum.* The megagametophyte is characterised as being anatropous, tenuinucellate and bitegmic, but only the outer integument forms the seed coat of mature seeds.

The variation in embryogenesis was simplified into five developmental types characterised by differences in suspensor (number of cells, whether a single cell or a linear row or as filamentous strands) and the origin of cells in the embryo proper (apical cell or apical and basal). The *Diaphananthe* type exists in genera such as *Angraecopsis, Diaphananthe, Chamaeangis, Rangaeris, Cyrtorchis, Angraecum, Acampe,* and *Tridactyle.* The *Bulbophyllum* type occurs in *Oeceoclades, Bulbophyllum,* and *Cribbia.* The *Polystachya* type is present in *Polystachya, Ansellia, Aërangis, Liparis, Calanthe,* and *Microcoelia.* The *Eulophia* type is restricted to genus *Eulophia* and the *Epidendrum* type is only present in *Epidendrum* species.

Apomictic and cleavage polyembryonate embryos were observed in *Eulophia petersii, E. horsfallii* and *E. angolensis.* The embryos resulting from apomixis develop from cells of nucellus near the chalazal end while those of cleavage polyembryony are zygotic in origin. The reason for the rareness of multiple embryos in other taxa is not known.

Six suspensor types were identified and named based on their ontogeny and whether they are organised as single cells, filaments of cells or juxtaposed strands of cells; viz. types A, B, C, D, E, F and G were identified. The type A suspensor which was observed only in *Bulbophyllum* and is tubular, constricted at the middle portion with the nucleus at the rounded terminal end. Type B suspensors (found in *Liparis, Cribbia* and *Calanthe*) are broad at the base, narrowing towards the tip, with the nucleus situated at the base. Type

C suspensors, found in *Diaphananthe* and *Angraecum,* have four long strands of eight cells each, which are juxtaposed. Type D suspensors, occurring in *Oeceoclades* and *Eulophia streptopetala* var. *stenophylla,* are unicellular, bifurcating at the base to give long branches stretching to the micropyle, and are filled with granular substances. Type E suspensors, which have four short juxtaposed cells with prominent nuclei near the base, are found in *Cyrtorchis, Rangaeris* and *Angraecopsis*. The type F suspensors, found in *Eulophia,* are long filaments of about 12-20 cells. The Type G suspensor is single-celled, and is generally short and elongate, as in *Polystachya, Aërangis, Ansellia, Acampe, Chamaeangis, Tridactyle*, being massive only in *Microcoelia.*

Endosperm was not observed in any of the species but primary endosperm nucleus was observed in *Eulophia, Ansellia* and *Polystachya.* From this study, it is concluded that endosperm formation is rare in orchids.

Seeds were varied in size and morphology. Size was variable and overlap among populations of a species is common. The seeds are generally minute, although large ones are found in the genus *Epidendrum.* Seed size ranged from 174 to 2881 μm long and 34 to 291.6 μm wide. Variation in sizes was quite significant also in the cells, their morphometry ranging from 30.59 to 195.1 μm in length and 1.15 to 90.363 μm in width. Descriptive characters such as wavy micro-ornamentation of epiphytes as opposed to reticulate type found in terrestrial seeds, were highly diagnostic as were clinal walls, and testal extensions.

By use of diagnostic characters of seed coats for comparative purposes, seeds were clearly distinguishable into epiphytic and terrestrial ones using multivariate analyses. Furthermore, five distinct seed types were recognised in the study taxa. Type A seed was

found in *Aërangis brachycarpa*, *A. luteoalba*, *A. confusa*, *A. coriacea*, *A. thomsonii*, *A. ugandensis*, *Polystachya adansoniae*, *P. cultriformis*, *P. bennettiana*, *P. fusiformis*, *P. dendrobiiflora*, *P. spatella*, *P. campyloglossa*, *P. tessellata*, *P. vaginata*, *Angraecum decipiens*, *A. dives*, *Angraecum erectum*, *Angraecopsis amaniensis*, *Rangaeris amaniensis*, *Cribbia brachyceras*, *Chamaeangis odoratissima*, *C. sarcophylla*, *Microcoelia exilis*, *M. stolzii*, *M. koehleri*, *Bolusiella iridifolia*, *Cyrtorchis praetermissa*, *Oberonia disticha*, *Tridactyle bicaudata;* Type B belonged to *Diaphananthe lorifolia*, *D. tenuicalcar*, *Solenangis aphylla*, *S. wakefieldii*, *Polystachya bella* and *P. confusa* while Type C was found in *Epidendrum cinnabarinum*, *E. ibaguense*, *Oeceoclades saundersiana*, and *Ansellia africana;* Type D included seed of *Eulophia angolensis*, *E. horsfallii*, *E. ovalis*, *E. petersii*, *E. speciosa*, *E. streptopetala var. stenophylla*, *E. streptopetala var. streptopetala*, and Type E seed was found only in *Liparis bowkeri* and *L. desteli*.

Orchid roots are mycorrhizal and the identified fungal isolates from the roots belong to the groups Basidiomycotina or fungi imperfecti based on the monillioids or conidiophores produced in pure cultures. The mycorrhizal relationships in all cases revealed host-and-habitats specificity.

Reliable *in vitro* seed germination methods were developed for species of *Eulophia* and *Polystachya*. Symbiotic germination of the seeds showed that effective germination follows response to fungal infection. On germination in the symbiotic system under laboratory conditions a period of 60 days was required in order to develop protocorms, which is a shorter period than that required by asymbiotic germination of seeds in the control experiments. However, it was noted that the host-fungus relationship *in vitro* is

highly specific and germination is almost certain if the seeds and fungus were derived from the same orchid.

Both symbionts were highly selective in their requirement of nutrient agar with additives such as raw banana homogenate and activated charcoal. Although extending experiments to field conditions was not possible due to limited time of the study, it is possible that the production of seedlings *in vitro* and subsequent replanting *ex situ* may be an effective means of saving some orchids from extinction.

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TABLE OF CONTENTS

List of Tables

Page

List of Figures

Page

PREFACE

Flower in the crannied wall,

I pluck you out of the crannies;-

Hold you here, root and all, in my hand,

Little flower-but if I could understand

What you are, root and all, and all in all,

I should know what God and man is.

TENNYSON

Chapter 1: INTRODUCTION

Contents

1.1: Introduction to the Orchidaceae

The Orchidaceae are probably the largest family in the flowering plants, the only possible rival being Asteraceae (Schweinfurth, 1959; Cronquist, 1968; 1981; Hutchinson, 1973; Arditti, 1992). Estimates of the size of the family vary greatly: Garay (1960, 1972) records 30,000 species in 800 genera; other estimates include 18,000 species in 750 genera (Heywood, 1978), 30,000 species with unspecified number of genera (Smith, 1977), 20,120 species in 736 genera (Thorne, 1992), 25,200 species with number of genera unspecified (Rasmussen, 1985, 1986; Atwood; 1986), 19,500 species in 750-800 genera (Takhtajan, 1997); 25,000 species in an unspecified number of genera (Burns-Balogh and Funk, 1986), 17,000 in 750 genera (Hunt, 1967, 1969; Summerhayes, 1968), 25,000 to 35,000 species in 800 genera (IUCN, 1998), 15,000 to 20,000 species in 1000 genera (Cronquist, 1981), 19,500 species in 796 genera (Dressler, 1993) and 19,468 species in 777 genera (Linder and Kurzweil, 1999).

The difficulty in giving the exact number results not only from the continual discovery of new species but also from the periodic rearrangement of those that are known.

Interestingly, however, of all the known orchid species three-quarters are epiphytes (Atwood, 1986; Benzing, 1986; la Croix, 1997).

This family is also widely distributed, colonizing almost all habitats in the world except the most extreme: notably the Arctic, Antarctica, deserts, and alpine mountain peaks. However, the largest proportion of the species occurs within the limits of the tropics. Dressler (1981) estimates that there are about 8,200 species in tropical Central and South America and 6,800 throughout the whole of South-east Asia including New Guinea and Northern Australia. This wide habitat range is partly due to the presence of both terrestrial (tuberous or bulbous plants) and epiphytic species. The family can therefore be found both in grasslands and in forests.

Orchidaceae is a rapidly evolving family (Dressler, 1993). The orchid family is characterised by a profusion of species, many with a highly restricted geographical distribution range. The family is often singled out as particularly prolific in respect of speciation. An intuitive recognition of such patterns may be expressed in comments such as: "The orchid family is still in a state of evolutionary flux" (Garay, 1960), or "Orchidaceae have all earmarks of a group in active evolution; species, genera, tribes and subtribes are all difficult to delimit" (Dressler, 1993). Further, the adaptations that are associated with mycotrophy and pollinator syndrome may have been a driving force in the speciation of the numerous orchids that are known today.

Many orchids exist in small populations in very specialized habitats (Sanford, 1974), although their seeds can be dispersed over a long range. This sets a scenario in which small populations, perhaps consisting of only one individual, can easily become established distant from the mother population, making further exchange of pollen unlikely. This in turn could stimulate speciation by founder effects, differential selection pressure or genetic drift.

Dressler (1993) provides a detailed system of classification for the family, which is widely used particularly for the Epidendroid orchids with more than 15,000 species in 524 genera (F. N. Rasmussen, 1995), some of which form the study group for the present work. The species composition of the Epidendroideae is probably more than that of earlier records, as Linder and Kurzweil (1999) have recently estimated 15,747 species in 576 genera.

The orchids of East Africa have attracted much interest from internationally renowned taxonomists and hobbyists. These, together with local orchidologists, have made major contributions to making the orchids well known by depositing materials in the East African and Kew herbaria. Their publications in internationally reputable checklists, floras, *The American Orchid Society Bulletin* and the *Journal of East African Natural History Society* (*JEANHS*) have made the orchids widely known. In Kenya the first description of the family Orchidaceae was included in the Flora of Tropical Africa by Rolfe (1898) and Piers (1951) gave a more pertinent account of orchids in Kenya. Particularly distinguished, though, is the work of Copley *et al.* (1964) who for the first time constructed an artificial key for their collections of Kenyan orchids, which comprised 192 species in 30 genera. Similar extensive collection and documentation was reported by Piers (1968), who recorded 250 species in 55 genera for East Africa. The local orchidologists, mainly hobbyists, played a complementary role in keeping threatened species in gardens, with the assistance of the orchid societies in their respective countries. It must also be appreciated that through their cultivation of the local orchids many positive achievements have been recorded in newsletters, especially on the ecology of local orchid species. These efforts, especially by the Kenya Orchid Society (KOS), have led to the publication of a book on the orchids of Kenya, which

highlights the current distribution of some of the orchids in different vegetation types of the country (Stewart, 1996).

The list of orchids of East Africa has recently been revised and updated, which has provided a reasonably accurate assessment of the distribution and taxonomy of these orchids, as published in the *Flora of Tropical East Africa* (*FTEA*) (Summerhayes, 1968; Cribb, 1984, 1989) in which details of 76 genera in two subfamilies are given. The orchids are reported as distributed all over the floral region. They are adapted to tropical climates and to a wide range of ecological habitats. The FTEA lists 689 species of orchids as occurring in East Africa: 253 are found in Kenya, 537 in Tanzania and 304 in Uganda (Table 1.1). Although the distribution details of the orchids are far from complete, endemism in East Africa has been computed using endemic fraction (Bytebier, 1995), calculated as number of endemic species, divided by total number of species present in the country. Several species are only known from the type collection and hence automatically show up as endemics. There are *ca.* 212 orchids endemic to East Africa, Kenya having 14 endemics, Uganda 25 and Tanzania 118 (of which two are endemic to Zanzibar Island).

Table 1.1: Number of orchid species per country, and total for East Africa (Adapted from Bytebier, 1995).

Tribe	Kenya	Tanzania	Uganda	Total
Epidendreae	81	189	121	247
Neottieae	4	10	7	11
Orchideae	91	215	84	253
Vandeae	77	123	92	178
Total	253	537	304	689

To date 25 rare orchids, six endangered, one vulnerable and one indeterminate (Table 1.2) have been documented for East Africa in the International Red List of threatened plants (International Union for Conservation of Nature and Natural resources (IUCN), 1998). The IUCN report underscores the fact that the orchids are threatened globally because of horticultural collection and trade.

Table 1.2. IUCN list of orchids of East Africa and their conservation status. The names of authors follow the Flora of Tropical East Africa. Ex = presumed extinct, E = endangered, V = vulnerable, R = rare, I = indeterminate (After IUCN, 1998).

Species	Conservation status						
	country	Ex	Ex/E	E	V	R	I
1. *Bonatea eminii* (Kraenzl.) Schltr.	Tanzania					*	
2. *Bonatea rabaiensis* (Kraenzl.) Rolfe	Kenya Tanzania					* *	
3. *Bonatea stereophylla* (Kraenzl.) Summerh.	Tanzania					*	
4. *Bonatea tentaculifera* Summerh.	Kenya					*	
5. *Bonatea volkensiana* (Kraenzl.) Rolfe						*	
6. *Brachycorythis tanganyikensis* Summerh.	Tanzania					*	
7. *Cynorchis usambarae* Rolfe	Tanzania					*	
8. *Disa longilabris* Schltr.	Tanzania						*
	Tanzania			*			
10. *Disa walteri* Schltr.	Tanzania					*	
11. *Disperis aphylla* Kraenzl. ssp. *bifolia* Verdc.	Tanzania					*	
12. *Disperis decipiens* Verdc.	Tanzania					*	
13. *Disperis egregia* Summerh.	Tanzania					*	
14. *Disperis parvifolia* Schltr.	Tanzania					*	
15. *Habenaria apiculata* Summerh.	Tanzania					*	
16. *Habenaria burtii* Summerh.	Tanzania					*	
17. *Habenaria busseana* Kraenzl.	Tanzania					*	
18. *Habenaria cordifolia* Summerh.	Tanzania					*	
19. *Habenaria haareri* Summerh.	Tanzania			*			
20. *Habenaria keniensis* Summerh.	Kenya				*		

21. *Habenaria nyikana* Rchb. f. ssp. *pubipetala* Summerh.	Tanzania			*			
22. *Habenaria occlusa* Summerh.	Tanzania					*	
23. *Habenaria odorata* Schltr.	Tanzania					*	
24. *Habenaria richardsiae* Summerh.	Tanzania					*	
25. *Habenaria thomsonii* Reichb. f.	Kenya			*			
26. *Holothrix pentadactyla* Krzl.	Kenya			*			
27. *Holothrix pleistodactyla* Krzl.	Tanzania			*			
28. *Holothrix triloba* (Rolfe) Krzl.	Tanzania					*	
29. *Pterygodium ukingense* Shltr.	Tanzania					*	
30. *Satyrium aberrans* Summerh.	Tanzania					*	
31. *Satyrium comptum* Summerh.	Tanzania					*	
32. *Satyrium kermesiana* Krzl.	Tanzania					*	
33. *Satyrium monophyllum* Krzl.	Tanzania					*	

The orchids of Kenya have been a major concern of conservationists because many of them are listed in the endangered category of the Kenyan Red Data Book ratings. It is estimated that in addition to the orchids listed in the IUCN report, another one hundred species are vulnerable according to the recent estimates (Khayota, 1989; Patel, 1992). This constitutes 37.7 percent of all the local species. In addition, several species may already be extinct. For example, *Polystachya fischeri* Schltr. was last collected in 1885, and since then its original habitat has been destroyed (Plant Conservation and Propagation Unit (PCPU), 1995). It is also feared that *Bonatea tentaculifera* Summerh., which has only been found once (in 1945), is already extinct (Mungai *et al.*, 1996). However, it is to be questioned whether such species, known only from the type collection as occurs in virtually every flora, are indeed "good" species, or just odd plants. More realistic as endangered species are those, which are well recorded from the past, but which, have now disappeared.

The three threats for extinction of the Kenyan orchids are:

1. Deforestation: Because *ca.* 119 Kenyan epiphytes are dependent on forest habitats their future is seriously endangered by forest destruction. Forests in Kenya are considered to be areas of high potential for agriculture. Therefore, in most parts of the country, deforestation is carried out to create farmlands for the growing population. Currently statistics (Republic of Kenya, 1998b) show that an average of 500 hectares of forests are being lost annually. Thus most of the epiphytic orchids have been destroyed. The terrestrial orchids that grow within the forest vegetation or in grasslands are usually destroyed when actual preparation and cultivation for crops takes place. In some regions the exposure of cleared forest land to heavy grazing by cattle and goats destroys the terrestrial orchids when the animals trample on the orchid pseudobulbs, so that

regeneration of young shoots is not possible. A survey of the orchids of the Taita hills (Beentje and Ndiangui, 1988) revealed that six out of 27 species are locally rare: *Polystachya albescens* Lindl., *Angraecum erectum* Summerh., *Platylepis glandulosa* (Lindl.) Reichb. f., *Ansellia africana* Lindl., *Tridactyle cruciformis* Summerh., and *Tridactyle tanneri* Cribb. This rarity was attributed to increased burning and clearing of forest cover for cultivation. An earlier study carried out in the Ngong hills (Khayota, 1987) revealed that of the 21 species in 13 genera previously recorded by the East African herbarium, only 17 species in 11 genera were still in existence. Eight species in seven genera which were known to have previously existed in the area were not re-located while four species were new to the area.

The encroachment on forests by a rapidly growing population to create farms is a long-standing problem. Based on a growth rate of 2.7 % in 1995 (instead of the earlier 3.3 % after the 1989 census), Kenya's population is expected to touch the 30 million mark by the turn of the century (Republic of Kenya, 1998a). This has serious implications for the conservation of orchids as more agricultural land will be required to settle people. Trees are logged as a source of timber (Lovett, 1989; Beentje, 1990). In the open forests, particularly in the Northern and Eastern provinces, the scattered trees, shrubs and scrubs are cut for wood fuel and charcoal-making. It is through these deforestation activities, as detailed in Faden *et al.* (1988), that some orchids are endangered. The rate of loss of habitat type to agriculture appears to be accelerating.

2. Destruction of epiphytic habitats: Removal of moss plants destroys the epiphytic habitat of many forest orchids. The moss plants plucked off the trees by vendors are sold to wreath makers in the major cities of Kenya. This leaves the orchid plants scattered on the forest floor and later they die. Moss plants are known to have several supportive advantages to orchid life more especially in sustaining the fungi that form symbioses

with epiphytic orchids. They also form a mantle for orchid root attachment and retention of moisture for nutrient recycling.

3. Overcollection of the orchids in the wild by professional collectors: Since restrictions prohibiting the collection of orchids in the wild in Kenya have not yet been successfully implemented there is illegal collection of orchids for sale as ornamental plants. The government's conservation agent, the Kenya Wildlife Services (KWS), requires that a collection permit be obtained before any collection is done in forest reserves or national parks. Even with this provision, both foreigners and locals still do poach orchids. As no steps have been taken to punish the offenders, collection of plants for sale in major cities of the country is rampant. The people most notorious for this are the hotel owners, most of whom want orchids to display in the hotel gardens and on trees in the grounds to attract tourists, who themselves are orchid lovers. They use young unemployed boys to collect orchids from the wild. Most of the specimens end up overseas, as reported for *Ansellia africana* (Khayota, 1989). The whole family Orchidaceae is listed in Appendix 2 of International Convention of Trade in Endangered Flora and Fauna (CITES) so that, contrary to what many people seem to believe, trade in wild and artificially propagated specimens is allowed, subject to a licence. As yet no African species of orchid is listed in Appendix 1 which are species in which trade is completely prohibited (la Croix, 1997).

Ansellia africana Lindl., for example, occurs in very restricted habitats in the great Rift Valley and the Indian Ocean coastal area and has featured greatly in conservation studies. The species, because of its spotted large flowers, is frequently uprooted for sale in hotels and, according to studies of Khayota (1996); commercial collection of the plant has contributed to its rarity today. My own survey in Mombasa and Nairobi (Ochora, unpublished data) has shown the following orchids to be in great demand in the local

flower vendor markets: *Ansellia africana* Lindl., *Angraecum erectum* Summerh., *A. eburneum* Bory, *Bulbophyllum* spp., *Aërangis* spp., *Eulophia horsfallii* (Batem.) Summerh., *E. angolensis* (Lindl.) Reichb. f., *Polystachya* spp., especially. *P. bella* Summerh.

1.2 Conservation status

The International Union for Conservation of Nature and Natural Resources (IUCN) (1986) has emphasized the danger to plants, including orchids, due to the habitat destruction world-wide. This has great implications on the survival of orchids as they cannot exist without the host forests since these provide specialized microclimatic conditions and the protection for the orchids. As a result the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) (1979) [cited in Paradhan (1982)] has listed the entire family Orchidaceae as an endangered group. Efforts to conserve rare and endangered orchids on a global scale have only been possible with some success through *in vitro* techniques developed by earlier workers, as summarised in Arditti (1982).

Conservation in Kenya has, in the past been directed towards resources that are of immediate and direct economic value, such as wildlife, water catchment areas and a few plant groups such as *Aloe* spp. (Kang'ethe, 1989). Recently the need to conserve species of Orchidaceae in Kenya, mainly for their horticultural value, was highlighted (Plant Conservation and Propagation Unit (PCPU), 1992) [cited in Simiyu (1996)]. Conservation achievements by PCPU as far as orchids are concerned (Simuyu, 1996) are *ex-situ* propagule accessions of *Angraecum eburneum* Bory ssp. *giryamae* (Rendl.) Cribb & Senghas and *Ansellia africana* Lindl., and asymbiotic germination of epiphytes. The major hindrance to PCPU's conservation work is the lack of understanding of

mycorrhizal relationships (Bytebier *et al.*, 1996). These authors consider the study of symbiotic relationships, especially mycorrhizae, essential to any conservation work in Africa, since most of the indigenous species display these relationships. The role of symbiotic seed germination in orchid conservation has not been examined in detail for the Kenyan orchids.

Since there is rapid destruction of natural habitats and considering the great interest in cultivating orchids, it is natural to try to save orchid species in gardens by using *in vitro* techniques such as the ones described in this thesis. It is also realised that unless orchids provide income of some sort at the national or local level, orchid conservation will be an uphill task. For this reason orchids for scientific study should be grown in botanic gardens and supported by the local community through awareness programs aimed at educating them about the usefulness of orchid conservation. The rare and endangered species need to be propagated and distributed or sold at reasonable prices to as many orchid lovers as possible to discourage uncontrolled collection from the wild. It also seems that growing of these orchids, especially the large flowered, genera by local peasants or flower vendors can succeed as a horticultural venture only if they do it collectively because the investment is costly, involving construction of greenhouses and use of expensive chemicals. There is also the need to employ professional growers in order to get the best results. The gardening techniques already exist among the Kenya Orchid Society (KOS) members, most of whom are licensed to grow exotic hybrids and local orchids as a hobby. In a broader sense, as a supplementation to conservation by growing orchids *ex situ,* hilltops should be reserved and targeted because of their great plant biodiversity and their lesser demand for growing crops by farmers. Other target sites should be the protected nature reserves and game parks because they are gazetted by

the government and almost free from human encroachment (see Fig. 1.1 and Fig. 1.2). The potential for conservation in such safe sites is high (Molski, 1979).

1.3 Orchid seeds

Nearly all orchids have dust-like tunicate seeds (Dahlgren & Clifford, 1982). The term tunicate is applied to orchid seeds because only the uniseriate epidermis of the outer integument persists in the mature seed. This forms a hollow space in which the embryo is found. This seed structure is undoubtedly related to the mycotrophic lifestyle of orchids and has apparently evolved under selective pressure to ensure post-dispersal infection by fungi (Leake, 1994).

Seed morphology in Orchidaceae has been studied in various genera in order to elucidate their systematic position and has revealed a tremendous variety of seed morphological features (Arditti *et al.*, 1979, 1980, 1992; Healey *et al.*, 1980; Barthlott and Ziegler, 1981; Tohda, 1983, 1985, 1986). The sculpturing of the seed coat is regarded as being systematically and phylogenetically useful at or above subtribe level (Barthlott, 1976; Barthlott and Ziegler, 1981; Dressler 1981, 1986, 1993).

Considerable interspecific variation in seed coat ornamentation has been found in many orchids. Seed morphology even shows clinal variation within a genus, which seems to be maintained by climatic selection in different geographic ranges and ecological diversities. Morphology and development of seeds need to be seen in a functional and ecological context. Morphological adaptations of seeds to various habitats needs investigation because the possible ecological correlates have not been adequately documented.

Growing orchids from seed is a complex matter that has led to extensive literature. The problems have been linked to the mycotrophic nature of the orchid seeds. Perhaps the

evolution of mycotrophic seed as well as production of enormous numbers of very minute seeds (up to 4 million in one seed capsule) is the major reason for the successful life of orchids (Saskena, 1960; Rasmussen, 1995). Uniqueness of orchids from other angiosperms is noticed even in their reproductive capacity. For example, efficiency in the utilization of resources of reproduction of most Angiosperms demands that, for any particular type of habitat, a species of flowering plant should produce seeds of a size optimal for seedling establishment in the given habitat (Salisbury, 1942). Thus, species of open habitats, such as grassland, typically produce a large number of small seeds, because the young seedling soon reaches light of a sufficient intensity for it to become self-supporting, and so a relatively small store of food material in the seed is adequate for establishment. But for species of closed habitat, e.g., woodlands where young seedlings grow up in poor light under an often dense canopy of tree foliage, it needs to be supported from the food reserves in the seed for a much longer period. Orchids differ from all other angiosperms by producing large quantities of seeds devoid of stored food in both habitats and which take a long time to develop leaves and thereafter self supporting.

1.4 Embryology

Embryology is important not only in systematic consideration of Orchidaceae but also in genetic diversity. Studies of orchid embryology, though generally rare, are very useful in understanding the different patterns found in the family. Similarities in ontogenetic studies of embryonic structures can be used as evidence for close relationship among taxa. It was embryology (nuclear endosperm formation), for example, which was used to link Orchidaceae with the Liliales (Dahlgren and Rasmussen, 1983).

The need to have a comprehensive knowledge about orchid embryos has arisen because of the paramount importance currently attached to seeds as sources of genetic diversity for conservation of orchids (Clements, 1982, 1995; Clements *et al.*, 1986; World Research Institute (WRI), United Nations Environmental Program (UNEP) and International Union for Conservation of Nature and Natural Resources (IUCN) (1992); Rasmussen, 1995). The main objective of conservation is to conserve genetic diversity, which is obtainable only through seeds in the wild or tissue culture, as opposed to clonal tissue culture whereby plant material is genetically identical. These genetic factors are found in the embryos hence the need to study orchid embryos. Moreover the first step for *in vitro* conservation involves storage of seeds in seed banks to provide seedlings for growing *ex situ* or *in situ* (IUCN, 1992). The reduced size of the seeds has meant that the standard methods for seed testing seed germinability in stored seeds using 2,3,5-triphenyl-tetrazolium chloride as a staining reagent does not work for orchid seeds (Harvais and Hadley, 1967).

It is not always easy to tell when a capsule or the seed is ripe or nearly so except by way of embryology. The only guidelines for ripening of orchid capsules (in months) have been given by la Croix (1997). The approach works for some orchids but not others, and this may result in storage of seeds with premature or aborted embryos in seed banks. Records of seeds without embryos are increasing (Stort and Pavanelli, 1984). Such a situation could be avoided by a detailed study of the embryos to determine the appropriate stage of embryo maturity before accession to seed banks.

Previously Johansen (1950), Veyret (1974) and Clements (1995) have reported great variation in orchid embryos and grouped them into various types. However, the number of publications on this subject for the Orchidaceae is far too small for such a big family. Nevertheless, the mature embryos are often described as small, being composed of

similar cells bounded by an epidermal layer, and these have been referred to as immature embryos by some authors (Coulter and Chamberlain, 1903; Davis, 1966) because the embryo does not reach the stage of differentiating organs. Investigations have shown that organs develop only after germination (Veyret, 1977). Interestingly, the embryo ontogeny takes different pathways, which if investigated for the entire family could be phylogenetically informative. It will be emphasised in this thesis that embryological work in the family should move from the purely descriptive aspects to encompass other areas especially ecology, conservation, and phylogeny.

1.5 Symbiotic seed germination

The processes involved in germination of orchid seeds and subsequent development of seedlings, including association of mycorrhizal fungi, have been well documented (Myers, 1979; Clements, 1982; Arditti *et al.*, 1982; Arditti and Ernst, 1993; Hagsater and Stewart, 1986; Koopowitz, 1986; Koopowitz and Kaya, 1983; Harley and Smith, 1983; Rasmussen, 1995; Zettler *et al.*, 1999). Nevertheless, many aspects of this subject have not been exhaustively covered and warrant further investigation. Mycorrhizal symbiosis is a subject of research that is complex, partly because fungal taxonomy is difficult. An objective of the present study was to assess whether symbiosis is of much importance in the conservation of Kenyan orchids.

1.6 Phylogeny

Phylogenetic studies using parsimony so far indicate that the Orchidaceae are a monophyletic family in which members share a suit of characters that set them apart from other angiosperms. The monophyly of Orchidaceae is supported by descriptions of many taxonomists (Garay, 1960; Dressler and Dodson, 1960; Vermeulen, 1966; Gray, 1972; Dressler, 1981, 1993; Rasmussen, 1985, 1986; Burns-Balogh and Funk, 1986; Szlachetko, 1995).

The first cladistic study of the Orchidaceae as a whole was that of Burns-Balogh and Funk (1986) whereby 42 floral characters for 26 tribes of the family were used. Although the resultant cladogram was used to develop a classification and a natural key to the tribes, the classification suffered several shortcomings. The classification was not explicit in the methods used to generate the tree shown; neither was it clear whether it was the only tree at that level of optimality. The tree does not wholly correspond to the data matrix presented. Vegetative characters were excluded from the analysis and there are significant questions about character-state recognition and interpretation. Nevertheless, the analysis (Burns-Balogh and Funk, 1986) revealed seven subfamilies divided into 20 tribes: Apostasioideae, Cypripedioideae, Neuwiedioideae, Spiranthoideae, Neottioideae, Orchidoideae, and Epidendroideae. Included in the subfamily Epidendroideae were ten tribes and the *Pleurothallis* group. The vandoid orchids were treated as a group within the subfamily Epidendroideae and not as a separate subfamily. These authors considered the treatment of the Epidendroideae as tentative because they lacked complete information on column structure.

A cladistic analysis of Orchidaceae for 98 genera using 71 morphological characters supported the monophyly of Apostasioideae, Cypripedioideae, Spiranthoideae and Epidendroideae (Freudenstein and Rasmussen, 1999). Even though the analysis faced problems related to scoring characters and complete exclusion of some characters like degree of style fusion and rostellum shape, Orchidoideae in the broad sense were found paraphyletic.

Molecular-based phylogenetic trees from both parsimony and maximum likelihood methods suggest that subfamily Epidendroideae is monophyletic with Neottieae as sister (Neyland and Urbatsch, 1996). However, similar studies by Kores *et al.* (1997) suggest that only Apostasioideae and Cypripediodeae are monophyletic. The most recent

cladistic parsimony analyses of *rbcL* nucleotide sequence data (Cameron *et al.*, 1999) divide the Orchidaceae into five primary monophyletic clades: apostasioid, cypripedioid, vanilloid, orchidoid, and epidendroid.

The findings of Cameron *et al.* (1999) recognise that within the Epidendroid clade the tribe Epidendreae is grossly polyphyletic, but Maxillarieae and Vandeae are monophyletic. Malaxideae is sister to a clade of genera from Cymbideae. Their work, however, faces major criticisms especially on the use of successive weighting to discuss the phylogenetic relationships without comparison with the topological relationships of the strict consensus tree before successive weighting. The closeness of the Vanilloid clade to the Orchidoideae clade rather than to the Epidendroid clade is seen as unrealistic since it was previously treated in the Epidendroideae (Freudenstein *et al.*, 1999).

1.7 Classification

All systematic studies have shown that Epidendroideae have been the most difficult to classify and interpret phylogenetically. For instance, Dressler (1981, 1986) has made repeated attempts to divide Epidendroideae into several natural groups. These attempts included segregating the orchids with erect anthers and soft pollinia into a distinct subfamily, Neottioideae, and placing those with stipes and superposed pollinia into Vandoideae. Dressler (1989) listed the characteristics that had previously been used to distinguish the vandoid orchids from the Epidendroid orchids: (1) early bending of the anther in floral development, (2) lateral inflorescence, (3) reduced number of pollinia (two or four), (4) superposed pollinia (if any), (5) well-developed viscidium, and (6) tegular stipe. As Kurzweil's (1987) floral developmental studies demonstrated that anther ontogeny in the vandoid orchids is similar to that in the epidendroid orchids, Dressler (1989) concluded that anther development is not a character that distinguishes the vandoids from epidendroids. These findings were reflected in Dressler's (1990)

revision in which he eliminated the subfamily Vandoideae and distributed its tribes within subfamily Epidendroideae. In that revision, Dressler recognised 19 tribes in the Epidendroideae that were segregated into three informal categories i.e. primitive tribes, the cymbidioid phylad, and the epidendroid phylad. The vandoid orchids were distributed among both the cymbidioid and the epidendroid phylads. Specifically, tribes Malaxideae, Calypsoeae, Cymbideae, and Maxillarieae comprised the cymbidioid phylad, whereas Vandeae was placed in the epidendroid phylad. Dressler (1993) contended that the vandoid orchids as a group are polyphyletic in that the tribe Vandeae is not closely related to other vandoid tribes.

In his treatment of Epidendroideae, Dressler (1993) elected to retain Neottiodeae and Vandoideae within Epidendroideae, but proposed splitting the more advanced tribes into two units: a cymbidioid phylad and an epidendroid phylad, the latter containing a dendrobioid subclade. This classification was based on vegetative features rather than floral characters, on which the division into epidendroid and vandoid orchids was based (Table 1.3).

The cymbidioid phylad essentially corresponds to those orchids with cormous growth habit and includes Maxillareae, Cymbideae, Calypsoeae, and Malaxideae. The epidendroid phylad contains Arethusaeae, Epidendreae, Coelogyneae, Glomereae, and the dendrobioid subclade containing Dendrobieae, Podochileae, and Vandeae. Most members of Epidendroid group possess a reed-stem growth form that Dressler interpreted as secondarily derived (Dressler, 1990).

According to the system of Dressler (1993) the Epidendroideae and Vandoideae, earlier regarded as separate subfamilies (Dressler, 1981 and Rasmussen, 1985), contain the majority of species within Orchidaceae, with more than 15,000 species in about 520 genera. According to Dressler (1981), Epidendroideae is less specialised than

Vandoideae. He contended that the one feature that unites the two groups is the incumbent anther that opens ventrally, and is erect in the young flower bud but subsequently bends downwards over the apex of the column until it is at right angles to the column axis at maturity.

As a result of the systematic problems recognised in the current literature regarding Epidendroideae more critical evaluation of the group is needed, using anatomy, cytology, morphology, phytochemistry, and molecular analysis.

Table 1.3: Recent classification systems of subfamilies Epidendroideae and Vandoideae. The ¤ means that the system of classification does not recognise the treatment of Vandoideae as a distinct subfamily.

Source	Epidendroideae	Vandoideae
Dressler (1993)	Malaxideae	¤
	Calypsoeae	
	Cymbideae	
	Maxillarieae	
	Stanhopiinae	
	Oncidiinae	
	Arenthuseae	
	Coelogynae	
	Epidendreae (New World)	
	Epidendreae (Old World)	
	Podochileae	
	Dendrobieae	
	Vandeae	
Burns-Balough and Funk (1986)	Malaxideae	¤
	Maxillarieae	
	Arethuseae	
	Coelogyneae	
	Epidendreae	
	Dendrobieae	
	Vandeae	
	Vanilleae	

	Gastroidieae	
	Triphoreae	
	Pleurothailis Group	
	Malaxideae	Maxillarieae
Rasmussen (1985)	Arethuseae	Cymbideae
	Coelogyneae	Vandeae
	Epipogieae	Polystachyeae
	Epidendreae	
	Vanilleae	
	Gastrodieae	
	Malaxideae	Malaxideae
Dressler (1981)	Calypsoeae	Cymbideae
	Arethuseae	Vandeae
	Coelogyneae	Polystachyeae
	Epidendreae	
	Vanilleae	
	Gasrodieae	
	Epipogieae	
	Cryptarrheneae	

1.8 Aims and scope of study

1.8.1 Aims

The fundamental aim of the research described in this thesis was to investigate the variation in embryology (megasporogenesis, megagametogenesis, and embryogenesis) and seed coat morphology in the Kenyan representatives of the orchid subfamily Epidendroideae. Members of this taxon are most "vulnerable", even though they are widespread in the country, and from the literature it is evident that they have not been studied much beyond their vegetative and floral morphology. Embryology and seed coat variation may be related to the mycological symbioses, which would be important for seed germination. The specific objective was to provide information that would be of assistance for *ex situ* conservation and cultivation of orchids.

1.8.2. Scope and limitations

The broader scope of the study was to investigate all aspects of seed ontogenesis and also symbiotic seed germination. Ultimately the results are to be used as a model for conservation where orchid seeds are kept in seed banks. When there is a need for plants – either for re-establishment in the wild, or to provide stock material for horticulture or *ex situ* conservation in botanic gardens- the plants can be grown from these seed stocks.

As only one subfamily has been studied, the results can probably not be extrapolated to the whole family. The ideal situation would be to study species representative of all the subfamilies in a comparative manner. Nevertheless, the research will provide a basis for further investigations into other Kenyan species in other subfamilies.

Figure 1.1: Map of Kenya showing location of the major hilltop forests

Figure 1.2: National Parks and Forest Reserves list.

1. Nairobi National park
2. Nairobi forest reserve
3. Mount Kenya
4. The Aberdares
5. Samburu, Buffalo Springs and Shaba National Park
6. Meru National Park, Bisadi and Rahole National Park
7. Lake Turkana
8. Lake Nakuru National Park
9. Lake Naivasha
10. Maralal National Sanctuary
11. Lake Victoria
12. Ruma National Park
13. Masai Mara National Park
14. Lake Turkana and Sibiloi National Park
15. Nasalot and South Turkana National Park
16. Marsabit National Park and Reserve
17. Tsavo National Park
18. Taita Hills Sanctuary
19. Eastern National Park
20. Amboseli National Park
21. Mt. Elgon National park
22. Malka Mari National Reserve

CHAPTER 2: EMBRYOLOGY

Contents

2.1 INTRODUCTION

The first embryological work on orchids was by Treub (1879), but possibly due to lack of suitable techniques and enthusiasm to study tiny ovules (35-50 × 50-65 μm) and embryos (3-10 × 13-30 μm) many aspects of the embryology of orchids are still insufficiently understood. Moreover, certain substances, probably tannins, that are common deposits on seed surfaces, hinder the use of traditional microtomy methods in studying orchid embryos. However, newer clearing procedures have made it possible to determine embryological patterns in Orchidaceae (Swamy, 1979; Clements, 1995).

