Pollination and Evolution of the genus *Mystacidium* (Orchidaceae)

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ABSTRACT

The morphology, anatomy and pollination biology of *Mystacidium* Lindl., a small, epiphytic genus of orchids, was investigated within a phylogenetic context. Morphological and anatomical studies were carried out in order to obtain characters for a cladistic analysis of the genus using *Cyrtorchis arcuata* (Lindl.) Schltr. as an outgroup. The phylogenetic analysis indicated that the genus may not be monophyletic. Two species of the closely related genus *Diaphananthe* Schltr., *D. caffra* (H.Bol.) Linder and *D. millarii* (H.Bol.) Linder, appear to be nested within *Mystacidium*. *Mystacidium* species grow in habitats varying from mistbelt forest to dry savanna. Analysis of stable isotope composition ($\delta^{13}C$ values) of leaves and roots showed that all *Mystacidium* species, as well as *D. caffra* and the outgroup *C. arcuata,* employ CAM photosynthesis. The $\delta^{13}C$ values were significantly negatively correlated with mean annual rainfall at the collection sites. Breeding system experiments revealed that *Mystacidium* is dependent on pollinators for fruit set, and that self-pollination results in substantially reduced seed set due to either inbreeding depression or partial self-incompatibility. Field observations revealed that *M. venosum* Harv. ex Rolfe and *M. capense* (L.f.) Schltr. are hawkmoth-pollinated, and that *M. gracile* Harv. and *M. pusillum* Harv. are pollinated by settling moths. The spurs of the flowers contain dilute, sucrose-dominant nectar. *Mystacidium venosum* and *M. capense* showed evidence of nectar reabsorption. Nocturnal emission of scent occurred in all species except *M. aliceae* H. Bolus and *M. brayboniae* Summerh., which are unscented, and was composed largely of a combination of monoterpenes and benzoids. Despite substantial variation in spur length (1 - 4.7 cm) among species, no evidence for directional selection on spur length was found in *M. venosum, M. capense* or *M. gracile*. Hand pollinations significantly increased fruit set in *M. capense* in two consecutive seasons at different sites, indicating pollen limitation. Although pollen removal was greater than pollen receipt in *M. venosum*, *M. capense* and *M. gracile*, suggesting transport loss or insufficient visitation, a remarkably high percentage of

removed pollen reached stigmas (35 – 50%). Experiments on *M. venosum* revealed that flower longevity is reduced by pollination, and that pollinia removed from flowers remained viable for up to 20 days under field conditions. The phylogeny indicated that long-spurred, hawkmoth-pollinated species are basal within the genus, and that shorter-spurred species pollinated by noctuid moths are derived.

PREFACE

I hereby declare that this project, submitted in the fulfilment of the requirement for the degree of Master of Science in the School of Botany and Zoology, Department of Botany, University of Natal, Pietermaritzburg, is the result of my own investigation, except where acknowledgement of other work is specifically indicated in the text.

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Dr S.D. Johnson

(Supervisor)

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INTRODUCTION

Angiosperms display an enormous diversity in floral characteristics which reflect adaptations to the myriads of insects and vertebrates that pollinate the majority of them. The radiation of the angiosperms has been attributed in part to these adaptations to pollinators (Grant and Grant 1965, Faegri and van der Pijl 1966, Stebbins 1970). Convergent patterns of common floral characters across unrelated plant taxa have been termed "pollination syndromes" (van der Pijl 1961, Faegri and van der Pijl 1966). The concept of a syndrome therefore, refers to a suite of recognizable floral characters which are a result of an adaptation to a pollinator's behaviour and characteristics (van der Pijl 1961), thereby enabling the distinction between, for example, a 'bee-flower' or a 'moth-flower'. Underlying this view of "syndromes" is that selection should favour floral traits that result in successful pollen transfer (O'Connel and Johnston 1998).

Orchids in particular, display remarkably diverse floral specializations, and their interactions with pollinators have led to intricate and extraordinary pollination mechanisms. The diverse morphology in orchids has been attributed to their close association with specific pollinators (Nilsson 1992). The successful radiation of orchids may also relate to the packaging of pollen into a compact unit (Johnson and Edwards 2000), which adheres to the body of the pollinator thereby reducing loss during transport (Harder 2000).

According to Waser (1983), competition for pollinators may exist when pollinators are infrequent. Reproductive success in orchids is often limited by pollinators (Zimmerman and Aide 1989, Calvo and Horvitz 1990, Johnson and Bond 1992, Burd 1994, Neiland and Wilcock 1994), and results in the potential for selection to favour floral structures and attractants that appeal to more reliable pollinators. According to Proctor *et al.* (1996), pollinator-limited reproductive success makes adaptive shifts to new pollination systems "rather easy". Stebbins' (1970) "most effective pollinator principle" encompasses this view and is illustrated in a number of studies. For example, Johnson (1997) demonstrated a shift from bee to hawkmoth pollination in the two subspecies of *Satyrium hallacki*. However in the absence of a "most effective pollinator", generalization, as opposed to specialization, in pollination systems may be favoured (Waser *et al.* 1996).

Some studies have claimed that the "pollination syndrome" concept is not useful in predicting pollination systems, and often does not reflect adaptations to flower visitors (Herrera 1996, Torres and Galetto 1998). Similarly, Waser *et al.* 1996 propose that the

adaptive link between floral characters and pollinators is actually generalized. However the documentation of specialist pollination systems is very widespread (Faegri and van der Pijl 1966, Nilsson *et al.* 1985, Cox *et al.* 1991, Johnson and Steiner 2000, Johnson *et al.* 2002). According to Stebbins' (1970) principle of "selection along the lines of least resistance", evolutionary pathways depend on the limitations imposed by the existing flower structure. For example, the long corolla tubes found in most flowers pollinated by long-tongued vectors can only evolve from corollas that are already tubular. Since phylogenetic constraints limit floral diversity (Johnson and Steiner 2000), pollination syndromes can appear to be less sharply circumscribed. For example, Torres and Galleto (1998) found that flowers of *Mandevilla pentlandiana*, which have typical 'moth flower' traits, were visited by a large array of diurnal pollinators, such as bees and bumblebees.

A critical means of assessing floral adaptations in relation to pollinators, is by mapping pollination systems onto phylogenies. This method is a way of elucidating how floral traits evolve if shifts in pollination systems have taken place (Armbruster 1992, Johnson *et al.* 1998, Johnson *et al.* 2002). For example, Armbruster's (1992) phylogenetic analysis of *Dalechampia* provided a means of investigating transitions in pollination systems (fragrance collecting and resin-collecting bees), and McDade's (1992) phylogenetic analysis of *Aphelandra* indicated that pollination by long-billed hermit hummingbirds was ancestral to pollination by short-billed trochiline hummingbirds.

On the basis of floral syndromes, Dodson (1962) predicted that 50% of the African orchids are pollinated by Lepidoptera. Moth flowers are generally white or pale-coloured, night-scented, and produce dilute sucrose-rich nectar concealed in long spurs. Studies by Johnson (1995, 1997) have confirmed pollination by hawkmoths in African terrestrial orchids, and hawkmoths have been shown to pollinate a number of epiphytic species in Madagascar (Nilsson *et al.* 1985, Nilsson and Rabokonandrianina 1988, Nilsson *et al.* 1992, Wasserthal 1997). The epiphyte *Mystacidium venosum*, which occurs in South Africa is also hawkmoth-pollinated (Luyt and Johnson 2001).

Floral traits, such as flower colour, spur length, fragrance and nectar chemistry must correspond closely with the requirements of pollinators in order to stimulate repeated visitation and increase the likelihood of successful pollination. Because reproductive success in orchids is often limited by pollinators (Neiland and Willcock 1994), floral traits important for pollination efficiency may be subject to selection pressures. Others argue that selection can proceed through the male component of floral evolution even when pollen receipt is not limiting to seed production (Bell 1985). Studies of pollination

ecology are therefore important for understanding the evolutionary processes in plant populations. Floral and vegetative morphology may also be related to ecophysiological adaptations related to habitat. For example, Búrquez and Corbet (1991) suggest that nocturnal nectar secretion may be linked with stomatal opening and translocation in CAM plants. Thus, the evolution of moth pollination and nocturnal nectar secretion, may be more likely to occur in CAM plants. Studies of ecophysiological adaptations are therefore also important for understanding evolutionary processes, as they may, in turn, be related to pollination ecology.

Epiphytic orchids are abundant near the equator, diminishing in number pole-wards, and make up only one-tenth of the total number of orchid species in South Africa (Stewart *et al.* 1982). Epiphytic orchids are able to flourish in inhospitable habitats, and their success is partly due to the evolution of CAM photosynthesis (Goh and Kluge 1989), which has been documented in a number of studies (Winter *et al.* 1983, Kluge *et al.* 1997).

The genus Mystacidium

Mystacidium Lindl. (Aerangiinae: Aerangidinae) is a genus of small epiphytic orchids which occur in southern and tropical Africa. Although it consists of only eight recognized species, the genus is poorly known. Even though epiphytic orchids are generally known to occupy a wide geographical range, with many southern African species extending from more northern tropical African countries, six of the eight *Mystacidium* species are endemic to the southern African region (Linder and Kurzweil 1999) (Plate 1).

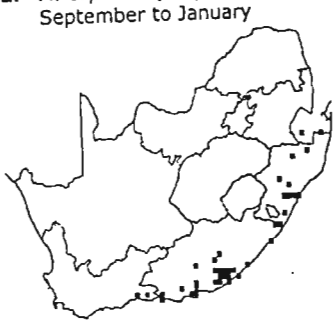
A. *M. aliceae* H. Bolus
November to March

B. *M. gracile* Harv.
August to October
Also in Zimbabwe

C. *M. braybonіae* Summerh.
November to January

D. *M. pusillum* Harv.
June to July

E. *M. capense* (L.f.) Schltr.
September to January



F. *M. venosum* Harv. ex Rolfe
April to July
Also in Mozambique

G. *M. flanaganii* (H. Bolus) H. Bolus
December to March

H. *M. tanganyikense* Summerh.
February to March
Zimbabwe, Zambia, Malawi, Tanzania.

Plate 1a-h. Distribution maps (based on specimens from the following herbaria: BOL, KEI, NGB, NH, PRE, PRU and UNP) and flowering times of *Mystacidium* Lindl. *Mystacidium tanganyikense* does not occur within the scope of the maps presented here.

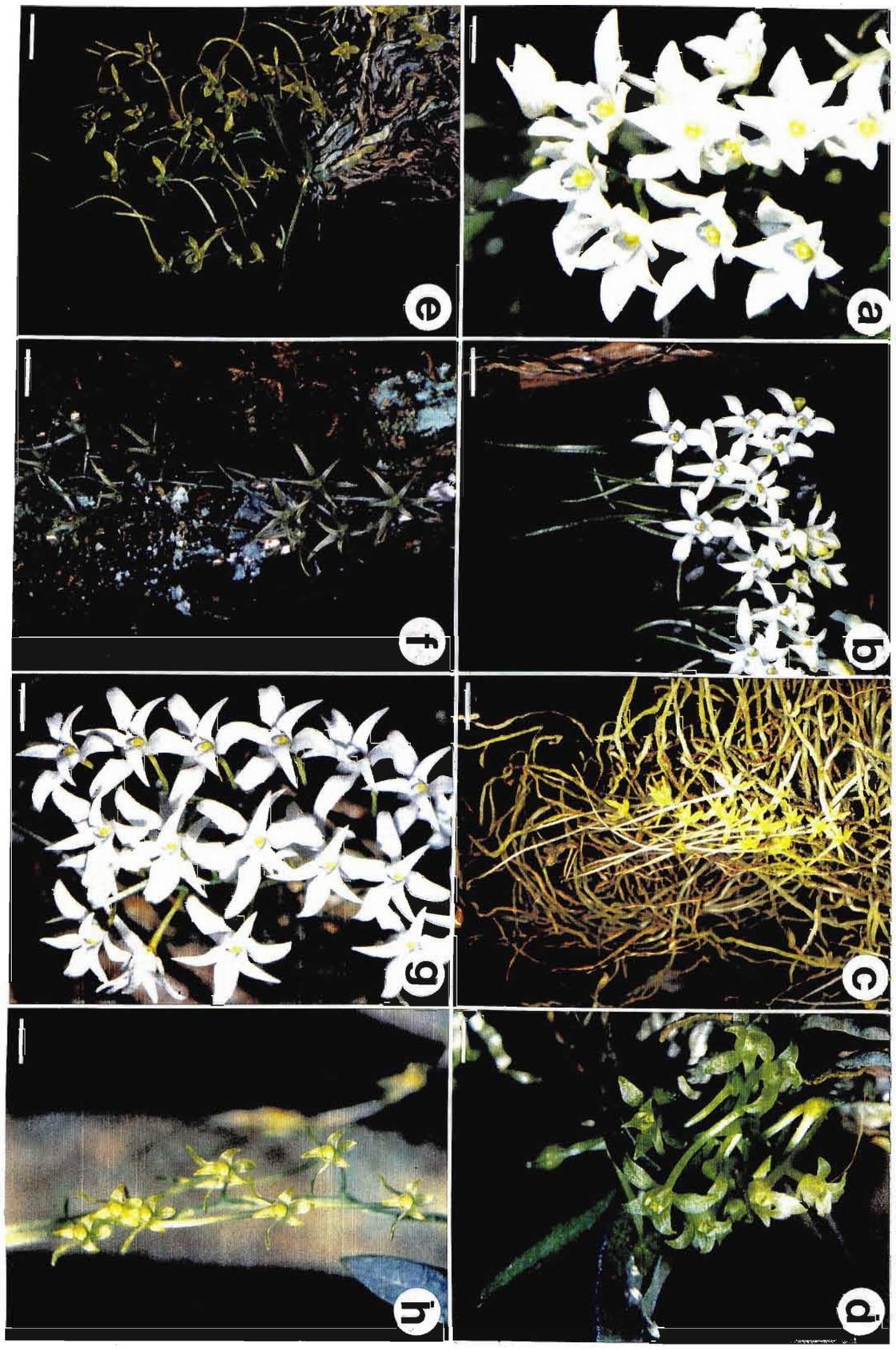


Plate 2a-h. *Mystacidium* Lindl. **a.** *M. braybonіae* (Soutpansberg). Scale bar = 60 mm. **b.** *M. venosum* (Verulam). Scale bar = 120 mm. **c.** *M. gracile* (Mbona Mountain Estate). Scale bar = 150 mm. **d.** *M. aliceae* (Vernon Crookes Nature Reserve). Scale bar = 30 mm. **e.** *M. pusillum* (Harold Johnson Nature Reserve). Scale bar = 60 mm. **f.** *M. tanganyikense* (Malawi). Scale bar = 85 mm. **g.** *M. capense* (Bishopstowe). Scale bar = 90 mm. **h.** *M. flanaganii* (Mbona Mountain Estate). Scale bar = 50 mm.