These techniques have come into use so recently that only a very small proportion of orchids have been investigated by these means. The Orchidaceae being one of the largest families of flowering plants, there are thousands of species which have not yet been studied embryologically.

In spite of the general neglect of studies in orchid embryology, there has been a recent survey that revealed that the tiny embryos are quite variable, and that this variation tracks the phylogenetic patterns in the family (Clements, 1995). This embryological diversification is further elucidated by the presence of different ontogenetic pathways involved in the development of embryos of different groups of orchids. There appears to be little variation among species within a genus, but unrelated genera may have very different embryo structures and ontogenies (Clements 1995). This variation may either be due to phylogenetic (historical) factors, or ecological factors resulting from adaptations of orchids to their habitats and geographic regions. Unfortunately little is known about the effect of ecological factors such as sun-shade, temperature, moisture and host-tree/vegetation on embryology of orchids. Such information would be helpful, especially because orchids sporadically speciate or colonise new habitats, giving rise to questions as to whether they retain ancestral embryological characters or develop new characters in response to ecological signals in those habitats.

In this chapter the relationship between embryological characters and phylogeny or ecology is assessed. The present study has been undertaken with the objective of ascertaining whether orchids collected in different habitats (e.g. in dense forests, open bushed woodland, grasslands, swamps or dry areas) in Kenya show similarities or differences in embryological characters by virtue of merely occupying these habitats. These studies will resolve whether embryological variation is correlated primarily to the ecological gradients or phylogenetic affinities.

2.1.1 Embryology or Orchidaceae

The original discoveries of pollen grains on the stigmas and pollen tubes in the ovaries were elucidated using some orchids and asclepiads (Brown, 1831, 1833). Other early studies by Amici (1824, 1830) on the actual role of pollen in fertilization, observed in *Portulaca oleracea, Hibiscus syriacus*, and other plants, created controversies. His interpretation that the pollen tube, after emerging from the pollen grain on the stigma surface, grew until it came into contact with the ovule was highly criticised by among others Schleiden (1837). It was only after he demonstrated his observations using

species of *Orchis* (Orchidaceae) that he commanded support from other workers (Amici, 1847). Further research by Hofmeister (1849) traced the formation of the embryo from the egg right up to its maturity in the seeds of many plants including *Orchis*. This provided the basis for other discoveries on plant sexuality, e.g. embryogenesis in *Capsella* and *Alisma* (Hanstein, 1870), actual fusion of the male gamete with the female gamete in *Monotropa* (Strasburger, 1884), and double fertilization in *Fritillaria* and *Lilium (*Newaschin, 1898).

After the pioneering work of Treub (1879), several other workers became interested in studying embryology in various representatives of the Orchidaceae and the field has been reviewed by many authors (Schnarf 1931; Swamy 1949a, b; Johansen, 1950; Wirth and Withner, 1959; Davis 1966; Abe 1972; Veyret 1974; Vij and Sharma, 1986; Haig, 1990; Arditti, 1992).

2.1.2 Megasporogenesis

There are hardly any publications in which the first initiation of ovule and integument primordia are mentioned except the paper by Hofmeister (1849) on ovule and integument initiation in Orchidaceae, particularly *Orchis morio, O. variegata, O. latifolia, O. angustifolia* and *O. palustris*. The assertion in his paper that ovules of orchids, unlike other angiosperms, were initiated dermally faced criticism from Strasburger (1872), who studied ovule initiation in *Orchis, Gymnadenia* and many other angiosperms. Strasburger considered the ovule of angiosperms, including orchids, to be initiated subdermally and this conclusion was supported by a series of subsequent publications (Warming, 1872, 1873, 1874; Haenlein, 1875; Kny, 1876; Barcianu, 1874; Solms-Laubach and Zu, 1874; Vermoessen, 1911). However, disagreement still prevails over the interpretation of the orchid ovule since the question has not been resolved as to whether the initiation originates by the activity of a single subdermal initial, or several such initials (Bouman, 1974). It should be noted that Strasburger's work was founded on Hanstein's (1868) histogene theory, which no longer features in modern terminology of the nucellar and integumentary studies. The cellular initiation of ovules and integuments is unknown for Epidendroid orchids and the present study is the first to establish the initiation process and take note of any variations that can be of taxonomic importance.

2.1.3 Megagametogenesis

The process of megagametogenesis as described in many published works takes place only after successful pollination. Surprisingly, this process in Orchidaceae may take several months before its completion hence the long periods required for accomplishment of fertilization (Wirth and Withner, 1959; Arditti, 1992). This slow response, exclusively observed in orchids, makes them unique among the angiosperms (Yeung and Law, 1989). However, Veyret (1975, 1981) has made rare observations of the development of an ovule before pollination in Orchideae and Neottieae. The process of megasporogenesis, once completed, produces anatropous, tenuinucellate and bitegmic ovules (Swamy, 1944, 1949a; Johansen, 1950; Davis, 1966; Abe, 1967). The archesporial cell/megaspore mother cell may form monosporic, bisporic or trisporic megaspores (Swamy, 1949a; Abe 1967, 1972a, b). Occasional occurrence of monosporic and bisporic embryo sacs in the same species is observed (Stort and Pavarelli, 1971). However, the number of nuclei in the embryo sac varies in different genera of orchids as exemplified in Table 2.1. A survey of the research conducted on the family Orchidaceae leaves no doubt that megagametogenesis has been the most extensively investigated phenomenon.

Table. 2.1. Summary of records of megagametogenesis in the family Orchidaceae. Final embryo sac configuration: egg apparatus:polar nuclei: antipodals.

Taxon	Number of nuclei in embryo sac	Final embryo sac configuration	Reference
Vanilla planifolia	6	3:1:2	Swamy, 1947
Epipactis helleborine	8	3:2:3	Vij & Sharma, 1986
Epipactis pubescens	6 or 8	3:2:3	Brown and Sharp, 1911
Epipactis veratrifolia	8	3:2:3	Vij & Sharma, 1986
Neottia listeroides	6, 5, or 7	3:3, 3:2 or 3:4	Tuschnjakowa, 1929, Vij & Sharma, 1986
Neottia nidus-avis	6	3:2:1	Modilewski, 1918
Spiranthes pseudocordigera	6 or 7	3:3:1 or 3:2:2	Baranov, 1915
S. australis	6 or 7	3:3:1 or 3:2:2	Baranov, 1915
S. gracilis	5, 6, or 8	3:1:1	Pace, 1914
Listera microglottis	8	3:2:3	Vij and Sharma, 1986
Listera ovata	6	3:1:2	Tuschnjakowa, 1929
Corallorhiza maculata	6	3:2:1	Sharp, 1912
Broughtonia sanguinea	6	3:2:1	Sharp, 1912
Achroanthes monophyllos	6 or 8	3:2:1 or 3:2:3	Stenar, 1937
Phaius grandifolius	4, 6, or 8	3:3	Sharp, 1912
Bletia shepherdii	4, 6, or 8		Sherp, 1912
Geodorum densiflorum	6, 7, or 8		Swamy, 1947 a
Galeola septentrionalis	8	3:2:3	Kimura, 1971
Eulophia epidendreae	6, 7 or 8	3:2:1	Swamy, 1943 a
E. nuda	6, 7, or 8	3:2:1, 3:2:2 or 3:2:3	Swamy, 1943 a
Cymbidium bicolor	6 or 8	3:2:1 or 3:2:3	Swamy, 1942 a
Bulbophyllum mysorense	6	3:2:1	Swamy, 1949 a
B. neilghense	6	3:1:2	Swamy, 1949 a
Oncidium praetextum	5 or 6	3:1:1	Afzelius, 1916
Paphiopedilum insigne	6	3:1:2	Afzelius, 1916
Cypripedium guttatum	5 or 6	3:1:1	Prosina, 1930
C. parviflorum	5 or 6	3:2 or 3:2:1	Carlson, 1945
C. bicolor	8	3:2:3	Swamy, 1942
Calypso bulbosa	6	3:2:1	Stenar, 1940
Microstylis wallichii	8	3:2:3	Sood & Rao, 1986
Malaxis saprophyta	8	3:2:3	Sood, 1992
Doritis pulcherrima	8	3:2:3?	Yasugi, 1983
Cypripedium spectabile	6, or 8	3:2:1, or 3:2:3	Swamy, 1945

2. 1. 4 Embryogenesis

Although embryogenesis in many angiosperms has been widely studied in the past five decades (Maheshwari, 1950; Johansen, 1950; Davis, 1966; Bhojwani & Bhatnager, 1983; Natesh & Rau 1984; Johri, 1984; Johri *et al.*, 1992) that of Orchidaceae has been grossly neglected. Only about 100 species have been examined in any appreciable detail (Wirth and Withner, 1959; Veyret, 1965, 1967, 1972, 1974, 1976, 1982; Savina, 1965, 1972, 1974, 1978; Corrias and Villari, 1983; Karanth *et al.*, 1981; Yasugi, 1983). Johansen (1950) argued that it is very difficult to typify the embryogenesis of Orchidaceae since the exact sequence of cell divisions in the late proembryo and early embryo is imperfectly known. Matters are greatly complicated by the fact that no histogenes except the epidermis are easily identified by the time the embryo is mature (Swamy, 1949b). Studies in orchid embryogenesis are lacking to the extent that even the standard published works do not cover all major taxonomic groups in this large family of plants (Veyret, 1976).

The earliest general scheme for classifying angiosperm embryos was devised by Souèges (1936-1939)[cited in Veyret, 1974]. This utilises embryogenic laws and how they apply to the divisions of a zygote and the contribution of zygotic cells to mature embryos. Essentially the zygote divides into a small apical cell [designated *ca*] and a large basal cell [designated *cb*] and embryo types are determined by the contributions of each cell to the embryo proper. Ever since the publication of Treub (1879) illustrating the embryo segmentation sequences in a small but diverse group of orchids, the suggestion to use embryogenesis to classify orchids has been considered. Swamy (1949b) attempted the first classification of orchid embryos using Souèges's laws of embryogenesis and recognised three groups: Group A (*Asterad* type), Group B (*Onagrad* type), and Group C (*Cymbidium* form). In the *Asterad* type the suspensor initial, middle cell, and terminal cells participate in the formation of the mature embryo, whereas in the *Onagrad* type the suspensor initial gives rise to the suspensor while the mature embryo is organised from derivatives of the terminal and middle cells. The *Cymbidium* form is characterised by irregular divisions both in the terminal and basal cell. This classification has been criticised because it does not provide a clear sequence of cell divisions, so that only a few species can be assigned to definite types, many of them to the *Cymbidium* form (Johansen, 1950).

Johansen (1950) followed a different approach: he suggested basing the classification of orchid embryos on the suspensor characteristics. On the basis of the presence or absence of embryonal tubes and whether they arise directly or indirectly from suspensor cell, he recognised 13 patterns in 13 Type genera, although his scheme does not take into consideration the facts from ontogeny. Each of the patterns was interpreted, as representing an embryo type typified by a particular genus and species whose development was fully followed. For example, orchids without a suspensor or those with a single-celled suspensor were classified as Type 1, the *Epipactis palustris* pattern. Species possessing relatively uniform suspensors were classified as Type 2, the *Orchis latifolia* pattern and so on. However, generally, many of the features cited by Johansen fall within the range of variations, often within a species, rendering the classification difficult to use. For example, (1) the number of embryonal tubes varies within a species, often within the ovules of one and the same ovary; (2) the direction of growth of embryonal tubes varies; and (3) the number and shape of the terminal cells of the suspensor that extend out of the micropyle have been reported to have variation within the same genus (Johansen, 1950).

Most authors who have studied orchid embryos have found difficulties in applying either the Swamy or the Johansen systems of embryo classification to their specimens. The scheme of Veyret (1955a, b; 1956; 1957a, b; 1961a, b, 1974) adopted Souèges' (1936-1939) system to classify orchid embryos. Her system, which is considered laborious and concentrates only on developmental details of the embryo proper to ascertain well-definable patterns to mature embryo stage, is nevertheless rather popular. The system identifies four proembryo tetrads, A1, A2, C1 and C2, as represented in the Orchidaceae. Further development is followed using conventional abbreviations in a series of stages from the tetrad to a mature embryo. This system of classifying embryos will be considered in some detail in the present study on Orchidaceae.

The uniqueness of orchid embryos is due to the fact that at maturity they are reduced, lacking the differentiation of embryonic organs found in other angiosperms, for instance, the cotyledon, hypocotyle and apical meristems (Maheshwari, 1950, Johansen, 1950, Davis, 1966, Bhojwani and Bhatnarger, 1974). Attention to the formation of embryonic organs in Orchidaceae is rare, but as Veyret (1983) briefly reports, it takes place at germination.

2. 1. 5 Polyembryony and apomixis

Polyembryony and its genetical implications are of considerable interest to the plant breeder, horticulturist and conservationist (Maheshwari and Sacher, 1963). The phenomenon of polyembryony has attracted much attention since its discovery in oranges (*Citrus* sp.) by Leewenhoek in 1779 [cited in Maheshwari, 1950]. Polyembryony as recorded for the Orchidaceae results almost exclusively from sexually produced embryos by cleavage, budding or proliferation during early stages of development or through apomixis (Weber, 1940; Stebbins, 1941; Swamy, 1942; 1943b; 1946b; 1949b; Gustafsson, 1946, 1947a, b; Johansen, 1950; Wirth and Withner, 1959).

It is a well known fact that the simplest method of increase in the number of embryos is through cleavage of the zygote or proembryo into two or more units (Maheshwari, 1950). This type of polyembryony is less common in orchids. Further, it has been observed that seeds of many genera and species of orchids, when planted on nutrient medium, proliferate into masses of undifferentiated cells, which can give rise to considerable numbers of plants, indicating the presence of cleavage embryony in orchids during germination. Usually polyembryonate seeds of orchids contain two embryos (Ansari, 1977; Chual and Rao, 1978; Singh and Thimmappaiah, 1982; Stort and Pavanelli, 1985) and cleavage polyembryony occurs only sporadically in Orchidaceae (Swamy, 1943). In only one genus, *Vanda,* does polyembryony occur during germination (Rao, 1964). Zygotic polyembryony as authentically illustrated in Orchidaceae shows consistency and is hence an important taxonomic character (Swamy, 1949b). The recorded instances of polyembryony show that in orchids a wide diversity of processes may be found, and that polyembryony is found throughout the family (Table 2.2).

Table 2.2. The occurrence and nature of polyembryony in the Orchidaceae (After Swamy, 1949b).

Species	Subfamily	Nature	Reference
Cymbidium bicolor	Epidendreae	Vertical or oblique division of zygote	Swamy, 1942
Habenaria platyphylla	Orchidoideae	Vertical or oblique division of zygote	Swamy, 1942
Eulophia epidendraea	Epidendroidreae	Irregular division of zygote-embryos from chalazal end	Swamy, 1943
Spathoglottis plicata	Epidendroideae	Cleavage embryony	Ansari, 1977
Geodorum densiflorum	Epidendroideae	Cleavage embryony	Ansari, 1977
Cephalanthera damasonium	Epidendroideae	Parthenogenesis	Hagerup, 1947
C. latifolia	Epidendroideae	Parthenogenesis	Hagerup, 1947
Listera ovata	Epidendroideae	Parthenogenesis	Hagerup, 1947
Orchis strictifolia	Orchidoideae	Both synergid and egg develop to maturity	Hagerup, 1947
Spiranthes australis	Spiranthoideae	Cells of nucellus develop into embryo	Swamy, 1946
S. cernua	Spiranthoideae	Inner integument produces apomictic embryos	Swamy, 1948
Nigritella nigra	Orchidoideae	Adventive embryony	Afzelius, 1928

A number of orchid species are known that produce polyembryonic seeds without the process being known. They are *Orchis latifolia, O. morio, Goodyera tessellata, G. pubescens, Aplectrum hiemale, Habenaria tridentata, H. blephoriglottis, Satyrium nepalense,* and *Cymbidium calceolus* (Withner, 1974).

Apomictic polyembryony, which is less reported in members of the Orchidaceae, has consequently received less attention for consideration in taxonomic studies. The formation of apomictic embryos is variable in terms of the ovular tissues that form them. Swamy (1949b) and Karanth *et al.* (1981) observed diploid apomictic embryos from the innermost layer of cells of inner integuments in *Zeuxine* and from nucellar epidermis in *Nigritella*. Dyad cells resulting from megaspore mother cell are also recorded as sources of parthenogenetically produced diploid embryos of orchids (Afzelius, 1928, 1932; Swamy, 1946b, 1948b). Alternatively haploid apomictic embryos form from the unfertilized eggs (Hagerup, 1944, 1945, Veyret, 1967, 1982) or synergids (Hagerup, 1947) of the embryo sac. Haploid embryos may be initiated prior to penetration of the embryo sac by the pollen tube or soon before fertilization (Seshagiriah, 1934, 1941). All forms of apomixis can be placed in three categories, termed diplospory, apospory, and adventive embryony (Maheshwari, 1950; Bhojwani and Bhatnager, 1983, Koltunow, 1991; Stort and Pavanelli, 1985). The key issues that have not been looked into in detail are whether the apomictic embryos have ontogenetic events of development that are similar to those produced through sexual means or whether polyembryony in general can provide information necessary for understanding the interrelationships among taxa.

2. 1. 6 Ontogenesis of suspensor

The suspensor in the Orchidaceae and angiosperms in general arises from the basal cell (*cb*) resulting from the first division of the zygote. Traditionally the suspensor was believed to function by positioning the embryo in a nutritionally favourable position in relation to endosperm, a nutritive tissue (Maheshwari, 1950; Bhojwani and Bhatnager, 1983). Most recent literature supports the speculation that the suspensor supports development of the embryo by absorption and transportation of nutrients from ovular tissues to the embryo proper (Yeung, 1980, 1993; Yeung and Meinke, 1993). However, the suspensor is absent in a minority of plants including some orchids (Treub, 1879; Swamy, 1949b; Veyret, 1956a, b, 1974; Natesh and Rau, 1984; Johri *et al.*, 1992). Treub (1879) concluded from his studies on orchids that the suspensorless forms are less resistant in the struggle for existence than those other orchids which possess a suspensor.

His findings were strongly supported by Coulter and Chamberlain (1903) especially for *Listera*, *Epipactis*, *Goodyera*, and *Spiranthes,* all of which are suspensorless.

The suspensor shows great diversity with regard to its size, shape, and the number of component cells. Treub (1879) described a number of forms assumed by the suspensor in six genera of Orchidaceae. According to his interpretations the suspensor grows out of the micropyle, often branches, and embeds itself in adjacent placenta (in *Phalaenopsis grandiflora*) or turns towards the embryo and finally envelops it (in *Peristylus grandis*). Ward (1880) reported other interesting forms in *Gymnadenia conopsea,* in which the first division of the fertilized egg is transverse, the basal cell forming a chain-like suspensor of eight to ten elongated cells that push through the micropyle into the ovary cavity. Sometimes the suspensor is formed from two very long cells with tips embedded into the placenta or consists of six or seven cells extending to the integuments or tissues of the funiculus (Leavitt, 1901). Since these early studies many more variations in suspensor morphology have been observed and described (Swamy, 1949; Johansen, 1950; Veyret, 1974; Chua and Rao, 1978; Corrias and Villari, 1983). As existing studies of suspensor are based on a small number of mainly European orchids it was decided in the present study to investigate further suspensor ontogenesis and suspensor organisation in mature seed, and to elucidate possible types found in the less studied Epidendroid orchids.

2.1.7 Endosperm

Endosperm, believed to be a product of a double fertilization process due to fusion of polar nuclei with the second sperm nucleus, is frequently reported to be absent or suppressed in Orchidaceae, as in Trapaceae and Podostemaceae. However, a few instances have been reported of Orchidaceae where the primary endosperm nucleus, contrary to earlier records, undergoes slight further development (Swamy, 1949b). The earliest observations of development of endosperm in orchids were made in *Cypripedium* (Pace, 1907; Prosina, 1930), *Paphiopedilum* (Afzelius, 1916), *Chamorchis* (Afzelius, 1916) and *Vanilla* (Swamy, 1947). Endosperm formation, when it occurs, is nuclear but only two to four endosperm nuclei are formed except in *Vanilla planifolia* where ten nuclei are reported (Swamy, 1949b). However, the concept of double fertilization has not been proved, as the actual fusion of polar nuclei with the sperm nucleus has not been observed in any recent studies but is only inferred from cellular contents of the embryo sac. Cronquist (1968) questioned why the second sperm should fuse with two other nuclei instead of only one and holds as wholly speculative that triple fusion initiates food

storage tissue in angiosperms. Sargant (1900)[cited in Lopes and Larkins, 1993] had hypothesized that endosperm originated from production of ancestral angiosperms from gymnosperms, later evolving into a storage organ. Despite the support of this hypothesis by studies on *Ephedra trifurca* (Lopes and Larkins, 1992), double fertilization in angiosperms is poorly understood empirically.

Recent detailed investigations show that lack of endosperm in the orchids may be due to single fertilization. Terasaka *et al.* (1979), working on *Spiranthes sinensis,* and Albert (1992), using fluorescent microscopy and computer imagery techniques for *Cypripedium cordigerum,* have raised doubt about double fertilization in orchids. There may also be endosperm reduction after triple fusion (Teryokhin and Kamelina, 1969, 1972). Studies of early phases of development using other modern techniques especially electron microscopy (Zhukova and Savina, 1978) and confocal laser scanning microscopy (Friedrikson, 1990, 1991; Fredrikson *et al.*, 1988), noted the occurrence of double fertilization in orchid specimens. Nevertheless, studies of development of endosperm or lack of it are important, as this is the only distinguishing character Dahlgren *et al.* (1985) used to separate members of the order Orchidales from their relatives in the Liliflorae. In the present study, lack of development of endosperm during embryogenesis has been examined.

In this thesis the embryo ontogeny of diverse taxa of the subfamily Epidendroideae (*sensu* Dressler, 1993) will be traced and those that have identical ontogenetic sequence will be grouped together into embryo types. The main aim is to utilise data from embryogenesis in explaining relationships of the Kenyan taxa of the subfamily Epidendroideae.

2.2 MATERIALS AND METHODS

2.2.1 Collection, fixation, and preservation of plant material

2.2.1.1 Collection sites

The localities for orchid sampling across Kenya were identified with the help of information obtained from the herbarium specimens deposited in the East African Herbarium (EA) housed by the National Museums of Kenya, Nairobi. The records showed details of location, habitat description, altitude (elevation), and grid reference figures for the geographic position. However, in the course of doing field work, new

localities were found for some orchids and these were described and locations determined with the aid of contour maps (SK81 KENYA, Scale ÷1:1 000 000, 1st edn. printed and published by the survey of Kenya, 1972). Voucher specimens were deposited in the East African Herbarium, Nairobi. Two species of the tribe Diseae were collected from Table Mountain, Cape Town, South Africa and also used for the study.

The species were assigned to the phytochoria designated by White (1983). The chorological information on the major physiognomic divisions of the vegetation of Africa was used to denote habitats. The vegetation types of White were also used. The classification of White (1983) maps the dominant Kenyan vegetation into four divisions (Table 2.3). The synopsis of the main composition of vegetation types in the divisions is thus: forest, woodland, bushland, thicket, shrubland, grassland, wooded grassland, desert afroalpine, scrub forest, transitional woodland, scrub woodland, mangrove, herbaceous, and halophytic.

Detailed information on the nature of orchid habitats for both terrestrial and epiphytic orchids was recorded. For terrestrial species, information on whether they grow in red or black cotton soil in grassland, bushland, wooded area, rocky area, open forest or dense forest was noted. In most cases, the host trees for the epiphytic orchids were identified and records of whether the orchid grows on the lower tree trunk or twigs and whether it was collected from open or dense forest were made.

Sixty six species in 24 genera from the subfamily Epidendroideae Lindl. and four species in two genera from subfamily Orchidoideae Lindl. were investigated (Appendix 1).

2.2.1.2. Species choice

Species were chosen to include both epiphytic and terrestrial species from as wide a range of habitats as possible. Great attention was paid to the diversity of the orchids in Kenya. The species belonging to the subfamily Epidendroideae occurring in Kenya and further classified into eleven subfamilies in four tribes (Appendix 1) were considered. As many species as possible in these taxa were selected for the study.

Table 2.3: List of the major floristic regions of Kenya showing the main vegetation and dominant habitats in four regional phytochoria (After White, 1983). The synopsis of the main composition of vegetation types in the divisions is thus: forest, woodland, bushland, thicket, shrubland, grassland, wooded grassland, desert afroalpine, scrub forest, transitional woodland, scrub woodland, mangrove, herbaceous, and halophytic

Table 2.3: List of the major floristic regions of Kenya showing the main vegetation and dominant habitats in four regional phytochoria (After White, 1983).

Division / Phytochoria	Main vegetation types	Habitats
XII. Lake Victoria Regional Mosaic. Is the meeting-place of five distinct floras: Guineo-Congolian, Sudanian, Zambezian, Somalia-Masai and Afromontane.	Transitional rain forest and woodland, swamp forest, scrub forest, wooded grassland, or evergreen and semi-evergreen bushland and thicket	Dense forests, open forests, wooded bushlands and thicket, wooded grasslands, swamp forest.
IV. Somalia-Masai region	*Acacia-Commiphora* deciduous bushland and thicket, secondary grassland and wooded grassland. Often forms transition between the Somalia-Masai and Afromontane Regions	Wooded bushland and thicket, wooded grassland, grassland, montane forests.
VIII. Afromontane archipelago-like regional center of endemism	Afromontane rain forest, bamboo, ever-green bushland and thicket, Afromontane and Afroalpine forest	Montane forests, wooded bushland and thicket, montane scrubs and grasslands.
XIII. Zanzibar-Inhambane Regional Mosaic	Lowland rain forest, transitional rain forest and undifferentiated rain forest	Scrub forests, swamp forests, woodland, bushland and thicket, edaphic grassland, wooded grassland.

2.2.1.3 Fixation and preservation

Ovaries and fruits at various developmental stages were collected in natural habitats and fixed on the spot in FPA_{50} (formalin, propionic acid and 50 % ethanol, 5:5:90 v/v/v). After 24-48 hr the specimens were first freed from soil particles, floral parts and any debris then transferred directly to 70 % ethanol (Johansen, 1940) in screw-top vials with internal seals for storage until use. Large fruits, such as those of *Eulophia*, were cut lengthwise using a clean scalpel in order to fit in the vials; these were also fixed in FPA_{50} solution before being transferred into 70% ethanol. As wide a range of materials as possible was collected in the field to ensure that all pre-and postfertilization stages of embryological development in each species were represented. The specimens were placed in portable storage boxes and kept in the laboratory at 10°C, which in the long run proved to be excellent conditions for preserving cellular integrity. In principle, materials preserved in this manner should be processed and observed as soon as possible, usually within one week. However, in my case the treatment of the materials and storage conditions preserved them for analysis 14 months later at the University of Cape Town, South Africa.

2.2.1.4 Clearing techniques and clearing agents

Embryological details were studied using the clearing-squash technique (Herr, 1971) in which materials are cleared until they become nearly transparent in order to be observed for embryological character variation with phase contrast optics. Optical sagittal sections through the ovules resemble microtome sections in that the protoplasts are slightly shrunken away from the cell walls. This method has been used with success in Papilionaceae (Prakash and Herr, 1979), Ulmaceae and Leguminosae (Herr, 1982), Poaceae (Linder and Rudall, 1993; Verboom *et al.,* 1994) and Orchidaceae (Clements, 1995). In the present study the ovules and seeds were dissected from the specimens in

70% ethanol and then cleared in a clearing fluid (Herr, 1971), which consisted of 85 % lactic acid, chloral hydrate, phenol, clove oil, and xylene in a weight ratio of 2:2:2:2:1. After 1-3 hr of treatment, the seeds and ovules were carefully placed on specially made slides, which were modifications of the Raj slides of Smith (1973). Two size "0" coverslips were temporarily mounted approximately 2 mm apart, with 3 drops of Herr's fluid, on a clear glass slide. The material to be studied was placed between these coverslips, in Herr's fluid. A third coverslip was placed on top in order to cover the material, without squashing or distorting the dissected out ovules.

For the present study, there were three modifications to the original technique for examining embryological tissues, which were as follows:

a). The ovules and seeds were dissected from the ovaries and fruits before clearing. The initial design permits for clearing of whole ovaries, after which ovules are dissected and transferred directly to the Raj slide in a drop of fluid. This did not work for the orchids as the fluid failed to penetrate the ovary tissues.

b). The basal cover slips were placed 2 mm apart instead of the 10 mm originally recommended for the Raj slides (Herr, 1971). It was found that this space was more suitable relative to the size of orchid ovules and seeds, which are minute. With larger (>2 mm) spaces air bubbles formed and these are difficult to expel and interfere with overall microscopic observations.

c). The cover glasses used here were not permanently affixed with either permount or balsam to the glass slides. These mountants were replaced with 3 drops of clearing fluid, which made the procedure easier.

2.2.2 Microtomy

The preserved fruits at different stages were serially sectioned according to the schedule of Sass (1958) and the slides observed under the light microscope in order to corroborate

variation in embryological characters revealed also by the Herr's clearing techniques. The material was subjected to the customary method of dehydration by infiltration through an alcohol-xylene series, and was embedded in paraffin wax. Longitudinal and transverse sections of different stages were cut to a thickness of 7 microns. The sections were stained in Heidenhain's iron alum haematoxylene and counterstained by fast green. Permanent slides of the sections were prepared for microscopic observation.

2.2.3 Microscopic observations

The cleared ovules and seeds, nearly transparent, were transferred, five to ten specimens at a time, to a drop of clearing fluid on the glass slides to be observed for structural variation of the megasporophyte, megagametophyte, embryo and endosperm. They were covered by a "0" cover glass and examined under the oil immersion objective of a Leitz Diulux 20 photomicroscope equipped with differential-interference-contrast (DIC) optics (Fredrickson and Frankson, 1988; Fredrickson, 1990, 1991). By focusing at various planes, it was possible to obtain a series of relatively undistorted optical sections through portions of intact specimens. Photographs were taken using Kodak Technical Pan film. Pictures were produced using Adobe Photoshop image processor software version 4.0. Negatives were scanned by a Nikon film scanner LS-4500 AF and digitized on a SAMPO/ALPHA scan 17 mx IBM-PC. Photographic images were observed in Photoshop using the TWAIN-32 option of the same PC and printed using Nikon Codonics image printing software on a NP-600 Nikon Codonics photographic network printer at a brightness of camma 1.5 and a contrast of 40.

2.3 RESULTS

Embryology results were obtained for 70 species in 24 genera of the subfamily Epidendroideae drawn from the tropical habitats in Kenya. Observations for *Vanilla*

polylepis, *Oberonia disticha* and *Ypsilopus longifolius* were incomplete, due to the absence of mature fruits. Furthermore, suitable material for analysis of megagametogesis was not available for a number of species investigated. In all the investigated taxa embryonic details are monotonously uniform in all species of a genus, differing only in minor details related to sizes of embryo sac and embryos or persistence of the "cap" (degenerated megaspores usually situated near the micropyle pore of the inner integuments).

Observation of material by both the clearing-squash technique using DIC optics and microtomy technique using a light microscope produced similar results. However, sectioning of most fruits was hampered by the tannins present in the seed coats. Furthermore, the embedding procedure affected the orientation of most ovules and fruits.

2.3.1 Megasporogenesis

2.3.1.1 Nucellus

Initiation of female gametophyte is found to be uniform in all the species. The nucellus, derived from the placenta, is a filamentous row of seven cells in species of the genera *Liparis*, *Oberonia*, *Cribbia*, *Diaphananthe*, *Chamaeangis*, and *Angraecopsis*. The remaining orchids in the four tribes, except the open forest *Vanilla polylepis*, with 13 cells, have nine cells bounded by single nucellar epidermis (Fig. 2.1). This finger-like nucellus projects outward from the placenta into the ovary locule. Cells of the nucellus are prominent with dense cytoplasm. There is no correlation between habitat and nucellus types.

2.3.1.2 Archesporial cell/megaspore mother cell

The terminal nucellar cell functions directly as an archesporial cell/megaspore mother cell, without cutting off any parietal cell (Fig. 2.2). The cell either elongates or, as in the

case of the open forest species of *Angraecum, Cribbia, Polystachya,* and *Microcoelia,* becomes rounded in outline. The remaining cells of the filament below the archesporial cell do not undergo any divisions and may persist in the mature embryo (Figs. 2.21). The persistent cells are two in number in *Angraecum, Polystachya,* and *Oeceoclades,* all from open forests, or three in all other genera (Fig. 2.26). These persistent cells are present throughout the ontogenesis and are well organised in the mature embryo stage although their nuclei disintegrate.

2.3.2 Megagametogenesis

2.3.2.1 Integuments

The inner integument is initiated earlier than the outer one by periclinal division of dermal layer. Cells of the epidermis surrounding the third cell below the megaspore mother cell differentiate as integumentary primordia for the inner integument. The cells divide actively forming the inner integument, which is two cell layers thick. The outer integument, comprising either two layers (Fig. 2.2) or rarely three layers (Fig. 2.7), is organised by the sub-epidermal cells resulting from divisions of nucellar filament cells. At this stage the structure bends through an angle such that the micropyle and the funiculus lie parallel to each other. The outer integument outgrows the inner one and has large elongated cells that stretch towards the base of the funiculus, where it amalgamates with cells of the funiculus. Inner integuments are always broader at the micropylar end and thinner towards the chalazal end. In mature seeds the inner layer of inner integuments is completely degenerated although a few crushed cells may persist at the micropylar end in some species. Cells of the outer layer of the inner integuments as they become highly stretched with their nuclei disintegrated in the mature embryonal stage

form the carapace, which covers the entire embryo. Ultimately the outer integuments form the seed coat.

2.3.2.2 Embryo sac

In the process of formation of the megasporangium, the megaspore mother cell (Fig. 2.3) undergoes two meiotic divisions to organise four cells. The three micropylar megaspores degenerate leaving the chalazal one (Fig. 2.4) to form the monosporic eight-nucleate embryo sac (Fig. 2.5). In most species the cytoplasm of the disintegrated megaspores persists in the form of a "cap-like" structure. The mature megagametophyte is anatropous, bitegmic and tenuinucellate. The observed embryo sacs are eight-nucleate in *Eulophia, Polystachya, Aërangis* and *Epidendrum,* with the exception of species of *Bulbophyllum*, which have six nuclei. Persistence of antipodals until the young proembryo stage was noticed in *Polystachya, Aërangis, Eulophia* and *Rangaeris* (Fig. 2.8).

2.3.3 Fertilization

As no evidence for the actual process of fertilization taking place was observed, it was difficult to infer at what stage it occurs. However, the zygote was always observed to possess a dense cytoplasm and nucleus associated with a cap-like structure of degenerated synergids at the micropylar pole of the embryo sac.

2. 3. 4 Embryogenesis

Five distinct pathways of embryo ontogenesis (embryogeny) were recognised among the investigated taxa. Despite individual differences in terms of size and shape of the embryos the overall developmental processes and subsequent cellular arrangement remained consistent within each group. The progenitor cell (in all cases the zygote) (Fig. 2.5, 2.10, 2.25) divides by a transverse wall (Fig. 2.6, 2.11, 2.26) to give a larger basal

(*cb*) and a shorter terminal or apical cell (*ca*). The basal cell becomes the suspensor initial and the terminal cell the initial for embryo proper. Further development in each group is described below under a set of "types". The names for embryonic types were derived from one of the generic names of the species in each category. The suspensor in mature embryos assumes different shapes even among genera that share the same mode of embryogenesis.

Group A: *Diaphananthe* type.

The embryonogeny of the species of the epiphytic genera *Angraecopsis, Diaphananthe, Chamaeangis, Rangaeris, Cyrtorchis, Angraecum, Acampe* and *Tridactyle* belong to this group.

The suspensor initial (*cb*) divides by transverse wall to form two cells, a daughter basal cell (*ci*) and terminal cell (*m*), resulting in a proembryo of three cells (Fig. 2.12). This is followed by a vertical division in the terminal cell (*ca*) into two daughter cells. The *ci* divides by oblique or nearly vertical wall into two juxtaposed cells (Fig. 2.13). The *ca* embryonal cell undergoes a vertical division into two juxtaposed cells. The suspensor cells divide yet again by another oblique wall, forming four juxtaposed cells which give the tiny proembryo a roundish shape, most pronounced in *Cyrtorchis* (Figs. 2.14, 2.15 and 2.16). Afterwards, *m* undergoes a longitudinal division to organize two cells in the middle portion of the embryo. Suspensor cells develop four long embryonic tubes or strands of eight cells each that elongate to the micropylar tip and persist in the mature embryo (Fig. 2.32c). In a few observations the suspensor cells of *Diaphananthe* form a single embryonal tube instead of eight cells, in which case the terminal cell is conspicuously rounded with a prominent nucleus. The resultant embryo proper at this stage is composed of four cells and is solely constructed from derivatives of *ca* and *m* cell. Further development of the embryo proper is somewhat irregular by oblique and

transverse walls to form a mature ovoid embryo with undifferentiated cells. At mature seed stage the embryo is ovoid, with a straight axis, and occupies 2/3 of the internal volume with a well developed suspensor. Suspensors of Group A are somewhat convoluted in the micropylar space of the seeds (Fig. 2.16).

Group B: *Bulbophyllum* type.

Orchids with embryogenesis type in Group B include species of *Oeceoclades*, *Bulbophyllum*, and *Cribbia*.

The suspensor initial (*cb*) (Fig. 2.27) does not undergo any further division. Therefore all development of the proembryo originates from the terminal or apical cell (*ca)*. The *cb* grows towards the micropyle and develops into a uninucleate suspensor. The terminal cell divides transversely, generating two daughter cells: (*cd*) and (*cc*), and the proembryo organises three cells (Figs. 2.28-2.29). The middle and terminal cells, by subsequent irregular divisions by vertical walls of proembryo give rise to the mature embryo with undifferentiated cells except in the well developed epidermal layer all round it. The suspensor develops one or two embryonal tubes, which differ in shape and length. That of *Oeceoclades* has two, one elongated towards the micropyle and the other to the chalaza, with heavy deposits in their cytoplasm and with their nuclei at the base (Fig. 2.30). The embryonal tubes in *Bulbophyllum* are shorter, with their nuclei at the rounded tip, while the embryonal tubes of *Cribbia* are also short with their nuclei at the base. The embryo occupies 2/3 of the seed volume. It is almost round in *Bulbophyllum* and ovoid in all other taxa.

Group C: *Polystachya* type.

Orchids in Group C include *Polystachya, Ansellia, Aerangis, Liparis, Calanthe,* and *Microcoelia.* The suspensor initial (*cb*) divides by a transverse wall to form two cells, a daughter basal cell (*ci*) and terminal cell (*m*), resulting in a proembryo of three cells (Fig. 2.19). The terminal cell or apical initial (*ca*) undergoes transverse division to organise two daughter cells that are superposed (Fig. 2.20). Subsequent divisions, which are random in transverse and vertical planes, seem to be more in the terminal and middle (*m*) cells, leading to a massive ovoid or oblong embryo (Fig. 2.22, 2.23, and 2.24). The cell towards the micropyle (*ci*) enlarges and develops into a single-celled suspensor, only recognisable from embryonic cells by its position at the tip, which is occasionally massive as in *Microcoelia.* The mature embryo, with a straight axis, is ovoid with undifferentiated cells and occupies 2/3 of total seed volume.

Group D: *Eulophia* type.

In *Eulophia* the suspensor initial is not necessarily the basal cell (*cb*) or its derivatives. In this genus embryogenesis is similar to that in Group C (*Polystachya* type) in the production of a three-celled proembryo (Figs. 2.6-2.7). Variation is observed whereby the latter divisions in the middle and basal cells are by similar walls to group C, but in a very disorganised pattern that is difficult to discern. Cells of the massive proembryo that are situated towards the micropyle develop into the suspensor (Fig. 2.8). The suspensor in most species is a filamentous row of 12-20 cells (Fig. 2.9). In addition to the suspensor being long it breaks through the integument into the placental tissues of the fruit.

Group E: *Epidendrum* type.

The suspensor initial (*cb*) divides by transverse wall to form two cells, a daughter basal (*ci*) cell and terminal cell (*m*), resulting in a proembryo of three cells. The terminal or apical initial (*ca*) undergoes a transverse division to organise two daughter cells, a middle cell (*cd*) and a terminal cell (*cc*). Derivatives of cell *cb* undergo transverse divisions to form suspensor, but those of cell *ca* divide by vertical walls in irregular pattern. The mature embryo is tiny, occupying only 1/10 of the seed volume, and undifferentiated (Fig. 2.31).

2.3.5 Embryo abortion

Occasionally aborted embryos were observed in *Microcoelia exilis* and *Chamaeangis odoratissima.* Only six aborted embryos were observed in the former species and one aborted embryo for the latter species. Abortion of embryos seems to occur at the late proembryo stage. Collapse of embryo sac and cellularization of seed was noted as in Figure 2.21.

2.3.6 Polyembryony and apomixis

Apomictic embryos were observed in *Eulophia petersii,* resulting from nucellar cells near the chalazal end, and their sizes are smaller than that of the zygotic embryos of the same species. Polyembryony of zygotic embryos is common in the exclusively terrestrial grassland species *E. horsfallii* and the swamp orchid *E. angolensis.* All evidence shows that their formation is by splitting of the zygote. The zygotic embryos lie side by side and are of equal sizes and orientation.

2.3.7 Ontogenesis of suspensor

Six types of suspensor were observed (Fig. 2.32a-g), and these are easily discernible in all mature embryos examined. The type of suspensor in *Polystachya, Ansellia, Acampe,*

Chamaeangis, Tridactyle, Aërangis and *Microcoelia* is fairly uniform (Fig. 2.32g). In these epiphytic genera the suspensor initial undergoes neither division nor any significant modifications and remains at the micropylar apex of the embryo (Figs. 2.23, 2.24). However, the suspensor of *Microcoelia* is more massive and twisted. *Eulophia* as shown by the examples of *E. petersii* and *E. angolensis* (Figs. 2.9, 2.32f), has a filamentous suspensor of 10-12 elongated cells. An exception is *Eulophia stenophylla*, where the suspensor is formed by the cells at the micropyle end and continuous with the embryo as branched, unicellular long filaments. In many species the suspensor penetrates the placental tissue. The suspensor in *Diaphananthe* and *Angraecum* (Fig. 2.32c) has four long strands of eight cells each, which are juxtaposed. The strands appear as a twisted mass (Fig. 2.17), which can easily be stretched to considerable length. In three species of *Cyrtorchis*, *Rangaeris*, and *Angraecopsis,* the four juxtaposed suspensor cells are unicellular, short to massive (Fig. 2.32e). That of *Bulbophyllum* is tubular (Fig. 2.32a), with the nucleus at the rounded terminal end. Tubular suspensors are common also in *Liparis, Cribbia* and *Calanthe* (Fig. 2.32, 2.32b) but their nuclei are located at the base. *Oeceoclades* and *Eulophia stenophylla* have a branched unicellular suspensor with embryonal tubes that elongate to the micropylar end (Fig. 2.32d). Details of suspensor morphology of *Epidendrum* are very difficult to discern from their base at the young proembryo through to embryo stage, although there is a filamentous extension of it to the micropylar region.

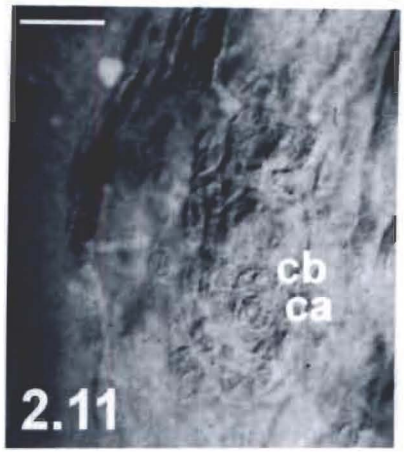
2.3.8 Endosperm

Endosperm tissue was not observed but its progenitor, the primary endosperm nucleus (PEN), was observed in *Ansellia africana*, *Polystachya odorata* and *Eulophia horsfallii*. Despite its prominence and persistence to the tetrad stage of the proembryo (Figs. 2.5, 2.18), the PEN does not develop further and is absent in the mature embryo.

2.3.9 Embryology and ecology

A synthesis of the embryological data detailed above reveals that, in general, the development of embryological structures of orchids is uniform at the generic level (Table 2.3). However, it also indicates that different genera sharing the same geographic regions, elevation and habitats do not necessarily have common developmental patterns in their embryology (Table 2.4).


2.10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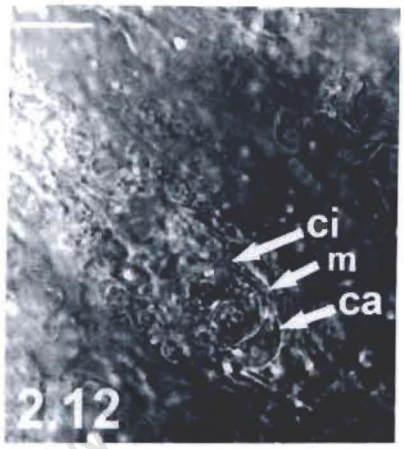
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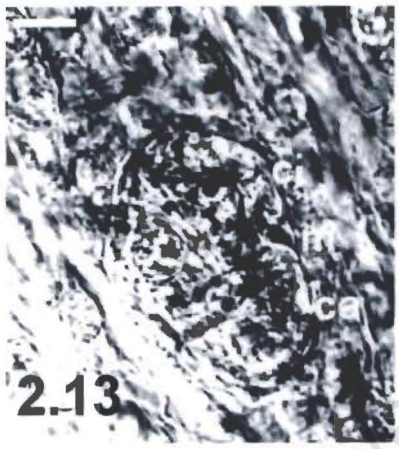
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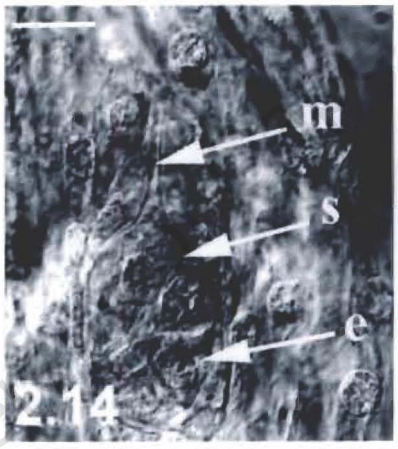
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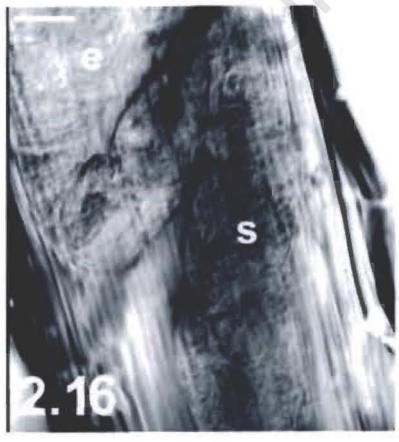
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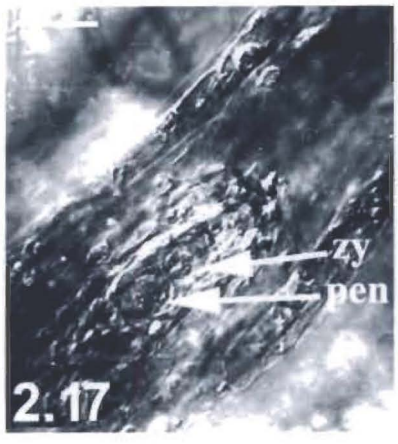
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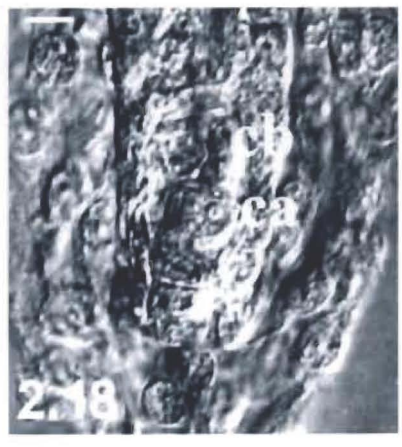
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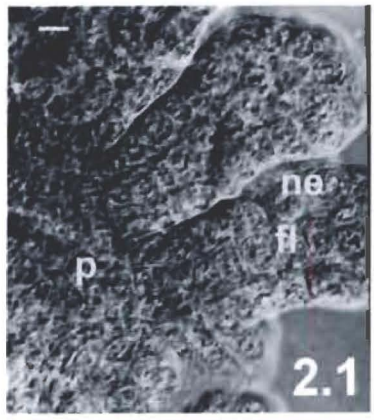
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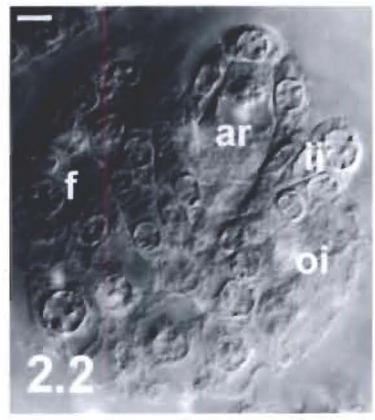
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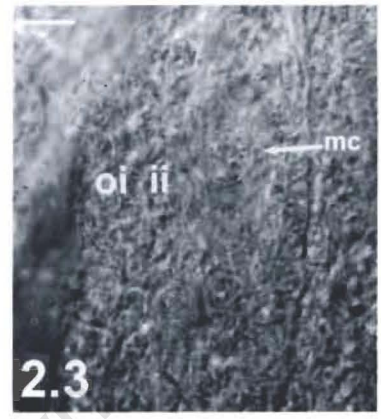
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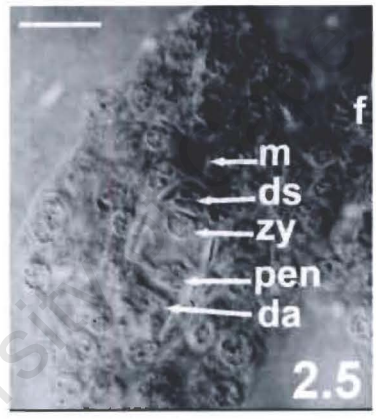
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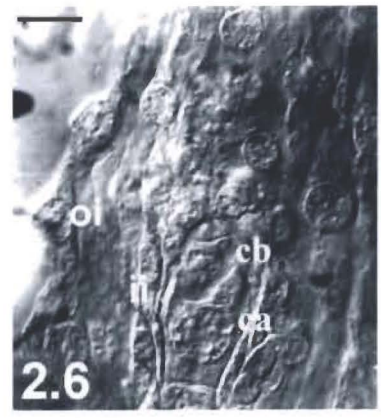
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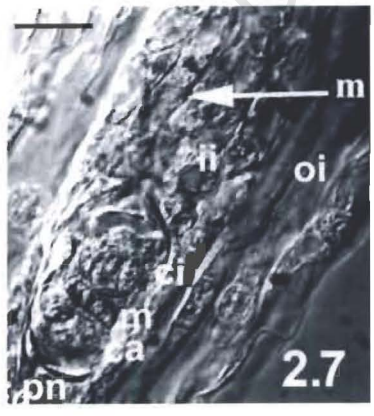
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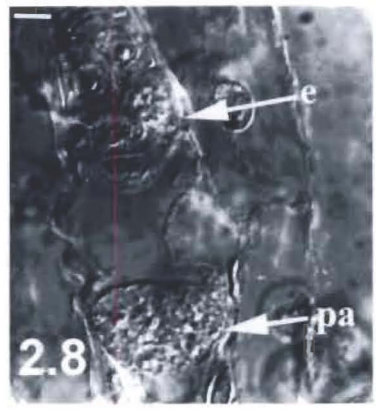
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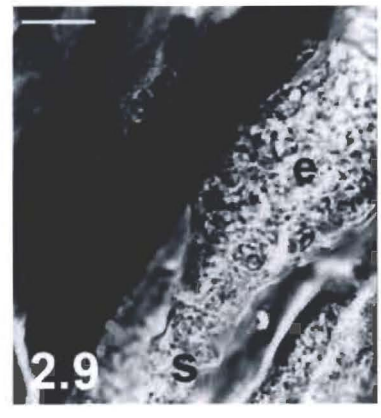
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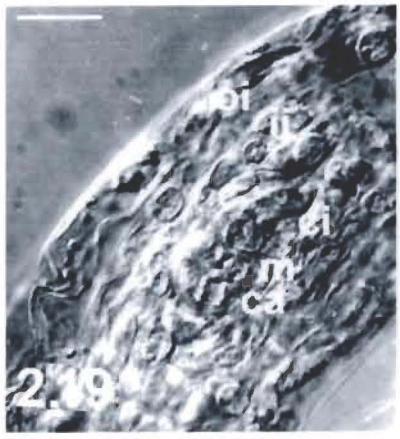
Figures 2.10-2.18. Embryogenesis in Epidendroideae. fig. 2.10 Zygote of *Cyrtorchis arcuata* (Bar = 10 μm), Zy = Zygote with dense cytoplasm; fig. 2.11 two-celled stage in *Tridactyle furcistipes* (Bar = 10 μm), cb = basal cell, ca = apical cell; fig. 2.12 three-celled proembryo ci = basal daughter cell of cb, m = daughter terminal cell of cb, ca = terminal cell; fig. 2.13 proembryo in *Cyrtorchis arcuata* (Bar = 10 μm); fig. 2.14 four-celled proembryo, m = micropyle formed by inner integuments, s = two-celled suspensor formed by cb (note vertical division of cb into separate cells), e = embryo; fig. 2.15 mature embryo and suspensor in *Rangaeris amaniensis* (Bar = 10 μm), s = suspensor, e = embryo; fig. 2.16 mature embryo and suspensor in *Diaphananthe rutila* (Bar = 10 μm), s = suspensor, e = embryo; fig. 2.17 Zygote in *Polystachya dendrobiiflora* (Bar = 10 μm), zy = zygote, pen = primary endosperm nucleus; fig. 2.18 Two-celled proembryo in *Aërangis ugandensis* (Bar = 10 μm), cb = basal cell, ca = apical cell.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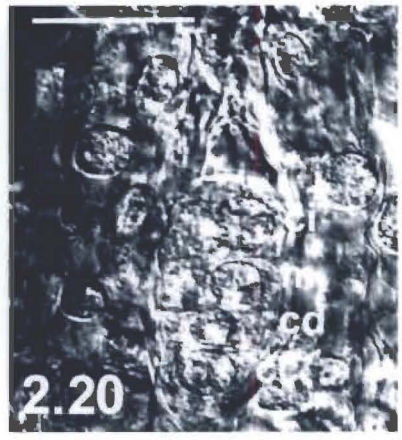
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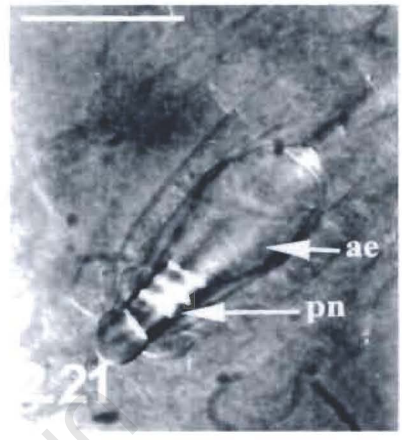
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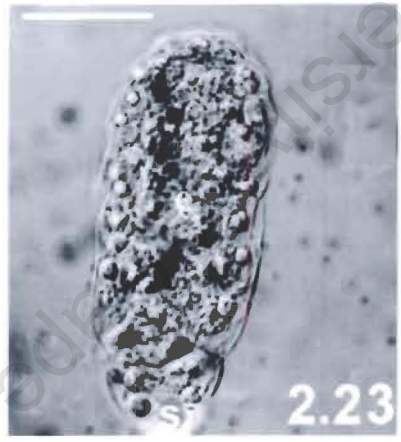
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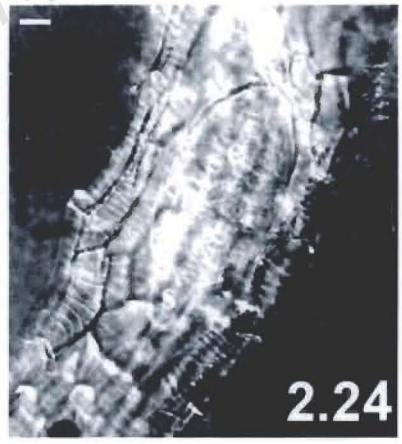
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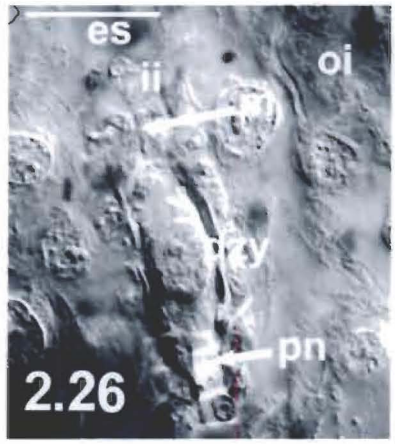
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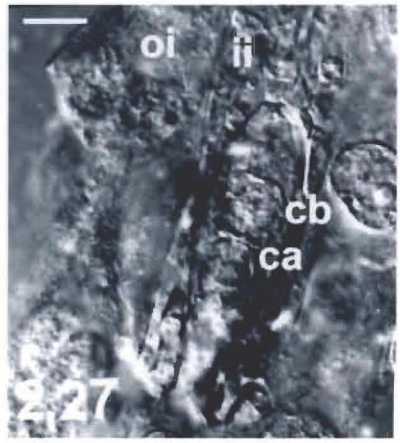
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Figures 2.19-2.27. Embryogenesis in Epidendroideae. fig. 2.19 three-celled proembryo in *Polystachya odorata* (Bar = 10 μm), ca = apical cell, m = middle cell (derived from cb), ci = basal daughter cell of cb, i.i = inner integument, o.i = outer integument; fig. 2.20 four-celled stage proembryo in *Polystachya odorata* (Bar = 10 μm), ci = daughter basal cell of cb, m = daughter terminal cell of cb, cd = daughter basal cell of ca, cc = daughter terminal cell of ca; fig. 2.21 aborted embryo of *Microcoelia exilis* (Bar = 10 μm), ae = arboted embyo, four persistent nucellar cells; fig. 2.22 mature embryo in *Aërangis brachycarpa* (Bar = 10 μm), e = embryo, s = suspensor; fig. 2.23 mature embryo in *Polystachya odorata* (Bar = 10 μm), e = embryo, s = suspensor; fig. 2.34 mature embryo of *Liparis bowkeri* (Bar = 10 μm), e = embryo, s = suspensor; fig. 2.25 Zygote in *Bulbophyllum cochleatum* (Bar = 10 μm), zy = zygote, fig. 2.26 dividing zygote in *Bulbophyllum cochleatum* (Bar = 10 μm), dzy = Zygote, pn = two persistent nucellar cells, m = micropyle formed by the inner integuments.

Figure 2.26. Two-celled proembryo of *Bulbophyllum intertexcum* (Bar = 10 μm), ca = apical cell, cb = basal cell, i.i = inner integument, o.i = outer integument, fig. 2.27 two-celled proembryo of *B. intertextum.*

Figures 2.28-2.31: Embryogenesis in Epidendroideae. Fig. 2.28 three celled-proembryo of *Oeceoclades saundersiana* (Bar = 10 μm), cb = basal cell, cd = basal daughter cell of ca, cc = terminal daughter of ca; fig. 2.29 three-celled proembryo of *Bulbophyllum cochleatum*, cb = basal cell, cd = basal daughter cell of ca, cc = terminal daughter; fig. 2.30 mature embryo of *Oeceoclades saundersiana* (Bar = 10 μm), e = embryo, s = suspensor; fig. 2.31 mature embryo of *Epidendrum cinnabarinum* (Bar = 10 μm), e = embryo, s = suspensor.

Figure 2.32. Diagrammatic representation of suspensor types in the Epidendroideae. In all cases Bar = 2.5 μm. a = type A; b = type B; c = type C; d = type D; e = type E; f = type F; g = type G.

Table 2.4: A summary of the distributions, habitats and embryological results. R= Floristic region (IV = Somalia-Masai region, XIII = Zanzibar-Inhambane region, VIII = Afromontane archipelago-like regional center of endemism, XII = Lake Victoria regional Mosaic), nf = number of cell of nucellar filament, ac = archesporial cells (megaspore mother cell), pnc = number of persistent nucellar cells, es = number of embryo sac nuclei, emg = embryogenesis, pe = polyembyony, s = suspensor type. The - means the character was not observed.

Species	R	Habitat	nf	ac	pnc	es	Emg	pe	s
Tribe Vandeae Lindl. **Subtribe Aerangidinae** Summerh.									
Aërangis brachycarpa (A. Rich.)Th. Dur. & Schinz	IV	open forest	7	rounded	2	8	*Polystachya* type	absent	type g
A. confusa J. Stewart	VIII	montane open forest	7	rounded	2	8	*Polystachya* type	absent	type g
A. coriacea Summerh.	XIII	open bushland	7	rounded	2	8	*Polystachya* type	absent	type g
A. luteoalba (Kraenzl.) Schltr.	IV	shrubs in open bushland	7	rounded	2	8	*Polystachya* type	absent	type g
A. thomsonii (Rolfe) Schltr.	XIII	dense forest	7	rounded	2	8	*Polystachya* type	absent	type g
A. ugandensis Summerh.	IV	dense forest	7	rounded	2	8	*Polystachya* type	absent	type g
Angraecopsis amaniensis Summerh.	IV	open wooded bushland	7	elongate	3	8	*Diaphananthe* type	absent	type e
A. breviloba Summerh.	XIII	open wooded bushland	7	elongate	3	8	*Diaphananthe* type	absent	type e
A. gracillima (Rolfe) Summerh.	IV	wooded bushland	7	elongate	3	8	*Diaphananthe* type	absent	type e

Species	R	Habitat	nf	ac	pnc	es	Emg	pe	s
Chamaeangis odoratissima (Reichb. f.) Schltr.	XII	open wooded bushland	7	elongate	3	-	*Diaphananthe* type	absent	type g
C. sarcophylla Schltr.	VIII	montane dense forest	7	elongate	3	-	*Diaphananthe* type	absent	type g
C. vesicata (Lindl.) Schltr.	IV	dense forest	7	elongate	3	-	*Diaphananthe* type	absent	type g
Cribbia brachyceras (Summerh.) Senghas	XIII	open wooded bushland	7	rounded	3	-	*Bulbophyllum* type	absent	type b
Cyrtorchis arcuata (Lindl.) Schltr.	XIII	montane open bushland	9	elongate	3	-	*Bulbophyllum* type	absent	type e
C. preatermissa Summerh.	IV	open woodland forest	9	elongate	3	-	*Bulbophyllum* type	absent	type e
Diaphananthe rutila (Reichb. f.) Summerh.	VIII	montane dense forest	7	elongate	3	-	*Diaphananthe* type	absent	type c
D. lorifolia Summerh.	XIII	open wooded bushland	7	elongate	3	-	*Diaphananthe* type	absent	type c
D. subsimplex Summerh.	IV	dense forest	7	elongate	3	-	*Diaphananthe* type	absent	type c
D. tenuicalcar Summerh.	IV	montane open forest	7	elongate	3	-	*Diaphananthe* type	absent	type c
D. xanthopollinia (Reichb. f.) Summerh.	IV	open forest	7	elongate	3	-	*Diaphananthe* type	absent	type c

Species	R	Habitat	nf	ac	pnc	es	Emg	pe	s
Microcoelia exilis Lindl.	XIII	open bushed woodland	7	rounded	3	-	*Diaphananthe* type	absent	type g
M. koehleri (Schltr.) Summerh.	IV	open forest	7	rounded	3	-	*Diaphananthe* type	absent	type g
M. moreauae L. Jonsson	IV	open bushland	7	rounded	3	-	*Diaphananthe* type	absent	type g
M. smithii (Schltr.) Summerh.	IV	open wooded bushland	7	rounded	3	-	*Diaphananthe* type	absent	type g
M. stolzii (Schltr.) Summerh.	IV	open woodland	7	rounded	3	-	*Diaphananthe* type	absent	type b
Rangaeris amaniensis (Kraenzl.) Summerh.	IV	open woodland	7	elongate	3	-	*Diaphananthe* type	absent	type e
R. muscicola (Reichb. f.) Summerh.	IV	dense forest	7	elongate	3	-	*Diaphananthe*	absent	type e
Tridactyle bicaudata (Lindl.) Schltr.	XII	dense forest	7	rounded	3	-	*Diaphananthe* type	absent	type g
T. scotellii (Rendl.) Schltr.	XII	open forest	7	rounded	3	-	*Diaphananthe* type	absent	type g
Ypsilopus longifolius (Kraenzl.) Summerh.	IV	bushed woodland	9	rounded	3	-	-	absent	-
Subtribe Aeridinae									

Species	R	Habitat	nf	ac	pnc	es	Emg	pe	s
Pfitzer									
Acampe pachyglossa Reichb. f.	IV	bushed woodland	-	-	-	-	*Diaphananthe* type	absent	type g
Subtribe Angraecinae									
Angraecum decipiens Summerh.	IV	open bushed woodland	9	elongate	2	-	*Diaphananthe* type	absent	type c
A. eburneum Bory subsp. *giryamae* (Rendl.) Senghas & Cribb	XIII	rocky open bushland	9	elongate	2	-	*Diaphananthe* type	absent	type c
A. dives Rolfe	XIII	coastal wooded forest	9	elongate	2	-	*Diaphananthe* type	absent	type c
A. erectum Summerh.	IV	open forest	9	elongate	2	-	*Diaphananthe* type	absent	type c
Tribe Cymbideae Pfitzer **Subtribe Eulophiinae** Bentham									
Eulophia angolensis (Reichb. f.) Summerh.	IV	swamp among sedges	7	elongate	3	8	*Eulophia* type	absent	type f
E. horsfallii (Batem.) Summerh.	XII	River valley among sedges	7	elongate	3	8	*Eulophia* type	absent	type f

Species	R	Habitat	nf	ac	pnc	es	Emg	pe	s
E. ovalis Lindl.	IV	open bushed grassland	7	elongate	3	8	*Eulophia* type	absent	type f
E. petersii Reichb. f.	IV	open bushland and thicket	7	elongate	3	8	*Eulophia* type	present	type f
E. speciosa (Lindl.) Bolus	IV	open bushland and thicket open scrubland	7	elongate	3	8	*Eulophia* type	absent	type f
E. streptopetala Lindl. var. *stenophylla* (Summerh.) Cribb	IV	silted rocky grassland	7	elongate	3	8	*Eulophia* type	present	type d
E. streptopetala Lindl. var. *streptopetala* (Summerh.) Cribb	VIII	montane rocky grassland	7	elongate	3	8	*Eulophia* type	absent	type f
Oeceoclades saundersiana (Reichb. f.) Garay & Taylor	IV	open wooded forest	-		-	-	-	absent	type d
Subtribe Cyrtopodinae Bentham									
Ansellia africana Lindl.	VIII	rocky bushed land	9	elongate	3	-	*Polystachya* type	absent	type g
Tribe Malaxideae Lindl. **Subtribe Liparidinae**									
Liparis bowkeri Harv.	XII	dense forest	7	elongate	3	-	*Bulbophyllum*	absent	type b

Species	R	Habitat	nf	ac	pnc	es	Emg type	pe	s
L. deisteli Schltr.	XII	dense forest	7	elongate	3	-	*Bulbophyllum* type	absent	type b
Oberonia disticha (Lam) Schltr.	IV	open wooded bushland	9	elongate	3	-	-	absent	-
Tribe Epidendreae **Subtribe coeliinae**									
Epidendrum cinnabarinum Salmann	IV	open grassland	9	rounded	3	8	*Epidendrum* type	absent	-
E. ibaguense HBK	IV	open bushed grassland	9	rounded	3	8	*Epidendrum* type	absent	-
Subtribe Polystachyiinae Pfitzer									
Polystachya adansoniae Reichb. f.	XII	open forest	9	elongate	3	8	*Polystachya* type	absent	type g
P. bella Summerh.	XII	dense forest	9	elongate	3	8	*Polystachya* type	absent	type g
P. bennettiana Reichb. f.	XII	dense forest	9	elongate	3	8	*Polystachya* type	absent	type g
P. bicarinata Rendl.	XII	dense forest	9	elongate	3	8	*Polystachya* type	absent	type g

Species	R	Habitat	nf	ac	pnc	es	Emg	pe	s
P. campyloglossa Rolfe	VIII	montane bushland	9	elongate	3	8	*Polystachya* type	absent	type g
P. confusa Rolfe	IV	open bushland	9	elongate	3	8	*Polystachya* type	absent	type g
P. dendrobiiflora Reichb. f.	XIII	open xerophyta forest	9	elongate	3	8	*Polystachya* type	absent	type g
P. eurychila Summerh.	XII	dense forest	9	elongate	3	8	*Polystachya* type	absent	type g
P. fusiformis (Thou.) Lindl.	XII	montane bushland	9	elongate	3	8	*Polystachya* type	absent	type g
P. piersii Cribb	IV	open dry forest	9	elongate	3	8	*Polystachya* type	absent	type g
P. steudneri Reichb. f.	XII	dense forest	9	elongate	3	8	*Polystachya* type	absent	type g
P. spatella Kraenzl.	VIII	montane dense forest	9	elongate	3	8	*Polystachya* type	absent	type g
P. tenuissima Kraenzl.	XII	dense forest	9	elongate	3	8	*Polystachya* type	absent	type g
P. tessellata Lindl.	XIII	open wooded forest	9	elongate	3	8	*Polystachya* type	absent	type g
P. transvaalensis Schltr.	IV	bush and woodland	9	elongate	3	8	*Polystachya* type	absent	type g
P. vaginata Summerh.	IV	open xerophyta	9	elongate	3	8	*Polystachya*	absent	type g

Species	R	Habitat	nf	ac	pnc	es	Emg	pe	s
		woodland					type		
Tribe Dendrobieae Endlicher **Subtribe Bulbophyliinae** Schlechter									
Bulbophylum cochleatum Lindl.	XII	open forest	7	elongate	3	6	*Bulbophyllum* type	absent	type a
B. encephalodes Summerh.	VII	open forest	7	elongate	3	6	*Bulbophyllum* type	absent	type a
B. intertextum Lindl.	XII	dense forest	7	elongate	3	6	*Bulbophyllum* type	absent	type a
B. maximum (Lindl.) Reichb. f.	XII	open forest	7	elongate	3	6	*Bulbophyllum* type	absent	type a
Tribe Arethusae Lindl. **Subtribe Bletiinae** Bentham									
Calanthe sylvatica (Thou.) Lindl.	XII	dense forest	7	elongate	3	-	*Bulbophyllum* type	absent	type b
Tribe Vanilleae Blume **Subtribe Vanillinae** *Lindl.*									
Vanilla polylepis Summerh.	IV	open forest	13	elongate	3	-	-	absent	-
Subfamily Orchidoideae **Tribe Diseae** Dressler									

Species	R	Habitat	nf	ac	pnc	es	Emg	pe	s
Subtribe Satyriinae Schlechter									
Satyrium crassicaule Rendl.	XII	swamp among sedges	-	elongate	2	-	Orchidoid type	absent	type a
S. volkensii Schltr.	XII	swamp among sedges	-	elongate	2	-	Orchidoid type	absent	type a
Subtribe Disinae SW.									
Disa ferruginea SW.	V	Fynbos	7	round	2	-	Orchidoid type	absent	type a
Disa graminifolia SW.	V	Fynbos	7	round	2	-	Orchidoid type	absent	type a

2.4 DISCUSSION

The present study confirms that the clearing-squash method and use of differential interference contrast optics can be very useful in studying tiny embryos to greater depths, as indicated by Clements (1995). Therefore, it is possible to provide more accurate interpretation of orchid embryology. Additionally, the clearing method allows us to scan more embryos in a short time than the sectioning method would, although both give the same results.

2.4.1 Megasporogenesis

In the structure of the nucellus and ovule initiation, species of Epidendroideae are very similar to all other members of the Orchidaceae investigated by Swamy (1949a). Despite the fact that Swamy studied a small sample, 25 species in 14 genera, his work has had profound influence on our interpretation of orchid embryology.

Reports in the literature often pay little attention to the process of ovule initiation (Bouman, 1974). In *E. ibaguense* (Yeung and Law, 1989) repeated anticlinal divisions in the placenta epidermis coupled with the periclinal divisions in the subepidermal layer produce the dichotomous branches that bear ovules at their tips. According to Bouman (1974), this pattern of ovule initiation is dizonate and the resulting ovule tends to be small. However, the discovery that the cells making up the nucellar filament vary depending on the lineage of the orchid has afforded a criterion of very important utility in systematics. In this study, which is the second observation of nucellar filament in the Orchidaceae after that of Swamy (1949a), genera with seven, nine or 13 cells have been recorded. In the only previously reported information about the structure (Swamy, 1949a), illustrated by *Eulophia nuda,* cells were found to range from six to seven,

covered by a single epidermis. Future embryological work focusing on this character might yield more variation in the number of filamentous cells in many other taxa.

The present work indicates that Orchidaceae are unique in the Angiosperms as there may not be the formation of an archesporial cell in the family. According to Swamy (1949a) the archesporial cell functions directly as megaspore mother cell. Since there is no evidence of a sporogenous cell (terminal nucellar cell) undergoing meiosis in the orchid megasporophyte to form the archesporial cell, there is doubt as to the formation of archesporial cell; instead the cell functions directly as megaspore mother cell. Perhaps what Swamy (1949a) regards as archesporial cell is according to the present study a terminal nucellar cell, which apparently enlarges and functions as a megaspore mother cell. The other difference is that Swamy noticed elongation of "archesporial cell" long before meiosis and the present investigation reveals that at both very early and subsequent stages the terminal nucellar cells can be either round or elongate depending on the genus. This finding is amplified by the surprising observation made by Afzelius (1916) for *Oncidium praetextum,* in which one of the cells of the nucellar epidermis functions as archesporial cell/megaspore mother cell.

The bitegmic integuments are organized by epidermal cells below the archesporial cell/megaspore mother cell and only the inner integument seems to form the micropyle. Although for Orchidaceae integumentary studies have been considered in some detail (Hofmeister, 1848; Warming 1878 [cited in Bouman 1974]) the present work is only the second which establishes the dermal derivation of the inner integuments and the development of the outer integuments from daughter cells of the filament (hence subdermal) is fundamental for the subfamily. The inner integument does not form a part of the mature testa since it becomes highly stretched and resorbed as the seed matures. But histochemical changes have been reported for the inner integumentary tissues soon

after fertilization for *Epidendrum ibaguense* (Yeung and Law, 1988). At that time cells of the inner integument become more cytoplasmic giving a strong protein reaction and hence playing a nutritive role for the developing embryo. There is perhaps influence of orchid habitat in organization of the outer integument in mature seed, which remains thin in the terrestrial species but which becomes highly thickened in epiphytes. Without biochemical analysis no obvious explanation can be found for the integument thickening in epiphytes, except speculations that integumentary cells become sclerotic and impregnated with a dark material (Swamy, 1947, 1949a).

2.4.2 Megagametogenesis

In the material studied eight-nucleate embryo sacs were commonly observed except in the genus *Bulbophyllum* with 6-nucleate embryo sacs. This observation for the Kenyan species contradicts the publication by Swamy (1949b) regarding a six-nucleate embryo sac in *Eulophia epidendrea*.