The small, monopodial epiphytes have white to pale green flowers (Plate 2). Several inflorescences bear 5 to 15 flowers. The sepals and petals are reflexed, and the lip, which is three-lobed in most species, is ecallose. A narrow, nectar-producing spur tapers from a wide mouth to an acute apex. *Mystacidium* is characterized by a three-lobed rostellum, with well-developed lateral lobes. The lateral lobes, which are more or less the same length as the central lobe, are minutely hairy (Linder 1989). In fact, the name of the genus, a derivation of the Greek word "mystax", which means moustache, refers to the hairy rostellum lobes. Two pollinia are attached to separate viscidia by slender stipes.

The taxonomy of *Mystacidium* is unclear and in need of revision. In particular, the relationship of the genus to *Diaphananthe* Schltr. has been problematic. In order to understand the problems related to the taxonomy of the genus, a brief summary of the literature relating to it follows:

The presence of a pencillate callus in the mouth of the throat of the spur was regarded by Schlechter (1918) as a key character for *Diaphananthe*, while *Mystacidium* is ecallose. In 1989, *M. millarii* H. Bol and *M. caffrum* H. Bol (synonym *Margelliantha caffrum*) were reduced to *Diaphananthe* (Linder 1989). The callus is present in the spur of *D. caffra* (= *M. caffrum*), and even though the finger-like process is lacking in the throat of the spur of *D. millarii* (= *M. millarii*), the lip is raised in a long callus along its midrib, from the throat of the spur to half-way to the apex (Linder 1989). According to Linder (1989),

> "*Mystacidium* is characterised by the well-developed lateral rostellum lobes, more or less as long as the central lobe, and tuberculate. The spur mouth is wide, and the spur tapers to an acute apex, while the sepals and petals are more or less reflexed, and acute. *Diaphananthe* is partially characterised by the finger-like process in the entrance to the spur, and a variable rostellum and spur configuration."

In 1836, John Lindley described the genus *Mystacidium*, based on *M. capense* (then *M. filicorne*) which he had previously described as *Angraecum capense*. He observed that:

> "With the habit of an *Angraecum* this curious little plant has characters of so marked a kind as to render it impossible to combine it with the genus. The curious two-legged caudicula, each point of which is inserted into the middle of a transparent gland, the naked rostellum, on which the legs of the caudicula are placed without any protection from the anther, and the two very curious bearded appendages that stand forward from the upper angles of the column, are all at variance with the structure of *Angraecum* and its allies."

At first, Bolus (1896) was of the impression that *Mystacidium* should not be distinct from *Angraecum*. However in his later remarks in 1896, he expressed the opinion that it would be acceptable to retain *Mystacidium*, but to restrict it to plants having the "peculiar bearded appendages to the rostellum" first described by Lindley (1836). In 1905, Bolus reconsidered the weight given to the "bearded appendages", and following Pfitzer (1889) and Rolfe (1898), concluded that the chief distinguishing characters of *Mystacidium* were two pollinia on two distinct stipes with separate viscidia. As a result, Bolus (1905) enlarged the limits of the genus. In his account of *Mystacidium* in 1905, which by then included *M. filicorne* (= *M. capense*), *M. gracile* and *M. pusillum*, Bolus transferred three species, *M. caffrum* (= *D. caffra*), *M. flanaganii* and *M. gerrardii* (= *D. xanthopollinia*) to *Mystacidium* from *Angraecum*, and described *M. millarii* and *M. peglerae* (= *D. xanthopollinia*) for the first time. Bolus (1911) also described *M. aliceae*, and Rolfe (1912) described *M. venosum* from Harvey's unpublished remarks on the genus. According to Rolfe (1912), *Mystacidium* at that time, consisted of approximately 50 African species, nine of which were endemic to South Africa. Summerhayes (1945, 1949) described *M. tanganyikense* and *M. brayboniae*. *Mystacidium* currently consists of only eight recognized species, five of which were included in those species listed as endemic to South Africa by Rolfe (1912).

The genus *Diaphananthe* was described by Schlechter in 1914. He derived the name from the diaphanous nature of the flowers, which are pale yellow-green and semi-transparent. The genera *Rhipidoglossum* and *Sarcorhynchus* were previously recognised as distinct from *Diaphananthe*, however Cribb (1989) has argued for their inclusion in the single genus *Diaphananthe*. This followed a review by Summerhayes (1960), who surveyed the species of *Diaphananthe* and *Rhipidoglossum*, originally distinguished by the "presence or absence of a foot to the column and the presence of a tooth-like callus in the mouth of the spur", and concluded that "it is not possible to divide the species into groups of more than sectional status on the basis of the column structure". Summerhayes (1960) therefore recognized the sect. *Diaphananthe*, in which the pollinia are attached by separate stipes to a common viscidium, the rostellum is bifid and tapering, and a prominent callus is present in the mouth of the spur; and sect.

Rhipidoglossum (Schltr.) Summerh., where each pollinia is attached by separate stipes to separate viscidia, the rostellum is obscurely 3-lobed with a prominent, fleshy middle lobe, and the callus is obscure.

Four of Bolus's (1905) *Mystacidium* species have since been assigned to *Diaphananthe*. *Mystacidium gerrardii* and *M. peglerae* are conspecific with *D. xanthopollinia* (Summerhayes 1960), and *M. caffrum* and *M. millarii* are now recognized as *D. caffra* and *D. millarii* respectively (Linder 1989).

Aims and rationale of this study

The radiation of *Mystacidium* in southern Africa, and the success of this inconspicuous epiphytic genus is remarkable. The broad objective of this study was to examine the morphology and reproductive biology of *Mystacidium* within a phylogenetic context in order to determine the pattern of evolutionary radiation.

In summary, the aims of this study were:

(1) To study the morphology and anatomy of *Mystacidium*
(2) To determine the incidence of CAM photosynthesis in *Mystacidium*
(3) To document the floral mechanism and pollination biology
(4) To carry out breeding system experiments for each species
(5) To investigate general nectar properties of each species
(6) To confirm whether *M. venosum* is capable of reabsorbing sugar following pollination, and whether this affects fruit quality (Appendix I).
(7) To investigate the incidence of pollen limitation
(8) To investigate processes of natural selection within the genus
(9) To determine the patterns of trait evolution in *Mystacidium*
(10) To carry out a cladistic analysis of *Mystacidium* using both morphological and anatomical data

CHAPTER 1
MORPHOLOGY AND ANATOMY

The morphology and anatomy of eight *Mystacidium* species, *Cyrtorchis arcuata, Diaphananthe caffra* and *D. millarii* was investigated. *Mystacidium* is characterised by a three-lobed rostellum, with well-developed, minutely hairy lateral lobes. The central lobe is either glabrous or slightly papillose. The three-lobed rostellum of *D. caffra* and *D. millarii* differs from that of *Mystacidium* in that the lateral lobes are poorly developed and glabrous, and the central lobe is stout. The beaked rostellum of *C. arcuata* is bifid. *Mystacidium* and the two *Diaphananthe* species have two pollinia attached by stipes to separate viscidia. The viscidia lie apart in *M. aliceae, M. brayboniae, M. tanganyikense, D. caffra* and *D. millarii,* and close together in the remaining species. In *C. arcuauta*, two pollinia are attached by stipes to a single viscidium. Three anatomical features were identified as being taxonomically valuable: the number of velamen layers, the size of the cells of the outer velamen layer in relation to the inner layers, and the presence of a complete mid rib bundle sheath. Seed surface ultrastructure is uniform in *Mystacidium*.

INTRODUCTION

The taxonomy of *Mystacidium* is unsatisfactory and in need of revision (see Introduction). The species of *Mystacidium* are not clearly separated by morphological characters; instead too much emphasis has been placed on flowering times (Linder and Kurzweil 1999). For example, most of the morphological criteria of *M. venosum* overlap with *M. capense*, and flowering time is therefore used as the final criterion for distinguishing between the two species: *M. capense* flowers early in summer, while *M. venosum* flowers from May to July. The morphological difference between *M. pusillum*, which flowers in winter, and *M. flanaganii* (H. Bolus) H. Bolus (summer flowering) has also not been well understood, and flowering time is generally also used to differentiate between the two species (Linder and Kurzweil 1999). Anatomical information on the genus is non-existent, and studies of the column morphology have only been undertaken in *M. venosum* (Luyt and Johnson 2001). The minute flowers make classification from pressed specimens very difficult, especially since the column, which provides the orchid taxonomist with many useful characters, is only between 0.5 mm and 2 mm tall. Fresh or preserved material is therefore essential for examination.

The aim of this study was to investigate the morphology of the columns, anatomy of the roots and leaves, and seed surface ultrastructure in *Mystacidium* in order to obtain characters for a cladistic analysis, using *Cyrtorchis arcuata* as an outgroup (Chapter 4). The morphological and anatomical studies included *C. arcuata, Diaphananthe caffra* and *D. millarii*, which also formed part of the cladistic analysis.

MATERIALS AND METHODS

Material was collected in the field or obtained from cultivated plants. Both newly collected material and the existing holdings of the University of Natal, Pietermaritzburg Herbarium (UNP) were used. Newly collected material is liquid-preserved and stored at UNP. The following is a list of the voucher specimens.

Mystacidium capense (L.f.) Schltr.: Luyt1; *M. venosum* Harv. ex Rolfe: Luyt2; *M. aliceae* H. Bolus: Luyt3; *M. gracile* Harv.: Luyt4; *M. pusillum* Harv.: Luyt6; *M. flanaganii* (H. Bolus) H. Bolus: Luyt7; *M. tanganyikense* Summerh.: Johnson s.n.; *M. braybonіae* Summerh.: Edwards s.n.
Diaphananthe caffra (H.Bol.) Linder: Edwards s.n., Luyt9; *D. millarii* (H.Bol.) Linder: Luyt8.
Cyrtorchis arcuata (Lindl.) Schltr.: Luyt5

Morphological Investigation of Columns

In order to produce high-quality images of the columns and to prevent too much shrinkage of the delicate samples, the technique developed by Gerstberger and Leins (1978) and described by Kurzweil (1991), was used. Plant material was fixed in FAA (formaldehyde:70% ethanol:glacial acetic acid 5:90:5) for at least 48 hrs and transferred to 70% ethanol for storing. The alcohol-preserved plant material was dissected and then transferred into 100% formaldehyde-dimethylacetale (FDA) overnight for chemical dehydration. The samples were then critical-point dried directly from FDA using CO_2 as a transitional fluid in a Hitachi HCP-2 critical point drier. The dry columns were mounted onto brass viewing stubs and sputter-coated with gold palladium in a Polaron E-5100 sputter coater. The samples were viewed and photographed in a Hitachi S-570 scanning electron microscope at an accelerating voltage of 10kV.

Leaf and Root Anatomy

Transverse sections of leaves and roots were embedded in resin using the standard Epon-Araldite procedure developed by the EM unit at the University of Natal, Pietermaritzburg. Fresh material was fixed in 3% Glutaraldehyde in 0.05 M NaCacodylate buffer for a minimum of 8 hours. Material previously fixed in FAA was first rinsed in distilled water. The plant material was subsequently rinsed twice, for a period of 30 minutes for each rinse, in 0.05 M NaCacodylate buffer, and fixed for approximately 3

hours in 2% osmium tetroxide in 0.05 M NaCacodylate buffer. This was followed by a rinse (2 x 30 minutes) in 0.05 M NaCacodylate buffer. The leaf and root material was then dehydrated in an alcohol series of 10 minute rinses in 30, 50, 70 (overnight) 80, 90 and 3 x 100% ethanol, and rinsed in propylene oxide (2 x 30 minutes). The embedding process involved placing the material into a mixture of 25% Epon : 75% propylene oxide for 2 hours, followed by 50% Epon : 50% propylene oxide for 2 hours, followed by 75% Epon : 25% propylene oxide overnight. The material was then transferred to 100% Epon for 24 hours. For each step, 5 drops of DMP per 5 ml Epon were added to the mixture. Finally the material was transferred to fresh 100% Epon (plus DMP) and placed in an oven for 48 hours at 70° C. Transverse sections of the leaves and roots were sectioned on an LKB Ultratome III microtome at a thickness of approximately 2µm , and stained with Ladd's Multi-stain. The sections were viewed under bright field under an Olympus AX70 light microscope, and the images were saved using the SIS program. Freehand sections of fresh leaf and root material were prepared and viewed in conjunction with the resin embedded sections in order to confirm certain anatomical features.

Seed Surface Ultrastructure

Seed surface ultrastructure was investigated by mounting several air dried seeds onto brass viewing stubs. The stubs were sputter-coated with gold palladium in a Polaron E-5100 sputter coater. The samples were viewed and photographed in a Hitachi S-570 scanning electron microscope at an accelerating voltage of 10kV.

RESULTS AND DISCUSSION

Column Morphology

The basic column of *Mystacidium* is erect, bearing two pollinaria at the apex beneath a removable anther cap (Figures 1.1a-h). The pollinia are attached by stipes to separate viscidia, which are situated at the terminal end of a three-lobed rostellum. The well-developed lateral lobes are minutely hairy, and are either longer than the central lobe (*M. capense, M. venosum* and *M. gracile*, Figures 1.1a, c and g), shorter than the central lobe (*M. flanaganii* and *M. aliceae,* Figures 1.1e and h), or the same length as the central lobe (*M. brayboniae, M. tanganyikense* and *M. pusillum,* Figures 1.1b, d and f). The stigmatic cavity is notched at the apex and lies directly behind the rostellum. The two viscidia are either situated close together (*M. brayboniae, M. capense, M. gracile, M. pusillum* and *M. venosum*), or apart (*M. aliceae, M. flanaganii* and *M. tanganyikense*).

The central lobe of the rostellum is either minutely papillose (*M. tanganyikense, M. flanaganii, M. brayboniae* and *M. capense*), or glabrous (*M. gracile, M. pusillum, M. venosum* and *M. aliceae*), and is either acute (*M. gracile, M. pusillum, M. venosum, M. aliceae, M. brayboniae* and *M. capense*), round (*M. flanaganii*), or bifid (*M. tanganyikense*).