Even though materials for studies in megagametogenesis were not adequate for an exhaustive research in the subfamily, there is a very substantial literature, all of which consistently indicates that an 8-nucleate embryo sac is more common in the Orchidaceae than a 6-nucleate embryo sac (Swamy, 1949a; Sood and Rao, 1986, 1987, 1988). This aspect of the embryo sac has been the most studied in orchids and has been documented for a diverse group of orchids in different countries and locations globally. Much of this success in embryo sac studies is due to easy accessibility to greenhouse orchids, for which materials can be collected at close intervals, as megagametogenesis is invariably a very quick process (Catling, 1981). However, all these publications confirm that the eight-nucleate embryo sac is the more common than the six-nucleate embryo sac (Swamy, 1942, 1943, 1945, 1947, 1949; Sharp, 1912, 1937; Brown and Sharp, 1911,

Baranov, 1915, 1918; Swamy, 1945; Alzelius, 1916; Carlson, 1945, 1954; Modilewski, 1918; Stenar, 1937; Prosina, 1930; Afzelius, 1916; Yasugi, 1983; Rao and Rao, 1984; Vij at al, 1982)

2.4.3 Embryogenenesis

Embryogenesis provides an insight into the evolution of the different zones of the embryonic mass. Different pathways of evolution of these zones might be indicative of the phylogenetic affinities of the species. Orchid embryogenesis is so diverse that it has not been possible to develop a single method of classification for embryos. The major contributing factor is that the embryos themselves are tiny, requiring special methods for studying them; an additional problem is to establish the destiny of all the embryo blastomeres (Treub, 1879; Swamy, 1949b; Johansen, 1950; Veyret, 1974).

From the point of view of embryo ontogenesis the species studied here can be grouped into five types based on the differences brought about by the variety of ontogenetic pathways exhibited by the zygote. The embryonic pathways are determined by the four embryogenic laws as Souèges (1936-1939)[cited in Veyret, 1974] defines them: laws of origin, of number, of disposition or arrangement, and of destinies of the first four cellular generations. Only the law of destinies cannot be determined in the development of the orchid since the embryo at the time of fruit maturity is, in general, morphologically undifferentiated. In the Souèges classification, the embryos are grouped into megarchetypes, these being defined by the sum of the constructive potential of the apical cell and basal cells, resulting from the first transverse segmentation of the zygote.

The first application of embryogenic laws in an attempted classification of orchids by Swamy (1949b) revealed consistency in categorising embryo types in both terrestrial and epiphytic orchids. The present study, like those of Swamy (1949a, b), deals with both

example, all the embryo types conform to Swamy's group B because they possess a suspensor. However, although the *Diaphananthe*-type of embryogenesis up to the four-celled proembryo stage resembles the *Onagrad*-type embryogenesis of Swamy (1949b), the *Bulbophyllum-, Polystachya-, Eulophia-* and *Epidendrum*-types are neither *Asterad* nor *Onagrad* and cannot be precisely defined as *Cymbidium*-type. Therefore, in the present study only representative generic names are used to designate the newly established embryo type.

The most interesting variation noted in this study is the construction of the embryo proper by the terminal cell (*ca*), or in some cases terminal cell and middle cell (*m*), or in yet others both *ca* and *cb*. This variation in embryogenesis and resultant embryo tetrads as presented here compares well with the A2, C1, and C2 embryo types proposed by Veyret (1974) using laws of embryonomy propossed by Souèges (1936-1939)[cited in Veyret, 1974].

The observations show that cell divisions in the first generation are uniform in all studied species, followed by a high degree of variation beyond the two-celled proembryo as the cells enter into the construction of embryo and suspensor. Therefore, because of inconsistency of sequence and planes of division subsequent to the first two cell generations, misinterpretations of the course of embryogenesis always result. The second difficulty in studying divisions of mature embryos was observed in the epiphytic genus *Epidendrum,* in which the suspensor is multicellular, obscuring the details of the embryo even at the very young stages.

This study of embryogenesis has therefore emphasised the first two cell generations and most mature embryos. As the findings of this study suggest, it is adequate to employ embryos of four or five cells in discussions of orchid taxonomy, as demonstrated by Swamy (1979). Swamy rightly argues about the observational and interpretative

inadequacies that can result from embryo ontogeny beyond the tetrad stage, such as failure to critically determine the longitudinal planes of division. He further suggests that transverse serial sections are also very necessary in order to determine the relative quantum of the working sphere of the terminal tier that becomes involved in the organisation of the cotyledon and epicotyl. Similar problems had been highlighted much earlier by Wirth and Withner (1959) who pointed out that the tremendous variation in the pattern of division in cell tiers beyond the tetrad stage and their position in different planes leads to inconsistency and is the main reason why interpretation of embryo ontogeny in Orchidaceae is problematical.

It is now well known that in the majority of orchids development of the embryo up to differentiation of cotyledon and epicotyl does not take place and the ontogeny becomes arrested at some step or the other during the early phases (Veyret, 1956, 1957a, b). Therefore the consistency of early ontogeny in the embryogenesis recorded in this investigation becomes a very vital contribution to the field of orchid taxonomy. Worse still, as recognised in this study, the mature embryo is almost featureless with only an undifferentiated mass of cells. Thus the Orchidaceae provide classic examples of an embryo being immature at the time of seed dispersal (Swamy, 1979). The taxa studied belong to this category and because of this limitation it is difficult to follow up the loci of initiation of the cotyledon and epicotyl.

The filamentous suspensors with haustorial extensions found in the present study are consistent with those reported in earlier publications regarding embryology of Epidendroid orchids (Swamy, 1949b; Jonsson, 1981). The results reveal that suspensors of all the species develop in the same way in that the tissue originates with the basal cell, but wide variation is exhibited in different groups. The variation in the structure and shape of suspensors fits largely into five types, most of which were also observed by

Swamy (1949b). The suspensor associated with the *Diaphananthe* type embryo is the first on record and is rather complicated, with four juxtaposed eight-celled embryonic tubes which may be short or tremendously long, but are coiled in the seed. Although their role is thought to be actively assisting in growth and development of the embryo proper (Yeung and Meinke, 1993), suspensors are known to vary in their morphology in almost all angiosperms, possibly to suit this function (Yeung and Meinke, 1993). Suspensor formation does seem to be a usual phenomenon in Orchidaceae; although suspensorless species also occur in the family. Treub in 1879 described forms in which the filamentous suspensor grows out and develops to enormous proportions. Similar results have been published by Swamy (1942) for *Cymbidium bicolor*. The structure of the suspensor is so consistent in orchids that Swamy (1949b) labelled it an admirable tool for classification of orchid embryos. In fact the classification of orchid embryos by Johansen (1950) is based entirely on the suspensor, as he identified 13 patterns of development on the basis of the presence or absence of suspensor. Swamy (1949b) dismissed the idea of suspensor haustoria and Johansen (1950) introduced the term embryonic tubes to denote haustoria-like features. The present study reinforces the evidence for the haustorial nature of the suspensor as it is present in mature embryos, often penetrating the tissues of the placenta, and does not shrive or die. This condition is common in many orchids (Rao and Sood, 1979).

The present study of embryo development has strengthened our understanding about relationships within the Epidendroideae, for example the strange clustering of *Cribbia* with *Oeceoclades* and *Bulbophyllum, Aerangis* clustering well with *Polystachya*, *Aërangis, Ansellia, Liparis, Microcoelia* and *Calanthe*, and *Eulophia* separates from *Oeceoclades*. Therefore the recognition of *Oeceoclades* might be supported.

2.4.4 Polyembryony and apomixis

As shown in Table 2.3 (section 2.3) the first report of polyembryony through cleavage was made by Swamy (1942) in *Cymbidium bicolor*, where the resulting seeds contained two embryos. Similar results were reported by Ansari (1977) in *Spathoglottis plicata* and *Geodorum densiflorum.* In *Cymbidium* the first division of the zygote may be oblique or vertical; in either case the resulting cells may separate and develop into separate embryos. In the present study the production of zygotic embryos as well as apomictic nucellar embryos was confined exclusively to three terrestrial species of *Eulophia.* Of the three species only *E. angolensis* exhibits both cleavage and adventive embryony in the same seed whereas *E. horsfallii* and *E. petersii* develop cleavage embryos. That occurrence of polyembryony in epiphytic species is uncommon is apparent from previously published results (Swamy, 1949a; Chua and Rao, 1978; Stort and Pavanelli, 1985)

In orchids the production of multiple embryos is rare (Webber, 1940; Stebbins, 1941; Swamy, 1942a, 1943b, 1946b, 1949b; Gustafsson, 1946, 1947; Johansen, 1950; Wirth and Withner, 1959) but it has been documented in diverse genera such as *Cymbidium, Eulophia, Habenaria,* (Swamy, 1942, 1943, 1946, 1949), *Cephalanthera, Listera, Orchis, Platanthera, Epipactis* (Hagerup, 1945, 1947), *Spiranthes* (Maheshwari and Narayanaswami, 1953) and *Vanda* (Rao, 1964). In *Spiranthes* and *Zeuxine* apomictic embryos develop from the nucellus and integuments (Rao, 1964; Swamy, 1946, 1949; Withner, 1959). Cleavage polyembryony has been reported for *Spathoglottis aurea* (Chua and Rao, 1978).

In the present work the *Eulophia* embryos did not exceed two in the seeds although a large number, ranging from two to nine, have been recorded in *Epidendrum nocturnum* (Stort and Pavanellii, 1985; Veyret, 1996). The occurrence of two embryos is common

in *Spathoglottis* (Chua and Rao, 1978). Apomictic embryos were observed in the present study for only one species of *Eulophia*. Apomictic polyembryony, being less reported in members of the Orchidaceae, has consequently received less attention for consideration in taxonomic studies.

2.4.5 Endosperm

Reports on endosperm formation in Orchidaceae as published by many authors have been contradictory. Garay (1960) and Veyret (1974) do not confirm formation of endosperm. Prior to their findings such tissue had been reported as forming in *Cypripedium spectabilie* and *C. parviflorum* (Pace, 1907), *C. guttatum* (Prosina, 1930), *Paphiopedilum insigne* and *Chaemorchis alpina* (Afzelius, 1916), and *Vanilla planifolia* (Swamy, 1947). The present findings have led to speculation that in populations of three species from different habitats in which primary endosperm nucleus (PEN) was observed double fertilization takes place. It is probable that for some reason the PEN does not develop further to form endosperm and it seems to disintegrate early in ontogenesis. For other species where PEN was not observed it is also speculated that double fertilization possibly takes place but the PEN degenerates very early, before first division of the zygote. There is strong evidence for double fertilization from the present work, contrary to suggestions that single fertilization might be responsible for the lack of endosperm formation (Terasaka *et al.*, 1979; Albert, 1990). Double fertilization seems to be the only mode of PEN formation (Zhukova and Savina, 1978; Fredrikson, 1990, 1991; Fredrikson *et al.*, 1988) but this is possibly followed by a reduction of PEN (Teryoklin and Kamelina, 1969, 1972; Rao and Rao, 1984; Sharma and Shekhar, 1982). Previous reports about endosperm formation confirm that the tissue, if present at all, does not develop beyond the 16-nuclear state (Swamy 1947, 1949b; Dahlgren and Clifford, 1982; Arditti, 1992).

Future work may explain the factors leading to the inability of PEN to develop to mature endosperm. In the aphyllous epiphyte *Microcoelia* it was found (Jonsson, 1981) that, even though endosperm does not form, the embryo is richly provided with fatty acids as nutritive material. Fatty acids have slightly more than double the energy content of starch (Lehninger, 1975), and thus to some extent balance the loss of energy potential by the reduction of starch-containing endosperm. Rao and Sood (1979) noticed that cells of the mature embryo of *Herminium angustifolium* contain abundant starch. Furthermore, it has been shown that embryos cells themselves contain substantial reserves of lipid and protein in *Disa, Disperis* and *Huttonaea* (Manning and Staden, 1987). Earlier estimations by quantification methods (Knudson, 1929) showed that that in *Cymbidium* the food reserves in embryo cells contain 32 % lipid and 1 % sugar, but no starch.

Chapter 3: SEED COAT MORPHOLOGY

Contents

3.1 INTRODUCTION

Seed coat morphological characters were investigated in order to reveal various characters e.g. size, shape and the diverse microornamentation on the seed surface of Epidendroideae. Interesting characteristics of seeds reported for the subfamily are that they are usually of minute size, and have unique surface sculpturing, testal extensions

and wax deposits of various shapes. The seed coat layer consists of radially stretched, tangentially elongated cells formed from the epidermal layer of the outer integument. Cells of the inner integument do not participate in construction of the seed coat since they are crushed and resorbed early in the ontogeny of seeds.

Several investigations of the variation present in the seed coat of orchids have provided systematically meaningful morphological data of both qualitative and quantitative nature. Such studies have become more numerous with the advent of the technique of scanning electron microscopy (SEM), with which seeds are observed with considerable depth of field. Some of these studies have revealed a wide variety of seed morphological features (Arditti *et el.*, 1979, 1980; Healey *et al.*, 1980; Barthlott and Ziegler, 1980, 1981; Rauh *et al.*, 1975; Tohda, 1983, 1985, 1986). Examples of detailed SEM studies of seeds from Epidendroideae include those of members of the Epidendreae and Neottieae (Clifford and Smith, 1969), Catasetinae (Chase and Pippen, 1990), Oncidiinae and related subtribes (Chase and Pippen, 1988), *Chiloschista lunifera* (Barthlott and Ziegler, 1980), *Microcoelia* (Jonsson, 1981), *Nervilia* (Petterson, 1991) and *Vanilla* (Cameron and Chase, 1998).

The most comprehensive study of seed coats in Orchidaceae is the doctoral dissertation by Ziegler (1981) on the subfamily Orchidoideae, of which a very small section of the results have been published (Barthlott and Ziegler, 1981). Ziegler recognised 20 seed types based on overall morphology, and these have been employed in subsequent studies of orchid phylogeny and classification by Petterson (1991), Dressler (1993) and Molvray and Kores (1995).

Many systematic studies have given varied suggestions regarding the taxonomic rank at which seed coat characters can be informative. Barthlott (1976, 1984a, b) was the first to express the opinion that the taxonomic-phylogenetic significance of orchid seed

morphological data is rather limited at the species level but could be useful at subtribal and tribal levels. Increased sampling and examination of variation in species that exhibit a particular seed type, e.g., the *Maxillaria* type of Oncidiinae (Chase and Pippen, 1990), have shown that the utility of seed coat morphology as a distinguishing feature does not usually extend below the taxonomic level of tribe and is only rarely valuable for intergeneric studies. Contrary to these findings, however, Cameron and Chase (1998) have recently reported that seed morphology clearly distinguishes genera of vanilloid orchids. The only study where seed morphology was useful in delimiting species is that of Wildhaber (1972) where distinction between the species of the genus *Orchis* was made by means of their seed coat characters.

From the functional point of view the morphology of the seed coat is said to have properties affecting aerodynamics and wettability (Rauh *et al.*, 1975; Kurzweil, 1993a, b; Barthloth, 1976; Murren and Ellison, 1998) or mycotrophy (Rasmussen, 1995; Leake, 1994). However, little is known about the ecological aspects of seed morphology in Orchidaceae. Certain unique seed coat characters, for example, hooked seeds (Chase and Pippen, 1988), were recorded for epiphytes but whether these form as a result of ecological influence through adaptation is not established. Research work has concentrated on the evolutionary specialization of the seed coat sculpturing without mention of their ecology, which would indicate the functional morphology of the seeds.

The Kenyan species of the subfamily Epidendroideae, which are the subject of the present study, are morphologically heterogeneous and found in habitats that range across the country's elevation, being found both in lowland and montane vegetation.

Although studies of seed coats in a small number of tropical orchids with close relatives found in Kenya have been conducted e.g. *Liparis loeselii* (Burgeff, 1936), *Bulbophyllum elisae, Calanthe veratrifolia,*(= *C. triplicata*) *Epidendrum* (hybrid), *Liparis reflexa,*

Oberonia iridifolia (Clifford and Smith, 1969); *Eulophia guineensis, Vanilla* sp., *Epidendrum nocturnum, Cyrtorchis* sp., *Diaphananthe* sp. (Barthlott, 1976), *Eulophia alta* and *E. ecristata* (Chase and Pippen, 1990), *Microcoelia* spp. (Jonsson, 1981a, b), and *Vanilla* (Cameron and Chase, 1998), it is considered that the data obtained in the present study will be a significant contribution towards a comparative study of the Kenyan seeds with those earlier studied. Several questions have been posed: for example, how does the varied ecology of orchids affect seed development or what role does common ancestry have in the evolution of seed coat characters?. Another important area which has been considered in the present study is the possibility of using the variation in seed coat morphology to discuss the relationships to ecology and systematics.

The aim of the present study is to utilise both quantitative and descriptive SEM characters of the Kenyan Epidendroid orchids (Dressler, 1993) to examine at species level whether variation of seed coat morphology is determined by shared habitats or phylogeny of the species.

3.2 MATERIALS AND METHODS

3.2.1 Species studied and seed sources

Species were sampled to reflect the full range of habitats in Kenya (from coastal forests to montane forest, from arid shrubland/scrubland to mesic grassland, from black cotton soil to sandy soil substrate). In addition, an attempt was made to obtain seed from all the subtribes of the Epidendroideae in Kenya. This would allow the testing of the hypothesis that ecology or phylogeny drives the seed coat morphology.

Fifty species from 20 genera and four tribes of the Kenyan Epidendroideae were studied. Where possible, several collections per species were included, to assess intraspecific variation.

Seeds were collected from mature capsules of living plants in the field: the vouchers and localities are summarised in Table 3.1. Fully developed seeds were isolated from mature capsules that had dried under natural conditions in the field. Seeds were removed from the capsules by opening the capsules fully with the aid of a scalpel and collecting the seeds on white papers, from which they were transferred into clear glass tubes. Seed colour was determined subjectively. All seeds were then stored in specially designed small manila envelopes placed over calcium chloride ($CaCl_2$) in a sealed container, and preserved in a refrigerator at 10°C for future handling i.e. for scanning electron microscopy. These storage conditions were found suitable for keeping the seeds intact so that no physiological or morphological changes occurred in them.

Table 3.1: Collection data for orchid seeds used for Scanning Electron Microscopy (SEM). The specimens are listed in alphabetical order in each subtribe following the classification of Dressler (1993).

Species	Collector & collection no.	Locality
Tribe Vandeae		
Subtribe Aerangidinae		
Aërangis brachycarpa (A.Rich.) Th. Dur. & Schinz	B. Bytebier 0018	Langata forest (1°22'S.,36°43'E.)
	J. M. Ochora 26	Olchore Onyore (0°45'S.,35°22'E.)
A. confusa J. Stewart	J. M. Ochora 26	Mathews Range (1°12'N.,37°22'E.)
A. coriacea Summerh.	B. Bytebier 0038	Tigoni (1°07.5'S.,36°40'E.)
A. luteoalba (Kraenzl.) Schltr.	B. Bytebier 0044	Lolgorian (1°14'S.,34°48'E.)
A. thomsonii (Rolfe) Schltr.	B. Bytebier 0075	Tigoni (1°07.5'S.,36°40'E.)
A. ugandensis Summerh.	B. Bytebier 0017	Kakamega forest (0°16'N.,34°55'E.)
	J. M. Ochora 40	Kakamega forest (0°16'N.,34°55'E.)
Angraecopsis gracillima (Rolfe) Summerh.	J. M. Ochora 66	Kuja Valley (0°47'S.,34°60'E.)
Chamaeangis odoratissima (Reichb.f.) Schltr.	J. M. Ochora 41	Kuja valley (0°47'S.,34°60'E.)
C. sarcophylla Schltr.	B. Bytebier 0072	Mt. Kenya (0°19'S.,37°31'E.)
C. vesicata (Lindl.) Schltr.	J. M. Ochora 72	Langata forest (1°22'S.,36°43'E.)
Cyrtorchis praetermissa Summerh.	J. M. Ochora 59	Mara N. Sanctuary (1°12,S.,35°05'E.)
Diaphananthe lorifolia Summerh.	B. Bytebier 0095	Saiwa Swamp N. Park (1°10'N,35°05'E.)
D. tenuicalcar Summerh.	J. M. Ochora 48	Aberdares Range (0°06'S.,36°28'E.)
Microcoelia exilis Lindl.	B. Bytebier 0015	Ukunda (4°17'S.,39° 33.5'E.)
M. koehleri (Schltr.) Summerh.	J. M. Ochora 44	Kuja valley (0°47'S.,34°60,E)
M. stolzii (Schltr.) Summerh	A. Foresti 109B.	Mathare (1°15'S.,36°49'E.)
Rangaeris amaniensis (Kraenzl.) Summerh.	B. Bytebier 0117	Mount Kulal (2°48'N.,37°05'E.)
Solenangis aphylla (Thou.) Summerh.	B. Bytebier 0008	Taru (3°46'S.,39°07'E)
S. wakefieldii (Rolfe) Cribb & J. Stewart	B. Bytebier 0009	Taru (3°46'S.,39°07'E)
Tridactyle bicaudata (Lindl.) Schltr.	J. M. Ochora 28	University of Nairobi (1°19'S.,36°48'E.)
Subtribe Aeridinae		
Cribbia brachyceras (Summerh.) Senghas	J. M. Ochora 43	Kuja Valley (0°47'S.,34°60'E.)

Subtribe Angraecinae		
Angraecum decipiens Summerh.	B. Bytebier 0112	Aberdares N. Park (0°10'S.,36°07'E.)
A. dives Rolfe	B. Bytebier 0011	Gazi (4°25'S.,39°30'E.)
A. erectum Summerh.	J. M. Ochora 16	Fourteen falls (1°10'S.,37°13E.)
Subtribe Eulophiinae		
Bolusiella iridifolia (Rolfe) Schltr.	B. Bytebier 0068	Aberdares N. Park (0°18'S.,36°07'E.)
Eulophia angolensis (Lindl.) Reichb.f.	J. M. Ochora 38	Milimaini swamp (10°4'S.,36°51'E.)
E. horsfallii (Batem.) Summerh.	B. Bytebier 0039	Kericho swamp (0°29'S.,35°04'E)
	J. M. Ochora 37	Rupingazi River (0°22N.,39°28'E.)
E. ovalis Lindl.	J. M. Ochora 21	Ngong hills (1°24'S.,36°31'E.)
E. petersii Reichb.f.	A. Foresti 103	Embu (0°27'S.,37°30'E.)
	J. M. Ochora 12	Kilima Kiu (1°33'S.,37°24'E.)
E. speciosa (Lindl.) Bolus	J. M. Ochora 30	Kilima Kiu (1°33'S.,37°24'E.)
E. streptopetala Lind. var. *stenophylla* (Summerh.) Cribb	J. M. Ochora 11	Fourteen Falls (1°10'N.,35°05'E.)
E. streptopetala Lindl. var. *streptopetala*	B. Bytebier 0064	Uashin Gishu (1°38'S.,35°30'E.)
Oeceoclades saundersiana (Reichb.f.) Garay & Taylor	B. Bytebier 0057	Mwache forest (4° 00'S.,39° 32'E.)
	J. M. Ochora 71	Kakamega forest (0°16'N.,34°55'E.)
Tribe Cymbideae		
Subtribe Cyrtopodinae		
Ansellia africana Lindl.	B. Bytebier 722	Hell's Gate N. Park (0°54'S.,36°21'E.)
Tribe Malaxideae		
Subtribe Liparidinae		
Liparis bowkeri Harv.	J. M. Ochora 5	Saiwa swamp (1°10'S.,35°05'E.)
L. deisteli Schltr.	J. M. Ochora 4	Saiwa swamp (1°10'S.,35°05'E.)
Oberonia disticha Lindl.	D. Patel 78	University of Nairobi (1°19'S.,36°48'E.)
Tribe Epidendreae		
Subtribe Coeliinae		
Epidendrum cinnabarinum Salzmann	J. M. Ochora 14	Lanet (0°24'S.,36°08'E.)
E. ibaguense HBK	J. M. Ochora 70	Langata (1°24'S.,36°43'E.)
Subtribe Polystachyinae		
Polystachya adansoniae Reichb.f.	J. M. Ochora 29	Kuja valley (0°47'S.,34°60,E)
P. bella Summerh.	B. Bytebier 0034	Kericho (0°24'S.,35°33'E.)
P. bennettiana Reichb.f.	B. Bytebier 0089	Taita hills (3°21'S.,38°24'E.)
	B. Bytebier 0091	Saiwa swamp N. Park (1°10'N.,35°05'E.)
P. campyloglossa Rolfe	A. Foresti 744	Mua hills (1°29'S.,37°10'E.)
P. confusa Rolfe	B. Bytebier 0062	Mount Kulal (2°48'N.,37°05'E.)
P. cultriformis (Thou) Sprengel	A. Foresti 735	Narok (1°04'S.,39°09'E.)
P. dendrobiiflora Reichb.f.	L. Newton 5595	Rukinga hills (3°49'S.,38°41'E.)
P. eurychila Summerh.	B. Bytebier 445	Saiwa Swamp N. Park (1°04'N.,35°06'E.)

P. fusiformis (Thou.) Lindl.	J. M. Ochora 53	Manga Range (0°33'S.,34°57'E.)
P. spatella Reichb.f	J. M. Ochora 18	Aberdares Range (0°06'S.,36°28'E.)
P. tessellata Lindl.	J. M. Ochora 61	Kinango (4°19'S.,39°19'E.)
P. transvaalensis Schltr.	A. Foresti 736	Mau escarpment (1°02'S.,36°08'E.)
P. vaginata Summerh.	A. Foresti 771	Naivasha (0°49'S.,36°14'E.)

3.2.2 Microscopy

Due to the small size of the seeds, all morphological observations were done by scanning electron microscope (SEM). Light microscopy (LM) simply does not provide a sufficiently resolved and detailed three-dimensional image of the seed coats. Details of seed coat structure become much more apparent under the SEM, especially fine details such as cell-wall lignification patterns and wax deposits, which are hardly discernible under the light microscope. Even if seed coat colour was readily determined by mere observation in the field or when seeds are poured in to small clear glass tubes in the laboratory (Barthlott, 1984a, b), a dissecting microscope is necessary for additional confirmation of this significant character.

Two instruments were used to examine the seeds. At the East African Herbarium, Kenya, a JEOL JSM-100 scanning electron microscope at an accelerating voltage of 25 kV was used, with a working distance of 48 mm. All electron micrographs were recorded from the photographic cathode-ray tube with Ilford HP 5 roll film. Material for this analysis was mounted on double-sided adhesive tape, and the sides of the stubs were coated with silver paste, to enhance electron flow, and the stubs gold coated in a fine ion sputter JFC-1100 vacuum evaporator. At the Electron Microscopy Unit, University of Cape Town, seeds were mounted on aluminium stubs using carbon paste mixed with glue at a ratio of *ca.* 1:1. The stubs were coated with gold and examined with a Cambridge S200 scanning electron microscope, at a filament voltage of 10 kV, micrographs being taken using Ilford 125 FP4 roll films. No differences due to voltage were observed, and magnification of entire seeds varied from 150 to 980 ×.

For the SEM analyses five to ten air-dried seeds of each species were mounted on the stubs. All the samples were observed for any intraspecific variation.

3.2.3 Morphometry

Measurements of seed coat attributes (Table 3.2) were done from the SEM images of seeds using Leoscan software on a LEO S400 computer-assisted measurement TV using the point to point option. In this procedure the SEM images are transmitted to a stereoscan S440 computer for scanning. The images are focused on the TV screen, and measurement options of the Leoscan software are used. Measurements were performed on at least three specimens on each stub. All the seed sizes were displayed on the screen and recorded on the stereoscan in micrometers (μm).

The studied specimens were selected and quantified on the TV screen to establish any differences between individuals of the same species. Data collected included seed and cell lengths, breadths, and cell numbers. Cells were counted over the entire length and widest regions of testae in absolute units. The seeds and cells were then measured in their longest and widest axes in micrometers. Only average sizes of variables were recorded and used in further analyses. Standard deviations (SD) representing variation of the individual seeds of a species were calculated and these proved particularly useful in length and width measures.

The outlines of the seeds and cells were traced from SEM micrographs on to transparent cartographic papers and shape differences were determined in terms of their two-dimensional symmetry, using the terminology of Stearn (1992). Data were recorded from all the electron micrographs taking into account the differences in magnification.

3.2.4 Qualitative data

In addition to raw measurement and count data, qualitative (meristic) characters showing morphological variation of the seeds were generated by SEM examination of the seed coats (Table 3.2). Characters associated with testa cells, such as arrangement of the cells, their middle lamella, secondary sculptures, epicuticular waxes on seed coats, and general micro-ornamentation, were very conservative and highly informative from seed to seed in a species.

3.2.5 Photographic imaging

SEM micrographs were produced using Adobe Photoshop image processor software, version 4.0. The SEM negatives of seeds were scanned by a Nikon film scanner LS-4500 AF and digitized on a SAMPO/ALPHA scan 17 mx IBM-PC. Photographic images were observed in Photoshop using the TWAIN-32 option of the same PC and

printed using Nikon Codonics image printing software on a NP-600 Nikon Codonics photographic network printer at a brightness of camma 1.5 and a contrast of 30.

3.2.6 Analysis of data

In order to simplify the discussion of the variation in the seed coats, it is advantageous to establish seed groups. Rather than form an intuitive classification of the seed coats into "types", or to use one or two striking seed coat attributes, it is better to use multivariate analysis which can take all the variation into account. To this end both cluster analysis and ordination were employed utilizing the characters listed in Table 3.3. Three complementary ordination algorithms were used: Principal Components Analysis (PCA), Multidimentional Scaling (MDS), and Principal Co-ordinates Analysis (PCO).

3.2.6.1 Cluster analysis

Data were entered directly on to a computerised spreadsheet program (Microsoft Excel version 5.0) for easy accessibility by other computer analytical programs. The patterns of variation within the matrix were analysed using both clustering techniques and ordinations. As no special (evolutionary) significance was attached to particular characters, all were given an equal weighting in the assessment of similarity between species. A data matrix of seeds was developed using 39 attributes and analysed using the numerical phenetic program NTSYS-PC for Windows version 2.0 (Rohlf, 1997). The character data were standardised (Sneath and Sokal, 1973) using the STAND (standardization) subroutine so that each character would have a sample mean of zero and a sample standard deviation of unity. The distance matrices determining relationships of taxa in the matrix were calculated by the SIMINT (similarity interval) subroutine in terms of "taxonomic" distance. This gives an indication of the inter-taxon phenetic relationship or affinity (Dunn & Everitt, 1982). Distance coefficients also appear to have the advantage that they is less affected by the number of operational taxonomic units (OTU's) included in the study (especially when the number of OTU's is $\geq$ 50). The sequential, agglomerative, hierarchical, and nested clustering method (SAHN) subroutine was used to perform the unweighted pair group method with arithmetic averages (UPGMA) clustering pattern (Sneath and Sokal, 1973). UPGMA was selected to cluster OTU's, as it has been shown to minimise distortions of inter-OTU distances during clustering (Rohlf, 1970). The COPH (Cophenetic) subroutine was used to test the goodness-of-fit between the distance implied in the clustering matrices and the

original distance or correlation matrices. Cophenetic correlation below about 0.8 may indicate such severe distortion in the dendrogram at lower linkages that the diagram is misleading.

3.2.6.2 Ordination

3.2.6.2.1 Principal Component Analysis (PCA)

PCA was performed to determine which characters are most important in defining the relationships between the seeds in reduced dimensions of multivariate analysis. PCA assumes that the variation in each character is continuous. The data set analysed here contains binary and meristic characters, which do not satisfy this assumption. Consequently, to establish the patterns of relationships Multidimensional Scaling and Principal Co-ordinates analyses were performed: these derive the co-ordinates of each OTU in hyperspace from the distance matrix, not from the original standardized matrix, and hence do not contain the assumption that the data varies continuously.

3.2.6.2.2 Multidimensional scaling (MDS)

The symmetrical (dis)similarity distance coefficients matrix data used in the cluster analysis was subjected to a nonmetric multidimensional scaling analysis, nonmetric-MDS (Kruskal, 1964; Shepard, 1980). This ordination technique was used to represent the dissimilarity among the objects (seeds) in hyperspace so that the interpoint distances in the dimensional space correspond as well as possible to the observed distances between the objects. The ordination ranks similar objects close together and dissimilar objects far apart in selected dimensions.

The statistic, stress, which takes the place of the criterion of "variance accounted for" in PCA measures the fitness of distances in the configuration space to monotone function of the original distances and for the purpose of the present study the measure that was used is STRESS 2 in three dimensions. The stress value shows how well the graphic distances fit the distance values in the original data. The ranking achieved was then compared to those in the original similarity matrix, giving a measure of stress, which is used to calculate the goodness-of-fit or badness-of-fit of the results with original data. Stress value is optimized through iterations i.e. with increasing iterations stress values are improved.

3.2.6.2.3 Principal co-ordinates (PCO) analysis

The symmetric (dis)similarity distance coefficients were transformed to a scalar product by using the double centre module of NTSYS-PC. In this way it was possible to compute eigenvalues and eigenvectors (resulting in a principal co-ordinates analysis, Gower, 1966, 1967, 1969, 1971) for the quantitative and binary data.

The EIGEN module was used to compute the eigenvectors of the transformed matrix. The resulting vectors, the principal co-ordinate axes, were then utilized in producing graphic representation of the objects by the projection module.

3.3 RESULTS

3.3.1 Seed variation

3.3.1.1 Seed size

Trends in seed dimensions were discerned in this study and revealed that size variation occurs even within a given fruit. The seeds are generally minute, varying from ≈ 174 to 2818 µm in length and 34 to 291 µm in width, with the majority of them having length in the 440-800 µm range. The largest seeds are found in two species of the genus *Epidendrum* (Figs. 3.29-3.32), with an extraordinary seed length of 1598 to 2818 µm. There is a marked difference in width of seeds, with *E. cinnabarinum* seeds (width 287 µm) being broader than the rest of the species. Seeds of species of *Eulophia* and *Ansellia africana* are rather long in size, measuring 644 to 896 µm, while the smallest seeds, <500 µm, are most commonly observed in epiphytic species.

3.3.1.2 Seed shape

In shape the seeds show differences according to genera or sometimes species. Ovoid seeds are observed in *Polystachya spatella* (Fig. 3.13), *P. adansonii* (Fig. 3.23), *Liparis bowkeri* (Fig. 3.27), *Oberonia disticha* (Fig. 3.44) and *Cyrtorkis praetermissa* (Fig. 3.43). Oblong seeds are found in the majority in the study group as exemplified by *Cribbia brachyceras* (Fig. 3.5), *Angraecopsis gracillima* (Fig. 3.7), *Polystachya campyloglossa* (Fig. 3.9), *P. transvaalensis* (Fig. 3.11), *Angraecum erectum* (Fig. 3.19), *Rangaeris amaniensis* (Fig. 3.21), *P. tessellata* (Fig. 3.25), *Rangaeris muscicola* (Fig. 3.41) and *Chamaeangis odoratissima* (Fig. 3.45). Seeds with narrowly oblong shapes, though fewer compared with other shapes, occur in *Microcoelia koehleri* (Figs. 3.1), *Microcoelia exilis* (Fig. 3.3), and *Solenangis*. Seeds with fusiform shapes differ from all

others by tapering towards both poles as in *Epidendrum cinnabarinum* (Fig. 3.29), *E. ibaguense* (Figs. 3.31), *Oeceoclades saundersiana* and *Ansellia africana*. The seeds of the genus *Eulophia* are uniformly club-shaped to almost fusiform (Figs. 3.33, 3.35, 3.37, 3.38). Occasionally more than one seed shape is encountered in a genus as is prominently demonstrated in *Polystachya*, where both ovoid and oblong shapes are exhibited.

3.3.1.3 Colour

The seeds have a variety of colours; white, yellow, brown, orange, light brown, dark brown and shiny yellow. Though typically uniform in most genera, colour varies to differing extents among species of a genus, as observed, for example, in *Eulophia* and *Aerangis* (Table 3.2).

3.3.1.4 Seed coat ornamentation

General microornamentation of the seed surfaces fall into two categories, the reticulate pattern (Figs. 3.27-3.40) found in terrestrial species, and the wavy pattern of epiphytes (Figs. 3.1-3.26, 3.41-3.46). Interesting additional features to the general patterns of ornamentation in twig epiphytes are the prominent testa extensions formed by transverse anticlinal walls, which were first observed in vandoid orchids (Chase and Pippen, 1988). These form hooks in *Cribbia brachyceras* (Figs. 3.5-3.6), and trichome-like processes in species of *Microcoelia* (Figs. 3.1-3.4) and *Solenangis*. Similar extensions in the form of ridge-like structures are also found in the monotypic *Ansellia africana*. In *Oberonia disticha* (Fig. 3.44), *Polystachya spatella* (Fig. 3.13), some populations of *Ansellia africana*, and species of *Chamaeangis* (Fig. 3.45) seed coats are characteristically twisted clockwise.

3.3.2 Cell variation

3.3.2.1 Cell structure

The seed coats consist of dead cells with concave periclinal walls. The main part of the seed coat is made up of 4-65 cells on the long axis and 4-25 cells on the short axis. Two categories of cells are evident: the large elongate cells of terrestrial species and the narrow slender, almost fused cells of epiphytes. The large cells are found more in the median portion of the seeds than at the ends.

3.3.2.2 Cell shape

The diversity of cell shapes especially in the median portion of the seeds is considerable; a rectangular shape is found in *Ansellia africana* and *Oeceoclades saundersiana*, a hexagonal shape in *Eulophia speciosa* (Figs. 3.35-3.36), hexagonal and rectangular in *Liparis* (Figs. 3.27-3.28), and the rest of *Eulophia* species (Figs. 3.33, 3.34, 3.37-3.40), irregularly elongate shapes in species of *Microcoelia, Cribbia brachyceras* and *Oberonia disticha* (Figs. 3.9-3.14, 3.41-3.46), a dumbbell shape in *Angraecopsis gracillima* (Figs. 3.7-3.8) and spatula-like shapes in *Aerangis* (Fig. 3.15-3.24).

3.3.2.3 Cell end walls

The cell end walls are flat in *Eulophia, Liparis* and *Oeceoclades*, arched and raised above the seed surface in *Polystachya* and many of the epiphytes, or transversely form trichome-like features, as in *Microcoelia* and *Solenangis*, hook-like features (*Cribbia brachyceras*), or ridge-like structures (*Ansellia africana*).