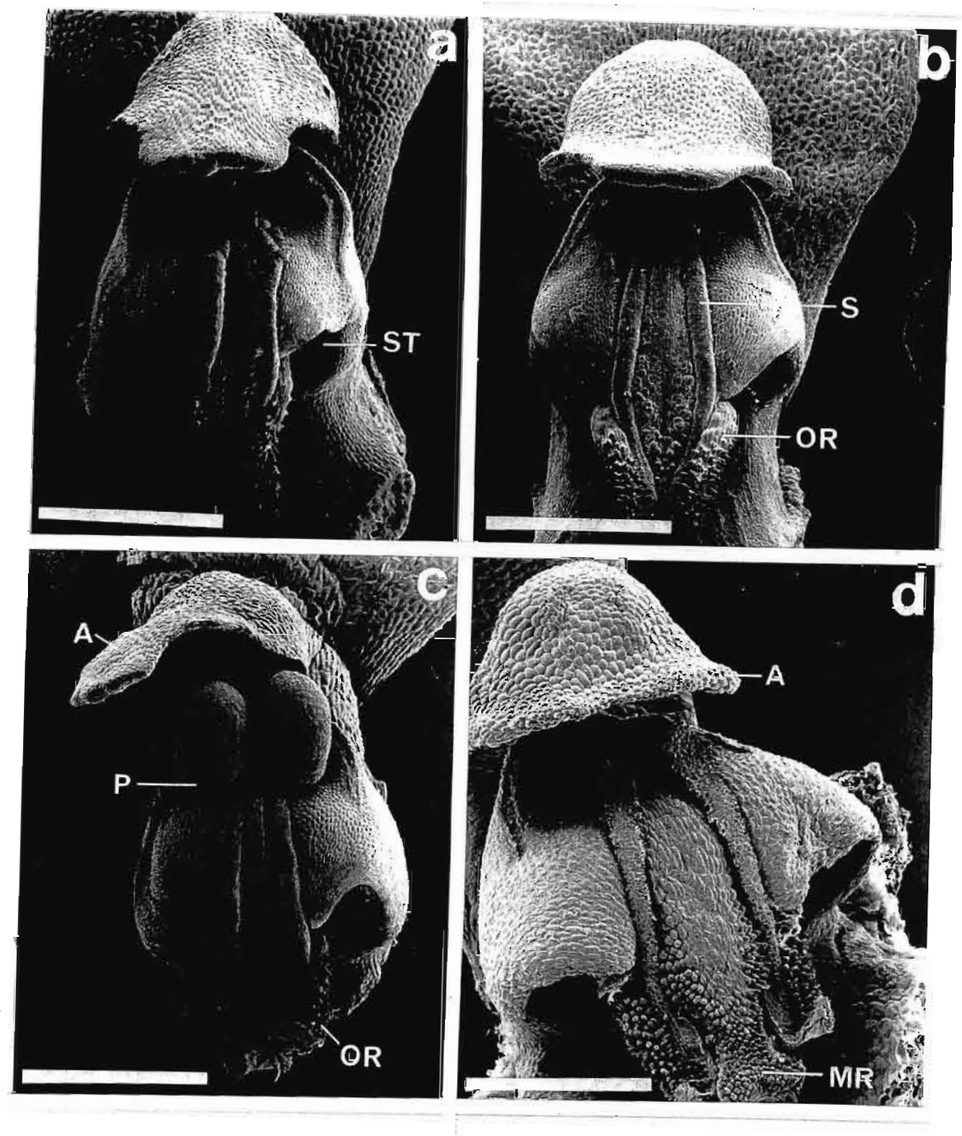
The stout columns of *D. caffra* and *D. millarii* (Figures 1.1i and j) also bear two pollinaria beneath removable anther caps which are green in colour. Stipes attach the pollinia to separate viscidia. Unlike in *Mystacidium*, the rostellum is three-lobed and the lateral lobes are short and poorly developed, with the central lobe being long and stout. The central lobe of *D. caffra* forms an acute apex (Figure 1.1i), while the apex of the central lobe of *D. millarii* is rounded (Figure 1.1j).

The elongated, beaked rostellum of *C. arcuata* is deeply bifid, and minutely papillose (Figure 1.1k). The two pollinia are attached by spathulate stipes to a single viscidium. The viscidium consists of two parts, with an "indurated, saddle-shaped upper part and a hyaline lower part " (la Croix and Cribb 1998).

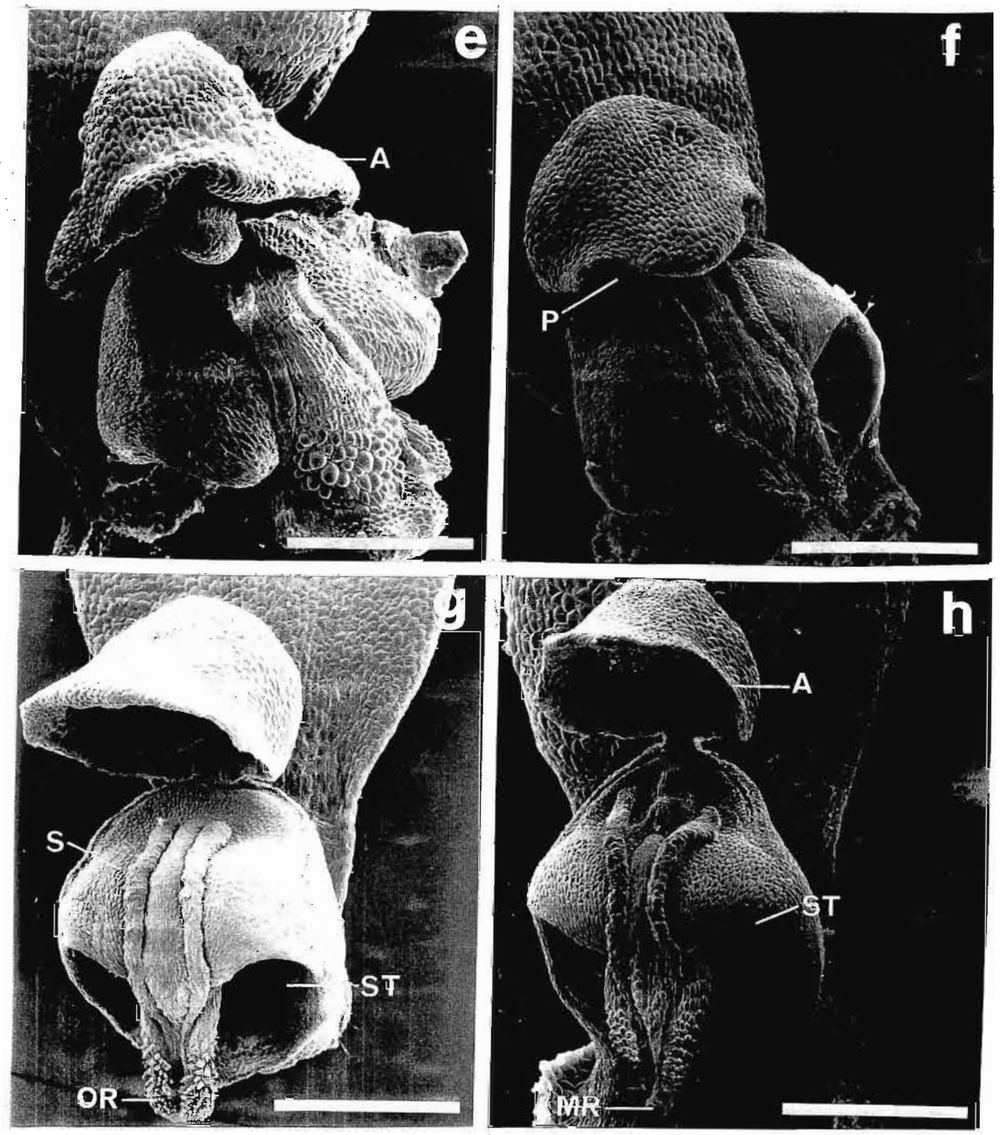
Leaf Anatomy

The leaves of *Mystacidium, C. arcuata* and *D. caffra* are distinctly conduplicate (Figures 1.2a-j). The transverse sections show that the V-shape is broader and rounder in *M. brayboniae, M. capense, M. gracile, M. flanaganii, M. pusillum, M. tanganyikense, D. caffra and C. arcuata*, and more acute in *M. aliceae* and *M. venosum*. The leaf outline of *M. pusillum* is distinct from the other species in that the V is exceptionally shallow (Figure 1.2a), and the leaf outline of *M. gracile* is unique in that it is 'convoluted' (Figure 1.2d).

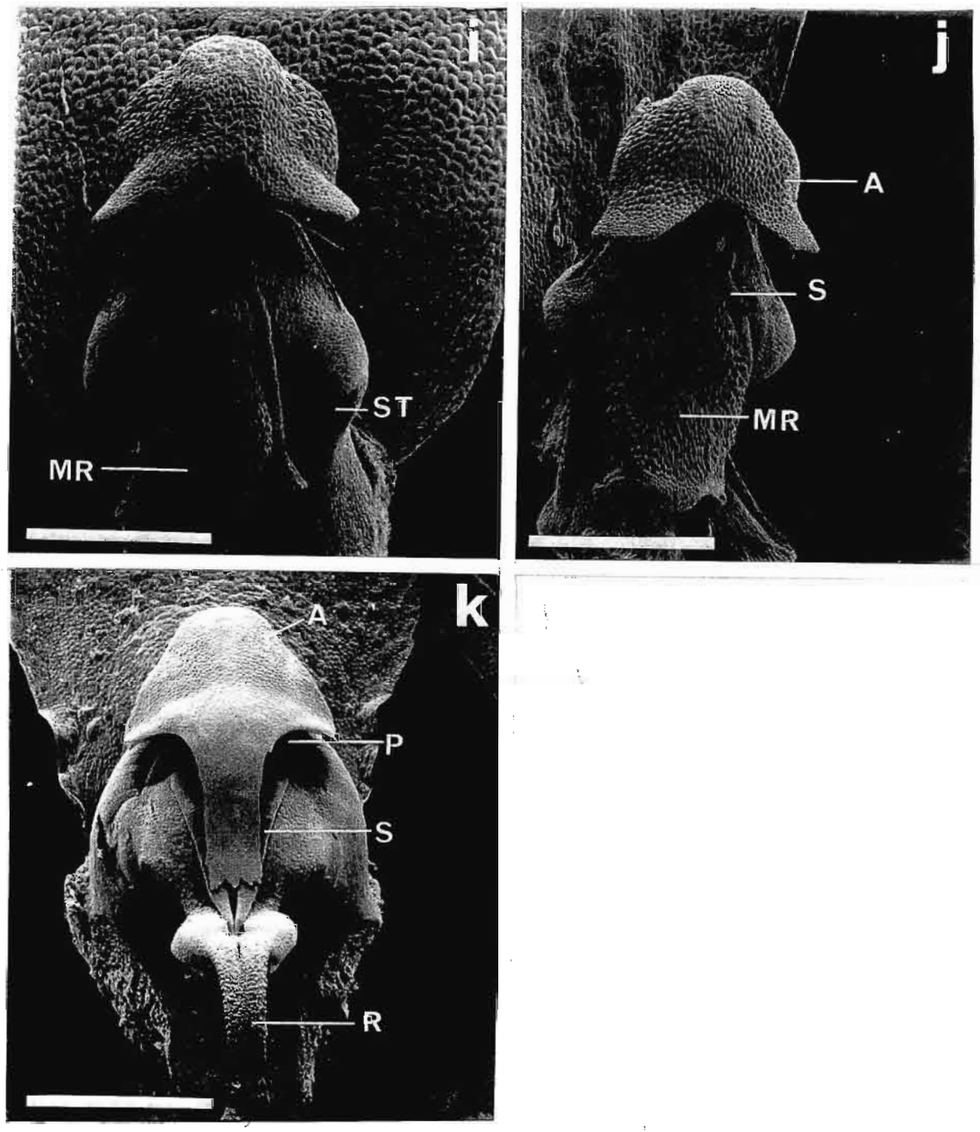
The cuticle ranges from approximately 2 μm to 5 μm in thickness. The epidermis is always uniseriate and the epidermal cells are smaller than the mesophyll cells. Upper epidermal cells are slightly larger than the cells of the abaxial epidermis, and both abaxial and adaxial epidermal cells are horizontally elongated, except in the abaxial epidermal cells of *M. aliceae, M. venosum, D. caffra* and *C. arcuata* (Figures 1.2c, h, i and j), where they appear to be more rounded-square. The anticlinal walls are straight.



Figures 1.1a-d. Scanning electron micrographs of columns. **a.** *Mystacidium capense* (Luyt1). Scale bar = 1mm. **b.** *M. braybonіae* (Edwards s.n.). Scale bar = 1mm. **c.** *M. venosum* (Luyt2). Scale bar = 750µm. **d.** *M. tanganyikense* (Johnson s.n.). Scale bar = 500µm. ST = stigma, S = stipe, OR = outer rostellum lobe, A = anther cap, P = pollinium, MR = middle rostellum lobe.