3.3.2.4 Cell endings

The cell endings occur independently in the seed coat rather than being joined to the longitudinally neighbouring cell by a middle lamella, connecting with those of adjacent cells or transversely lying side by side in the seed coat (Figs. 3.14, 3.27-3.40). In rare instances three or four cell endings are associated together by a common middle lamella at the fusing junctions of cells (Fig. 3.7-3.8). The wall-to-wall adhesion zone is completely or partially fused. The middle lamella is visible in most species or sunk in with a sunken line between the walls, as in *Eulophia* and *Epidendrum*. All species possess a pore at the micropylar tip with variable diameter depending on the genus.

3.3.2.5 Anticlinal walls

The thickened anticlinal walls of adjacent cells of epiphytic species are highly fused. Other species have seed surfaces that consist almost entirely of anticlinal walls (notably in *Polystachya dendrobiiflora*). The longitudinal walls are straight in most species or irregularly elongate. Transverse anticlinal walls of the epiphytes are arched and raised above the seed surface except in *P. dendrobiiflora* and *Tridactyle bicaudata* where they are flat. The fusion of anticlinal walls in species of *Eulophia, Oeceoclades*, *Epidendrum*, and *Liparis* is incomplete, leaving a groove, in the form of a concavity, at the corners where three cells meet. Their transversal walls are flat, appearing raised only because

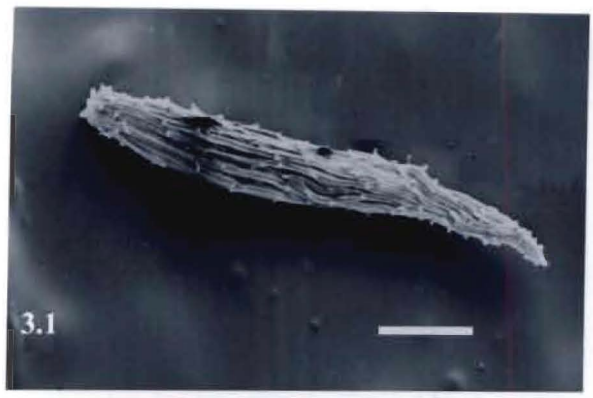
the outer periclinal walls are sunken. In *Ansellia africana* anticlinal walls are completely fused and covered with a membrane such that their dual nature is undetectable. The anticlinal walls are fairly uniform in thickness, measuring 1.7-5.6 µm.

3.3.2.6 Periclinal walls

Periclinal surfaces are concave and sunken to take a basin-like appearance in the seeds of terrestrial species but in epiphytes they are almost completely covered by the thick anticlinal walls and are visible only from cell ends. They exhibit different kinds of sculpturing, which reveals a clear distribution pattern only in terrestrial species. Sculptures in the form of longitudinal striations occur exclusively in *Ansellia africana*. Transverse striations of species of *Eulophia*, *Oeceoclades* and *Liparis* vary greatly in thickness, abundance and distance from one another. The widely spaced striations of *Liparis* and *Oeceoclades* join the anticlinal walls at right angles, and in the closely spaced striations of *Eulophia*, except *E. streptopetala*, they vary greatly in angle of attachment to the anticlinal walls. These features are more or less uniform in each genus. The periclinal walls between these striations are smooth in *Ansellia africana* and warty in all other species. The periclinal surfaces of the seeds of epiphytes are warty, except those of *Oberonia disticha* and *Cyrtorchis praetermissa*, both of which have smooth periclinal surfaces.

3.3.2.7 Epicuticular secretions

Secretions, mainly of waxy deposits, are observed in many epiphytic species. Verrucose wax deposits along the entire length of anticlinal walls are widespread in *P. dendrobiiflora, P. transvaalensis, P. confusa and P. bella*. Verrucose waxes also occur in *Bolusiella iridifolia* and *Tridactyle bicaudata*. Other deposits in the form of wax caps, first referred to by Ziegler (1981), are present in *Microcoelia, Solenangis, Diaphanathe, P. tessellata*, and *Ansellia africana*. In *Ansellia africana* the secretions are also observed along the longitudinal striations of periclinal walls.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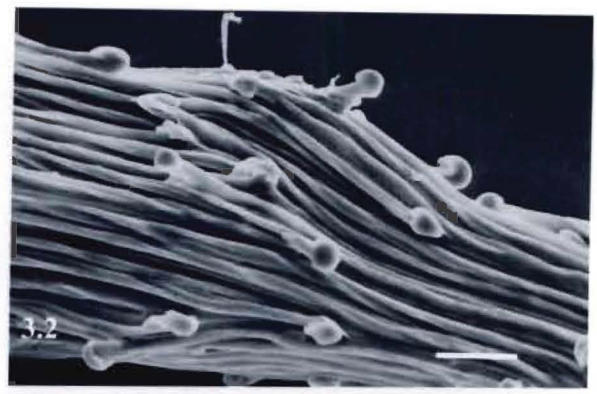
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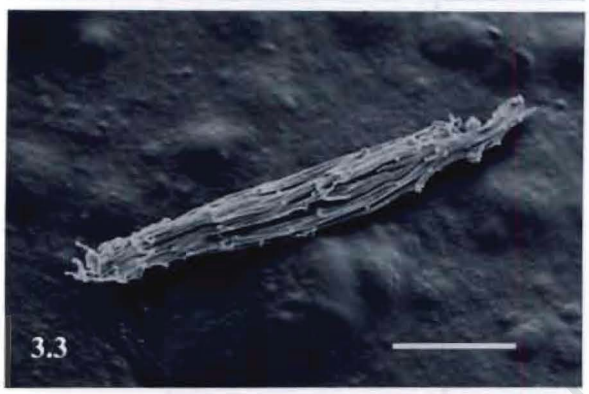
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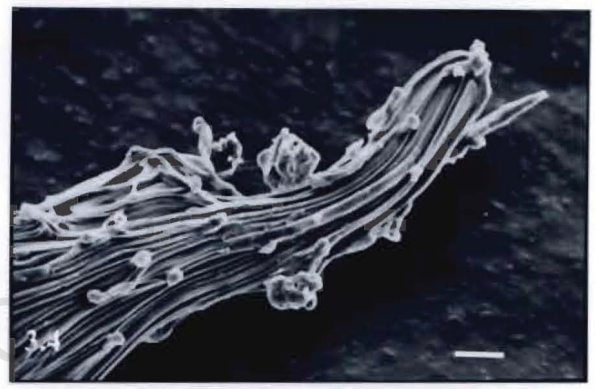
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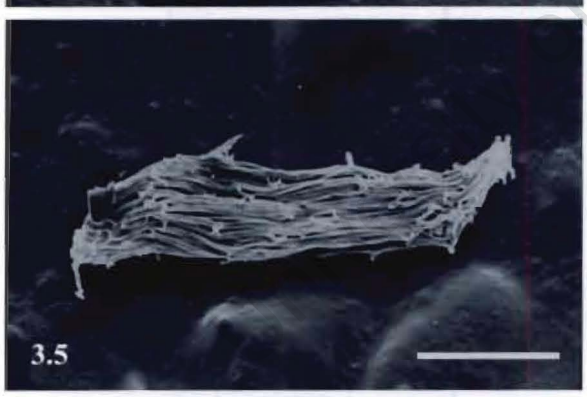
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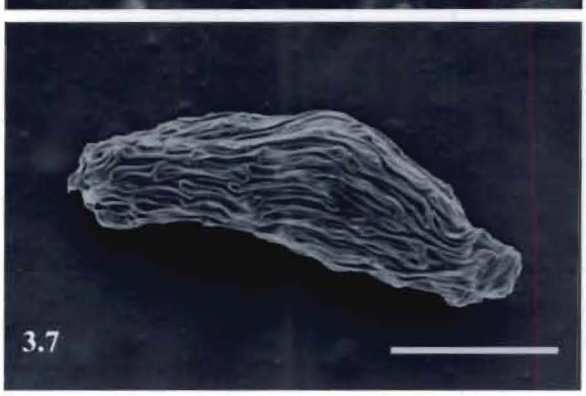
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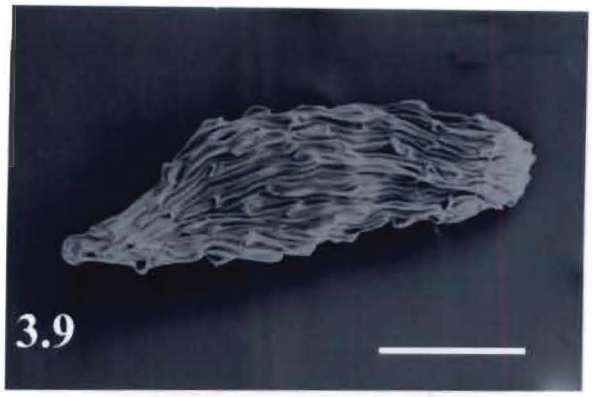
3.8

Figures 3.1-3.8: Scanning Electron Micrographs (SEM) of mature seed coats (In all cases scale bar = 100 μm for entire seed and scale bar = 10 μm for details of seed surface). Figs. 3.1-3.4 *Microcoelia.* Figs. 3.1-3.2: *M. koehleri*, entire seed; Fig. 3.2, details of seed surface. Figs. 3.3-3.4: *M. exilis;* Fig. 3.3, entire seed; Fig. 3.4, seed surface. Figs. 3.5-3.6*: Cribbia brachyceras*; Fig. 3.5: entire seed; Fig. 3.6: seed surface details. Figs. 3.7-3.8: *Angraecopsis gracillima*; Fig. 3.7: entire seed; Fig. 3.8: seed surface details.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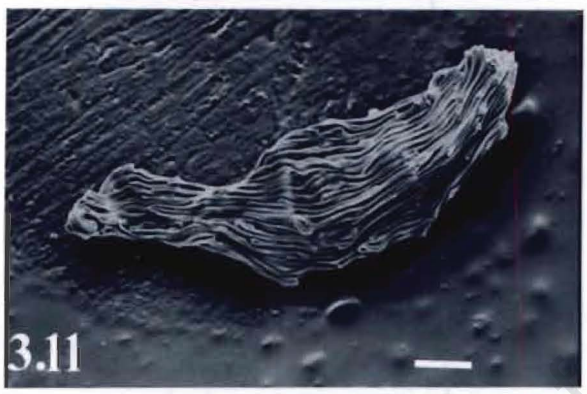
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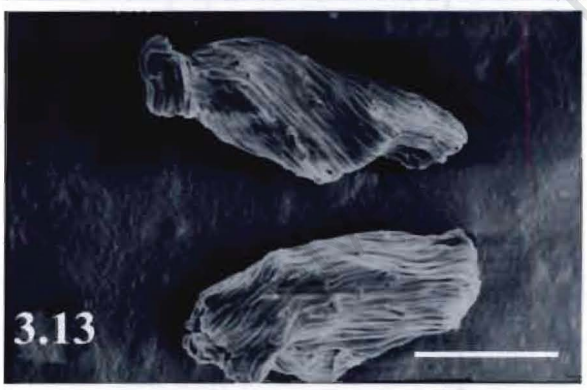
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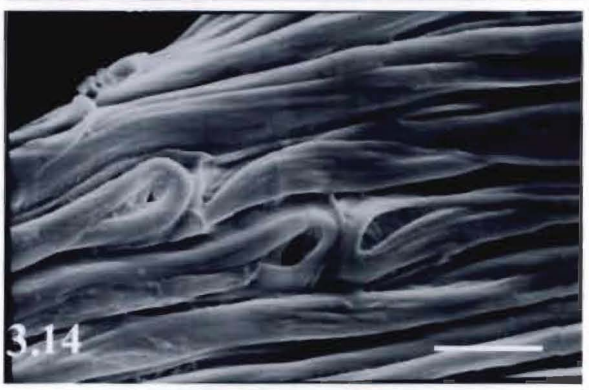
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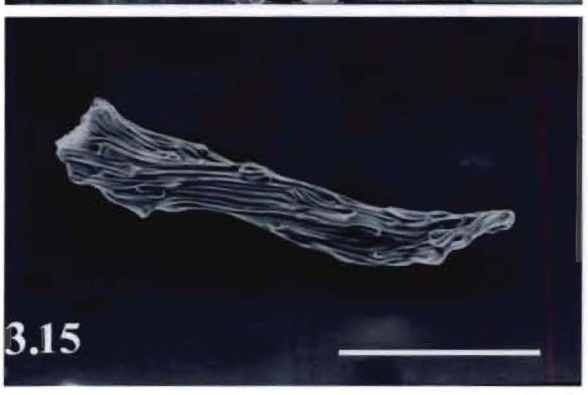
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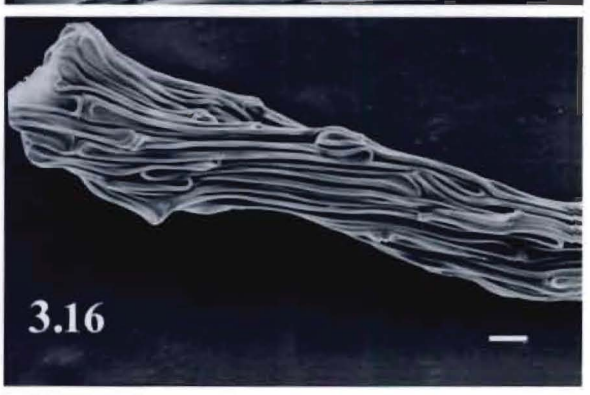
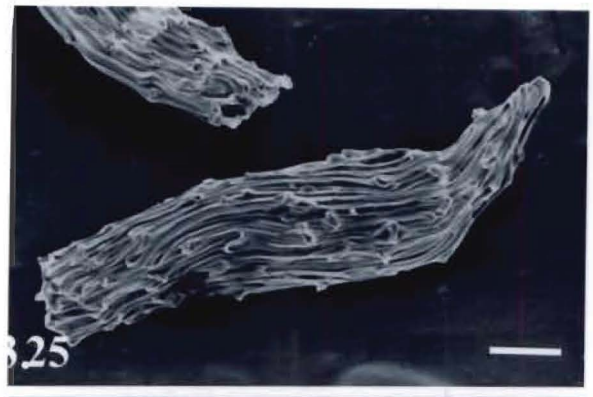

3.16

Figures 3.9-3.16: Scanning Electron Micrographs (SEM) of mature seed coats (in all cases scale bar = 100 μm for entire seed and scale bar = 10 μm for details of seed surface). Figs. 3.9-3.11. *Polystachya;* Fig. 3.9: *P. campyloglossa*, entire seed; Fig. 3.10: seed surface details. Fig. 3.11: *P. transvaalensis*, entire seed; Fig. 3.12: seed surface details. Fig. 3.13: *P. spatella*, entire seed; Fig. 3.14. seed surface details. Figs. 3.15-3.16. *Aerangis* : Fig. 3.15, *A. brachycarpa*, entire seed; Fig 3.16: seed surface details.

Figures 3.17-3.24. Scanning Electron Micrographs (SEM) of mature seed coats (in all cases scale bar = 100 μm for entire seed and scale bar = 10 μm for details of seed surface). Figs. 3.17-3.18: *A. ugandensis*, entire seed (scale bar = 100 μm); Fig. 3.18: seed surface details (scale bar = 10 μm). Fig. 3.19-3.20 *Angraecum erectum*: Fig. 3.19: entire seed; Fig. 3.20: seed surface details. Figs. 3.21-3.22: *Rangaeris amaniensis.* Fig. 3.21: entire seed; Fig. 3.22: seed surface details. Figs. 3.23-3.24: 3.23: *P. adansoniae*, entire seed; Fig. 3.24: seed surface details.

Figures 3.25-3.32. Scanning Electron Micrographs (SEM) of mature seed (in all cases scale bar = 100 μm for entire seed and scale bar = 10 μm for details of seed surface). Fig. 3.25: *P. tessellata*, entire seed; Fig. 3.26. seed surface details. Figs. 3.27-3.28: *Liparis bowkeri*. Fig. 3.27: entire seed; Fig. 3.28: seed surface details. Figs. 3.29-3.32. *Epidendrum*. Figs. 3.29: *E. ibaguense,* entire seed; Fig. 3.30: seed surface details. Fig. 3.31: *E. cinnabarinum,* entire seed; Fig. 3.32: seed surface details.

Figures 3.33-3.40. Scanning Electron Micrographs (SEM) of mature seed coats (in all cases scale bar = 100 μm for entire seed and scale bar = 10 μm for details of seed surface). *Eulophia.* Fig. 3.33: *Eulophia streptopetala* var. *streptopetala* entire seed; Fig. 3.34: seed surface details. Fig. 3.35. E. *speciosa* entire seed; Fig. 3.36. seed surface details. Fig. 3.37: *E. horsfallii*, entire seed; Fig. 3.38: seed surface details. Fig. 3.39: *E. angolensis*, entire seed; Fig. 3.40: seed surface details.



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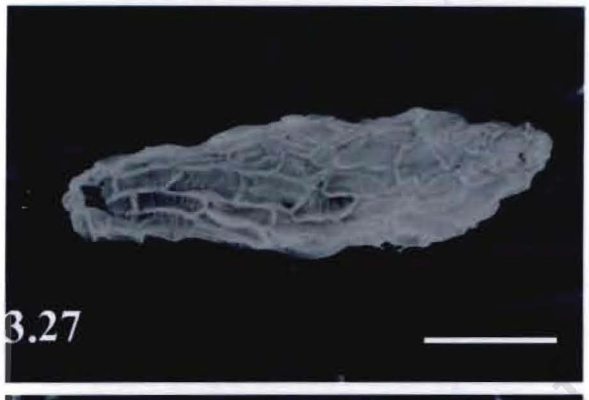

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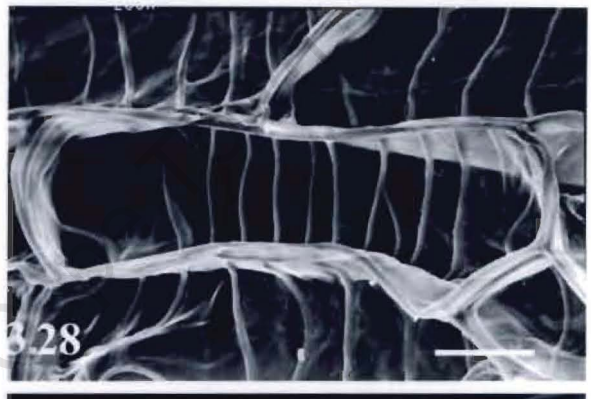

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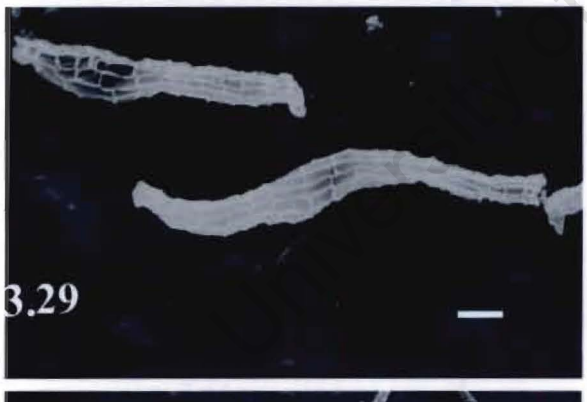

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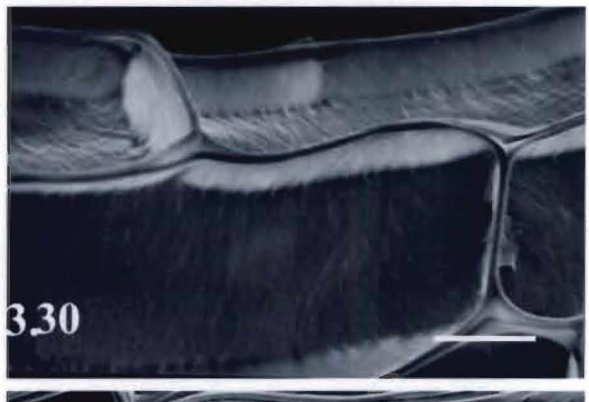

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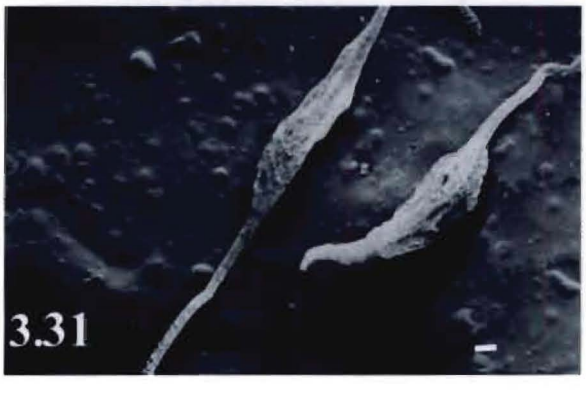

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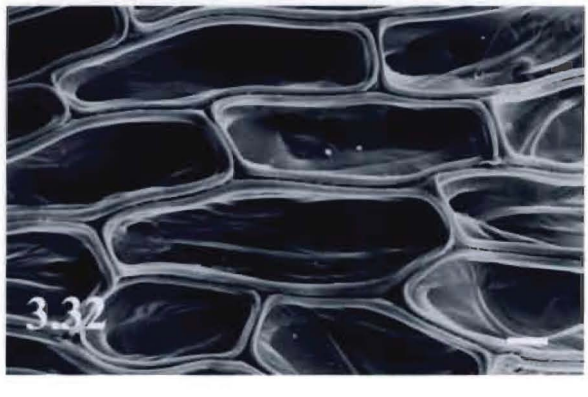

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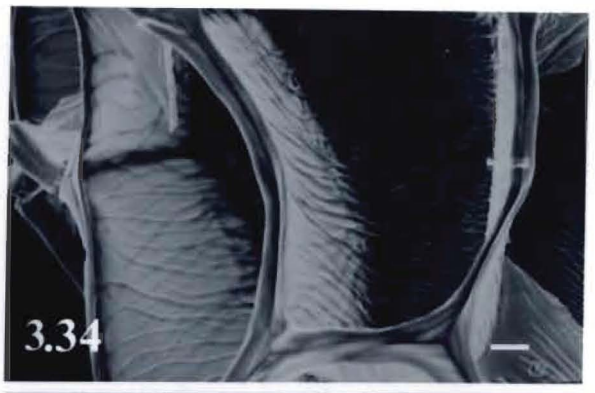
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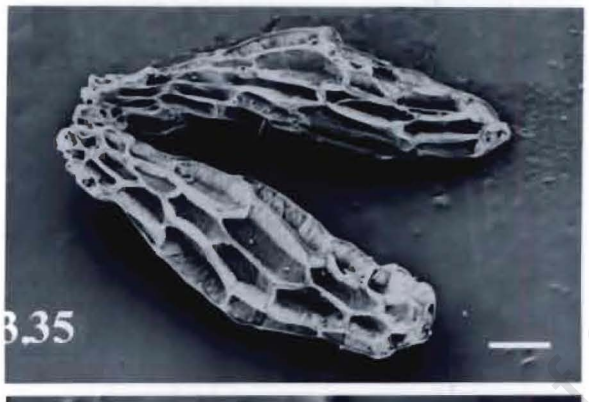
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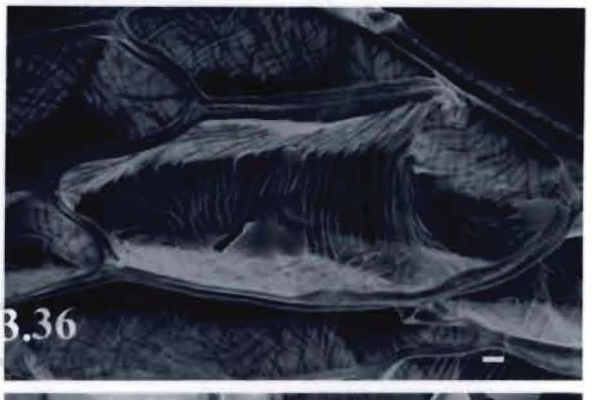
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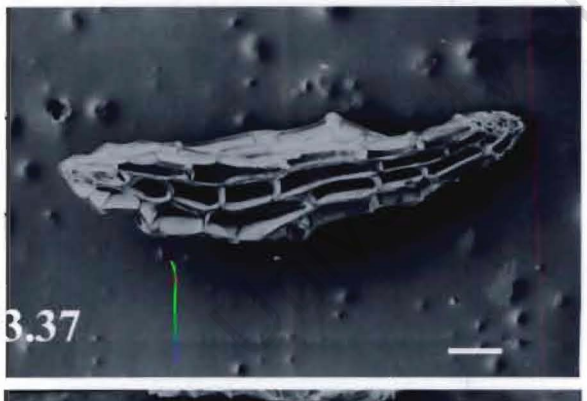
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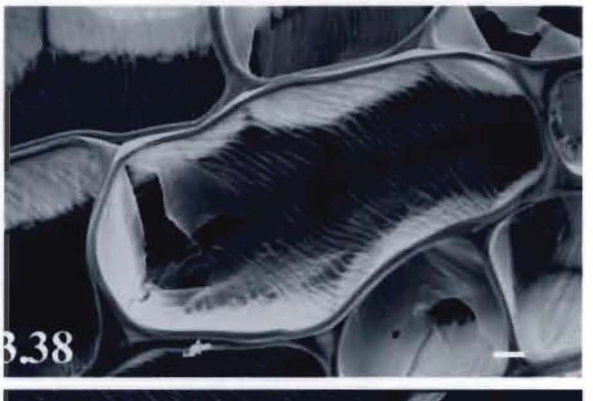
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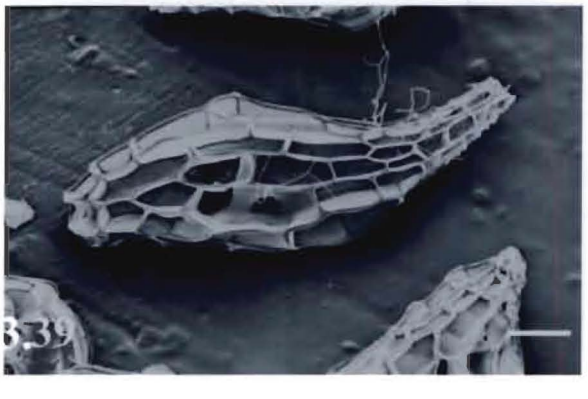
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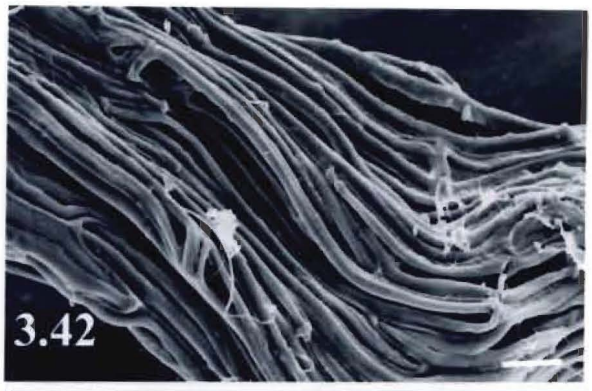
Figures 3.41-3.46. Scanning Electron Micrographs (SEM) of mature seed coats (in all cases scale bar = 100 μm for entire seed and scale bar = 10 μm for details of seed surface). *Rangaeris muscicola*. Fig. 3.41: entire seed; Fig. 3.42: seed surface details. Fig. 3.43: *Cyrtorchis praetermissa*. Fig. 3.44: *Oberonia disticha*. Fig. 3.45. *Chamaeangis odorattissima*. 3.46. *Polystachya fusiformis*.


3.41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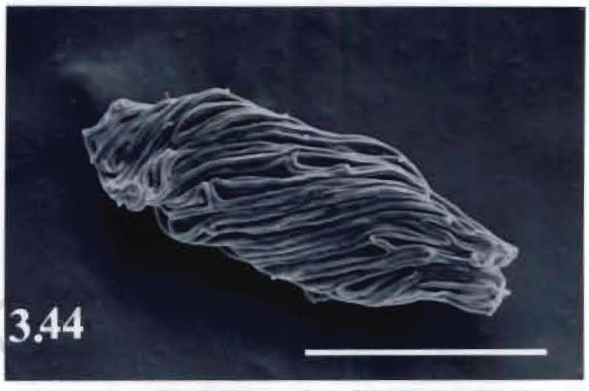
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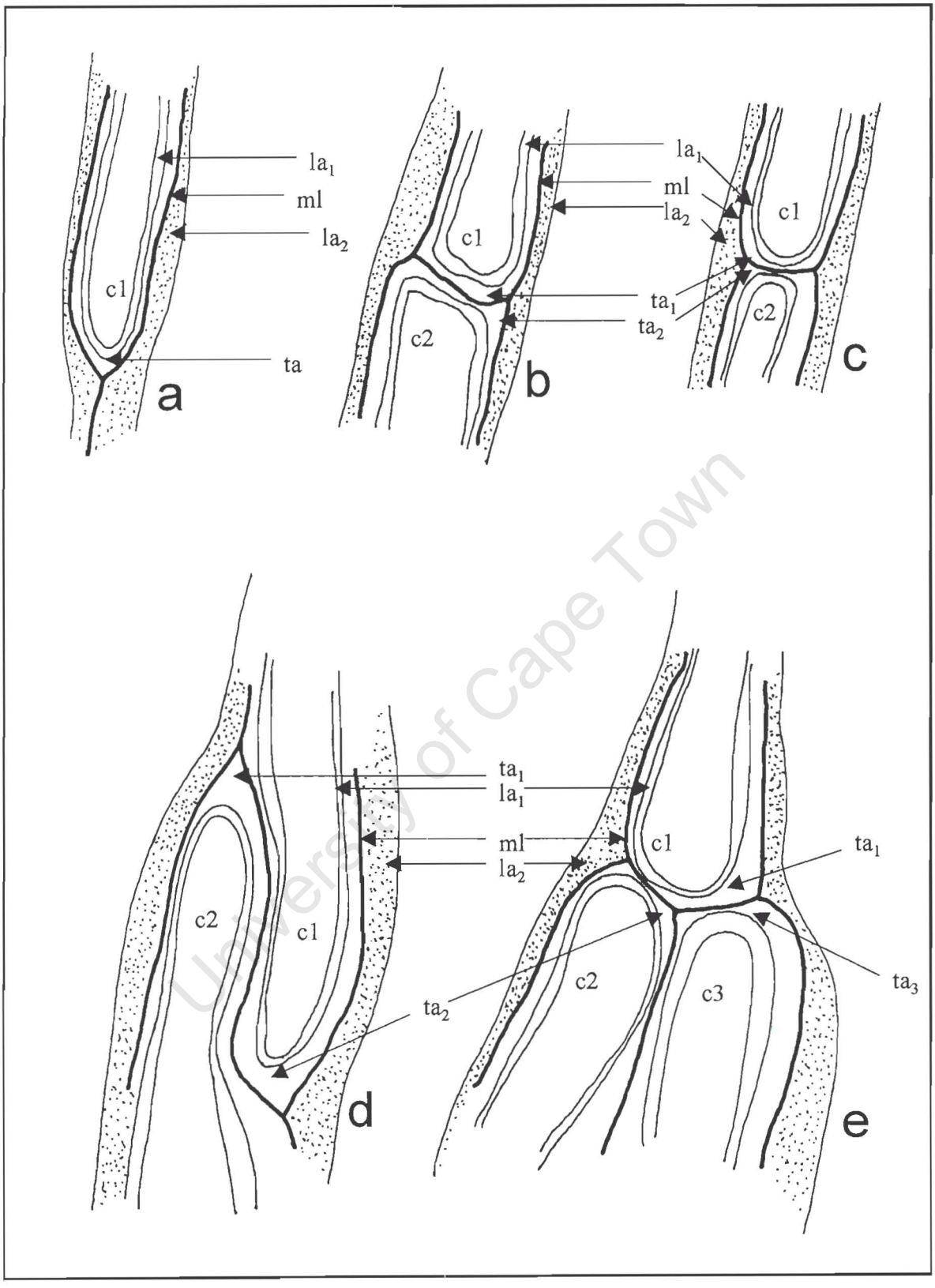


Figure 3.47: Illustrations of cell endings of mature seed coats. Fig. a. Independent cell endings, C1 = seed coat cell, ml = middle lamella, la_1 = longitudinal anticlinal wall, la_2 = longitudinal anticlinal wall of neighbouring cell, ta = transverse anticlinal wall. Fig. b. Connecting-arched cell endings, C1 = seed coat cell, C2 = second seed coat cell, ml = middle lamella, la_1 = longitudinal anticlinal wall, la_2 = longitudinal anticlinal wall of neighbouring cell, ata_1 and ata_2 = transverse anticlinal walls of connecting cells. Fig. c. connecting-flat cell endings, Fig. d. transversely lying side by side cell endings. Fig. e. three cell endings joined together.

Table 3.2: Colour and numerical parameters of orchid seeds; ns = number of specimens measured, ncl = number of cells at the longest axis of seed coat, ncw = number of cells at the widest axis of seed coat.

	Seed colour	ns	Seed length (mean, µm)	SD	Seed width (mean, µm)	SD	Ratio l/w (µm)	ncl	ncw	Cells/mm ($\mu m \times 10^3$) length of testa	Cell length (mean, µm)	SD	Cell width (mean, µm)	SD	Ratio cell l/w (µm)
1. *Aerangis brachycarpa*	light brown	4	354.0	55.41	50.5	15.5	7.0	5	11	14.1	101.7	13.1	5.5	2.65	18.53
2. *Aerangis confusa*	dark brown	3	386.9	6.0	99.0	9.5	3.9	5	10	12.9	114.5	3.2	1.2	0.03	99.98
3. *Aerangis coriacea*	dark brown	4	548.3	26.3	55.8	2.7	9.8	6	12	10.9	120.7	30.5	4.2	1.8	28.96
4. *Aerangis luteoalba*	white	4	366.6	73.3	46.6	4.4	7.9	6	14	16.4	61.1	21.9	3.4	1.9	17.77
5. *Aerangis thomsonii*	light brown	3	396.7	8.0	78.1	4.9	5.1	6	13	15.1	104.1	10.6	6.1	1.35	17.11
6. *Aerangis ugandensis*	light brown	4	465.3	62.4	65.3	9.3	7.1	6	12	12.9	110.9	24.1	4.8	3.11	23.28
7. *Angraecum decipiens*	light brown	3	415.0	10.8	44.2	12.5	9.4	4	15	9.6	182.5	5.4	3.2	0.62	57.63
8. *Angraecum dives*	light brown	3	380.2	78.0	45.6	12.2	8.4	4	11	10.2	87.6	62.8	2.6	1.01	33.4
9. *Angraecum erectum*	light brown	5	441.2	32.4	74.2	10.1	6.0	5	18	11.3	59.1	18.6	6.0	2.23	9.93
10. *Angraecopsis gracillima*	white	4	326.7	1.1	68.5	11.6	4.8	5	16	15.3	59.7	9.3	1.8	0.46	33.04
11. *Ansellia africana*	white	8	872.5	53.1	217.9	29.8	4.0	9	4	10.3	175.5	48.8	57.3	13.77	3.06
12. *Bolusiella iridifolia*	white	3	289.3	11.3	70.1	14.2	4.1	5	14	17.3	126.4	17.7	5.8	2.15	22.0
13. *Chamaeangis odoratissima*	white	3	361.1	44.4	60.5	3.4	6.0	7	18	19.4	92.4	28.9	2.3	0.63	40.03
14. *C. sarcophylla*	white	6	396.1	47.5	65.2	9.6	6.1	6	19	15.2	99.6	14.4	1.6	0.24	64.16
15. *Cribbia brachyceras*	white	3	404.2	18.0	75.0	23.3	5.4	4	18	9.9	87.5	18.4	2.1	0.71	41.87
16. *Cyrtorchis preatermissa*	brown	3	216.26	20.5	61.0	15.8	3.6	3	13	13.9	101.2	19.9	4.1	2.10	24.88
17. *Diaphananthe lorifolia*	white	3	342.8	7.2	78.0	1.9	4.4	9	11	26.3	97.6	1.9	2.4	0.85	41.0
18. *D. tenuicalcar*	white	3	459.1	14.9	84.0	6.6	5.5	10	12	21.8	137.5	27.0	2.3	0.83	60.57
19. *Eulophia angolensis*	shiny yellow	6	685.5	54.2	213.2	40.1	3.2	6	5	8.8	135.2	25.5	64.1	12.11	2.11
20. *E. horsfallii*	shiny yellow	5	872.4	115.1	291.6	71.9	3.0	7	4	8.1	240.9	55.5	127.0	64.08	1.9
21. *E. ovalis*	shiny yellow	6	690.2	203.1	215.5	82.5	3.2	6	4	8.7	125.1	17.6	45.8	6.09	2.73
22. *E. petersii*	shiny yellow	6	644.1	78.3	201.5	40.5	3.2	6	4	9.3	138.5	39.1	61.8	20.36	2.26
23. *E. speciosa*	shiny yellow	6	853.4	60.9	257.3	41.5	3.3	6	4	7.0	268.6	77.4	61.2	22.78	4.39
24. *E. stenophylla*	shiny yellow	5	896.2	46.2	218.3	24.8	4.1	6	4	6.7	195.1	48.1	98.8	85.68	1.98
25. *E. streptopetala*	yellow	6	849.6	70.6	223.9	29.5	3.8	7	4	8.2	180.3	50.4	90.4	57.56	2.0
26. *Epidendrum cinnabarinum*	white	7	2818.0	0.6	287.0	40.7	9.8	65	15	23.1	88.9	25.9	23.4	5.2	3.8
27. *E. ibaguense*	white	5	1598.0	0.6	145.0	30.2	11.0	35	4	21.9	80.3	3.0	28.2	2.41	2.85

28. *Liparis bowkeri*	white	5	278.4	52.2	68.6	28.1	4.1	8	5	28.7	55.0	4.1	16.7	2.72	3.3
29. *L. desteliii*	white	5	200.1	48.3	61.7	20.4	3.3	7	5	35.0	65.7	12.6	19.3	1.048	3.41
30. *Microcoelia exilis*	orange	5	558.8	71.4	51.6	3.4	10.8	5	16	9.0	36.1	9.1	1.2	0.01	31.26
31. *M. stolzii*	orange	3	543.0	63.3	49.4	6.0	11.0	5	18	9.2	30.6	7.4	1.2	0.02	26.6
32. *M. koehleri*	orange	6	496.6	7.5	50.8	3.5	9.8	7	16	14.1	37.2	8.6	1.2	0.01	32.1
33. *Oberonia disticha*	yellow	3	174.0	6.3	57.3	59.0	3.1	3	16	17.3	109.4	20.3	5.5	0.34	19.97
34.*Oeceoclades saundersiana*	white	6	586.3	11.8	51.9	7.8	11.3	12	4	20.5	41.0	7.2	5.8	1.5	7.08
35. *Polystachya. adansoniae*	yellow	4	258.8	17.1	87.9	9.5	3.0	6	12	23.2	144.0	13.3	1.6	0.2	87.71
36. *P. bella*	yellow	3	347.1	33.5	94.6	10.6	3.7	8	11	23.1	127.5	16.4	2.4	1.0	53.73
37. *P. bennettiana*	yellow	3	376.9	33.7	80.0	8.0	4.5	6	15	15.9	135.1	38.9	1.8	0.5	73.29
38. *P. campyloglossa*	cream	7	307.8	48.9	67.2	18.2	4.6	5	14	16.2	102.3	41.1	2.3	0.8	45.5
39. *P. confusa*	yellow	3	426.9	86.3	91.8	27.2	4.7	12	18	28.1	91.1	26.2	2.0	0.6	44.96
40. *P. cultriformis*	cream	6	241.5	133.2	77.8	8.6	3.1	6	12	24.8	68.7	13.0	6.5	0.3	10.53
41. *P. dendrobiiflora*	yellow	3	469.1	53.7	84.0	8.0	5.6	5	19	10.7	107.5	0.2	1.6	0.2	68.92
42. *P. fusiformis*	yellow	6	380.6	28.5	91.9	7.0	4.1	6	14	15.8	91.1	26.4	2.5	0.5	36.64
43. *P. spatella*	cream	6	268.0	56.1	93.5	10.8	2.9	3	18	11.2	90.5	21.4	4.3	1.37	20.9
44. *P. tessellata*	yellow	7	237.1	20.6	72.4	7.2	3.3	3	13	12.7	105.7	16.5	2.1	0.9	51.1
45. *P. transvaalensis*	yellow	6	370.0	71.2	78.4	5.4	4.7	7	15	18.9	97.1	16.4	1.8	0.5	54.7
46. *P. vaginata*	shiny yellow	3	430.4	19.9	83.5	7.7	5.2	3	12	7.0	166.8	47.1	2.9	0.2	57.9
47. *Solenangis aphylla*	yellow	4	225.8	53.8	38.4	9.6	5.7	15	17	66.6	71.4	18.4	1.3	0.3	54.11
48. *S. wakefieldii*	yellow	4	253.9	18.8	34.1	8.7	7.5	12	16	47.3	114.2	25.6	1.7	0.6	66.25
49. *Rangaeris amaniensis*	yellow	6	321.4	187.1	68.8	9.5	4.7	7	25	21.8	51.1	14.4	2.0	0.8	26.14
50. *Tridactyle bicaudata*	white	4	193.2	110.3	72.0	9.2	2.7	3	12	15.5	136.6	96.5	6.9	1.2	19.81

3.3.3 Phenetic analysis

The cophenetic correlation coefficient (r_{cs}) value, using distance measures is $r_{cs} = 0.8068$.