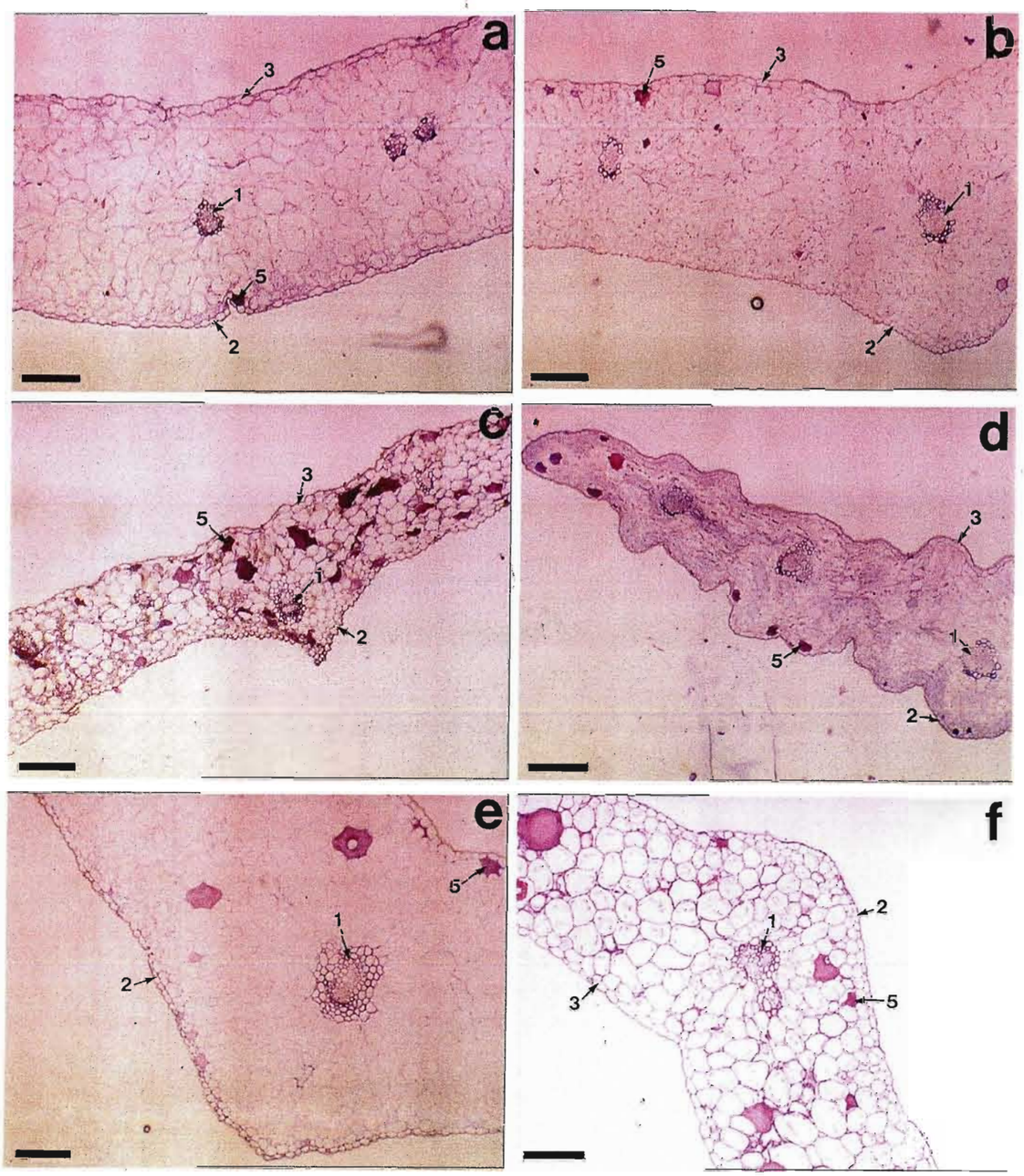
Figures 1.1e-h. Scanning electron micrographs of columns. **e.** *Mystacidium flanaganii* (Luyt7). Scale bar = 430µm. **f.** *M. pusillum* (Luyt6). Scale bar = 500µm. **g.** *M. gracile* Luyt4). Scale bar = 600µm. **h.** *M. aliceae* (Luyt3). Scale bar = 500µm. A = anther cap, P = pollinium, S = stipe, ST = stigma, OR = outer rostellum lobe.



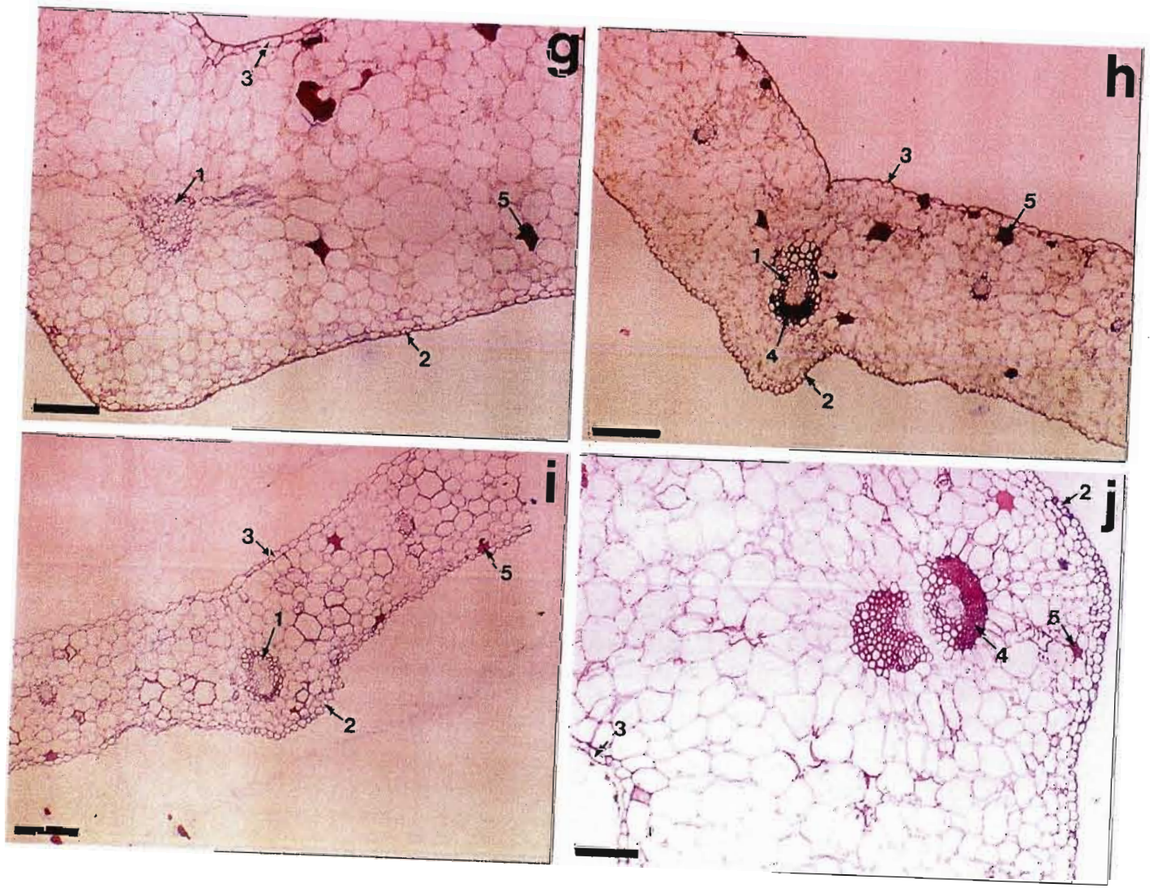
Figures 1.1i-k. Scanning electron micrographs of columns. **i.** *Diaphananthe caffra* (Edwards s.n.). Scale bar = 750µm. **j.** *D. millarii* (Luyt8). Scale bar = 1mm. **k.** *Cyrtorchis arcuata* (Luyt5). Scale bar = 1.76mm. MR = middle rostellum lobe, ST = stigma, A = anther cap, S = stipe, P = pollinium, R = rostellum

The mesophyll is always homogenous (palisade absent), consisting of 9 - 14 layers of cells. The cells are large and distinctly rounded in all species except *M. pusillum*, *M. flanaganii* and *M. gracile* (Figures 1.2a, b and d) where they are rather irregularly shaped. The cells adjacent to, and within approximately 3 layers of the abaxial epidermis of all the species are smaller and more compactly arranged than the cells adjacent to the adaxial epidermis. Chloroplasts are abundant in both adaxial and abaxial mesophyll cells, and raphide bundles occur throughout the mesophyll. The vascular bundles are collateral and are arranged in a single row across the width of the leaf, the number of which depends on the size of the leaf. The bundles alternate in size from the midrib bundle which is the largest, and occur half way between the lower and upper surfaces. All vascular bundles are free in the mesophyll. The bundle sheaths are sclerenchymous. The sheath of the midrib bundle is complete in all species except *M. flanaganii, M. pusillum, M. gracile* and *M. capense* (this is consistent with Gasson and Cribb 1985, who reported that the sclerenchyma bundle sheath of *M. capense* was incomplete). Sclerenchyma is also associated with xylem and phloem poles of the main vein in *M. flanaganii, M. venosum, M. brayboniae, M. aliceae, D. caffra* and *C. arcuata*. Sclerenchyma is associated with the phloem pole only in the main vein of *M. tanganyikense* (Figure 1.2f), and with the xylem pole only in *M. gracile* (Figure 1.2d). Fibres are not associated with the xylem or phloem poles in the main vein *of M. capense* (Figure 1.2g). Sclerenchyma fibres associated with the xylem and phloem poles of the main veins of *M. venosum* and *C. arcuata* are extensive (Figures 1.2h and j).

The leaf anatomy of *Mystacidium* is similar to that of other Angraecoid orchids described by Gasson and Cribb (1985). Gasson and Cribb (1985) found that a thick cuticle (up to 7 μm) and single row of vascular bundles seems to be universal in Angraecoid orchids, with the exception of *Podangis dactyloceras*, which has an isobilateral leaf with two rows of bundles. Gasson and Cribb (1985) found that a palisade layer was mostly absent in the Angraecoid orchids they surveyed, but present in *Angraecum conchiferum* and *Cyrtorchis ringens* (although absent in other *Cyrtorchis* species, such as *C. aschersonii* and *C. sedenii*). Furthermore, they found that raphide sacs were also abundant in most of the species they surveyed. According to Gasson and Cribb (1985), there is little variation in epidermal cell arrangement within *Orchidaceae*.



Figures 1.2a-f. Leaf transverse sections. **a.** *Mystacidium pusillum* (Luyt6). **b.** *M. flanaganii* (Luyt7). **c.** *M. aliceae* (Luyt3). **d.** *M. gracile* (Luyt4). **e.** *M. brayboniae* (Edwards s.n.). **f.** *M. tanganyikense* (Johnson s.n.). 1 = vascular bundle, 2 = abaxial epidermis, 3 = adaxial epidermis, 5 = raphide idioblast. Scale bars = 200 µm.

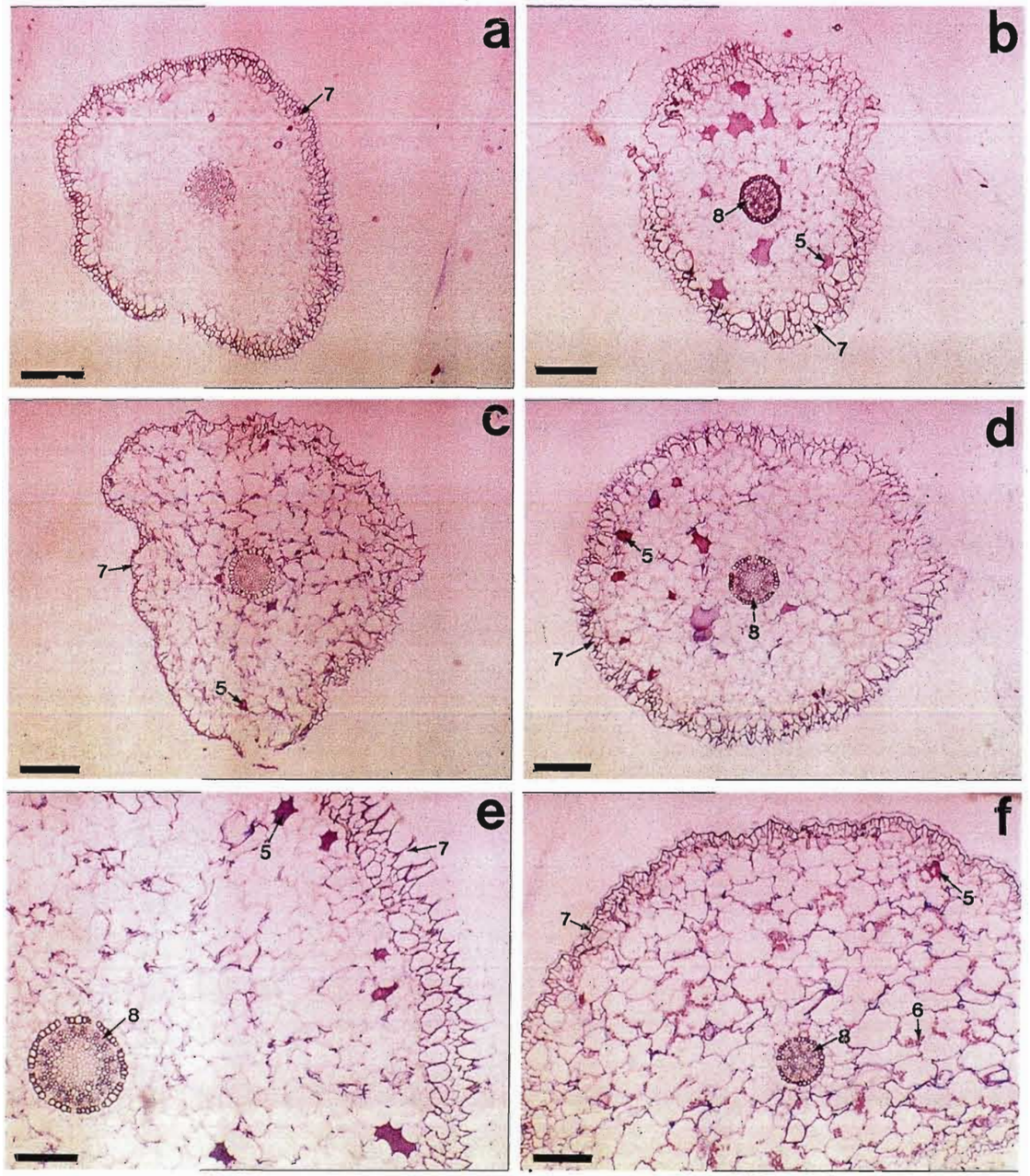


Figures 1.2g-j. Leaf transverse sections. **g.** *Mystacidium capense* (Luyt1). **h.** *M. venosum* (Luyt2). **i.** *Diaphananthe caffra* (Luyt9). **j.** *Cyrtorchis arcuata* (Luyt5). 1 = vascular bundle, 2 = abaxial epidermis, 3 = adaxial epidermis, 4 = sclerenchyma fibres, 5 = raphide idioblast. Scale bars = 200 µm.

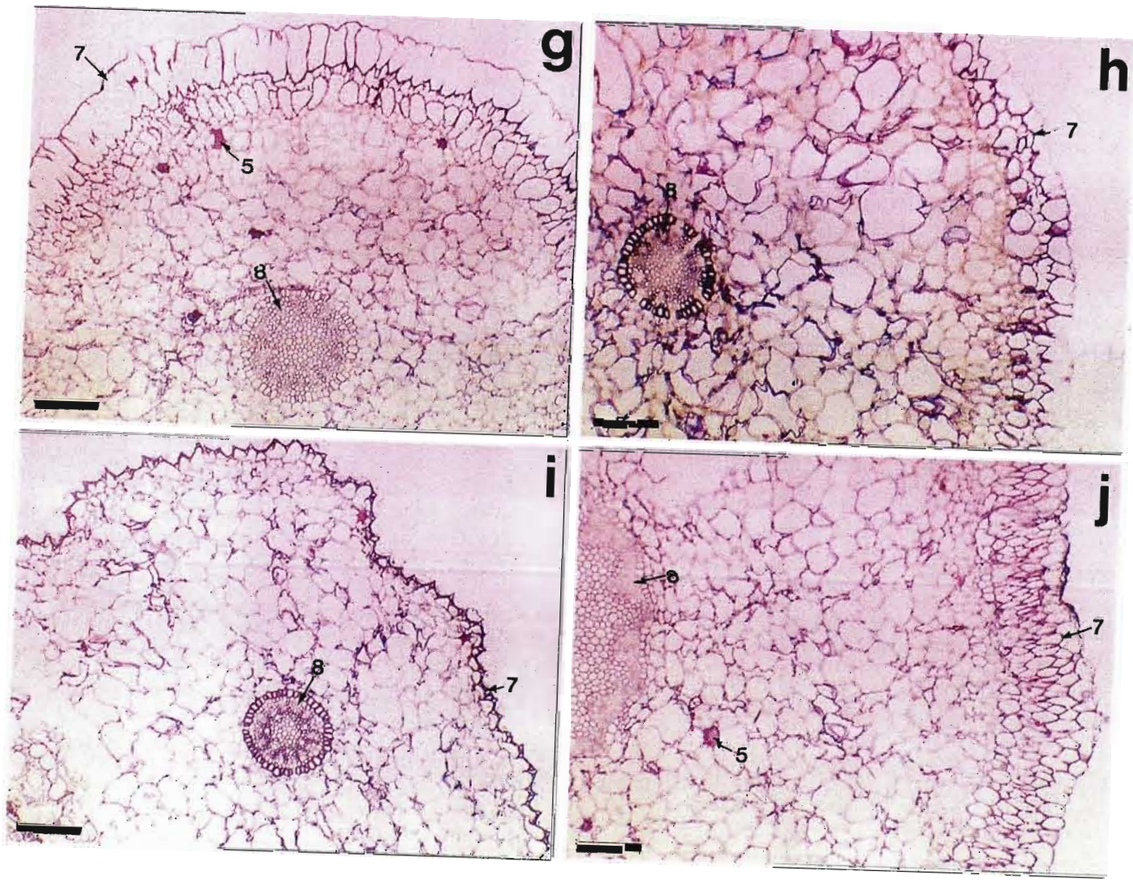
Root Anatomy

Velaminia, common in aerial roots of epiphytes, consist of a sheath of dead cells which originate by periclinal divisions from the root-dermatogen (Porembeski and Barthlott 1988). Porembeski and Barthlott (1988) specified a "vanda type" velamen, which they describe as being 3 to 5 layered, with the exodermis cells (the delimitation of the root cortex against the velamen) often much larger than the velamen cells, and cell size decreasing towards the exodermis. Some of the data reported here contradict observations by Porembeski and Barthlott (1988), for example, *D. caffra* (synonym *M. caffrum*) is reported by Porembeski and Barthlott (1988) to posses a 3-layered velamen; my observations show that the velamen of *D. caffra* consists of only one layer (Figure 1.3i). My observations also show that the velaminia of all the *Mystacidium* species are 3-layered, except in *M. aliceae* (Figure 1.3c) and *M. tanganyikense* (Figure 1.3f), where they have one layer. The velamen of *C. arcuata* (Figure 1.3j) is made up of 4 layers (consistent with Porembeski and Barthlott 1988, and Nseya and Arends 1995). The size of the cells making up each layer of the velamen vary between species. In *M. pusillum, M. flanaganii*, and *M. gracile* (Figures 1.3a, b and d), the cells of the middle and outer layers of the velaminia are similar in size, but smaller than the cells of the inner layer. The cells making up the outer layer of the velaminia of *M. brayboniae* (Figure 1.2e) and *M. capense* (Figure 1.3g) are much larger than the cells of the middle and inner layers, with the cells of the middle layer being smaller than those of the inner layer. In *M. venosum* (Figure 1.3h), the cells of the outer and inner layers are similar in size, but larger than the cells of the middle layer. The cells making up the 4 layers of the velamen of *C. arcuata* are similar in size.

The cortex comprises the greatest volume of the root, ranging from 7 - 10 layers of relatively large cells in *Mystacidium* and *D. caffra*, and about 15 layers in *C. arcuata*. Chloroplasts occur throughout the cells of the cortex, and raphides are abundant. Starch cells are present scattered throughout the cells of the cortex of *M. tanganyikense* (Figure 1.3f). The number of phloem bundles in the vascular cylinder ranges from 7 - 17.



Figures 1.3a-f. Root transverse sections. **a.** *Mystacidium pusillum* (Luyt6). **b.** *M. flanaganii* (Luyt7). **c.** *M. aliceae* (Luyt3). **d.** *M. gracile* (Luyt4). **e.** *M. brayboniae* (Edwards s.n.). **f.** *M. tanganyikense* (Johnson s.n.). 5 = raphide idioblast, 6 = starch cell, 7 = velamen, 8 = phloem bundle. Scale bars = 200 µm.



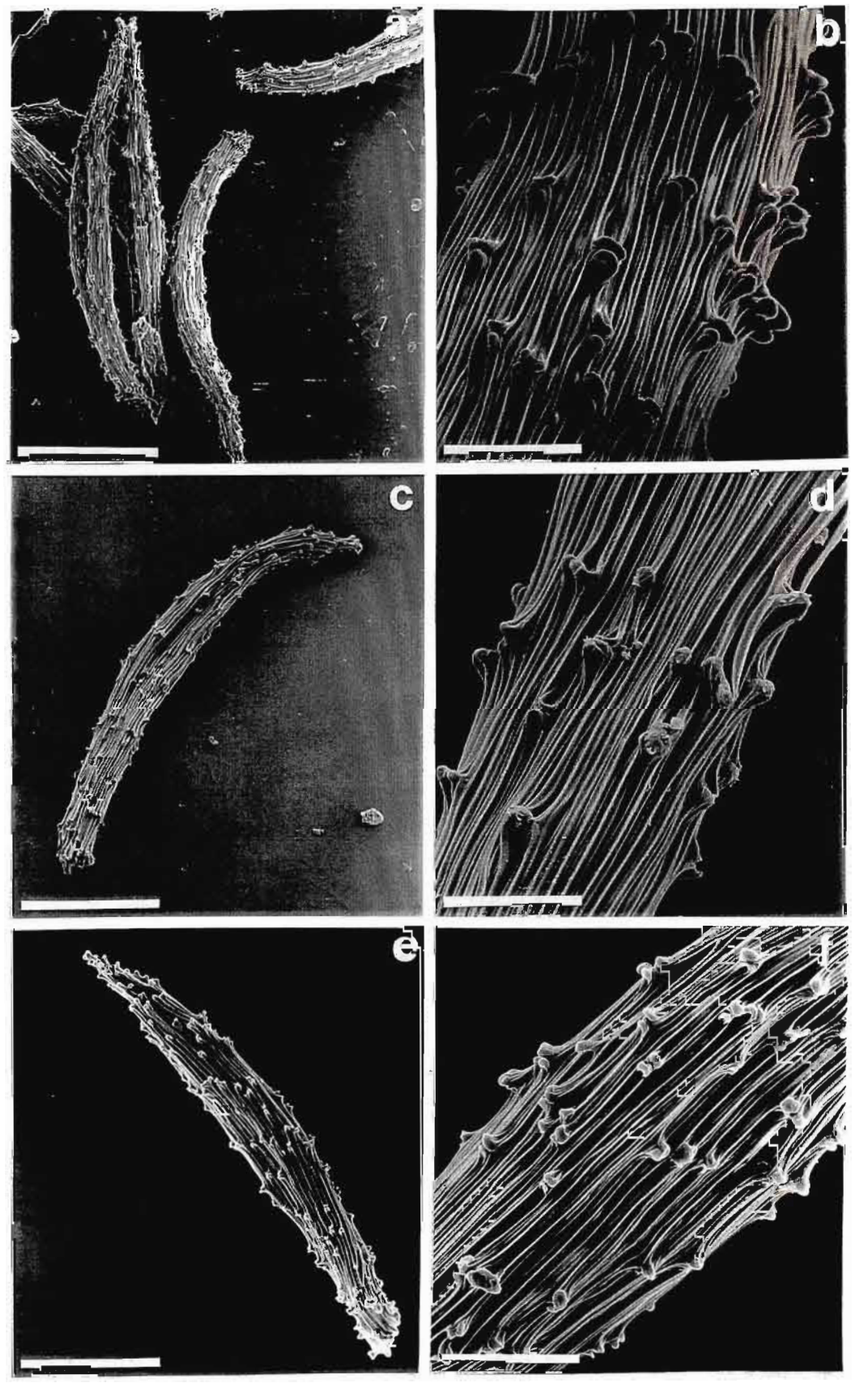
Figures 1.3g-j. Root transverse sections. **g.** *Mystacidium capense* (Luyt1). **h.** *M. venosum* (Luyt2). **i.** *Diaphananthe caffra* (Luyt9). **j.** *Cyrtorchis arcuata* (Luyt7). 5 = raphide idioblast, 7 = velamen, 8 = phloem bundle. Scale bars = 200 µm.

Seed Surface Ultrastructure

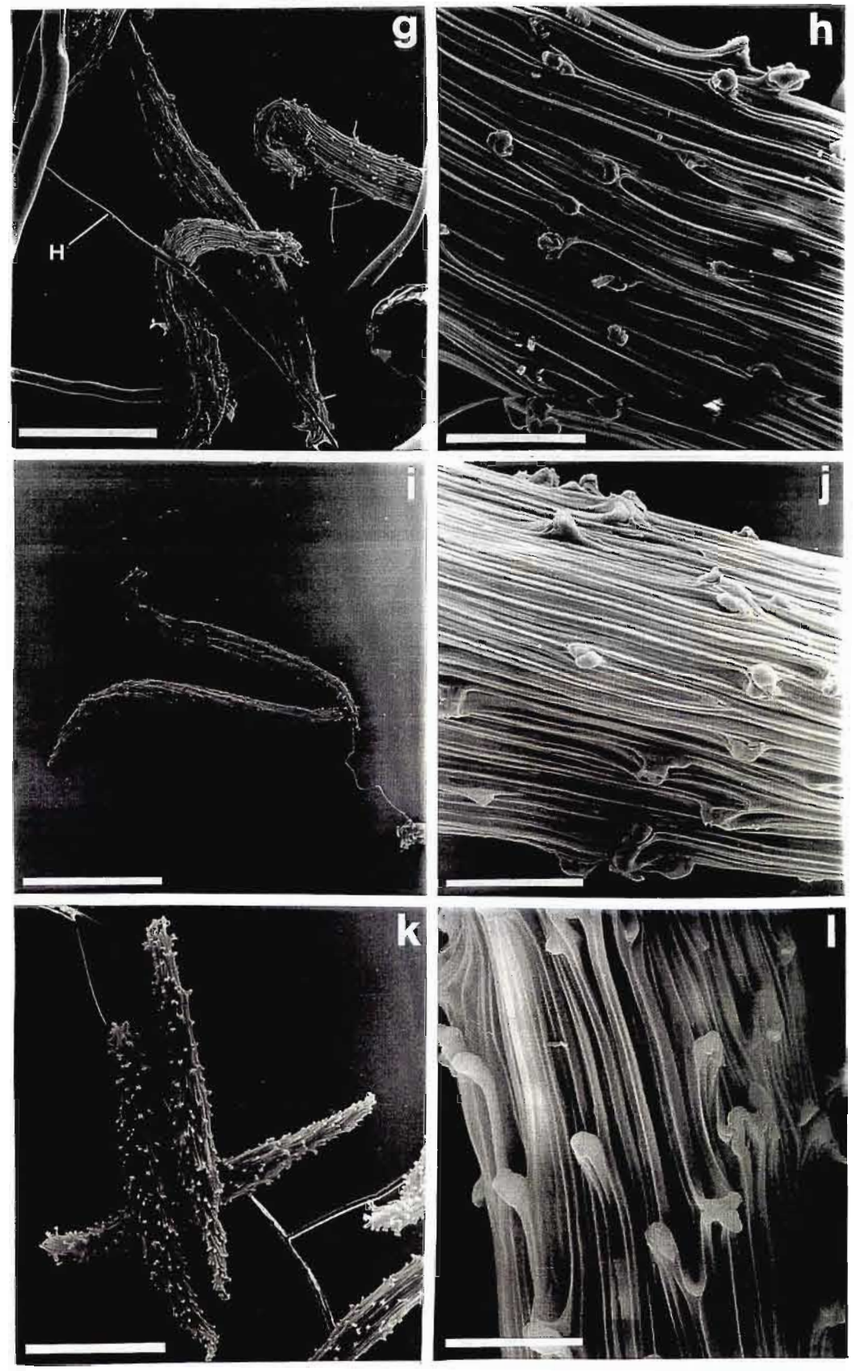
The sculpturing of the seed coat is regarded as being phylogenetically useful, especially at the subtribal and tribal levels (Barthlott 1976). The structure of the *Mystacidium* seeds that were studied fall under Dressler's (1993) "vanda type". The oblong dust seeds are golden brown, and the actual seed length ranges from 558 μm in *M. braybonіae* to 888 μm in *M. gracile* (Table 2). The testa cells of all the species are strongly elongate and periclinal thickenings are not seen. The surfaces of the seeds are made up of marginal ridges and bear wart-like structures where the terminal ends of the elongated testa cells meet (Figures 1.4a-l). The hairs interspersed among the seeds are hygroscopic and aid in dispersal. According to Dressler (1993), the seed coats of the "vanda type" are highly derived, and have probably evolved independently several times. The seed surface morphology is not taxonomically informative within *Mystacidium*.

Table 1. Seed lengths of *Mystacidium*

Species	*N*	Length (μm)	SD
M. capense	16	690	0.4
M. venosum	16	610	0.4
M. gracile	18	888	0.3
M. flanaganii	16	833	0.3
M. aliceae	15	654	0.4
M. braybonіae	15	558	0.3



Figures 1.4a-f. Scanning electron micrographs of seed surface ultrastructure. **a, b.** *Mystacidium capense*. Scale bars = 231µm, 30µm. **c, d.** *M. venosum*. Scale bars = 176µm, 30µm. **e, f.** *M. brayboniae*. Scale bars = 150µm, 50µm.



Figures 1.4g-l. Scanning electron micrographs of seed surface ultrastructure. **g, h.** *Mystacidium gracile*. Scale bars = 250µm, 27µm, H = hygroscopic hair. **i, j.** *M. flanaganii*. Scale bars = 380µm, 27µm. **k, l.** *M. aliceae*. Scale bars = 231µm, 25µm.

CONCLUSION

The velamen characters of *Mystacidium* are similar to those described by Porembeski and Barthlott (1988) for other species in the subtribe Aerangidinae (considered to have a "vanda type" velamen, which is usually 3-5 layered), with the exception of *M. aliceae* and *M. tanganyikense*, which have a single-layered velamen. The leaf anatomy of *Mystacidium*, *D. caffra* and *C. arcuata* in transverse section is also consistent with that of other Angraecoid orchids described by Gasson and Cribb (1985). The lateral rostellum lobes of *Mystacidium* species are uniquely papillose.