Table 3.3: List of character and character states of the orchid seeds used for phenetic analysis. The metric units and number of individual measurements studied are shown in Table 3.2.

1. Length of seeds (SLE) (μm).
2. Width of seeds (SWI) (μm).
3. Ratio of seed length to seed width (SRAT).
4. Number of cells at longest axes of testae (NCLA).
5. Number of cells at widest axes of testae (NCWA).
6. Number of cells/mm length of testa (NMLT).
7. Length of cells (CL) (μm).
8. Width of cells (CW) (μm).
9. Ratio of cell length to width of cells (CRAT).
10. Nature of periclinal walls (NPWALLS): 1 = smooth, 2 = warty.
11. Anticlinal walls (AWALLS): 0 = completely fused, 1 = partially fused.
12. Presence or absence of lamella perforations (LPERF): 0 = present, 1 = absent.
13. Middle lamella (MLAM): 1 = raised, 2 = sunk in.
14. Outer periclinal walls (OPWALLS): 1 = striated, 2 = not striated.
15. Micoornamentation of testae (MICRO): 0 = reticulate, 1 = wavy.
16. Nature of anticlinal walls (NAWALLS): 1 = flat, 2 = not flat.
17. Transverse anticlinal walls (TAWALLS): 0 = arched, 1 = not arched.
18. Testal extensions (TEXT): 0 = present, 1 = absent.
19. Light brown seed (LBSEED): 0 = present, 1 = absent.
20. Dark brown seed (DBSEED): 0 = present, 1 = absent.
21. White seed (WSEED): 0 = present, 1 = absent.
22. Yellow seed (YSEED): 0 = present, 1 = absent.
23. Shiny yellow seed (SYSEED): 0 = present, 1 = absent.
24. Orange seed (OSEED): 0 = present, 1 = absent.
25. Cream seed (CSEED): 0 = present, 1 = absent.
26. Fusiform seed (FSEED): 0 = present, 1 = absent.
27. Oblong seed (OBSEED): 0 = present, 1 = absent.
28. Narrowly oblong seed (NOSEED): 0 = present, 1 = absent.
29. Twisting of testae (TWIST): 0 = twisted, 1 = not twisted.
30. Presence or absence of intercellular spaces (IS): 0 = present, 1 = absent.
31. Dumbbell shaped cells (DCELL): 0 = present, 1 = absent.
32. Rectangular cells (RCELL): 0 = present, 1 = absent.
33. Rectangular and polygonal cells (RPCELL): 0 = present, 1 = absent.
34. Irregularly elongate cells (IECELL): 0 = present, 1 = absent.
35. Independent cell ends (IC): 0 = present, 1 = absent.
36. Spatula-like cell ends (SCELL): 0 = present, 1 = absent.
37. Connecting cell ends (CCELL): 0 = present, 1 = absent.
38. Presence or absence of wax deposits (WAXD): 0 = present, 1 = absent.
39. Seed type (ST): 1 = Group A, 2 = Group B, 3 = Group C, 4 = Group D, 5 = Group E.

The seeds cluster into two unequal groups: one with 15 species, all terrestrial, the other of 37 epiphytic species (Fig. 3.48). At the general level, five seed coat clusters were recognised and designated as A, B, C, D, E.

The seed coats comprising cluster A are of 30 epiphytic species belonging to different genera- *Aerangis brachycarpa*, *A. luteoalba*, *A. confusa*, *A. coriacea*, *A. thomsonii*, *A. ugandensis*, *Polystachya adansoniae*, *P. cultriformis*, *P. bennettiana*, *P. fusiformis*, *P. transvaalensis*, *P. tessellata*, *P. spatella*, *P. vaginata*, *Angraecum decipiens*, *A. erectum*, *P. dendrobiiflora*, *Angraecopsis amaniensis*, *Rangaeris amaniensis*, *Cribbia brachyceras*, *Chamaeangis odoratissima*, *C. sarcophylla*, *Microcoelia exilis*, *M. stolzii*, *M. koehleri*, *Bolusiella iridifolia*, *P. campyloglossa*, *Cyrtorchis praetermissa*, *P. tessellata*, *Oberonia disticha* and *Tridactyle bicaudata*.

The cluster B consists of seven seed coats of three different genera belonging to epiphytes- *Diaphananthe lorifolia*, *D. tenuicalcar*, *Solenangis aphylla*, and *S. wakefieldii*, *P. bella*, and *P. confusa*.

The seed coats of cluster C belong to four species- *Epidendrum cinnabarinum* (epiphytic species), *E. ibaguense* (epiphytic species), *Oeceoclades saundersiana*, and *Ansellia africana* (epiphytic species). The cluster of seed coats in group D is composed of six terrestrial species of the genus *Eulophia- Eulophia angolensis*, *E. ovalis*, *E. petersii*, *E. speciosa*, *E. streptopetala* var. *stenophylla*, *E. streptopetala* var. *streptopetala*.

The seed coats of cluster E comprises the two terrestrial species of *Liparis, L. bowkeri and L. deisteli*.

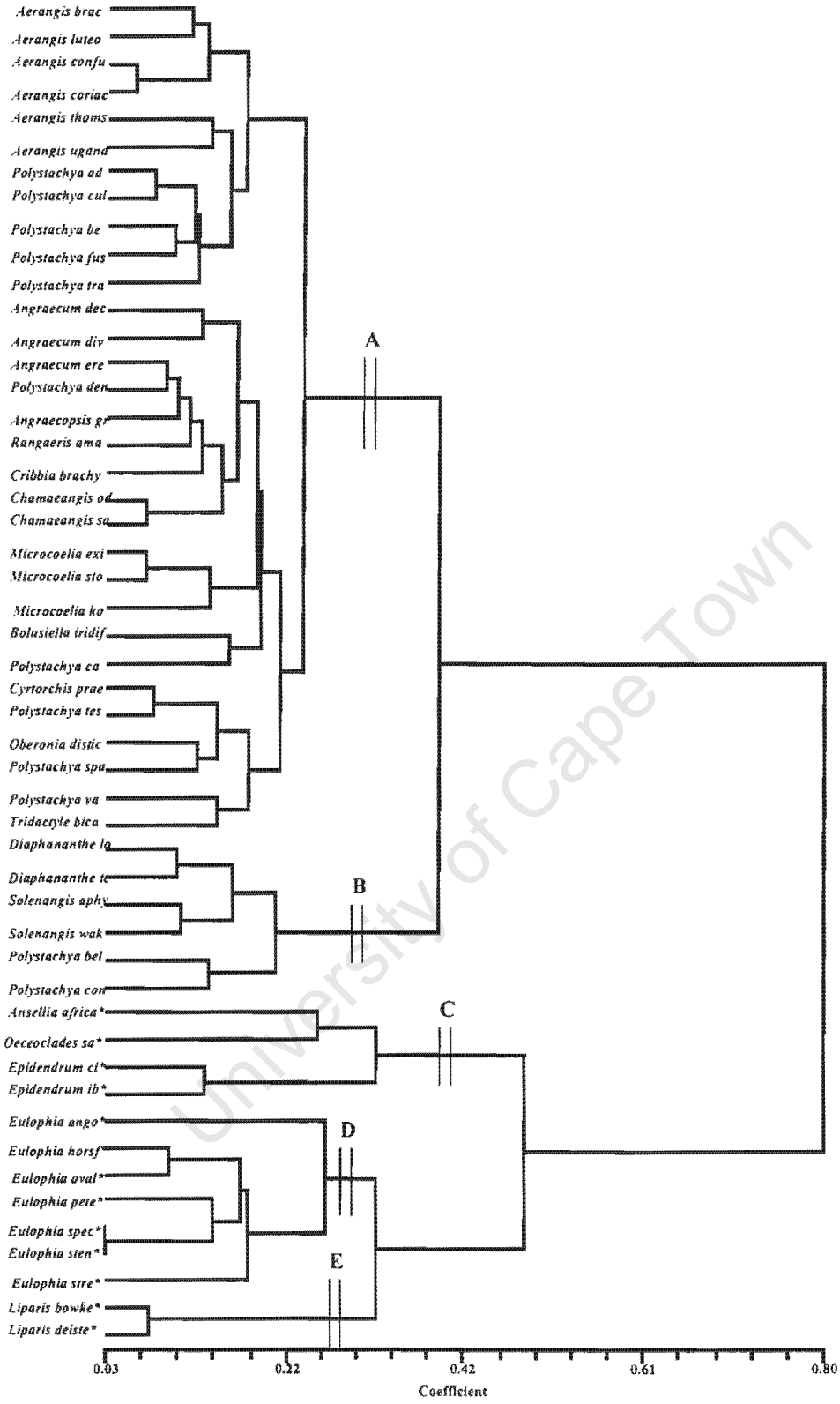


Figure 3.48. Dendrogram based on an unweighted pair group method of cluster analysis using arithmetic averages (UPGMA) for the orchid characters described in Table 3.3 based on 50 OTU's showing cluster dissimilarity. The dissimilarity scale (taxonomic distance from matrices of correlation) is shown as the abscissa. Names at the tips of the dendrogram are species names. The species marked with an asterisk were derived from terrestrial habitats and those without from epiphytic habitats. The clusters are labelled A, B, C, D and E. *Ansellia africana, Epidendrum cinnabarinum* and *E. ibaguense* are ecologically epiphytic orchids.

3.3.4 Ordinations

3.3.4.1 Principal Components Analysis

The data set was ordinated by PCA, showing that the first three axes account for 37.6%, 9.2% and 7.3% of the total variance respectively. The first axis reflects most of the character variation as is normal for biometric studies (Sneath and Sokal, 1973). The most important characters are marked with asterisks in Table 3.4, most of them qualitative.

Table 3.4: Cumulative variance and factor loadings extracted from correlation matrix for seed characters used for ordination analysis. Characters abbreviations are as in Table 3.3 and those marked with asterisks are the most important source of variation in the seed hence defining relationships of OTUs in ordination. Marked loadings are > .700000.

Eigenvalues:
Extraction: Principal components

	Eigenval	% total Variance	Cumul. Eigenval	Cumul. %
1	13.92718	37.64102	13.92718	37.64102
2	3.41205	9.22176	17.33923	46.86279
3	2.68332	7.25223	20.02255	54.11501

Factor Loadings:

	Factor 1	Factor 2	Factor 3
SLE	-0.588	0.448	-0.097
SWI	-0.869*	-0.087	0.089
SRAT	0.230	0.678	0.190
NCLA	-0.323	0.591	-0.342
NCWA	0.760*	0.064	-0.002
CL	-0.515	-0.574	0.197
CW	-0.850*	-0.171	0.225
CMLT	0.221	0.333	-0.305
CRAT	0.627	-0.211	0.013
NPWALLS	-0.649	-0.050	-0.134
AWALLS	-0.933*	0.083	-0.03
MLAM	-0.851*	-0.015	-0.033
OPWALLS	0.944*	-0.134	0.026
MICRO	0.944*	-0.134	0.026
NAWALLS	-0.944*	0.134	-0.026
TAWALLS	0.715*	0.085	-0.187
TEXT	-0.190	-0.584	-0.541
LBSEED	-0.126	0.113	-0.087
DBSEED	0.087	-0.330	0.613
WSEED	-0.240	0.095	0.103
YSEED	0.695	0.390	-0.357
SYSEED	-0.192	-0.522	-0.639
OSEED	-0.114	0.090	-0.006
CSEED	0.022	-0.181	0.154
FSEED	-0.885*	0.187	0.026
OSEED	-0.250	-0.515	-0.596
NOSEED	-0.154	0.138	0.329

TWIST	0.933*	-0.083	0.030
IS	-0.093	0.160	0.209
DCELLS	0.757	-0.361	0.247
RCELLS	0.447	0.337	-0.331
RPCELLS	-0.901*	0.142	-0.090
IECELLS	-0.195	0.102	-0.033
ICELLS	0.695	0.390	-0.357
SCELL	-0.432	-0.195	-0.443
CCELLS	0.850*	-0.204	-0.070
WAXD	0.204	-0.107	0.106
ST	-0.161	0.042	0.502
Expl.Var	13.927	3.412	2.683
Prp.Totl	0.376	0.092	0.073

3.3.4.2 Multidimensional scaling

Multidimensional scaling analysis using STRESS 2 computation (see Table 3.5) reached minimum iterations after five steps producing a goodness of fit value of 0.088, which is a very good result indicating that the variation accounted for by this correlation coefficient matrix compares to a great extent with the variation in original data. The two-dimensional scatter diagram by MDS of principal axes I and II separate the seeds largely into five subclusters (Fig. 3.49). The terrestrial seeds are distinctly separated from the epiphytic seeds with respect to distance and position in the character hyperspace. These clustering by MDS bears a close resemblance to the results of the cluster analysis (Fig. 3.48).

Table 3.5: Stress coefficients using STRESS 2 computation

Iteration	Stress	Srat	SRatAV	SfGr	Step
0	0.778	0.800	0.800	0.008	0.163
1	0.707	0.910	0.835	0.092	0.242
2	0.596	0.842	0.837	0.010	0.439
3	0.392	0.657	0.772	0.015	0.486
4	0.148	0.378	0.609	0.023	0.116
5	0.088	0.594	0.604	0.013	0.024

While the clustering by MDS gives similar patterns to the phenogram (Fig. 3.48), it was observed that in many cases seeds that are closely related phylogenetically are close together in the ordination space. At the same time, some of the unrelated groups also form clusters in this space. For example, the cluster labelled A comprises mixed seeds from three tribes:-tribe Vandeae (*A. brachycarpa*, *A. luteoalba*, *A. confusa*, *A. coriacea*, *A. thomsonii*, *A. ugandensis*, *Angraecum decipiens*, *A. dives*, *Angraecum erectum*, *Angraecopsis amaniensis*, *Rangaeris amaniensis*, *Cribbia brachyceras*, *Chamaeangis odoratissima*, *C. sarcophylla*, *Microcoelia exilis*, *M. stolzii*, *M. koehleri*, *Bolusiella iridifolia*, *Cyrtorchis praetermissa*, *Tridactyle bicaudata*), tribe Epidendreae (*Polystachya adansoniae*, *P. cultriformis*, *P. bennettiana*, *P. fusiformis*, *P. dendrobiiflora*, *P. spatella*, *P. campyloglossa*, *P. tessellata*, *P. vaginata*) and Malaxideae (*Oberonia disticha*). Cluster B is composed of tribe Vandeae (*Diaphananthe lorifolia, D. tenuicalcar, Solenangis aphylla, S. wakefieldii*) and Epidendreae (*Polystachya bella*, and *P. confusa*) while cluster C is formed of *Epidendrum* species (tribe Epidendreae), *Oeceoclades saundersiana* (tribe Vandeae) and *Ansellia africana* (tribe Cymbideae). The seeds in cluster D are of *Eulophia* seeds (tribes Vandeae) while cluster E is of *Liparis* seeds (tribe Malaxideae).

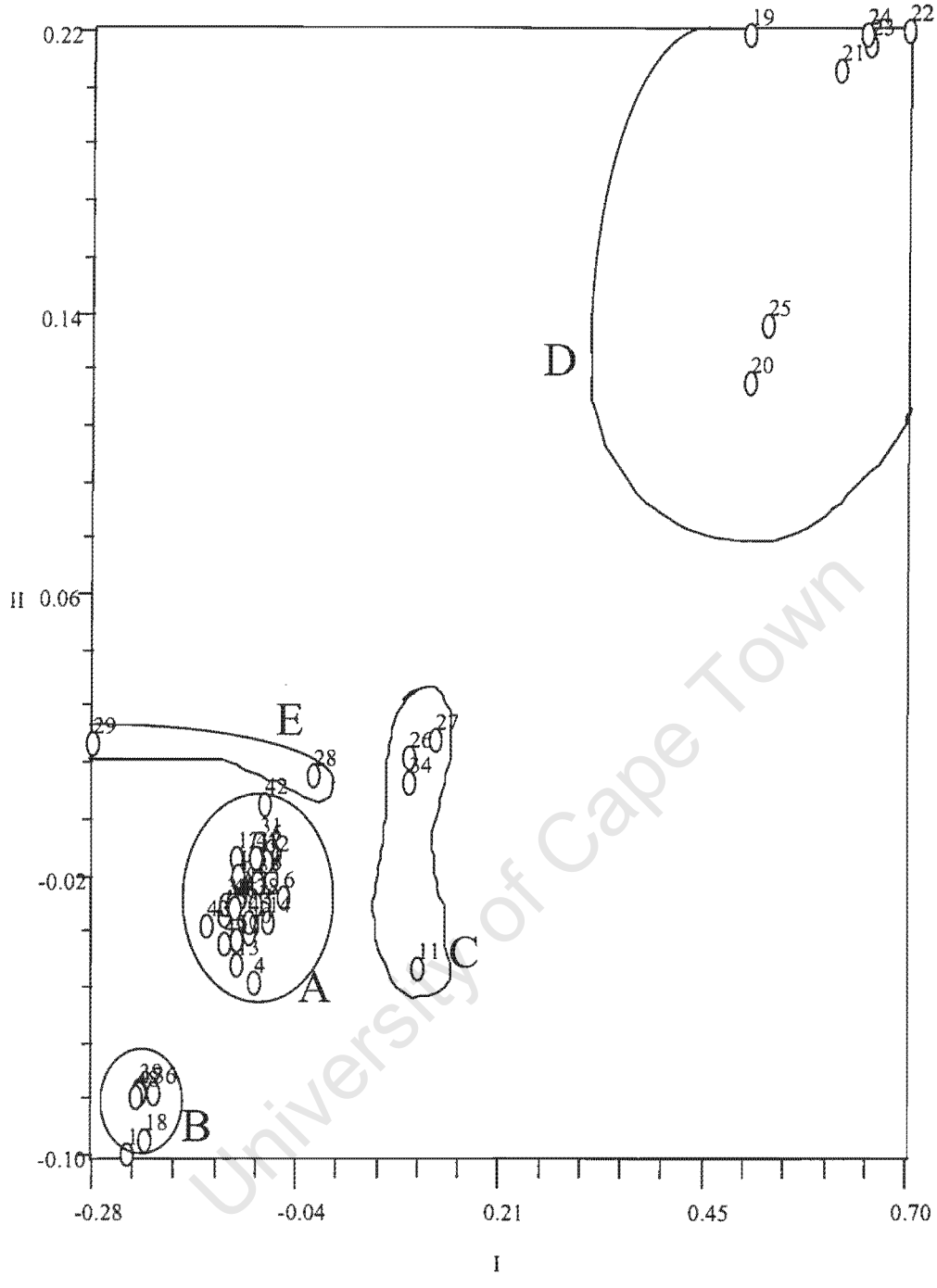


Figure 3.49. Scatter plot of two-dimensional MDS configuration of first and second axes for 50 taxa. The OTU numbering corresponds to that of species listed in Table 3.2. Groupings labeled as in fig. 3.48.

3.3.4.3 Principal co-ordinates analysis

Principal co-ordinates analysis using co-ordinates of individual characters yielded similar results to the cluster analysis and MDS analyses. The PCO (see Fig. 3.50) does not give any new results, but grouping is even clearer than in MDS ordination. The first and second axes further seem to separate the dense subcluster of 31 epiphytic seeds into three minor subclusters, comparing very well with the clustering patterns of the dendrogram. However, in MDS the internal minor clustering of this large subcluster is not as pronounced as in the PCO results. Therefore, the PCO also extracted mainly five groups of seeds as the other analyses did but went further in revealing three small groups of seeds nested in the major epiphytic cluster of 32 seed coats. In this study only the five major clusters are considered.

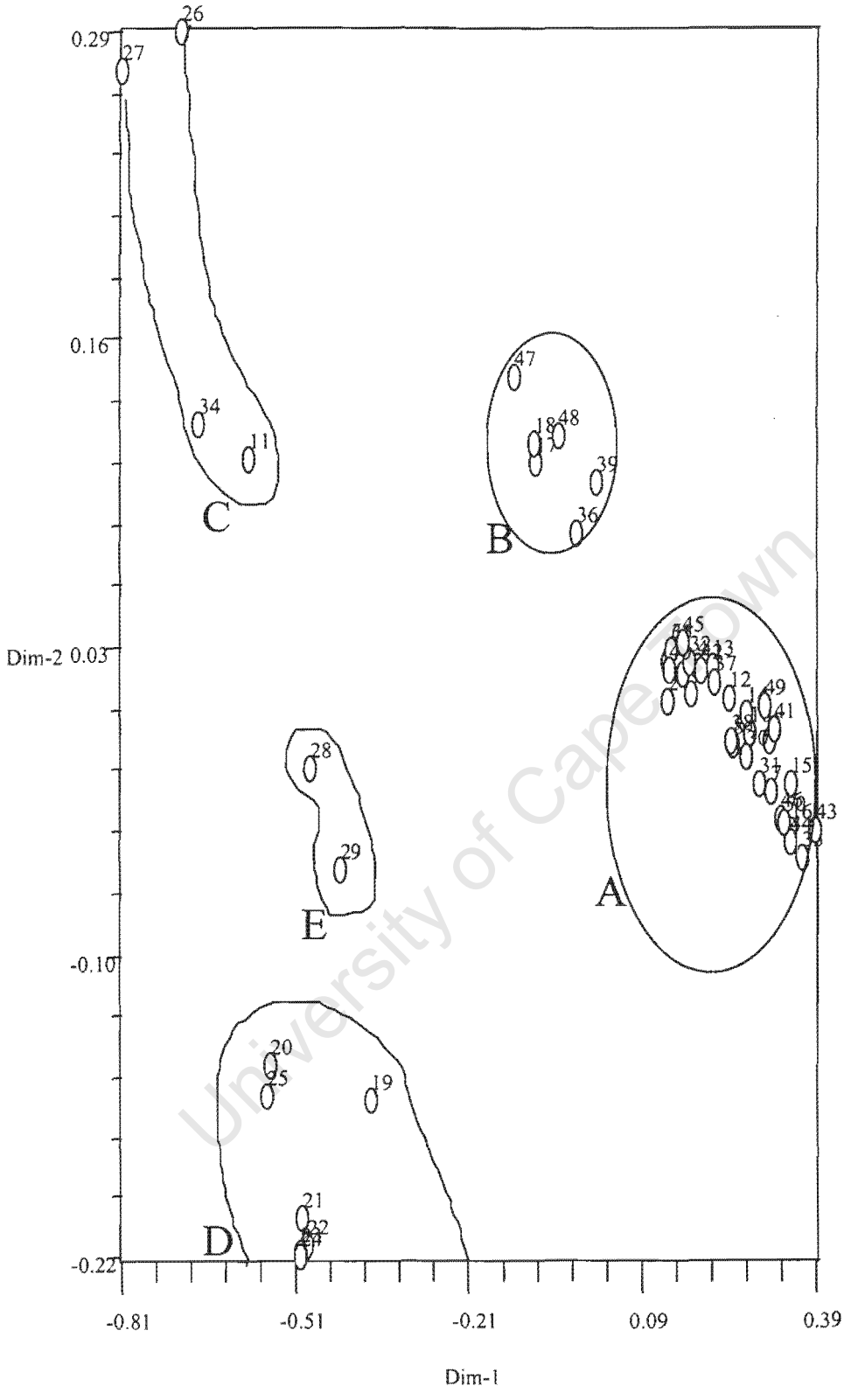


Figure 3.50. Scatter diagram of principal co-ordinate analysis for axes I compared with II for 50 orchid taxa. The OTU numbering corresponds to that of species listed in Table 3.2 and groups to Fig. 3.48.

3.3.5. Seed groups

Based on the multivariate analyses five groups can be recognised. These are listed below with the species that make them up:

Group A- *Aerangis brachycarpa*, *A. luteoalba*, *A. confusa*, *A. coriacea*, *A. thomsonii*, *A. ugandensis*, *Polystachya adansoniae*, *P. cultriformis*, *P. bennettiana*, *P. fusiformis*, *P. dendrobiiflora*, *P. spatella*, *P. campyloglossa*, *P. tessellata*, *P. vaginata*, *Angraecum decipiens*, *A. dives*, *Angraecum erectum*, *Angraecopsis amaniensis*, *Rangaeris amaniensis*, *Cribbia brachyceras*, *Chamaeangis odoratissima*, *C. sarcophylla*, *Microcoelia exilis*, *M. stolzii*, *M. koehleri*, *Bolusiella iridifolia*, *Cyrtorchis praetermissa*, *Oberonia disticha*, *Tridactyle bicaudata*.

Group B- *Diaphananthe lorifolia*, *D. tenuicalcar*, *Solenangis aphylla*, *S. wakefieldii*, *Polystachya bella* and *P. confusa*.

Group C- *Epidendrum cinnabarinum*, *E. ibaguense*, *Oeceoclades saundersiana* and *Ansellia africana*

Group D- *Eulophia angolensis*, *E. horsfallii*, *E. ovalis*, and *E. petersii*, *E. speciosa*, *E. stenophylla*, *E. streptopetala*.

Group E- *Liparis bowkeri* and *L. deisteli*.

3.4. DISCUSSION

There is a great degree of variation at species level exhibited in the seed coats of the 50 orchid species observed in the present study. The results from the study show that the seed coats of the Kenyan Epidendroideae are very varied in size, shape and texture. The variation exhibited by the seed coat features is useful in defining the different clusters of seeds.

Nevertheless it is also noteworthy to relate variation of seed coat morphology in the present study to embryological findings also established in the present work, which to some detail explains formation of seed coats of Epidendroideae from the outer integumentary cells of the ovules during embryogenesis. At the last stages of embryo formation the inner integuments become degenerated to form the embryo carapace, a protective layer of the mature embryo, leaving only the outer integuments intact, which ultimately become highly stretched to organizing the seed coats. However, the varied surface structures observed on seed coats are as a result of secondary micro-ornamentation e.g. thickening of clinal walls and wax deposition that takes place at the close of embryogenesis. These seed coats, however, besides their taxonomic value are crucial during dispersal (Rauh *et al.*, 1975; Kurzweil, 1993a, b; Barthloth, 1976; Murren and Ellison, 1998) and germination of the seeds (Rasmussen, 1995; Leake, 1994).

3.4.1. Character variation

The study reveals that, within the Epidendroideae, seeds are generally small in size, as already illustrated by previous workers (e.g. Clifford and Smith, 1969; Healey *et al.*, 1980), but at the same time, there are some very large seeds relative to those of other orchids, such as those of *Epidendrum cinnabarinum* and *E. ibaguense,* which appear to be exceptional in the subfamily.

Seed coat morphology also exhibits great variation in cell structure whereby epiphytes have narrow slender, almost fused cells, and terrestrial species possess large elongate cells. Using cell structure and seed coat ornamentation as criteria seeds can be readily identified as terrestrial or epiphytic in origin.

There is considerable diversity in morphological features diagnostic of orchid seeds, some of which have been reported earlier for other epidendroid orchids, e.g. testal extensions (Beer, 1863; Chase and Pippen, 1990) and twisted testa (Burgeff, 1936). Such projections were first noted by Beer (1863) although he inappropriately referred to them as spur projections. Chase and Pippen (1990) termed them "wax hooks" for species of Catasetinae but this term was only suitable in describing the testal extensions of *Cribbia brachyceras* in the present study. As the testal extensions of *Microcoelia* were neither "hook-shaped" nor "club-shaped" (Jonsson, 1981), the term "trichome-like processes" was introduced to describe them. "Trichome-like processes" was used also to denote those testal extensions found in *Solenangis* as they are morphologically similar to those of *Microcoelia* (Jonsson, 1979), and the term "ridge-like extensions" was adopted for those of *Ansellia africana*, which had not been studied before.

As generally no attention in the literature has been given to describing seed coat cells in any detail, new terminology has been developed here to provide a clearer description of the cells especially for the epiphytes, which have always proved problematical to delimit. Previously, the cell shapes of epiphytic seed coats were generally described as longitudinally stretched (Chase and Pippen, 1990). From the present study, however, it is recognised that the seed coat cells of epiphytes exhibit spatula-like, irregularly elongate and dumbbell-like shapes, which are totally different from the rectangular and polygonal cell shapes of terrestrial seeds. Cell endings are also described here as

connecting, independent or transversely lying side by side, and this also serves a useful purpose for categorising seed coats in general.

The considerable variation in seed morphology reported in this study could be accounted for in ecological or taxonomic terms, especially in view of the fact that seeds of epiphytes and terrestrial orchids can be distinguished with a high level of consistency using seed coat characters.

3.4.2. Multivariate methods

It is encouraging to note that the three multivariate analyses suggest five groups of seeds. The clustering method for determining relationships among taxa yielded good clustering of the 50 species with a reliable cophenetic correlation coefficient of 0.81 (section 3.4.3). The value was higher than those that are typical for taxonomic data, although values of 0.9 are not unusual for similar data (Rohlfe and Fisher, 1970).

The accounts of Rohlfe and Fisher (1968) concerning the values of the cophenetic coefficients is that they tended to decrease with the number of taxa and to be almost independent of the number of characters recorded, and that a value above 0.8 was usually sufficient to reject the null hypothesis. However, they emphasise that even a correlation near 0.9 does not guarantee that the dendrogram serves as a sufficiently good summary of the phenetic relationships because some distortions in terms of patterns in the original multidimensional characters space do occur in the analysis.

Interestingly, MDS and PCO produced similar results, that is to say, five seed groups with minimised distortions. The seed coat groups retrieved by MDS and PCO compare well with the groups or clusters produced by the cluster technique creating confidence in the results produced by the three different methods (see Figs. 3.48, 3.49, 3.50). However, the visual presentation of the OTUs by cluster analysis shows little distortions and is

much better than those obtained by MDS and PCO, as it is easier to account for clusters by means of the taxonomic distance scale shown as abscissae.

3.4.3. Seed groups

The separation of seeds into the five groups is well supported by numerical and descriptive characters used in multivariate analyses. However, epiphytic and terrestrial species formed groups of seeds corresponding to those habitats. Although epiphytes or terrestrial seeds cluster together *Epidendrum* and *Ansellia,* sometimes regarded as lithophytes have their seeds also clustering together. Seeds of *Oeceoclades* are very similar to the lithophytes as they are fusifom in shape and share many characters with the lithophytes. Other seeds, particularly those of epiphytes form clusters depending on the (dis)similarity of the seeds and not according to tribe or subtribe.

Considering that *Eulophia* and *Oeceoclades* were formerly treated together with respect to their seed details (Healey *et al.*, (1980); Molvray and Kores (1995), it is interesting from the present study to note that the two groups are separated based on seed size rather than clinal walls, which are largely similar. The grouping of *Eulophia* species with *Liparis* species seem to be more realistic since seed size, shape and the clinal walls in the two groups are much similar except that periclinal striations of *Eulophia* are close together. However, the scatter of some *Polystachya* species among the Vandeae is noteworthy, since taxonomically they are closely related and their seeds show similarity in size and micro-ornamentation.

Group A. This type of seed is present in *A. brachycarpa, A. luteoalba, A. confusa, A. coriacea, A. thomsonii, A. ugandensis, Polystachya adansoniae, P. cultriformis, P. bennettiana, P. fusiformis, P. dendrobiiflora, P. spatella, P. campyloglossa, P. tessellata, P. vaginata, Angraecum decipiens, A. dives, Angraecum erectum, Angraecopsis amaniensis, Rangaeris amaniensis, Cribbia brachyceras, Chamaeangis odoratissima, C.*

sarcophylla, Microcoelia exilis, M. stolzii, M. koehleri. Bolusiella iridifolia, Cyrtorchis praetermissa, Oberonia disticha, and *Tridactyle bicaudata*. Seeds very small, white or yellow in colour, ovoid, oblong or narrowly oblong in shape, 216.26-558.8 × 44.17-98.97 μm in dimensions, with a l/w ratio of 2.69-16.98. Cells, 3 to 12 in the long axis and 10 to 25 in the short axis, measure 30.59-136 × 0.63-6.9 μm, with ratio of 2.86-64.6. Cell shapes vary from irregularly elongate, spatula-like to dumbbell shapes. Anticlinal walls thick, highly stretched and often covered by verrucose wax deposits in species of *Angraecum, Bolusiella iridifolia, Polystachya dendrobiiflora, P. bella, P. confusa, Chamaeangis odoratissima* and *Rangaeris amaniensis*; transverse walls are raised above the seed surface forming testal extensions in *Cribbia* and *Microcoelia*. Cell end walls are connecting, independent and /or transversely lying side by side. In *Angraecopsis amaniensis* the middle lamella is raised and covered with wax deposits. Microornamentation is wavy.

Group B. Species exhibiting this seed type are *Diaphananthe lorifolia, D. tenuicalcar, Solenangis aphylla, S. wakefieldii, Polystachya bella* and *P. confusa*. Seed are white or yellow in colour, oblong in shape with those of Solenangis narrowly oblong, 225.29-459.07 × 34.05-94.45 µm in dimensions; with a l/w ratio of 3.671-5.862. Cells are irregularly elongate, spatula-like in shape, 8 to 15 in long axis, 11 to 18 in short axis, 47.26-137.5 × 2.2.7-91.12 µm; with a ratio of 44.96-60.573, and connecting or both connecting and independent cell endings present. Anticlinal walls are completely fused, transverse walls raised above the seed surface, and covered with verrucose wax deposits. Transverse walls of *Solenangis* are highly arched forming "trichome-like" testal extensions of about 12-21 µm long. Periclinal walls are concave, smooth, or warty. Microornamentation is wavy.

Group C. The seed type is found in *Epidendrum cinnabarinum*, *E. ibaguense*, *Oeceoclades saundersiana*, and *Ansellia africana*. They are characterised by being white in colour, fusiform in shape and the micropylar pole being narrow and longer than the chalazal pole. The seeds are 586.26-2818 × 46-218 μm in dimensions and 3.06-11.02 μm l/w ratio. Cells, 9 to 65 in the longitudinal axis, 4-15 in the short axis, are mainly hexagonal, with some rectangular cells, or entirely rectangular in *Epidendrum* species, they measure 38.7-88.87 × 4.93-28.23 μm and have a l/w ratio 2.85-7.08, connecting. Anticlinal walls are thin, and those of neighbouring cells are partially not fused in *Epidendrum* (evidence of their dual nature), usually with sunken middle lamella but raised and covered with a membrane in *Ansellia africana* and *Oeceoclades saundersiana*. Transverse anticlinal walls are raised in *A. africana* to form ridge-like structures, sometimes covered with wax deposits. Intercellular gaps are present as star-like features at cell junctions of *Epidendrum* species. Periclinal walls are concave, smooth in *E. cinnabarinum*, sparsely transversely striated in *E. ibaguense,* and *Oeceoclades saundersiana* and longitudinally striated in *Ansellia africana.* Verrucose wax deposits are present along the walls of striations in *A. africana*. Microornamentation is reticulate.

Group D. This type is present in the seven *Eulophia* species, *Eulophia angolensis, E. horsfallii, E. ovalis, E. petersii, E. stenophylla,* and *E. streptopetala.* Seeds are light yellow to yellow, balloon-shaped, 585.6-687.45 μm × 55.3-77.23 μm, with l/w ratio 3.1-3.24 μm. Cells are mainly hexagonal although rectangular cells are also present in *E. horsfallii* and *E. angolensis.* Their dimensions are 125.1-138.1 × 45.77-64.08 μm, with four to eight cells in the long axis and four to five in the short axis. Anticlinal walls of neighbouring cells are thin and not completely fused leaving a grove in the form of a triangular concavity at corners where three cells meet. Periclinal walls are sunken to

assume a basin-like appearance, transversely striated, and warty. Transverse striations are very close together in *E. speciosa* but fairly widely spaced in all other species. General microornamentation is reticulate.