CHAPTER 2
PHOTOSYNTHETIC PATHWAYS

Stable isotope composition ($\delta^{13}C$ values) of leaves and roots in *Mystacidium* range from -14.91 to -18.99, indicating that *in situ*, all species perform CAM photosynthesis. *Mystacidium aliceae, M. brayboniae, M. tanganyikense, M. capense*, as well as *C. arcuata* (-13.52) perform pronounced CAM, while the remaining species, including *D. caffra* (-22.02) perform moderate CAM. Only *M. aliceae* exhibited significantly different $\delta^{13}C$ values in leaves and roots. Leaf thickness is significantly correlated with $\delta^{13}C$ values. Species occupying cool mistbelt forests showed less pronounced CAM than species occurring in savanna or coastal forest habitats, and $\delta^{13}C$ values were significantly correlated with mean annual rainfall at the collection sites.

INTRODUCTION

The exposed epiphytic microhabitat is typified by moderate to high light intensities, and since the most common substrate, tree bark, is often limited in terms of its water holding capacity, many epiphytes may be subject to periodic water stress. A study by Winter *et al.* (1983) revealed that crassulacean acid metabolism (CAM) is an important mechanism enabling epiphytes to exist under such extreme conditions, and a number of studies have shown that CAM is widespread among epiphytic orchids (Winter *et al.* 1983; Kluge *et al.* 1997).

Plants functioning in CAM mode undergo four phases over a 24 hour cycle, which were summarised by Osmond (1978) as follows: Phase I - CO_2 fixation and acid synthesis occur in the dark period; Phase II - At the beginning of the light period, a peak of atmospheric CO_2 fixation takes place, at which time both PEP and RBP carboxylase may be active; Phase III - During the day, a depression of CO_2 uptake occurs - C_4 acid decarboxylation and refixation of CO_2 takes place while stomata are closed; Phase IV - Once deacidification is complete in the later part of the light period, fixation of atmospheric CO_2, mainly via RBP carboxylase, occurs as the stomatal conductance increases. The CAM pathway thereby results in carbon gain while minimizing water loss through CO_2-fixation at night when temperature and evaporation is low. CAM plants are therefore clearly adapted to arid or dry environments. According to Kluge and Ting (1978), nocturnal fixation of CO_2 contributes to a positive carbon balance, especially when water is in short supply. Kluge and Ting (1978) further demonstrated that in periods of good water availability, CAM plants have the ability to fix relatively high amounts of CO_2 during the day (via the C_3 pathway).

Kluge *et al.* (1997) analysed the incidence of CAM in the epiphytic genus *Angraecum*. Their experiments with *A. sesquipedale* revealed that drought and temperature are

important factors modulating CAM, and it was concluded that the ecological success of the genus is largely attributed to genotypic diversity and intraspecific plasticity of the photosynthesis behaviour (Kluge *et al.* 1997). The aim of this study was to investigate the incidence of CAM photosynthesis using stable isotope composition ($\delta^{13}C$ values) in *Mystacidium, Cyrtorchis arcuata* and *Diaphananthe caffra* in natural environments. The $\delta^{13}C$ value is correlated with the C_3 and C_4 pathways of photosynthetic metabolism, and can reliably distinguish between the two pathways (Osmond *et al.* 1975). The C_4 plants show a $\delta^{13}C$ value of approximately $-12^0/_{00}$, and C_3 plants exhibit a $\delta^{13}C$ value of about $-27^0/_{00}$ (Osmond *et al.* 1975). Since C_4 photosynthesis is not known to occur in orchids (Arditti 1992, Goh and Kluge 1989), and anatomical investigations carried out in Chapter 1 determined that Krantz anatomy does not occur in any of the species in this study, $\delta^{13}C$ values can be used to distinguish between CAM and C_3 photosynthesis. This distinction between CAM and C_3 plants has been successfully applied in previous studies on orchids (Winter *et al.* 1983, Earnshaw *et al.* 1987, Kluge *et al.*1997). The ecologically tolerant epiphytic orchids in this study occur across a wide range of habitats, from mistbelt forest to dry coastal forest, on a number of different host trees and in both exposed and shady areas. Species in the north and east of South Africa are generally subjected to cool, dry winters, and those in the south and west are subjected to hot, dry summers.

MATERIALS AND METHODS

Leaf material of *C. arcuata* and *D. caffra*, and leaf and root material of all eight species of *Mystacidium,* (except *M. brayboniae* and *M. tanganyikense*, where only leaf material applies) was collected in the field for the estimation of stable carbon isotope composition ($\delta^{13}C$). Leaf thickness was determined in the middle of the leaf blade (between tip and base, and between midrib and edge) (Winter *et al.* 1983). Material was dried for approximately 24 hours at 100° C. The dried material was ground to a fine powder and analysed by Professor W. Stock (University of Cape Town). Analysis was carried out on 10 mg samples, which were combusted in sealed quartz tubes. The carbon dioxide formed in the tubes was collected by cryogenic distillation, and the $\delta^{13}C$ values were determined on a VG Micromass 602E mass spectrometer using a reference gas calibrated against National Bureau of Standards isotope references. All isotope ratios are expressed relative to the Chicago Pee Dee Belemnite standard [$\delta^{13}C = ((R_{sample}/R_{PDB})-1) \times 1000$, where R is the ratio of ^{13}C to ^{12}C] (Bond *et al.* 1994). Following Kluge *et al.* (1997), $\delta^{13}C$ values between $-10^0/_{00}$ and approximately $-20^0/_{00}$ are considered as pronounced CAM,

whereas values more negative than -25$^0/_{00}$ are indicative of C_3 photosynthesis, and values between these extremes suggest facultative CAM (C_3/CAM intermediates). Mean annual precipitation for the sites from which plant material was collected was supplied by Craig Peter (University of Natal), using the database developed by Schulze *et al.* (1997).

RESULTS AND DISCUSSION

A list of the species examined, their habitat, leaf thickness, and $\delta^{13}C$ values of both leaves and roots is given in Table 2. The results indicate that all the species examined can be classified as CAM type plants, and are consistent with a number of studies on epiphytic orchids exhibiting CAM (Neurenbergk 1963, Winter *et al.* 1983, Lüttge 1987, Griffiths 1989, Kluge *et al.* 1997). According to Griffiths (1989), the evolution of CAM in epiphytes is related to growth in a potentially arid microclimate, where plants have limited, or no access to soil moisture.

The $\delta^{13}C$ values obtained from the leaf material range from -13.52$^0/_{00}$ in *C. arcuata* to -22.09$^0/_{00}$ in *D. caffra* (Table 2). The $\delta^{13}C$ value of *C. arcuata* is indicative of pronounced CAM, with fixation of external CO_2 occurring mainly at night by PEP carboxylase, whereas the more negative value in *D. caffra* indicates a substantial contribution of C_3-mediated CO_2 uptake. Teeri *et al.* (1981) reported a strong relationship between leaf thickness and leaf $\delta^{13}C$ values in species of Crassulaceae, and Winter *et al.* (1983) found that $\delta^{13}C$ values of orchids tended to be less negative with increasing leaf thickness. This is also evident when comparing the $\delta^{13}C$ values of *C. arcuata*, which has a leaf thickness of 1.64 mm, and *D. caffra*, which has a leaf thickness of 0.47 mm. Linear regression indicated that $\delta^{13}C$ values were significantly positively correlated with leaf thickness (Figure 2.1). However, this trend is not apparent in *Mystacidium*, especially when comparing the leaves of *M. aliceae* and *M. capense*, which have similar $\delta^{13}C$ values, and yet *M. capense* has leaves twice as thick as the leaves of *M. aliceae.* A regression analysis excluding *C. arcuata* revealed that the correlation between $\delta^{13}C$ values and leaf thickness was not significant ($r^2 = 0.16$, $P = 0.28$). Although leaf succulence is an important pre- requisite for CAM, Kluge and Ting (1978) outlined the importance of distinguishing between two types of leaf succulence, as they are related to different modes of photosynthesis. According to Kluge and Ting (1978), leaf-succulent orchids, where succulence is due to non-photosynthetic external or internal water-storing tissues with a non water-storing photosynthetic mesophyll, perform C_3 and are not capable of CAM, whereas leaf-succulent orchids, where succulence is due to a relatively homogeneous water-storing

photosynthetic mesophyll, exhibit CAM. Based on the anatomical studies in Chapter 1, all the species in this study exhibit the latter type of succulence. The leaves of all eight *Mystacidium* species, as well as those of *C. arcuata* and *D. caffra*, are uniformly composed of spherical to irregularly shaped cells, with no differentiation into palisade and spongy parenchyma. Furthermore, all the species in this study have large vacuoles in the same cells that have chloroplasts. Winter *et al.* (1983) found that the leaves of the epiphytic orchids *Bulbophyllum evasum* and *Phreatia baileyana* were over 2 mm thick and had $\delta^{13}C$ values of −27.4 and −30.6 $^{0}/_{00}$ respectively, because leaf succulence was due to water storage tissue lacking chloroplasts. The above mentioned species were two of the 29 species of epiphytic orchids out of a total of 82 species studied that were classified as C_3 type plants by Winter *et al.* (1983).

Table 2. $\delta^{13}C$ values in *Mystacidium*, *D. caffra* and *C. arcuata*. Numbers in brackets indicate sample size.

Species	Habitat	Leaf thickness (mm) ± S.D.	Leaves $\delta^{13}C$ ($^{0}/_{00}$) ± S.D.	Roots $\delta^{13}C$ ($^{0}/_{00}$) ± S.D.
M. aliceae	Coastal forest	0.53 ± 0.01 (10)	-15.00 ± 0.15 (6)	-19.66 ± 1.48 (5)
M. braybonniae	Greenhouse	1.2 ± 0.15 (10)	-17.01 (1)	-
M. capense	Dry thornveld	1.1 ± 0.16 (10)	-15.22 ± 0 (2)	-15.48 (1)
	Shaded, garden		-15.75 ± 0.63 (6)	-15.79 ± 0.56 (3)
M. flanaganii	Mist belt forest	0.85 ± 0.01 (10)	-18.99 ± 0.65 (5)	-17.32 ± 0.49 (3)
M. gracile	Mist belt forest	0.65 ± 0.01 (10)	-19.49 (1)	-19.85 ± 0.33 (4)
M. pusillum	Dry coastal thornveld	0.73 ± 0.02 (10)	-17.4 ± 0.70 (3)	-17.68 ± 0.52 (4)
M. venosum	Dry coastal thornveld	0.66 ± 0.02 (10)	-17.23 ± 0.29 (5)	-19.86 ± 1.25 (4)
M. tanganyikense	Montane forest	0.8 ± 0.02 (10)	-14.91 (1)	-
D. caffra	Mist belt forest	0.47 ± 0.01 (10)	-22.02 ± 0.09 (3)	-
C. arcuata	Shaded, garden	1.64 ± 0.16	-13.52 ± 0.15	-

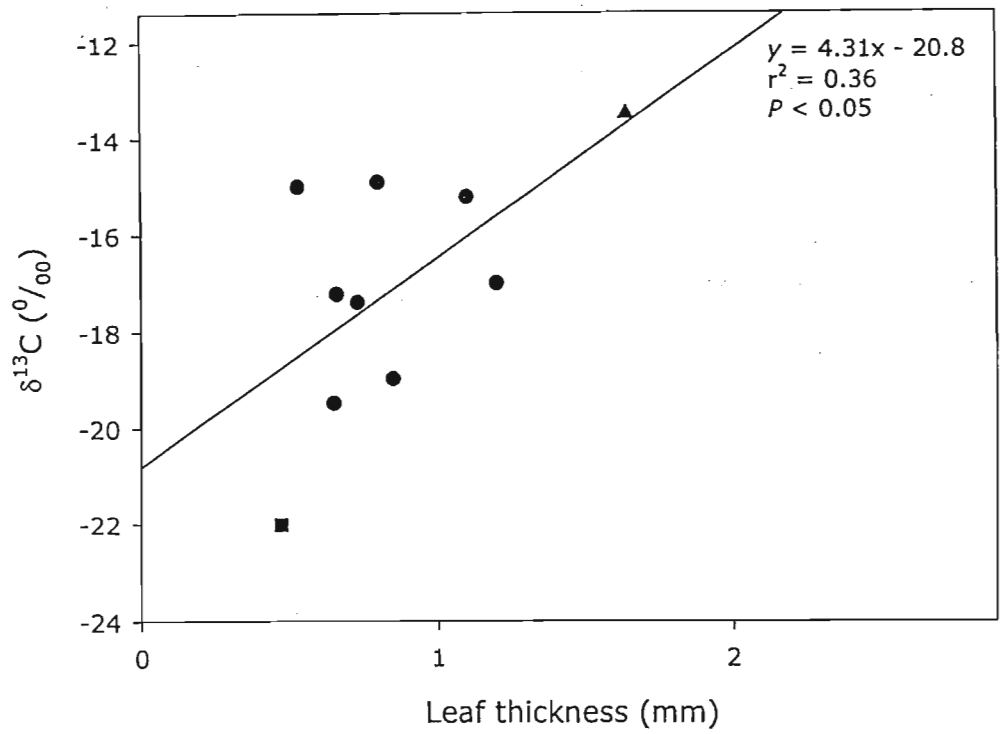


Figure 2.1. Relationship between leaf thickness and $\delta^{13}C$ values of *Mystacidium* (●), *C. arcuata* (▲) and *D. caffra* (■).

Linear regression indicated that $\delta^{13}C$ values of leaves were significantly negatively correlated with the mean annual precipitation for the sites from which plant material was collected (Figure 2.2). Kluge *et al.* (1997) found that *Angraecum* species occupying cool montane forests showed $\delta^{13}C$ values indicative of C_3 photosynthesis, and that species growing at lower altitudes with higher temperatures had typical CAM-type $\delta^{13}C$ values. The $\delta^{13}C$ values found in the species in this study are also related to their habitats (Table 2). The most negative $\delta^{13}C$ values were found in the species occupying mistbelt forests (*M. flanaganii*, *M. gracile* and *D. caffra*), which experience higher rainfall (*c.* 1354 mm) and cooler night time temperatures than coastal forests and thornveld (*c.* 803-937 mm). The less demanding environmental conditions in the mistbelt forests allow the orchids to harvest proportionally larger amounts of external CO_2 directly by the C_3 pathway during the day.