Group E. This group includes only *Liparis bowkeri* and *L. deisteli*. Seeds are ovoid in shape and white in colour, measuring 200-278.35 × 20.422-28.11 μm with a l/w ratio of 3.245-4.06. The cells are 7 to 8 in the longitudinal axis and 5 in the short axis; 55-65.657 × 16.667-19.259 μm in dimensions, with a l/w ratio 3.3-3.41. Both rectangular and hexagonal cells are present, connecting.

Anticlinal walls of neighbouring cells are not completely fused leaving a groove in the form of a triangular concavity at corners where three cells meet. The middle lamella is sunken between two cells. Periclinal walls are depressed to assume a basin-like appearance, transversely striated, and warty. Microornamentation is reticulate.

CHAPTER 4: MYCORRHIZAE AND SYMBIOTIC SEED GERMINATION

Contents

4.1 INTRODUCTION AND LITERATURE REVIEW

Orchids are known to depend on mycorrhizal fungi for their nutrition to germinate and at some stages of their development. Since most of the orchids grow and thrive in conditions of nutrient impoverishment, they consistently utilize fungi as an alternative means to obtain energy (H. N. Rasmussen, 1995). It has been suggested that the mycorrhizae play a central role in the absorption of organic matter besides other nutrients by mature mycotrophic orchids (Montforts & Küsters, 1940; Richardson, 1956; Benzing, 1986; Benzing & Atwood, 1984). The majority of mycotrophic orchids are found in forest habitats, with some found in a wide variety of woodland and scrub communities (Leake, 1994). Detailed habitat descriptions for the majority of species are lacking and knowledge of the kinds of soils and microclimatic conditions experienced by members of this family in Africa is incomplete.

Smith (1967) suggested that the orchids either depend on mycorrhizae for their lifetime or they can become independent at maturity. Later investigations showed that some orchids depend on mycorrhizae for considerable periods, for example, the terrestrial species *Cypripedium acaule* and *Orchis ustulata* stay underground for a long period of

time (Rasmussen, 1995). *Rhizanthella gardneri* in Australia lives entirely underground (Dixon, 1991).

Despite the paucity of information concerning the mycorrhizal dependence of orchids through their lifespan it has been shown that all orchids require mycorrhizal fungi for seed germination and seedling development (Bernard, 1899, 1909; Warcup, 1981a, b; Clements, 1982, 1988). Perhaps the induction of seed germination by fungi is the most important factor limiting conservation since this relationship is crucially important for the re-establishment of rare and endangered orchids by growing them from seed. Although much interest has been shown in recent years with regard to the general question of symbiosis between orchids and fungi, our understanding of the subject remains incomplete, particularly for orchid species from East Africa.

Early research on symbiotic seed germination was conducted by Bernard (1889), using seeds of the hybrid *Laeliocattleya* and various fungal strains isolated from the orchid. One of these, a species of *Rhizoctonia,* caused germination, which led Bernard (1909) and also Burgeff (1909) to conclude that germination of orchids can be caused only by the aid of endophytic fungi belonging to the genus *Rhizoctonia.* Although major breakthroughs have been made in identifications of mycorrhizal fungi, not all of them are of common occurrence, and even those that have been utilised for symbiotic seed germination of some orchids do not work for other orchids (Warcup and Talbot, 1967). The problem is compounded by the fact that, although many strains of fungi are recorded in the literature, most of them are not of common occurrence globally. The present state of knowledge in relation to host-fungus specificity in germination is far from comprehensive (Hadley, 1970; Warcup, 1971, 1973; Clements, 1981, 1982; Perkins and McGee, 1995).

The purpose of the present study was to explore the mycorrhizal relationship in the orchids of Kenya that have hitherto not been studied. The potential for mycorrhizal associations in symbiotic seed germination through tissue cultures as a means for conservation of rare and endangered orchids *ex situ* is explored, and the issue of orchid-fungus specificity in the relationships is addressed.

The present study is based on a detailed examination of 12 orchid species from different epiphytic and terrestrial habitats. The research focuses on orchid endomycorrhizae and symbiotic seed germination to answer questions regarding the spread of mycorrhizal fungi in orchids, the dependence on mycorrhizae for *in vitro* germination of orchid seeds and whether there is strong orchid-fungus specificity.

4.2 MATERIALS AND METHODS

4.2.1 Sampling sites

Thirteen sampling sites for roots and seeds were selected across Kenya from a wide range of habitats (Table 4.1). Habitats varied significantly in elevation and ecology within an area between 4° South and 5° latitude North of the equator, 34° and 42° East of Greenwich. Fieldwork to collect orchid seeds for storage in the laboratory was done in one-year (June 1996 to July 1997) at the same time studying fungal isolates using freshly obtained roots.

I. Roots

Healthy roots of mature, wild-growing orchids were excavated and maintained fresh and wet until the fungal isolation processes were carried out in the laboratory. The roots were sampled from two or more individual plants of the same population of orchids to assess if the orchid species harboured more than one fungus in the roots. Only roots heavily colonized by endomycorrhizae were used for the isolation of fungi.

II. Seeds

Seeds were collected from mature capsules that had dried under natural conditions in the field. Seeds were removed by opening the capsules fully with the aid of a scalpel on to white papers. The seeds spilt on each paper were then formed into a heap and stored over calcium chloride ($CaCl_2$) in small manila envelopes in a sealed container. The container was then placed in a refrigerator at 4°C for preservation of the seeds until they were used for *in vitro* germination.

Table 4.1: Sampling sites for the seeds and roots of Kenyan orchid species used in the study. Specimens of *Eulophia* species were collected from terrestrial habitats and *Polystachya* species from epiphytic habitats. In only six species were collected both seeds and mycorrhizal roots.

Samples	Voucher collection	Locality	Habitat
Seeds			
Eulophia angolensis	JMO38	Milimaini (1°04'S.,36°51'E.)	swamp
E. horsfallii	JMO37	Rupingazi (0°22'N.,37°28'E.)	river valley
E. petersii	JMO12	Kilima Kiu (1°33'S.,37°24'E.)	dry open bushland
E. speciosa	JMO30	Kilima Kiu (1°33'S.,37°24'E.)	dry open bushland
E. stenophylla	JMO11	Fourteen Falls (1°10'S.,37°13'E.)	silted rocky grassland
E. streptopetala	JMO10	Manga range (0°33'S.,34°57'E.)	montane rocky
Polystachya campyloglossa	JMO3	Ngong Hills (1°24'S.,36°31'E.)	open forest
P. cultriformis	JMO9	Kaheho (0°06'S.,36°28'E.)	thick forest
P. fusiformis	JMO53	Manga range (0°33'S.,34°57'E.)	montane *Vangueria*.sp.
P. spatella	JMO48	Kaheho (0°06'S.,36°28'E.)	thick forest
P. transvaalensis	FO736	Mau escarpment (1°2'S.,36°8'E.)	bushland *Acacia* ssp.
P. vaginata	FO127	Langata (1°16'S.,36°36'E.)	thick forest
Mycorrhizal Roots			
Eulophia stenophylla	JMO1	Arboretum (1°19'S.,36°43'E.)	open forest
E. streptopetala	JMO20	Manga range (0°33'S.,34°57'E.)	montane rocky grassland
E. streptopetala	JMO5	Kaheho (0°06'S.,36°28'E.)	thick forest edge
E. speciosa	JMO14	Kilima Kiu (1°33'S.,37°24'E.)	dry open bushland
E. petersii	JMO19	Kilima Kiu (1°33'S.,37°24'E.)	dry open bushland
E. streptopetala	JMO23	Ngong Hills (1°24'S.,36°31'E.)	open forest
E. stenophylla	JMO6	Fourteen Falls (1°10'S.,37°13'E.)	silted rock
Polystachya steudneri	JMO17	Saiwa swamp (1°10'N.,35°05'E.)	swamp edge forest
P. dendrobiiflora	JMO18	Rukinga Hills (3°49'S.,38°41'E.)	forest *Xerophyta*
P. campyloglossa	JMO24	Ngong Hills (1°24'S.,36°31'E.)	open forest
P. cultriformis	JMO15	Kaheho forest (0°06'S.,36°28'E)	thick forest edge
P. tenuissima	JMO16	Kakamega (0°16'N.,34°55'E.)	thick forest

4.2.2 Nutrient media

Three kinds of nutrient media for solid-substrate cultured inoculum were used in the experiments, one for isolation of fungi and two for symbiotic seed germination.

i) The fungal isolation medium is that of Clements *et al.* (1986) modified by Dijk and Eck (1994) with respect to nitrogen sources and antibiotics (Table 4.2). The composition of the solution was: 1.5 mM KH_2PO_4; 1.5 mM KNO_3; 2.0 mM $(NH_4)_2SO_4$; 1.0 mM $Ca(NO_3)_2$; 0.5 mM $MgSO_4$; 2.0 mM asparagine; 5.0 g sucrose; 8.0 g agar (purified); 0.5 g yeast extract; 28 mg $FeSO_4$. $7H_2O$; 38 mg Na_2EDTA; 20 mg penicillin; 10 mg streptomycin; 10 mg tetracycline; in 1000 ml. distilled water. It was found necessary to add tetracycline (10 mg. l^{-1}), which is not included in the original formulation, to the recipe to eliminate a white bacterium that was not sensitive to the other two antibiotics.

ii). The oat medium for seed germination (Clements *et al.*, 1986) consisted of: 200 mg $Ca(NO_3)_2.4H_2O$; 100 mg KCl; 200 mg KH_2PO_4; 100 mg $MgSO_4.7H_2O$; 100 mg yeast extract (Difco); 2 g sucrose; 3 g finely ground 2% Bokomo© oats; 12 g agar (purified); in 1000 ml. distilled water (Table 4.3). This oats medium of Rasmussen (1990) was modified from the oats solution of Clements (1986). Further modification of the medium relates to the variation in ingredients used in culture medium as explained below. The source of the carbon in the medium was varied by using sucrose (2 $g.l^{-1}$) (medium designated H2) or glucose (2 $g.l^{-1}$) (medium designated H4) for comparison of their effect on the rate of germination. In the formulation where glucose was utilized instead of sucrose, soluble $NH_4NO_3.4H_2O$ (1.0 $mM.l^{-1}$) was added and no $Ca(NO_3)_2.4H_2O$ (200 $mg.l^{-1}$). The main reason for varying the ingredients was to develop the medium to give optimum growth of both fungi and seeds for regular use.

iii). The third medium, designated as germination medium by Dijk & Eck (1995), comprised: 1.0 mM glutamine; 1.0 mM NH_4NO_3; 1.0 mM $(NH_4)_2SO_4$; 1.0 mM

$Ca(NO_3)_2$; 1.0 mM K_2SO_4; 0.5 mM $MgSO_4$; 0.1 mM $FeSO_4$; 0.1 mM Na_2EDTA; 6.0 g agar (purified); 1.0 g yeast extract; 10.0 g sucrose; 0.2 g inositol; in 1000 ml. distilled water (Table 4.4).

To meet the demands for vitamins, amino nitrogen and phosphorus of both orchid species (Mead & Bulard, 1975, 1979; Van Waes & Deberg, 1986) and mycorrhizal fungi (Stephen & Fung, 1971; Powell & Arditti, 1975; Hadley & Ong, 1978) all cultures were supplied with yeast (Difco) extract. Additionary, blended raw banana homogenate (10 $g.l^{-1}$) and activate charcoal (0.2 $g.l^{-1}$) were added as additives in H2 medium. The pH was varied between 5.0 and 6.3 by using concentrated KOH to raise it and HCl to lower it before autoclaving at 121°C for 20 minutes. The media were then dispensed into containers for sowing and subculturing orchids and fungi in a laminar air-flow cabinet.

Table 4.2: Composition of antibiotic medium used for isolation of orchid mycorrhizal fungi (Modified from Clements *et al.* 1986).

KNO_3	1.5 mM
$(NH_4)SO_4$	2.0 mM
$Ca(NO_3)_2$	1.0 mM
$MgSO_4$	0.5 mM
Asparagine	2.0 mM
Sucrose	5.0 $g.l^{-1}$
Agar (purified)	8.0 $g.l^{-1}$
Yeast extract (Difco)	0.5 $g.l^{-1}$
$FeSO_4.7H_2O$	28 $mg.l^{-1}$
Na_2 EDTA	38 $mg.l^{-1}$
Penicilline	20 $mg.l^{-1}$
Streptomycine	10 $mg.l^{-1}$
Tetracycline	10 $mg.l^{-1}$

Table 4.3: Composition of oat medium for germination of orchid seeds. Clements *et al.* (1986) medium modified by Rasmussen *et al.* (1990).

$Ca(NO_3)_2.4H_2O$	200 mg
KCl	100 mg
KH_2PO_4	200 mg
$MgSO_4.7H_2O$	100mg
Yeast extract (Difco)	100 mg
Sucrose	2 g $.l^{-1}$
Finely ground Bokomo oats	3 g $.l^{-1}$
Agar (purified)	12 g$.l^{-1}$

Table 4.4: Composition of the germination medium for germination of orchid seeds (From Dijk & Eck 1994).

Glutamine	1.0 mM
NH_4NO_3	1.0 mM
$(NH_4)_2SO_4$	1.0 mM
$Ca(NO_3)_2$	1.0 mM
K_2SO_4	1.0 mM
$MgSO_4$	0.5 mM
$FeSO_4$	0.1 mM
Na_2EDTA	0.1 mM
Agar (purified)	6.0 g.l-1
Yeast extract (Difco)	1.0 g.l-1
Sucrose	10.0 g.l-1
Inostol	0.2 g.l-1

4.2.3 Isolation of fungi from roots

The isolation of fungi from populations of the five terrestrial and fiveepiphytic orchid species collected in different habitats (Table 4.1) followed the schedule used by Warcup and Talbot (1967). Roots were washed gently with cold running tap water to remove any adherent soil particles and other debris and sterilized with 5% (m/v) $Ca(OCl)_2$. The roots were transversely sectioned and stained with 0. 05% trypan blue in lactophenol (20 ml phenol, 20 ml lactic acid, 40 ml glycerin, 20 ml water). They were checked under a stereomicroscope at low power (×40) for patterns of fungal colonization in the cells of cortices. Some roots were first cleared in 10% KOH and then similarly sectioned and stained to correlate the *in vivo* peleton morphology with that of correspondingly isolated fungal strains. The epidermal and outer cortex of those root sections with healthy fungal coils were cut away with a sterile razor blade. The sections were stirred gently in sterile distilled water for 30 minutes. They were then transferred into an antibiotic isolation medium (Djick & Eck, 1995). The fungi were regularly subcultured in three replicates of fresh medium to ascertain their purity.

4.2.4 Fungal identification procedures

Fungal cultures were examined at frequent intervals and the following details recorded:

1. Rate of growth, described as very slow, slow, moderate, or rapid. In most cases, averages calculated from measurements every 24 hrs. were used as indicators of growth rate (mm/hr).
2. Colony colour and colour changes, whether uniform or in zones or patchy.
3. Colour and colour changes of the reverse of the colonies.
4. Colour changes in the medium, whether confined to the area covered by the colony or diffusing.

5. Texture of the surface, whether loose or compact; plane, wrinkled or buckled; velvety; matted, floccose, hairy, ropy, gelatinous or leathery.
6. Odour, if any.
7. Character of drops of fluid (exudate) found on aerial growth.

Mature hyphae were aseptically removed from the plates and mounted on glass slides in a drop of 0.05% trypan blue in lactophenol according to the protocol of Boedijn (1956). Following this staining procedure, a small drop of the stain was placed at the centre of a clean glass slide. With a sterile needle a very small portion of hyphae was picked off from the culture and placed in the drop. Using a pair of needles the material was teased until it was well wetted before covering with a cover-glass. After the area immediately around the cover-glass was dry a clear nail vanish was applied to the cover-glass edge using a thin brush and allowed to dry.

The slides were observed under an Olympus BH-2 compound microscope at ×600, using bright-field optics for identification purposes. The following details were recorded:

1. Characteristics of aerial or submerged hyphae; presence or absence of septa, approximate diameter, characteristic of special structures if any present.
2. The stage at which fruiting structures develop.
3. The characteristics and disposition of the mature fruiting organs; whether sporangia, perithecia, pycnidia, sporodochia, coremia, or detached conidiophores; whether borne in the substratum, or on the surface, or on aerial mycelium.
4. Colour, size, and shape of mature fruiting organs or fruit bodies.

4.2.5 *In vitro* symbiotic germination tests

Seeds stored at 4°C belonging to six species each of *Eulophia* and six *Polystachya* (Table 4.1) were used in the symbiotic germination experiments. The seeds (150-200 per experiment) were pre-treated in 5% (m/v) $Ca(OCl)_2$ mixed with two drops of Tween 80 (0.05% v/v) for 10-20 minutes (H. N. Rasmussen, 1995), rinsed five times in sterile distilled water, and sown on the surface of 2% oat agar medium in slants (Djick & Eck, 1995). Each of the twelve fungal isolates (Table 4.5) of the ten orchids species (Table 4.1) was applied to seeds of each of the 12 orchid species, and each inoculation was replicated three times. In doing so, small blocks of isolation medium in which the fungi were well established were aseptically cut and added to the petri plates and slants containing seeds. In addition, three replicates of uninoculated seed were used as controls. The preparations were sealed with parafilm to control contamination.

The multiple cross-inoculation experiments as above were also performed using the germination medium, which has a higher concentration of sucrose (10 $g.l^{-1}$), in addition to glutamine and inostol which are not incorporated in the oats medium.

Germination conditions were set at 16h light at 22°C, followed by 8h dark at 18°C. Humidity was 70% and light intensity of 130 $\mu mol.m^{-2}.s^{-1}$. Seeds were subcultured every 28 days.

To monitor germination five to ten seeds from each of the cultures were mounted onto glass slides, stained with 0.05% trypan blue in lactophenol and covered with cover slips. These were observed under a microscope with low-power objective lenses to monitor germination. Swelling and differentiation of the embryos followed by seed coat rupture recognized germination. The entire process was graded as follows: - no germination, 0 swelling of embryo and inversion of fungus, + embryo differentiation/germination, ++ seed coat rupture, +++ protocorm development. Observations were made using ordinary

bright field optics on a Zeiss Dialux 20 photomicroscope. Photographs were taken using an Ilford Pan F film (bright field).

4.3: RESULTS

4.3.1 Fungal taxonomy

Twelve endomycorrhizal fungi from ten orchid species were isolated and identified. The fungal strains were first identified into their major taxonomic groups using fructification characters (Table 4.5) and using a combination of culture, fructification and hyphal morphology and the taxonomy of Parmeter and Whitney (1970), Sneh *et al.* (1991), Burgeff (1936), Curtis (1939), Currah *et al.* (1987), Smith (1969), Barnett and Hunter (1972), and Warcup and Talbot (1967, 1971, 1980) they were tentatively classified to the generic and where possible to the specific level (Table 4.6).

Table 4.5: A list of fungal strains isolated from Kenyan orchid species showing the major taxonomic groups of the fungi. E = fungal isolate from epiphytic orchids and T = fungal isolate from terrestrial orchids.

Strain	Taxonomic group of the fungus	E/T	Source orchid
E1	Fungi Imperfecti	T	*Eulophia angolensis*
E2	Basidiomycete	T	*E. streptopetala* var *streptopetala*
E3	Fungi Imperfecti.	T	*E. streptopetala* var. *streptopetala*
E4	Mycelia sterilia	T	*E. speciosa*
E5	Mycelia sterilia	T	*E. petersii*
E6	Mycelia sterilia	T	*E. streptopetala* var. *streptopetala*
E7	Fungi Imperfecti	T	*E. streptopetala* var. *stenophylla*
E8	Fungi Imperfecti	E	*Polystachya steudneri*
E9	Fungi Imperfecti	E	*P. dendrobiiflora*
E10	Basidiomycete	E	*P. campyloglossa*
E11	Basidiomycete	E	*P. cultriformis*
E12	Fungi Imperfecti	E	*P. tenuissima*

The orchids show great diversity in their fungal partners, ranging from Fungi Imperfecti especially, mycelia sterilia (the form-genus *Rhizoctonia*) to fruiting basidiomycetes. The terrestrial *Eulophia* species are mycorrhizal with soil saprophytes particurly *Rhizoctonia* spp. (Figs. 4.1 - 4.3), which are anamorphs of fungi in the group Basidiomycotina. However, one fungal strain, an isolate of *Eulophia streptopetala* var. *streptopetala* was found to form true basidiomycete fruiting bodies but since itsdiagnostic features never resembled any of the described fungi in the published literature it was difficult to do the identification to generic level. The Basidiomycetes isolated from the epiphytic *Polystachya* spp. were identified as *Ceratobasidium* spp. The fungi imperfecti marked with question marks (?) in the table 4.6 are probably new species.

Table 4.6: A list of endomycorrhizae isolated from epiphytic and terrestrial Kenyan orchid species. The fungi with question marks against them do not conform to the described species in the existing fungal taxonomy. E = fungal isolate from epiphytic orchids and T = fungal isolate from terrestrial orchids.

Strain	Endomycorrhiza	E/T	Source orchid
E1	*Cytosporella* sp.	T	*Eulophia angolensis*
E2	Basidiomycete sp.	T	*E. streptopetala* var *streptopetala*
E3	*Gilmaniella* sp.	T	*E. streptopetala* var. *streptopetala*
E4	*Rhizoctonia stahlii*	T	*E. speciosa*
E5	*Rhizoctonia sclerotica*	T	*E. petersii*
E6	*Rhizoctonia* sp.	T	*E. streptopetala* var. *streptopetala*
E7	*Mortierella* sp.	T	*E. streptopetala* var. *stenophylla*
E8	Imperfect fungus(?)	E	*Polystachya steudneri*
E9	Imperfect fungus(?)	E	*P. dendrobiiflora*
E10	*Ceratobasidium* sp.	E	*P. campyloglossa*
E11	*Ceratobasidium* sp.	E	*P. cultriformis*
E12	Imperfect fungus(?)	E	*P. tenuissima*

4.3.2 Nutrient media

The germination nutrient medium was most suitable for growth of both fungi and their compatible seeds. However, some setbacks were experienced on use of the 2% oats nutrient medium:

a) Oats nutrient medium showed the highest growth rate of fungi, so that the tiny orchid seeds were densely covered by the fungal mat, rendering it difficult to observe germination progressively necessitating frequent subcultures at intervals of 14 days.

b) The nutrient medium designated as H4 with glucose as a source of carbon (2 $g.l^{-1}$) and $NH_4NO_3.4H_2O$ (1.0 $mM.l^{-1}$) to supply inorganic nitrogen did not support germination of the seeds.

c) The medium designated H2 proved adequate only for seeds of *Eulophia* and not *Polystachya*.

The early germination process was found to be faster in the dark than in an illuminated environment and further development of the early protocorms required a photoperiod treatment. Both the germination and oats (H2) media were adequate for germination of only *Polystachya fusiformis* in the control experiments.

4.3.3 Symbiotic seed germination

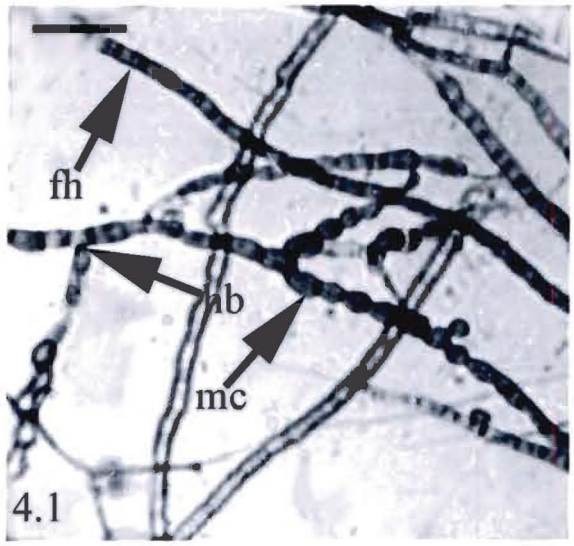
The twelve fungal isolates used for symbiotic germination differed in their ability to support germination of orchid species (Table 4.7). The experiment showed that a compatible orchid/fungus combination is required for germination and subsequent development of seedling (protocorms) *in vitro*. A compatible relationship is defined here, as in Clements (1988), as one where there is rapid development of protocorms and seedlings following infection by a mycorrhizal fungus (i.e., in > 75% of all viable seeds).

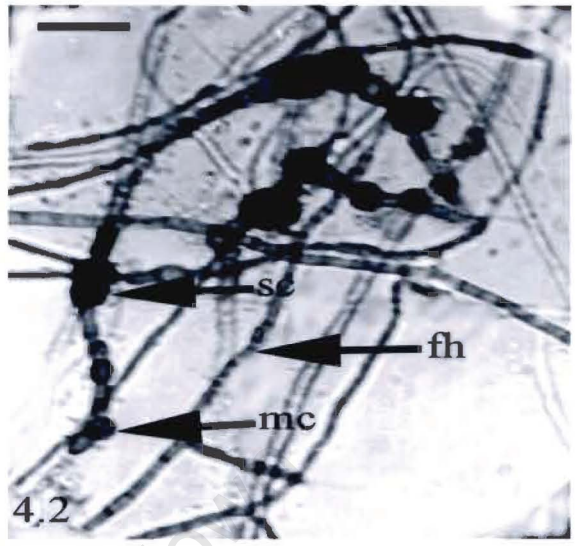
The data provide evidence that the fungi that caused germination of orchid seeds are not necessarily the ones isolated from the orchids bearing the seeds. At the same time these orchid species in all inocula exhibited peculiar requirements for fungal symbionts, making germination of all species by a common fungus difficult. However, efficient germination to protocorm stage was assured if the fungal symbiont and seeds were obtained from the same orchids. Although embryo differentiation/germination consistently occurred in all treatments successful germination to protocorm stage was observed only in five species, three of which had seeds and their fungal partners from same orchid species. However, a majority, i.e. eight orchid species, was reluctant to develop protocorms in the 120 days of the experiments. This was described as slight germination because embryos differentiated even though there was no development into protocorms. In the four cases where fungi did not cause any germination whatsoever only swelling of embryos was detected, with no significant morphological changes.

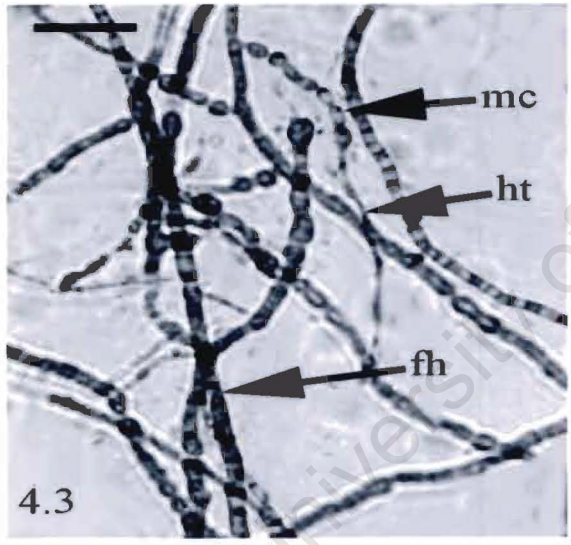
Some degree of specificity in terms of fungal requirement was displayed in the five orchids that germinated (*E. angolensis, P. adansoniae, P. campyloglossa, P. cultriformis* and *P. spatella*) while the rest were receptive to two or more fungi. In this study compatibility of the orchid/fungus combination did not depend on the habitats of the partners.

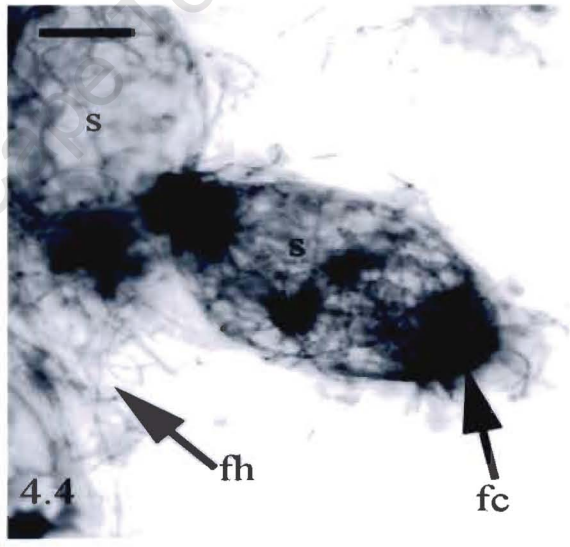
Table 4.7: Developmental stages of orchids grown symbiotically on modified oats medium and germination medium inoculated with a range of fungal isolates. Codes are as follows: - indicates no embryo differentiation/germination; 0 swelling of embryo and inversion of fungus; + embryo differentiation; ++ seed coat rapture; +++ protocorm development; and **underline** indicates those trials in which the fungus used was isolated from the species bearing the seeds.

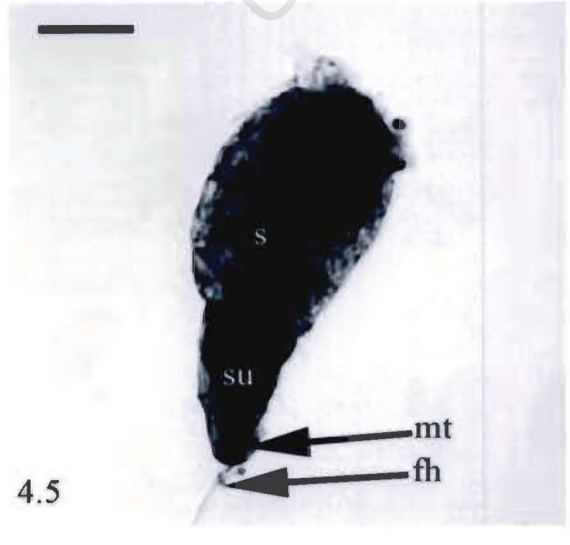
	FUNGAL STRAINS											
	Terrestrials						Epiphytes					
ORCHID SPECIES	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
Eulophia angolensis	-	-	-	++	-	-	-	-	-	-	-	-
E. horsfallii	+	-	-	-	-	-	0	-	-	-	-	-
E. petersii	++	-	-	-	-	-	+	-	-	-	-	-
E. speciosa	-	-	-	+	-	-	-	-	-	-	-	-
E. streptopetala var. *stenophylla*	-	-	-	-	+++	-	+++	-	-	-	-	-
E. streptopetala var.*streptopetala*	-	+++	-	-	-	+	-	-	-	-	+	-
Polystachya adansoniae	-	-	-	-	-	-	-	-	-	-	-	++
Polystachya campyloglossa	-	-	-	-	-	-	-	-	-	-	+++	-
P. cultriformis	-		-	-	-	-	-	-	-	-	-	+
P. fusiformis	-		-	-	+++	-	-	-	-	-	+	0
P. spatella	-	-	-	-	-	-	-	+	-	-	-	-
P. transvaalensis	-	-	-	-	-	-	-	-	+	-	-	0
P. vaginata	-	-	-	-	-	-	-	0	+	-	-	-

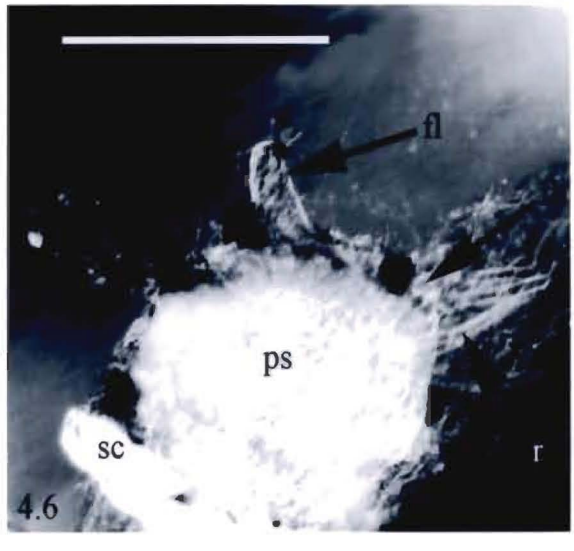












Figures 4.1-4.6: Mycelium of fungal symbionts isolated from the mycorrhizae of mature *Eulophia* species. Figs. 4.1-4.2: *Rhizoctonia sclerotica* of *Eulophia streptopetala* var. *stenophylla*; fh = fungal hypha, hb = hyphal branching, mc = branched monilliod cells; Fig. 4.3: Mycelium of *Rhizoctonia* sp. of *Eulophia streptopetala* var. *streptopetala;* mc = non-branched monillioid cells, ht = long extension of hyphal tip, fh = fungal hyphae.

Figures 4.4-4.6. Stages of symbiotic germination of seeds. Fig. 4.4: Formation of symbiosis between seeds of *Polystachya fusiformis* and *Rhizoctonia sclerotica* after 6 days of sowing; s = whole seeds showing carapace, fc = fungal colonisation at micropylar pole, fh = fungal hyphal extensions. Fig. 4.5: Embryo of *Eulophia streptopetala* var. *stenophylla* with pelotons formed *in vitro* with *Rhizoctonia sclerotica* after 14 days; s = whole seed, su = suspensor cells, mt = accumulation of fungal hyphae, fh = fungal hyphae. Fig. 4.6: Seedling of *Polystachya fusiformis* at 60 days after sowing; fl = first leaf, ps = young pseudobulbs, sc = remains of raptured seed coat after germination, r = young roots.

4.3.4 Time-scale and stages of seed germination

Analysis of progressive formation of symbiosis *in vitro* revealed that fungal strains form thick hyphal concentration on the outside of the micropylar end of the seeds of both *Eulophia* and *Polystachya*. The embryo at this stage is observed to be undifferentiated but once the hyphae penetrate into the seed, they grow all over the carapace, a structure that encloses the embryo (Fig. 4.4). An accumulation of fungal hyphae forming a thick tissue outside the suspensor forms at the tip of the suspensor about six days after fungal invasion for most seeds of both the species (Fig. 4.5). When the tissue establishes well hyphal penetration of the suspensor takes place and at this stage embryos differentiate.

A significant differentiation takes place in the region between the suspensor and the embryo proper. This results in embryo greening in this region and is commonly visible in seeds in culture medium. As the embryo increases in volume further differentiation takes place and it enters the protocorm stage. The shoot apical meristem organises and forms a green leaf in a period of about 90 days (Fig. 4.6).

In the control (uninoculated) experiments, the seeds of *Eulophia* either do not germinate in response to the media, or the time taken for germination is longer than 4 months relative to that of the treated seeds (Table 4.8). In *Polystachya fusiformis,* the only species which responded to the media, seed germination started on the 60th day after inoculation.

Table 4.8: Time-scale for *in vitro* germination of *Eulophia* and *Polystachya* embryos. - no embryo differentiation/germination, + embryo differentiation/germination, ++ seed coat split, +++ protocorm development.

	0-6 days	7-9 days	14-28 days	50-60 days	60-90 days	120 days
Eulophia	-	+	+	++	+++	+++
Polystachya	-	+	++	+++	+++	+++
Control						
Eulophia	-	-	-	-	-	-
Polystachya	-	-	-	-	+	+++

4.4 DISCUSSION

This study of the mycorrhizae and their role in symbiotic germination of the Kenyan orchids has shown the close relationship between orchid germination and its dependence on mycorrhizae in their natural habitats. Findings of the present study are consistent with those of earlier workers concerning the widespread formation of orchid mycorrhizae in natural habitats (Burgeff, 1936; Harley, 1969; Dörr and Kollmann, 1969; Arditti, 1967, 1979, 1984; Warcup, 1981; Clements, 1982). Host range, which is the diversity of plant species in field conditions with which a fungus can form mycorrhizae (Molina *et al.*, 1992), was wide in this study in that fungal symbionts are not host-restricted. For example, the three populations of *Eulophia streptopetala* (see Table 4.6) in different localities each harbour a different fungus. Therefore, there is no evidence from the present findings that a particular fungus forms mycorrhizae with all members of a species or genus. Likewise, in each locality populations of an orchid species are each associated with a different fungus, hence the lack of support for species specificity for the fungi.

The endomycorrhizae observed in *Eulophia* and *Polystachya* are comparable since *Ceratobasidium* spp. isolated from the latter frequently occurs in *Rizoctonia*-stages commonly present in the former genus. *Rhizoctonia* and the free-living soil imperfect fungi belonging to the orders Moniliales and Sphaeropsidales are commonly the endophytes of *Eulophia* according to the present study. Interestingly the genus *Polystachya* has fungal species of *Ceratobasidium* and species classified as members of Tulasnellaceae, which are not Fungi Imperfecti and could be of common occurrence in *Eulophia* in their *Rhizoctonia*-stages. The present observation is the first record of these fungi forming mycorrhizae with epiphytic *Polystachya* orchids.

Since only Eulophias possessed *Rhizoctonia* species as their associated mycorrhizae, it can be suggested that these fungal species are well adapted for terrestrial habitats. Published research reports *Rhizoctonia* as the most widely isolated fungi, especially from terrestrial orchids (Burgeff, 1936; Curtis, 1939; Downie, 1959; Alconero, 1969; Warcup, 1981; Warcup and Talbot, 1967, 1971, 1980; Warcup *et al.*, 1987). However, little attention has been given to the Fungi Imperfecti that form mycorrhizae with orchids. Perhaps the contribution of the present study together with earlier findings (Currah *et al.* 1987; Perkins *et al.,* 1995) is that Fungi Iimperfecti could be far more common in orchid mycorrhizae than was previously thought.

Endomycorrhizal species belonging to the genus *Ceratobasidium* identified in the present study are regular isolates of epiphytes(Warcup, 1981; Currah *et al.*, 1987; Smerciu and Currah, 1989; Uetake, 1997). It is interesting to note from the present study that *Ceratobasidium* although initially recorded as a terrestrial saprophyte, adapts well in both terrestrial and epiphytic habitats.

There is scant information in published literature on fungal taxonomy, making it very difficult to identify the fungi to specific level without any ambiguity. In this study, the problem was overcome by analysing the consistent diagnostic features to the generic level or a higher taxon when the distinguishing characters failed to permit further identification. Coupled with this problem and yet intriguing is the question of whether new species has been recorded in the present study. Confirmation will require a long time and the knowledge of an expert mycologist to identify. While there have been controversies i.e. the question of specificity of mycorrhizal associations in orchids (Bernard, 1909; Knudson, 1925; Curtis, 1939; Hadley, 1970) the data presented here fail to confirm that in the field the Kenyan orchids are usually associated with specific endophytes.

One of the questions to be answered in this research was whether populations of an orchid species collected from different sites share the same fungus. The findings of the study show that each orchid population was associated with a specific endophytes not found in other populations of the same orchid species elsewhere. The fact that orchids did not share the same fungal species, either as a result of shared terrestrial or epiphytic habitat or common ancestry, means that the formation of mycorrhiza is not an ecological phenomenon. However, it is important to gain more evidence on this topic by more sampling of orchid populations in shared sites within the habitats.

Fungal isolates of *Ceratobasidium* spp. were earlier reported to be restricted to the terrestrial habitat but were also found in epiphytic habitat according to the present study. This refutes the contention that mycorrhizal fungi are strictly habitat-specific. Curtis (1939) first indicated that mycorrhizae could show habitat-specificity, and later publications also verified that the association of mycorrhizae in the wild is highly habitat-specific (Warcup, 1971, 1990; Jones 1988; Masuhara and Katsuya, 1992). The present study, however, does not corroborate their findings probably due to lack of enough sampling.

The processes described for infection of *Eulophia* and *Polystachya* seeds by mycorrhizal fungi and subsequent germination of seeds *in vitro* show that the relationship between orchids and their associated fungi is complex. A significantly small number of orchid species in the present study formed protocorms through *in vitro* symbiotic techniques. The different combinations of orchid/fungus in germination tests (see Table 4.7) explains the fact that only compatible partners effected favourable germination. Fungi that stimulated germination of the seeds are not necessarily the ones isolated from those orchids in their natural habitats, as noted in the *E. streptopetala* var. *stenophylla* and *R. sclerotica* combination.

The precise relationship between fungi and orchids *in vitro* is unclear although Rasmussen (1995) mentions that fungi may stimulate germination of seeds. It only appears that penetration of the fungus into the seeds is necessary for embryo differentiation and continued germination (Warcup, 1981; Zelmer and Currah, 1997; Zettler and McInnis, 1993). Despite the reluctance by many authors (Masuhara and Katsuya, 1994; Rasmussen, 1995; Warcup, 1973, 1975, 1985a, b; Perkins and McGee, 1995; Perkins *et al.*, 1995) to conclude that the fungi cause actual embryo differentiation, and hence germination, the present findings show that the fungi stimulate embryo differentiation within the first two weeks of inoculation in the genera investigated. However, in the control treatments i.e. asymbiotic germination experiments *Polystachya fusiformis* seeds took 60 days to show germination/differentiation and *Eulophia* seeds did not germinate even after four months of incubation and subculturing. Clements (1982) also found that germination of seeds symbiotically was three times faster than the asymbiotic germination. The seeds of the taxa he investigated took four months to germinate symbiotically while the asymbiotic seeds took 10-20 months to germinate.

The nutrient medium used is important. The media developed by Clements (1982) and then modified by Djick and Eck (1994) and Rasmussen (1995) supported germination of infected orchid seeds of *Eulophia* on agar slopes. However, the media did not readily germinate epiphytic species of *Polystachya* unless additives of raw banana homogenate as opposed to 10% w/v ripe banana homogenate (Yam and Weatherhead, 1988) and activated charcoal (Rasmussen, 1995), were available in the media.

This difference in medium preference is due to the fact that the oat medium was initially developed for the Australian terrestrial orchid *Caladenia* with its fungus *Sebacina vermifera* (Clements & Ellyard, 1979). This medium has since supported symbiotic germination of many temperate terrestrial orchids (Clements, 1982, 1988; Zelmer &

Currah, 1997). The only epiphyte that has so far germinated in the oats medium is *Dendrobium discolor* Lindl. with the fungus *Tulasnella irregularis* (Warcup, 1981). However, in many other publications where this medium has been utilised as substratum for symbiotic partners, the medium is used in modified form. It also appears that the orchid seeds are in a sense specific to the kind of agar surface used. This is possibly the main reason why the special Davis type agar is needed in the original formulation of Clements (1982) oat medium. In our case the purified agar supplied by Sigma company as item EEC NO 232-658-1 was adequate for the seeds of both *Eulophia* and *Polystachya*.

The information obtained from this study has major implications in the conservation programmes of the Kenyan orchids. As most of the orchids in Kenya are rating high in the list of endangered and vulnerable plants due to destruction of habitats by the general public, it possible through the symbiotic techniques used in the present study to develop seedlings from seed and make them available to botanic gardens, commercial growers and conservationists. However, it should be realised that the ultimate goal of symbiotic germination trials in the present study is to make it possible for introduction of compatible orchid fungi into the protocorms of orchid since they will require mycorrhizae for adaptation in a soil mix or new habitats. Although germination trials of the seed stored in the national seed bank at the East African herbarium (EA) have not been done through this techniques, there is a possibility that most of them, which are now listed as rare and endangered will be germinated and relocated to afford them protection in safe sites or return them to their original habitats. Further study will focus on long-term preservation of seed useful mycorrhizal fungi together with using liquid nitrogen proposed by Pritchard (1984) for orchids.

But it must be appreciated that there are problems with the *in vitro* symbiotic seed germination method employed in the present study especially in regard to orchid-fungus combinations where only a small number of seedlings form protocorms. In the case of extremely rare orchid species, limited to a few small populations, the goal of conserving the entire species may be possible through habitat protection and *ex situ* preservation. The potential for *in vitro* symbiotic germination is great and promising as a conservation strategy for orchids *ex situ*. The time-scale for producing seedlings through these methods is short (90-120 days). It is encouraging for conservation reasons to note from the data that mycorrhizal infection and establishment *in vitro* depends highly on compatible seed/fungus partners. This implies that with appropriate media and fungi, seed germination is assured and since seedling formation is faster, huge supplies of orchids are possible for conservationists. In appears from the present study that the success of *ex situ* conservation through symbiotic seed germination depends much on preservation of both seeds and compatible fungi in seed banks. Interestingly, as projected from earlier work (Perkins *et al.*, 1995, Masuhara & Katsuya, 1994) that those fungi that form mycorrhizae with orchids in the laboratory are likely to associate with the same orchids in the field, there is hence a likelihood for survival of those orchids in the field.

CHAPTER 5: SYNTHESIS

Contents

5.1 General introduction

This chapter presents the synthesis of information described in the preceding four chapters in order to reflect the broader picture of seed development, symbiotic seed germination and conservation of the Kenyan orchids. Also highlighted in this chapter are limitations of the present study and possible areas for future research.

The present study on embryology, seed coat and symbiotic associations of the Kenyan orchid species, as presented in the preceding chapters, has provided information useful for the classification and understanding the evolution of the diversity of embryological and seed coat features. A close association with endophytes is necessary for conservation of the orchids especially through *in vitro* symbiotic seed germination.

5.2 Correlation between embryology, seed coat morphology and symbiotic associations

There is a relationship between the embryology, seed coats and mycotrophy. The seed coats are formed by the outer integuments during embryogenesis, and are probably under selection to enhance mycotrophy during seed germination. Therefore, in order to have an insight of the morphology of seed coats e.g. integumentary initiation in the ovule, the number of integumentary cell layers forming the seed coat, as well as lack of endosperm in mature seed prompting adaptation to mycotrophy, a study of embryology is absolutely of necessity. In this connection, size and organisational structure of the two or three cell-layer seed coats revealed by embryology, for example, indicate that the rough seed

surfaces with wavy and reticulate microornamentation evolve in such a way that it is an adaptation to mycotrophy in epiphytic and terrestrial habitats respectively. However, embryology does not provide adequate account of such structures of the seed coats viz. microornamentation, testal extensions, colour and epicuticular secretions observed in some orchids in the present study. Formation of these features e.g. in *Microcoelia* might be a result of ecological adaptation by the orchids and especially mycotrophy and long distance dispersal (Rasmussen, 1995). However, the aforementioned seed coat characters may have a direct bearing to mycorrhizal associations during germination (Carlson, 1935; Geitler, 1956; Wirth and Withner, 1959). Embryology in the present study shows that the tiny size of epidendroid seeds (174-2818 x 34-291 μm) also noted by Clifford and Smith (1969) might be due to these adaptational requirements.

The absence of endosperm is possibly due to failure of double fertilization to take place during embryogenesis (Pace, 1907; Prosina, 1930; Afelius, 1916; Swamy, 1947, 1949a) although presence of Primary endosperm nucleus (PEN) in *Ansellia*, *Eulophia* and *Polystachya* was noticed. The endosperm, the nutritive tissue of the embryo (Arditti, 1992), is not formed in any of the species studied. The lack of endosperm is thought to be the precise reason why the seeds require mycorrhizal symbiosis to enhance differentiation of the minute embryos and sustain their continued germination and nutrient uptake (Clements, 1988). The present study suggests that the role of endophytes in orchid germination is to induce differentiation of the embryos and possibly provide them with nutrients derived from the nutrient media *in vitro* for continued growth (Zettler, 1996). It is thus possible that the fungal pelotons formed in the protocorms *in vitro* also can continue surviving with orchid under natural conditions in the field or a soil mix (Hardley, 1970) and therefore useful in *ex situ* conservation. However, these

nutritional relationships between the embryos and mycorrhizae in relation to lack of endosperm need a further study.

5.3 Phylogeny

Very little is known about the relationship between of the variation in embryology, seed morphology, and phylogeny of the Epidendroideae (Clements, 1995). This study demonstrated that genera can be clustered together or separated depending on whether they possess uniform or different ontogenetic patterns of sporogenesis, gametogenesis, and embryogenesis. Within these patterns the structure of integuments, embryo sacs, gametophytes, embryos and suspensors, for instance, suggests that the data are useful for taxonomic purposes, thus corroborating Johri (1963) and Bhojwani & Bhatnagar (1983) comments. In addition, variation in these embryological features are correlated with variation in seed coat morphology, and mycorrhizal associations. Such correlations were already suggested by Rasmussen (1995).

Data from the present study show that initial assumption that seeds collected from diverse habitats will not show morphological and embryological similarity to form distinct clustering is not supported. Considering the fact that the present study reveals similarities among species defined by embryology, which is less influenced by geographic proximity or altitude it is possible to use taxonomy as an indirect measure of phylogenetic relatedness. This is in stark contrast to the patterns of variation in seed coat morphology. For these structures, the best correlations appear to be with habitat types, rather than the taxonomy. The discovery in the present study of embryological patterns existing in the orchids depending on their lineages rather than ecology is an indicator that a more complex embryological relationships exist among the epiphytic and terrestrial orchids.

Epidendroideae has been regarded as the most difficult subfamily of the orchids to interpret phylogenetically (Dressler, 1981, 1986, 1993; Rasmussen, 1986). Consistent with this, the present study of embryology, seed coat morphology and mycorrhizal associations indicate that the relationships in the presently circumscribed tribes viz. Vandeae, Epidendreae, Cymbideae, Malaxideae, Vanilleae, Arenthusae and Dendrobieae may not be correct. The variation of the aspects described for the different orchid species in the present study is largely as a result of common ancestry and to some extent ecology. Previous phylogenetic patterns (Burns-Balogh and Funk, 1986; Cameron *et al.*, 1999) were determined using vegetative, floral, and molecular characters. It is apparent that these characters in the present study might give a rearrangement of species if incorporated into a cladistic analysis for the entire subfamily Epidendroideae.

A strong conclusion cannot be made from the present study regarding the relationships of the Vanilloideae due to inadequate sampling. The tribe Vanilleae, by using seed coat morphology in addition to vegetative and molecular characters, has been elevated to a separate subfamily Vanilloideae (Cameron *et al.*, 1999). This is corroborated by the nucellus of *Vanilla polylepis*, which has with a length of 13 cells is the longest among the orchids studied here. Unfortunately, due to lack of suitable material in the present study, no further examination of embryology was described for the vanilloids. Consequently, it cannot be established whether such large nucelli are typical of the whoel subfamily, or only a portion of it. However, the Vanilloid seeds (Cameron & Chase, 1998) are morphologically different from those of Epidendroideae.

The remaining six tribes are rather uniform in their embryology and seed coat morphology, and should therefore be retained within the Epidendroideae as treated in Dressler (1990, 1993).

The abolition of the subfamily Vandoideae (Dressler, 1990), and inclusion of its members in the subfamily Epidendroideae, is supported by the findings of the present study, especially embryology and seed coats. I have been able to show that the vandoid embryology and seed coats are similar to those of some species of Dendrobieae, Malaxideae, and Cymbidieae. For instance, the nucellus, integuments, embryo sacs and embryo types including suspensor types strongly link the vandoids to the Epidendroids. The eight-nucleate embryo sacs observed for the vandoids, especially *Eulophia* and *Aërangis,* are also found in the species of Epidendreae, e.g. *Polystachya* and *Epidendrum*. With respect to embryogenesis, the *Diaphananthe* type is typical of the vandoids, with exception of some species e.g. *Cribbia brachyceras* that possess the *Bulbophyllum* type embryogenesis, which links it to the Cymbidioids and Dendrobioids. Other vandoids, particularly *Aërangis* and *Microcoelia,* share the *Polystachya* embryogenesis with species of Arethusae, Malaxideae, Epidendreae and Cymbideae. The exceptions are *Eulophia* and *Epidendrum,* which have a distinct type of embryogenesis. In addition, the variation of seed coats of 50 species show that species of 41 epiphytic vandoids and Epidendreae, i.e. seed groups A and B, are closely related strengthening this Vandoideae-Epidendroideae link.

Therefore, the characters studied, if included in a cladistic study together with vegetative, floral morphology, and molecular characteristics, may provide a clearer understanding of the phylogenetic patterns within the Epidendroideae. However, the monophyly of the Epidendroideae can only be established if the embryological and seed coat morphology in the present study is to be included in a cladistic analysis together with other characters.

5.4 Ecology

In the present study only seed coat morphology shows a stronger correlation with habitat types (listed in Appendix 1) than with their taxonomy hence necessitating further sampling to ascertain such similarity as the one of the epiphytic *Ansellia* and *Epidendrum* with terrestrial *Eulophia* and *Oeceoclades*. Despite the fact that structural and microsculptural features of seed surfaces frequently reflect important generic and phylogenetic differences (Barthlott, 1984b), the present study indicates that there is mainly an ecological basis in their divergence. These results show that ecological factors are important in the development of secondary structures of the seed coats. The characteristic forms of these surface cells are the result of differential thickening in their clinal walls, and this is revealed only through embryological studies. However, seeds with similar seed coats do not show resemblance in embryology, as illustrated by epiphytic seed groups A, B, and C from different genera (Chapter 3). Seeds in each of the categories mentioned are similar in their seed coat details but different in the structure of their nucellus, integuments, embryo sacs, embryogenesis and suspensors. The diversity of 39 morphological characters of Epidendroideae (Table 3.3) is linked with habitat and geographical information (Table 3.1) and shows that seed morphology is mostly habitat driven. Not surprising the least variable morphometric variation occurs among groups of species which belong to the same habitat, and seed coats of epiphytes form groups distinct from terrestrial ones. Within the terrestrial and epiphytic groups little variation is found. Nevertheless, as detailed somewhat at the generic level, e.g., *Polystachya* where variation is noted occasionally even at population level, the structure of seed coat surface confirms the fact that it may often be the rich source of phylogenetic signals (Barthlott and Voit, 1979). Since only the surface microstructure was studied it is possible that the internal organization of the seed coats is determined by common

ancestry (Manning, 1991), which according to the present study requires a further examination using SEM at the ovule stage to expose the systematic information at different taxonomic levels. The five distinct seed groups conveniently recognised as A, B, C, D, and E, based on variation in secondary thickening that have been observed in the 20 genera of Epidendroideae studied, indicates that seed surfaces are of systematic importance although genera tend to be uniformly either epiphytic or terrestrial. However, this diversity is also important in indicating that the seeds from epiphytic habitats have a different seed format from those of terrestrial habitats. This is not surprising with the dust-seeds of Orchidaceae, which depend on extremely specific ecological conditions for germination e.g. saprophytic-mycorrhizal conditions (Rauh *et al.*, 1975). It is worthwhile noting that the seed groups in the genera of either habitats are distinguishable except for the lithophytic genera, *Ansellia* and *Epidendrum* that possess a majority of characteristics of terrestrial seeds viz. seed size and microornamentation differing only in possession of longitudinal periclinal striations. It is possible, as suggested from the present study, that the seed coats have evolved in response to the specialised habitat on trees for epiphytes and on the ground for terrestrial species.

Although morphological adaptation of seeds to the epiphytic or terrestrial habitats is evident, other possible ecological correlates have not been investigated. From the functional point of view, other than mycotrophy of the seeds, seed coats in the present study may be very important in seed dispersal assisting the tiny seeds to be dispersed over long distances (Rasmussen, 1995) due to air spaces between the capacious testae and the tiny embryos (Ziegenspeck, 1935; Carlson, 1940) and rough sculpturing of testae which traps air bubbles (Rauh *et al.*, 1975). However, the aerodynamic properties of the

seeds responsible for their dispersal and the discriminate landing mechanisms of epiphytic seeds on trees and terrestrial seeds on the ground need further investigation.

5.5 The linkage to germination controls

The present study underscores the fact that orchid species, which occur in epiphytic and terrestrial habitats, depend on mycotrophy and are heavily infected with fungi in their thick, sparsely branched roots. The extremely simple roots (Brundrett & Kendrick, 1988) that are fleshy (Niewieczerzalowna, 1933) are heavily colonised by fungi probably from the protocorm cortex of the seedling stage. However, infection of mycorrhizae on the new roots has been suggested to be from outside the seedling (Hadley, 1982). Since the roots of an orchid species in the present study are infected by only one fungal species within a habitat it confirms the suggestion that mycorrhizae are habitat-related. Although one *Ceratobasidium* sp., previously isolated from terrestrial environment, was found in the epiphytic orchids used in this study, rarely do fungi of epiphytic orchids form mycorrhizae with terrestrial orchids, and vice versa. The results provide a basis for a detailed survey and classification of fungi and establishment of the host-range and host-specificity in mycorrhizal symbioses of Kenyan orchids.

As described in the present study, the linking of mycorrhizal associations with germination is crucial since the orchid embryos with diverse suspensors are undifferentiated and rely on fungi in order to germinate. Since the tiny embryos lack endosperm, hence reflecting a deficit of reserve nutritional resources made available for germination, external sources of nutrients by way of compatible fungi are essential. Therefore this ecological phenomenon indicates that the seed coats, which are well adapted for penetration of fungi to translocate the nutrients into the suspensors is very important for orchid management.

The present study established that seed coats play an important role in establishment of mycorrhizal symbioses by way of provision of the micropylar pores formed solely by the seed coat cells for penetration of fungi. This adaptation of the outer integument to mycotrophy by forming micropylar pores seems essential because the seeds, due to stretching of the mature seed coat and deposition of tannin-like substances on the surface (Chase and Pippen, 1990), seem to lose the micropyle of the outer integument. The micropyle seems to be lost due to extreme narrowing as a result of over-stretching of cells during maturation of seeds followed by deposition of tannins hence covering the micropyle of the outer integument completely. Therefore, according to the findings of the present study the mycorrhizal fungi penetrate the seeds through the micropylar pores to form mycotrophic tissues with the cells of the suspensor. In species of *Polystachya* and *Eulophia,* the only genera studied in detail with respect to mycorrhizae, concentration of fungal hyphae in the suspensor cells during embryo differentiation may be an indication of transfer of substances from the fungi to orchid.

The need for symbiosis for germination possibly results from the small and undifferentiated embryos and also the general absence of the endosperm in the seeds. However, the *Polystachya* embryos are selective in terms of nutrient requirements, especially banana homogenate and other additives. Since *Eulophia* type embryos did not require additives for symbiotic germination, it suggests that orchid embryos from different habitats could be media selective and hence have different nutritional requirement in the wild.

5.6 Effects on conservation

Due to pressures of expanding agricultural land in Kenya leading to destruction of natural habitats of orchids, conservation programmes should be intensified. Thus methods of perpetuating orchid species for *ex situ* conservation are required so that the

orchids can be protected either on hilltops or Government protected areas. This is the only opportunity left to manage and preserve the genetic diversity of Kenyan orchids. Accordingly, since most of the orchids are threatened in the wild and yet are of special interest to conservationists and horticulturists (World Resource Institute (WRI), International Union for Conservation of Nature and Natural Resources (IUCN) and United Nations Environmental Program (UNEP), 1992; PCPU, 1995), considerable conservation effort should be directed to these plants. Many of these species that are of horticultural importance should be targeted for commercial production and distributed to interested growers. The combined information of embryology, seed coat morphology and symbiotic associations in the present study gives the best understanding of the use of *in vitro* symbiotic propagation of orchids for *ex-situ* conservation.

Although the relationship between symbiotic fungi and germination of orchid seeds were examined for only *Eulophia* Lindl. and *Polystachya* Hook., from terrestrial and epiphytic habitats respectively, it is possible to extend the study to other orchids of an aforementioned importance. However, it should also be noted that cultures were easily initiated from the seeds grown on 2% oat medium and germination medium, alone, or oats medium supplemented with 10 g/l^{-1} banana fruit homogenate and 0.2 g/l^{-1} activated charcoal. The conservation implications are that as media formulated in the present study could be sufficient for germinating embryos of different taxonomic groups and seed types there might be orchids that are extremely media specific hence requiring different formulations. These warrant further research and fact-finding using a broad spectrum of Epidendroid orchids and different formulation of media.

As *in vitro* symbiotic germination of orchids developed in the present study becomes widely applied for epidendroid orchids, it therefore becomes possible to develop seed banks for securing seed germplasm of rare and endangered orchids for long-term and

active propagation programmes. However, from these studies it appears that the stage at which seeds are collected from the field and storage conditions are crucial and preference should be given to storing fully matured seeds at 4°C. Preservation of orchid seed germplasm in seed banks and subsequent propagation by means of symbiotic germination has been suggested previously (Clements, 1982, 1986). The knowledge of seeds and seedling biology established in the present study together with that of earlier workers (Zettler *et al.*, 1999) is essential if conservation of orchids is to succeed. For that reason, the present study emphasizes the use of symbiotic seed germination methods since the seeds take a shorter time to reach the seedling stage (60 days). However, since germination of seeds requires fungal endophytes these also need to be isolated and cultures maintained. Particularly fungi that are effective symbionts such as *Ceratobasidium* isolated from an epiphytic orchid, which germinated a terrestrial orchid, and likewise the terrestrial *Rhizoctonia*, which germinated an epiphytic orchid, should be the major focus. Hopefully in the future only a single compatible fungus or a few irrespective of source habitat may be required in order to germinate a wide range of orchid seeds. Owing to the unsuitability of preservation techniques, conservation of mycorrhizal fungi is largely dependent on continued sub-culture, which is rather laborious. However, spontaneous mutation of fungi and viability after storage needs investigation.

In conclusion, the results described in this thesis have provided useful information for a clearer understanding of the ontogenesis of Epidendroid seeds and their germination. The data on embryology, seed coat and *in vitro* symbiotic germination are useful for the classification of orchids in general and for the conservation programme of rare and endangered Kenyan orchids in particular.

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Appendix 1: Collection details of species used in the study of embryology, seed coat and conservation (vouchers deposited in the East African Herbarium (EA), Nairobi). The taxonomic treatment of the species follows the system of Dressler (1993).

Taxa	**Locality**	**Collector & collection no.**	**Altitude (m.a.s.l.)**	**Habitat**
Tribe Vandeae Lindl. **Subtribe Aerangidinae Summerh.**				
Aërangis brachycarpa (A. Rich.)Th. Dur. & Schinz	Olchore Onyore (0°45'S.,35°22'E) Langata forest (1°22'S.,36°43'E.)	B.Bytebier 0018 J.M.Ochora 26	1680	epiphyte in open forest by a permanent stream
A. confusa J. Stewart	Mathews range (1°12'S.,37°22'E.)	J.M.Ochora 27	2135	tree epiphyte in montane open forest
A. coriacea Summerh.	Jora forest (3°57'S.,38°48'E. Tigoni (1°07.5'S.,36°40E.)	L.Newton 5596 B.Bytebier 0038	872	epiphyte in open bushland
A. luteoalba (Kraenzl.) Schltr.	Lolgorian (1°14'S.,34°40'E.)	B.Bytebier 0044	1900	epiphyte on shrubs in open bushland
A. thomsonii (Rolfe) Schltr.	Tigoni (1°07'S.,36°40'E.)	B.Bytebier 0075	1930	epiphyte in dense forest
A. ugandensis Summerh.	Kakamega forest (0°16'N.,34°55'E.)	J.M.Ochora 40 B.Bytebier 0017	1516	low level trunk epiphyte in dense forest
Angraecopsis amaniensis Summerh.	Trans-Nzoia	A.Foresti 383	1500	epiphyte in open wooded bushland
A. breviloba Summerh.	Ngangao forest (3°23'S.,38°28'E.)	J.M.Ochora 62	850	epiphyte in open wooded bushland

A. gracillima (Rolfe) Summerh.	Kuja valley (0°45'S.,34°52'E.)	J.M.Ochora 43	1650	epiphyte in open wooded bushland near Kuja River
Bolusiella iridifolia (Rolfe) Schltr.	Aberdares N. Park (0°47'S.,36°07'E.)	B.Bytebier 0068	2300	epiphyte in montane dense forest
Chamaeangis odoratissima (Reichb. f.) Schltr.	Kuja valley (0°45'S.,34°52'E.)	J.M.Ochora 29	1650	open wooded bushland
C. sarcophyla Schltr.	Mt. Kenya (0°19'S.,37°31'E)	B.Bytebier 0072	2135	epiphyte in montane forest
C. vesicata (Lindl.) Schltr.	Langata forest (1°22'S.,36°43'E.)	J.M.Ochora 72	1400	epiphyte in dense forest near river
Cribbia brachyceras (Summerh.) Senghas	Kuja valley (0°45'S.,34°52'E.)	J.M.Ochora 43	1650	epiphyte in open wooded bushland
Cyrtorchis arcuata (Lindl.) Schltr.	Manga range (0°33'S.,34°57'E.)	J.M.Ochora 5	2135	low level trunk epiphyte in montane shrubs
C. preatermissa Summerh.	Mau National Sanctuary (1°12'S.,35°29'E.)	J.M.Ochora 58	1500	open woodland forest
Diaphananthe rutila (Reichb. f.) Summerh.	Saiwa swamp N. Park (1°10'S.,35°05'E)	A.Foresti 799	1900	epiphyte in montane dense forest
D. lolifolia Summerh.	Chemosoren (0°36'S.,35°17'E.)	B.Bytebier 0095	1930	epiphyte in open wooded bushland
D. subsimplex Summerh.	Bahati forest (0°15'S.,36°15'E.)	J.M.Ochora 31	1900	epiphyte in a dense forest
D. tenuicalcar Summerh.	Aberdare range (0°06'S.,36°28'E.)	J.MOchora 5	2200	epiphyte in montane open forest

D. xanthopollinia (Reichb f.) Summerh.	Chemosoren (0°36'S.,35°17'E.)	A.Foresti 803	1900	epiphyte in open forest
Microcoelia exilis Lindl.	Ngongoni forest (4°16'S.,39°34'E.)	J.M.Ochora 73	200	epiphyte in open bushed woodland
M. koehleri (Schltr.) Summerh.	Kuja valley (0°45'S.,34°52'E.)	J.M.Ochora 44	1650	epiphyte in open evergreen forest
M. moreauae L. Jonsson	Athi plains (1°22'S.,36°48'E.)	A.Foresti 388	1660	epiphyte in open bushland
M. smithii (Schltr.) Summerh.	Mau National Sanctuary (1°2'S.,35°29'E.)	J.M.Ochora 60	1500	epiphyte on trunks of short trees in open wooded bushland
M. stolzii (Schltr.) Summerh.	The Nyrobe Cottage's garden (1°15'S.,36°49'E)	A.Foresti 109	1680	epiphyte in open woodland
Rangaeris amaniensis (Kraenzl.) Summerh.	University of Nairobi (1°19'S.,36°48'E.) Mt. Kulal (2°48'S.,37°05'E.)	J.M.Ochora 54 B.Bytebier 0117	1930 2400	epiphyte in open woodland
R. muscicola (Reichb. F.) Summerh.	Langata forest (1°22'S.,36°43'E.)	J.M.Ochora 68	1400	epiphyte in dense forest
Solenangis aphylla (Thou.) Summerh.	Taru (3°46'S.,39°07'E)	B.Bytebier 0008	870	epiphyte in open forest
S. wakefieldii (Rolfe) Cribb & J. Stewart	Taru (3°46'S.,39°07'E)	B.Bytebier 0009	870	epiphyte in open bushland

Tridactyle bicaudata (Lindl.) Schltr.	Kericho (0°23'S.,35°28'E.)	J.M.Ochora 28	2350	epiphyte in dense forest
T. scotellii (Rendl.) Schltr.	Kuja valley (0°48'S.,34°54'E.)	J.M.Ochora 77	1650	epiphyte in open forest
Ypsilopus longifolius (Kraenzl.) Summerh.	Mau escarpment (0°56'S.,36°00'E.) Kaheho forest (0°06'S.,36°28'E.)	A.Foresti 779 J.M.Ochora 52	2000	low epiphyte in xerophytic woodland
Subtribe Aeridinae Pfitzer				
Acampe pachyglossa Reichb. f.	Misali island (5°14.3'S.,39°36'E)	L.Newton 5614	1-2	epiphyte in open bushed woodland
Subtribe Angraecinae				
Angraecum decipiens Summerh.	Aberdares N. Park (0°10'S.,36°07'E.)	B.Bytebier 0122	390	epiphyte in open bushed woodland
A. eburneum Bory subsp. *giryamae* (Rendl.) Senghas et Cribb	Taru forest (3°50'S.,39°12'E.)	J.M.Ochora 2	486	epiphyte on rocks of open bushland
A. dives Rolfe	Gazi (4°25'S.,39°30'E.)	B.Bytebier 0011	390	epiphyte of coastal wooded forest
A. erectum Summerh.	Fourteen Falls (1°10'S.,37°13'E)	J.M.Ochora 16	2050	epiphyte in open forest near Athi R.
Tribe Cymbideae Pfitzer **Subtribe Eulophiinae** Bentham				
Eulophia angolensis (Reichb. f.)	Milimaini swamp (1°04'S.,36°51'E.)	J.M.Ochora 38	1400	terrestrial in swamp among

Summerh.				sedges
E. horsfallii (Batem.) Summerh.	Irangi swamp (0°22'S.,37°28'E.) Kericho Swamp (0°29'S,35°04'E.)	J.M.Ochora 37 B.Bytebier 0039	1900	terrestrial in river valley among sedges
E. ovalis Lindl.	Ngong hills (1°24'S.,36°31'E.)	J.M.Ochora 12	2500	terrestrial in open bushed grassland
E. petersii Reichb. f.	Kilima Kiu (1°33'S.,37°24'E.) Embu (0°27'S.,37°30'E.)	J.M.Ochora 12 A.Foresti 103	1901 2135	terrestrial in bushland and thicket terrestrial in open bushed shrubland
E. speciosa (Lindl.) Bolus	Kilima Kiu (1°33'S.,37°24'E.) Kiaora reserve (1°19'S.,36°53'E.)	J.M.Ochora 30 J.M.Ochora 88	1901	terrestrial of bushland and thicket terrestrial of open scrubland
E. streptopetala Lindl. var. *stenophylla* (Summerh.) Cribb	The National Arboretum (1°19'S.,36°43'E.)	J.M.Ochora 11	1400	terrestrial of silted rocky grassland
E. streptopetala Lindl. var. *streptopetala* (Summerh.) Cribb.	Manga range (0°33'S.,34°57'E.)	J.M.Ochora 10	2000	terrestrial in montane rocky grassland
Oeceoclades saundersiana (Reichb. f.) Garay & Taylor	Kakamega forest (0°16'N.,34°55'E.) Mwache forest (4°00'S.,39°05'E.)	J.M.Ochora 71 B.Bytebier 0057	1800	terrestrial of open wooded forest
Subtribe Cyrtopodinae Bentham				
Ansellia africana Lindl.	Olkalian hill (0°45'S.,36°21'E.)	B.Bytebier 722	2300	epiphyte in rocky bushed land

Tribe Malaxideae Lindl. **Subtribe Liparidinae**				
Liparis bowkeri Harv.	Saiwa swamp National park (1°10'N.,35°05'E.)	J.M.Ochora 79	1800	terrestrial in dense forest by the swamp
L. deisteri Schltr.	Saiwa swamp National park (1°10'N.,35°05'E.)	J.M.Ochora 4	1800	terrestrial in dense forest by the swamp
Oberonia disticha (Lam) Schltr.	University of Nairobi (1°19'S.,36°48'E.)	J.M.Ochora 78	1560	cultivate epiphyte in open wooded bushland
Tribe Epidendreae **Subtribe coeliinae**				
Epidendrum cinnaparinum Salmann	Lanet (0°24'S.,36°08'E.)	J.M.Ochora 14	2000	cultivated in open grassland
E. ibaguense Humb. et Kunt	Langata (1°22'S.,36°43'E)	J.M.Ochora 70	1900	cultivated in open bushed grassland
Subtribe Polystachyiinae Pfitzer				
Polystachya adansoniae Reichb. f.	Kuja valley (0°45'S.,34°52'E.)	J.M.Ochora 29	1650	epiphyte of river valley open forest
P. bella Summerh.	Kericho (0°24'S.,35°33'E.) Taita hills (3°21'S.,38°24'E.)	J.M.Ochora 25 B.Bytebier 0089	1800 970	epiphyte in dense forest
P. bennettiana Reichb. f.	Kakamega forest (0°16'N.,34°55'E.)	J.M.Ochora 19	1800	epiphyte in dense forest

	Saiwa swamp National park (1°10'N.,35°05'E.)	B.Bytebier 0091	1900	
P. bicarinata Rendl.	Kericho (0°23'S.,35°28'E)	J.M.Ochora 24	2534	epiphyte in dense forest
P. campyloglossa Rolfe	Ngong hills (1o24'S.,36o31'E) Mua hills (1°29'S.,37°10'E.)	J.M.Ochora 3 A. Foresti 744	2135 2100	epiphyte in montane bushland with shrubs
P. confusa Rolfe	Mt. Kulal (2°48'S.,37°05'E.)	B.Bytebier 0062	2300	epiphyte in open bushland
P. dendrobiiflora Reichb. f.	Rukinga hills (3°49'S.,38°41'E.)	L.Newton 5595	329	epiphyte in open xerophyta
P. eurychila Summerh.	Saiwa swamp National park (1°10'N.,35°05'E.)	B.Bytebier 445	1800	epiphyte of dense forest
P. fusiformis (Thou.) Lindl.	Manga range (0°33'S.,34°57'E.)	J.M.Ochora 53	2000	epiphyte on montane grassland shrubs
P. piersii Cribb	Mt. Nyiru forest (2°05N.,36°50'N.)	B.Bytebier 112	2700	epiphyte of dry forest
P. steudneri Reichb. f.	Saiwa swamp National park (1°10'N.,35°05'E.)	J.M.Ochora 55	1800	epiphyte of dense forest
P. spatera Kraenzl.	Aberdare range (0°6'S.,36°28'E.)	J.M.Ochora 49	2000	epiphyte of montane dense forest
P. tenuissima Kraenzl.	Kakamega forest (0°16'N.,36°36'E)	J.M.Ochora 84	2300	epiphyte in dense forest
P. tesselata Lindl.	Kinango (4°19'S.,39°19'E.)	J.M.Ochora 61	329	epiphyte in open wooded forest
P. transvaalensis Schltr.	Mau escarpment (1°2'S.,36°8'E)	A.Foresti 736	2200	epiphyte in bushed woodland
P. vaginata Summerh.	Naivasha (0°49'S.,36°14'E.)	A.Foresti 771	1950	epiphyte of *Xerophyta* woodland

Tribe Dendrobieae Endlicher **Subtribe Bulbophyliinae** Schlechter				
Bulbophylum cochleotum Lindl.	Kakamega forest (Shidenya side) (0°16'N.,34°55'E.)	J.M.Ochora 15	2300	epiphyte in open forest
B. encephalodes Summerh.	Kakamega forest (Shidenya side) (0°16'N.,34°55'E.)	B.Bytebier 588	2300	epiphyte in open forest
B. intertexcum Lindl.	Mbororo forest	J.M.Ochora 81	300	epiphyte in dense forest
B. maximum (Lindl.) Reichb. f.	Chania gorge (1°10'S.,37°03'E.)	J.M.Ochora 39	2000	epiphyte in open forest near a Chania River
Tribe Arethusae Lindl. **Subtribe Bletiinae** Bentham				
Calanthe sylvatica (Thou.) Lindl.	Saiwa National Park (1°10'S.,35°5'E.)	J.M.Ochora 55	1800	terrestrial in dense forest
Tribe Vanilleae Blume **Subtribe Vanillinae** Lindl.				
Vanilla polylepis Summerh.	Chania gorge (1°10'S.,37°03'E.)	J.M.Ochora 39	2000	climber on trees by the riverside
Subfamily Orchidoideae **Tribe Diseae** Dressler **Subtribe Satyriinae** Schlechter				

Satyrium crassicaule Rendl.	Saiwa swamp National park (1°10'N.,35°05'E.)	J.M.Ochora 74	1800	terrestrial in swamp among sedges
S. volkensii Schltr.	Kakamega forest (0°16'N.,36°36'E)	J.M.Ochora 80	2300	terrestrial in swamp among sedges
Subtribe Disinae				
Disa ferruginea (Thunb.) Sw	Table mountain (34°59'S.,28°25'S.)	J.M.Ochora 90	700	terrestrial in the fynbos vegetation
Disa graminifolia Schltr.	Table mountain (33°59'S.,28°25'S.)	J.M.Ochora 91	730	terrestrial in the fynbos vegetation