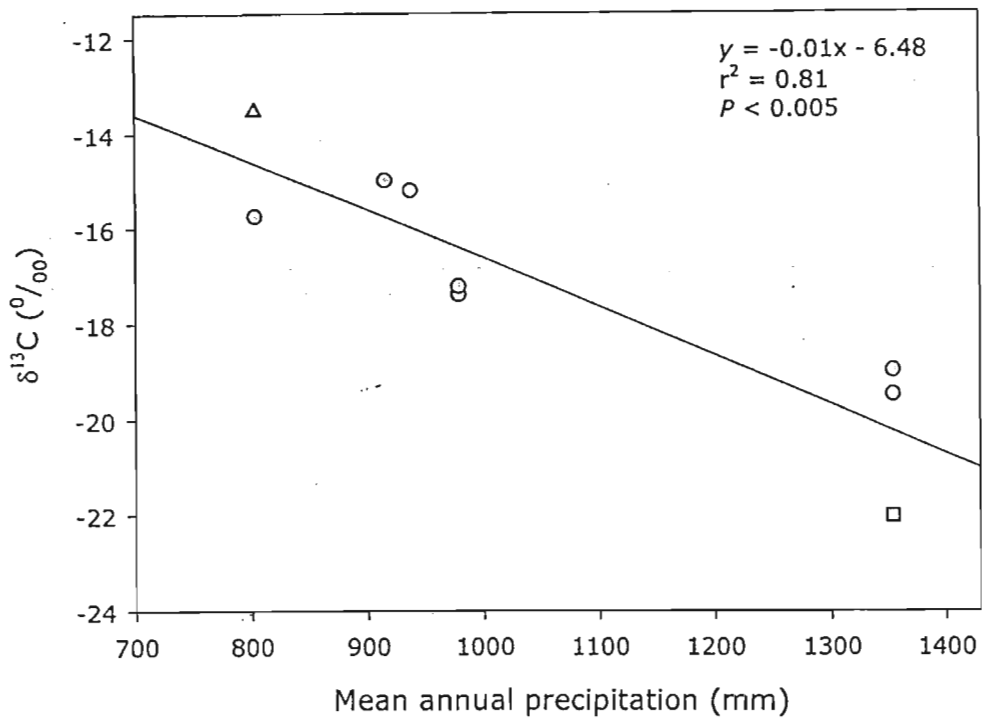


Figure 2.2. Relationship between mean annual precipitation for the various sites and $\delta^{13}C$ values of leaves of *Mystacidium* (●), *C. arcuata* (▲) and *D. caffra* (■).

The $\delta^{13}C$ values obtained from the chloroplast-containing roots of *Mystacidium* were generally similar to those obtained from the leaves, except in the case of *M. aliceae* (Table 2). While t-tests showed that there was no significant variation in $\delta^{13}C$ values between the roots and leaves in most of the species, $\delta^{13}C$ values in the roots of *M. aliceae* were significantly more negative than the leaves (t = 3.3, $P < 0.05$), suggesting greater contribution of day-time CO_2 uptake to total carbon gain. Although the roots of *Mystacidium* are photosynthetic, it is difficult to assess whether the $\delta^{13}C$ values of the roots of the remaining species are indicative of CAM photosynthesis, or a result of translocation of photosynthetic products between roots and leaves. Furthermore, it is possible that the $\delta^{13}C$ values obtained from the roots could be due to diffusional limitations of CO_2 uptake through the velamen and exodermis (O'Leary 1981), although the roots do possess pneumatodes. Other studies have shown that the roots of epiphytic orchids are capable of dark net CO_2 fixation (Benzing and Otto 1981), therefore it is also possible that most carbon assimilation may take place through the roots, and that carbon compounds are translocated to the leaves.

CONCLUSION

The $\delta^{13}C$ values of CAM plants range between those typical of C_3 and C_4, depending on the relative amount of CO_2 fixation at night (CAM mode), versus the direct fixation of atmospheric CO_2 during the day (C_3 mode). The $\delta^{13}C$ values of CAM are therefore used as a relative measure of night fixation through PEP carboxylase and day fixation of atmospheric CO_2 through RBP carboxylase (Edwards and Walker 1983). As a number of studies have shown (Kluge and Fisher 1967, Osmond *et al.* 1978, Kluge *et al.* 1997), some CAM plants exhibit photosynthetic plasticity, whereby, depending on environmental conditions, they can change their degree of day versus night fixation of atmospheric CO_2. For example, Kluge *et al.* (1997) demonstrated that drought forced *A. sesquipedale* and *A. eburneum* to operate in a pronounced CAM-mode as opposed to a moderate CAM-mode. The $\delta^{13}C$ values of *M. capense* in both dry and wet habitats were very similar, however, and indicative of pronounced CAM in both habitats. In order to test the plasticity of CAM in a single *Mystacidium* species more rigorously, laboratory experiments would need to be carried out with cessation of watering under controlled conditions, or $\delta^{13}C$ values from a wider range of habitats could be compared. Further studies would also be necessary to determine whether *Mystacidium* exhibits organic acid fluctuation and little, or no exogenous nocturnal CO_2 fixation, a phenomenon known as CAM-cycling. Nevertheless, the results reported here indicate that *in situ*, *M. aliceae, M. brayboniae, M. capense, M. tanganyikense* and *C. arcuata* operate in a pronounced CAM-mode, and that nocturnal CO_2 fixation in the remaining *Mystacidium* species and in *D. caffra*, which exhibit slightly more negative δ13C values, is supplemented by some CO_2 fixation during the day. The strong correlation between δ13C values and mean annual precipitation (Figure 2.2) may reflect plasticity of CAM, or localized adaptation of the photosynthetic system. CAM is therefore an important mechanism that enables these epiphytic orchids to exist in exposed microhabitats, which are often subject to periodic water stress since tree bark, their substrate, has a low water holding capacity.

CHAPTER 3
POLLINATION BIOLOGY

The pollination biology of the genus *Mystacidium* was investigated. The flowers of these orchids are pale green to white, and produce dilute, sucrose rich nectar concealed in spurs, ranging from 1-4.7 cm in length. Breeding system experiments revealed that *Mystacidium* species depend on pollinators for fruit set, and that self-pollination results in significantly reduced seed set. Field experiments showed that *M. venosum* and *M. capense* are visited by hawkmoths at dusk, and that *M. gracile* and *M. pusillum* are visited by settling moths. Both the hawk- and settling moths carried pollen along the length of their tongues. *Mystacidium venosum* and *M. capense* exhibited fluctuation of nectar properties that coincided with hawkmoth feeding periods, as well as evidence for nectar reabsorption in the early hours of the morning. Nocturnal emission of scent occurred in all species except *M. braybonіae* and *M. aliceae*, and was largely composed of a combination of monoterpenes and benzoids. Despite variation in spur length within species, there was no evidence for directional selection in *M. venosum*, *M. capense* and *M. gracile*. Hand-pollinations significantly increased fruit set in *M. capense* at the whole plant level in two consecutive seasons at different sites, indicating pollen limitation. Despite pollen removal being higher than pollen reciept in *M. venosum*, *M. capense* and *M. gracile*, suggesting pollen wastage, the percentage of pollen removed that reached subsequent stigmas at a particular site was much higher (c. 35-50%) than values reported for other plants. Flower longevity in *M. venosum* is reduced significantly by pollination, and pollinia removed from flowers remains viable for up to 20 days in natural conditions.

INTRODUCTION

Most of the species in the subtribe Aerangidinae, including those belonging to *Mystacidium*, conform to the syndrome of moth pollination (Dressler 1993). The white, long-spurred flowers of *M. venosum* are known to be pollinated by hawkmoths (Luyt and Johnson 2001) and *M. capense* has spurs that exceed 3 cm in length, suggesting pollination by hawkmoths. Pollen vectors for the remaining, shorter-spurred species in the genus cannot easily be predicted. Floral morphology of these species suggests pollination by settling moths, but bees, flies or even butterflies are also likely candidates.

Many flowers have evolved long spurs, traits that enable effective pollination by long-tongued pollinators. Hodges (1997) argued that, since spurs allow plants to adapt to more specialized pollination systems, the innovation of floral spurs has promoted radiation in a number of plant groups (Robertson and Wyatt 1990, Steiner and Whitehead 1990, Johnson and Steiner 1997). The mechanism by which long spurs evolved was suggested by Darwin (1862) and later experimentally tested by Nilsson (1988): selection for longer spurs will compel a long-tongued pollinator to insert its proboscis deep into the spur, thereby ensuring contact with the flower's sexual organs. Conversely, longer tongues are selected when they enable pollinators to reach deeply concealed nectar. The "evolutionary race" continues as pollinators with longer

mouthparts drive the selection for longer spurs, and vice versa. Nilsson (1988) showed that artificially shortened spurs of the moth-pollinated flowers of *Platanthera* received less effective pollination. Wasserthal's (1997) observation that the Malagasy hawkmoth *Xanthopan morgani praedicta*, which has a tongue length of *c.* 22 cm, pollinates *Angraecum sesquipedale*, which has a spur exceeding 30 cm in length, confirms Darwin's prediction that the orchid must be pollinated by a moth with a tongue length a little shorter than the spur.

Breeding Systems

Breeding systems of seed plants range from obligate outcrossing, to simultaneous outcrossing and selfing, to autogamy (Barrett *et al.* 1996). According to Barrett *et al.* (1996), long-lived species usually have high genetic loads resulting in strong inbreeding depression, which may constrain the evolution of selfing. Orchids, which are known to be long-lived (Clayton and Aizen 1996), show sophisticated floral adaptations to maximize outcrossing and reduce geitonogamy. Although self-incompatibility is rare in orchids, reduction in seed set usually results from self-pollination due to inbreeding depression (Johnson 1994, Johnson and Edwards 2000, Luyt and Johnson 2001). Bending movements of the caudicle or stipe after withdrawal from a flower has been interpreted as a mechanism to prevent geitonogamy (Darwin 1862, Johnson and Edwards 2000), and is evident in *M. venosum* (Luyt and Johnson 2001).

Nectar

Nectar is probably the most important floral reward offered by animal- and insect-pollinated plants (Simpson and Neff 1983). The volume, concentration, constituents and distribution of nectar among flowers plays a major role in the effectiveness of pollination (Kevan & Baker 1983). Because various pollinators have different energy needs, nectars are highly variable in sugar composition, concentration and caloric content (Kevan and Baker 1983). As a reward, nectar can be modified to increase pollinator constancy or specificity. Subtle alterations in the chemistry, volume, concentration and secretion of nectar can be correlated with different pollination systems (Simpson and Neff 1983). Nectar is therefore likely to be subject to selective pressures imposed by pollinators, often resulting in plants with the same pollinators having similar nectar characteristics (Pyke and Waser 1981, Baker and Baker 1982) through convergent evolution.

Conversely, pollinators display adaptations related to specific nectar-feeding behaviours,

such as elongated mouthparts that facilitate feeding from tubular corollas. Nectar viscosity rises with sugar concentration, and high viscosity reduces intake by nectar-feeders (Heyneman 1983). A high concentration of nectar sugar may therefore lower the energy intake rate. Hawkmoths use energetically expensive feeding techniques and so require relatively dilute nectar to enable minimal feeding time as well as maximize energy flux. Settling moths have high energy costs in traveling to the nectar source, but have low costs for feeding, and therefore exploit slightly more concentrated nectar (Scoble 1992). The most common sugars in nectar include sucrose (a disaccharide), and the hexose monosaccharides glucose and fructose (Baker and Baker 1983). The nectar of flowers pollinated by moths is generally sucrose rich or sucrose dominant, although settling moth nectars may have slightly higher ratios of monosaccharides than the nectar of hovering moth-pollinated flowers (Baker and Baker 1983).

Constituents of nectar other than sugars also vary among species, and Baker (1977) suggests that there is an adaptive reason for the presence of substances such as alkaloids, vitamins, proteins, amino acids and lipids. According to Southwick (1990), the components making up the nectar solution influence the taste of the nectar and may therefore be important in attracting certain pollinator groups. Scoble (1992) found that water, sugars, salts and amino acids are the substances most sought after by Lepidoptera. The presence of amino acids in nectar would be beneficial to insects that feed solely on nectar and have no other source of protein-building materials.

The temporal pattern of nectar secretion can also be correlated with pollinator class (Cruden and Hermann 1983), and the patterns of nectar production may be equally as adaptive as nectar chemistry. The timing of nectar secretion affects the foraging frequency of both efficient and inefficient pollinators. The selective pressures imposed by pollinators on nectar may influence aspects such as resource availability. For example, the high nectar sugar content required by some pollinators could be costly for a plant to produce. Adaptive mechanisms associated with nectar production, such as resumption of nectar production following nectar removal, cessation of nectar secretion when pollinators are inactive, and reabsorption may enable plants with high reproductive costs to maximize their fitness (Cruden *et al.* 1983, Koopowitz and Marchant 1998, Luyt and Johnson 2002).

Scent

According to Kaiser (1993), the enormous diversity in the scent of orchids reflects the equally diverse pollination systems found in the family. Floral fragrances are important as attractants for pollinators, and together with visual cues and rewards, influence pollinator behaviour (Dobson 1994, Varassin *et al.* 2001). Descriptions of floral scent in the past were subjective and vague; for example, moth-pollinated flowers were described as having a "heavy-sweet, very strong, not fruity" odour (van der Pijl and Dodson 1966). Current techniques allow for the objective chemical classification of floral fragrances (Kaiser 1993).

The different biosynthetic classes of volatile compounds that make up the complex compositions of floral fragrances include isoprenoids, benzenoids, phenyl propanoids (phenolic compounds) and fatty acid derivatives (aliphatic compounds) (Kearns and Inouye 1993, Proctor *et al.* 1996). According to Dressler (1993), the kinds of compounds that characterise a particular floral fragrance may reflect pollination syndromes rather than phylogenetic relationships. For example, sulphur-containing compounds distinguish bat-pollinated flowers from most species pollinated by other animals or insects (Kaiser and Tollsten 1995, Knudsen and Tollsten 1995), and monoamines and diamenes characterise the unpleasant odour of many fly-pollinated flowers (Dobson 1994). A number of studies examining floral fragrance composition in relation to pollinator type have focused on moth-pollinated flowers (Knudsen and Tollsten 1993, Miyake *et al.* 1998, Levin *et al.* 2001). Brantjes (1973) suggested that moths may discriminate between different floral scent blends and between individual floral scent compounds. Brantjes (1978) also demonstrated that floral scent plays an important role in the 'seeking flight' in a moth, and it is known that scent-production of hawkmoth-pollinated flowers coincides with the main period of moth activity at dusk and in the evening (Nilsson 1978, Luyt and Johnson 2001, Johnson *et al.* 2002). Floral scent therefore certainly does play an important role in the attraction and guidance of pollinating moths, and it has been suggested that convergent evolution for the attraction of hawkmoths in a number of floras may in fact involve scent chemistry (Kaiser 1993, Knudsen and Tollsten 1993, Miyake *et al.* 1998). However, Raguso (2001) reviewed physiological and behavioural responses of pollinators to scent, and concluded that floral scent ought to be defined as a product of a combination of the dynamics of biosynthetic pathways, phylogenetic constraints, and trade-offs between pollinator and herbivore attraction. But Knudsen and Tollsten (1993) found that floral scent chemistry is a valid component for defining moth-pollinated flowers in comparison with flowers of other pollination

syndromes, and that floral scent is selected by a specific group of pollinators that have similar sensory preferences.

Pollination Success

Because orchids possess pollinia, they are well suited for estimating both male and female reproductive success at the pollination stage (Nilsson 1992, O'Connel and Johnston 1998). Pollinia removal serves an estimate of male reproductive success, and pollinia receipt is a measure of female reproductive success (Nilsson 1992). Nilsson (1992) demonstrated that floral traits of *Platanthera* influence both male and female reproductive success. If fruit set in a population is pollen limited (which, as mentioned earlier, is common in orchids), then traits that influence the probability of pollen receipt (female function) should be under strong selection. This was demonstrated by Johnson and Steiner (1997), who showed that selection for longer spurs in *Disa* occurred through the female, rather than the male, function. In some cases, selection will favour floral traits that appeal to a more reliable pollinator, causing a shift from, for example, bee to hawkmoth pollination as was illustrated by Johnson (1997).

Low pollinator visitation in orchids may have resulted in selection for long-lived flowers (Arditti 1979). Primack (1985) suggested that long-lived flowers in orchids represent an adaptation for highly specialized pollination systems where the specific pollinator may be relatively scarce. Floral persistence thereby maximizes the opportunity for pollination to occur. Long-lived pollen is also important if pollinator visitations are low, or if pollinaria are transported by widely foraging insects, because eventual deposition can still contribute to reproduction (Neiland and Wilcock 1994). Flower and pollen longevity therefore maximize reproductive success through both the female and male function (Luyt and Johnson 2001).

Aims and Objectives

The objectives of this study were to investigate the breeding systems and floral biology, including nectar and scent chemistry, of the different species of *Mystacidium*, and to identify the pollinators of as many species of the genus as possible. Further aims were to investigate the incidence of pollen limitation and selection on spur length in selected species.

MATERIALS AND METHODS

Breeding System Experiments

In order to determine the compatibility system of the species of *Mystacidium* and the degree of dependence on pollinators for fruit set, breeding system experiments were conducted on all species, except *M. tanganyikense* (which does not occur in South Africa). Pollinators were excluded from flowers by fine mesh bags or a greenhouse that prevented access by flying insects. Individual flowers were randomly allocated to selfed and outcrossed treatments. Selfed flowers were pollinated with pollinaria from flowers on the same inflorescence, and outcrossed flowers were pollinated with pollinaria from a different plant. A third group of flowers were not hand-pollinated to test for autogamy. Breeding system experiments carried out in the field required bagging of flowers to exclude pollinators. The percentage of flowers which developed into mature fruits was determined. Fruit weight, and the percentage of seeds with embryos, was measured where possible.

Study Sites and Pollinator Observations

Pollinator observations were carried out in the field for all species of *Mystacidium* except *M. brayboniae*, which is endemic to remote parts of the Soutpansberg. All observations, except those carried out at Vernon Crookes Nature Reserve, took place from just before dusk until at least two hours later (Table 3). A flashlight was used to observe pollinators after dusk, and a 250 W mercury vapour light trap was run at the site of observation to attract pollinators. When possible the light trap was left to run overnight. An insect net was used to capture pollinators foraging on the flowers.

Table 3. Study sites and pollinator observations of *Mystacidium*

Species	Study site		Date	Total hours of observation	
	Breeding system	Pollinator observations		Daytime	Evening
M. alicea	Vernon Crookes Nature Reserve	Vernon Crookes Nature Reserve	9, 10, 12, 17, 18 Mar 2000	24	4
M. brayboniae	Greenhouse	-	-	-	-
M. capense	Bishopstowe	Ixopo	12 Nov 1999	-	60
		Richmond	19, 20, 24 Nov 1999		
		Wartburg	26, 30 Nov 1999 1, 3 Dec 1999		
		Bishopstowe	5, 6, 9, 11, 20, 30 Dec 1999 5, 6, 12 Jan 2000 4, 5, 12, 15, 26, 27 Dec 2000		
		Lion Park	14 Dec 1999 14, 18, 19 Dec 2000		
M. flanaganii	Mbona Mountain Estate	Mbona Mountain Estate	28, 29, 31 Jan 2000 1, 2, 4, 5, 6, 10, 11, 12, 13 Feb 2000 30, 31 Jan 2001 3, 4, 7, 8, 9, 10, 11, 12, 13, 15, 16 Feb 2001	-	50
M. gracile	Mbona Mountain Estate	Mbona Mountain Estate	16, 17, 23, 24 Sep 1999 1, 6 Oct 1999 10, 15, 16, 17, 19, 20, 21, 26, 27, 29 Sep 2000 2, 3, 5 Oct 2000	-	54
		Bulwer	11, 13, 18, 20, 22 Oct 1999		
		Gilboa	20, 21 Oct 2000 5 Oct 2001		
M. pusillum	Harold Johnson Nature Reserve	Harold Johnson Nature Reserve	12, 14 July 2000 29, 30 June 2001 1 July 2001	-	10
M. venosum	Greenhouse	Verulam	1, 4, 5, 9, 10, 15, 18, 19 June 1998 2, 11, 15, 22 June 1999	-	60
		Baynesfield	31 May 1999 8, 10, 18, 21, 30 June 1999 15, 17, 19, 20, 21, 22 June 2000		
		Karkloof	17 June 2000		
		Harold Johnson Nature Reserve	12, 14 July 2000 29, 30 June 2001 1 July 2001		
*M. tanganyikense**	-	Nkiya Plateau, Malawi	27 March 2000	-	2

*Observations of *M. tanganyikense* were undertaken by Dr S.D. Johnson

Spur Length and Nectar Measurements

In order to assess the average spur length of each species, the spur lengths of flowers from different plants were measured using a steel ruler. The volume of nectar in the spurs of each species was measured using a calibrated micropipette (Fisherbrand 1-5 µl), and the nectar concentration was determined with an Atago N1 0-32% pocket refractrometer. Measurements were taken from unvisited flowers shortly before dusk for all species except *M. braybonіae*, where measurements were taken in the morning and afternoon. In order to determine the sugar ratios of the various species, nectar samples of each species were applied as spots to Whatman no.1 filter paper. The nectar sugar composition of each species was analysed by Professor B-E van Wyk using basic HPLC techniques (van Wyk 1993). This entailed eluting the nectar with distilled water using a centrifuge and performing HPLC on a "Waters Sugarpack" column. A refractive index detector allowed accurate calculation of sugar compounds of each sample using peak heights.

Nectar was also tested for amino acids using the method developed by Baker and Baker (1975). Nectar drops were placed on Whatman No.1 chromatography paper, dried quickly, and then stained with ninhydrin (0.2% in acetone). Up to 24 hrs at lab temperature was allowed for a violet colour to come to a maximum. A comparison scale for colour depth was created by making spots from dilutions of a sucrose solution of histidine and staining these with ninhydrin. Every score was scored against the "histidine scale" where each unit advance represents a doubling in concentration. 3.9 mg/ml of histidine was dissolved in 20% sucrose solution. From this stock, which was referred to on a concentration scale of 10, a series of 50% dilutions was prepared down to a scale of 1. Eleven circles (*c.* 0.5 cm in diameter) were marked along a strip of Whatman No. 1 filter paper. One µl of each histidine concentration (1 to 10) was applied to the centre of each circle, and distilled water to the circle marked 0. Once the 'calibration scale' was dry, the histidine references were stained with 1 to 3 µl of ninhydrin (added to the centre of each circle). The filter paper strip was then dried at room temperature for at least 24 hours. The colour intensities of the nectar samples were compared with those of the calibration scale in order to evaluate amino acid concentrations in the nectar sample. It was not possible to perform nectar measurements on *M. aliceae* due to the extremely small quantity of nectar present in the flowers.

In order to assess whether nectar production in *M. venosum*, *M. capense* and *M. gracile* coincided with pollinator activity, the timing of nectar production of all three species was

determined by monitoring the change in nectar height, volume and concentration over a 24 hour period at four hour intervals. Nectar height measurements were taken from the same 10 flowers at each 4-hourly interval. Nectar volume and concentration measurements required destroying the flowers, therefore measurements had to be obtained from 10 different flowers every 4 hours. The conversion to nectar sugar weight (mg) was done according to Dafni (1992).

Scent Chemistry

Floral scents are made up of complex mixtures of volatile organic substances, and a wide range of compounds have been detected in them (Knudsen and Tollsten 1993, Kaiser 1993, Proctor *et al.* 1996). In order to determine which species of *Mystacidium* were scented, scent production was assessed qualitatively by sniffing several inflorescences at regular intervals throughout the day. In order to collect the scent of *M. venosum, M. gracile, M. capense,* and *M. pusillum* a trapping technique, which involves sorption on charcoal followed by solvent extraction (Kaiser 1993), was applied to flowers collected from the Verulam site, Mbona Mountain Estate, Bishopstowe and Harold Johnson Nature Reserve respectively. The whole inflorescence of a number of plants of the same species was placed in a glass vessel, and the scented air surrounding the flowers was drawn through the adsorption trap by a battery operated pump. The scent of *M. venosum* was collected on 23 June 1999 between 17h30 and 19h30 at a flow rate of 144.5 ml/min, and again on 5 July 1999 from 17h45 to 22h30 at a flow rate of 194.5 ml/min. The scent of *M. gracile* was collected on 18 October 1999 from 18h30 to 04h30 at a flow rate of 184.99 ml/min. The scent of *M. capense* was collected on 3 June 2000 from 17h30 to 22h30 at a flow rate of 191.67 ml/min. The scent of *M. pusillum* was collected on 9 July 2000 between 18h25 and 06h25 at a flow rate of 208.98 ml/min, and on 29 June 2001 a second sample was collected from 18h00 to 21h30 at a flow rate 190.59 ml/min. The traps, which contain a thin layer of 5 mg of charcoal embedded between two grids fused into the wall of a glass tube, were placed within the glass vessel. The traps were then sent to Dr Kaiser, where the adsorbed scent was recovered from the traps by extraction with 10-50 µl of carbon disulfide, and investigated by GC/MS. Scents with extremely polar compounds require further extraction with 10-50 µl ethanol for complete recovery.

Selection on Floral Spur Length

Studies were conducted to establish the relationship between spur length and pollination success in *M. venosum*, *M. capense* and *M. gracile*. The spur length of flowers of each

species was measured at the relevant sites, and the flowers were examined for the presence of pollinia in the stigmatic cavity and for removal of pollinaria from the anther. In December 2000, 60 inflorescences of *M. capense* were randomly selected from the Lion Park, and 30 from Bishopstowe. All flowers were checked for pollinaria removal or receipt to ensure that they had not previously been visited. The average spur length of each inflorescence was recorded. Randomly selected flowers from each inflorescence were treated in the following way: the spur of one flower was cut off at the tip; the spur of a second flower was artificially shortened to 2 cm from the entrance using duct tape; and the spur of a third flower was artificially shortened to 3 cm from the entrance using duct tape. A fourth flower was selected as a control and left unmanipulated.

Reproductive Success

In order to determine whether fruit set is limited by pollen receipt in *M. capense*, 94 flowers of five randomly selected plants at Bishopstowe were hand pollinated in December 1999. Plants with a similar number of flowers as each of the experimental plants were selected as controls, and the flowers were left unmanipulated. After approximately three months, the percentage fruit set of each experimental plant and that of the control plants was compared. This experiment was repeated in the following season in December 2000 at the Lion Park site, where 105 flowers of five plants were hand pollinated. Treatment × site effect were examined using two-way Analysis of Variance (Zar 1984).

Flower and Pollen Longevity

An experiment was carried out in the greenhouse to determine the effect of pollinaria removal and pollination on the subsequent lifespan of flowers of *M. venosum*. Treatments were implemented at the time of flower anthesis, and included : (1) Unmanipulated flowers (control); (2) Removal of both pollinaria only; and (3) Pollination using pollinia from a separate plant. The flowers were monitored every day until the end of the flower lifespan, which was marked by the closing or "collapse" of the corolla.

To determine how long pollen can remain viable for under outdoor conditions, pollinaria were removed from flowers of *M. venosum* at flower anthesis and stored attached to insect pins after recording the date. The insect pins carrying the pollinaria were kept upright on a polystyrene board which was placed in an open box. The box was covered with insect netting and left outdoors to approximate field conditions. Flowers 1, 5 and 10

days old were cross-pollinated by hand using either 1, 5, 10, 15 or 20 day old pollinia. Percentage fruit set and the percentage of seeds with embryos was recorded ± 5 months later when the fruits had fully matured.

RESULTS

Breeding System Experiments

All the species of *Mystacidium* are clearly dependent on pollinators for fruit set, as no fruits were produced by bagged unmanipulated flowers (Table 4). It was not possible to compare fruit and weight dimensions and seed viability of cross- and self-pollinated flowers of *M. aliceae*, *M. flanaganii* and *M. pusillum* as those flowers that set fruit were aborted before the fruits reached maturity. The final fruit set of experimental cross-pollinations was higher in all species except *M. brayboniae*, however χ^2 tests indicated that the differences between fruit set of cross- and self-pollinated flowers were not significant. However, significant differences in fruit dimensions and fruit weight between selfed and outcrossed flowers of *M. brayboniae, M. capense, M. venosum* and *M. gracile* (Table 4) do suggest that *Mystacidium* may suffer from some degree of inbreeding depression when self-pollinated. *Mystacidium* can therefore be described as being only partially self-compatible.

Pollinator Observations

A hawkmoth, identified as *Nephele accentifera accentifera* (de Beauvois),was caught whilst foraging the flowers of *M. venosum,* at 17h24 on 9 June 1998. Twenty pollinaria were attached to the proboscis which was 6.15 cm long. A hawkmoth was also captured whilst feeding from flowers of *M. venosum* at Baynesfield on 8 June 1999 at 17h30. There were no pollinaria attached to the 5.1 cm long proboscis. (See appendix II for details of hawkmoth activity).

A noctuid moth, identified as a species of *Cucullia* (Shrank), carrying pollinaria of *M. gracile,* was caught near the light trap at the Bulwer site at approximately 20h00 on 16 October 1999. Two pollinaria were attached along the length of the moths's proboscis, which was 3.5 cm long.

Table 4. Results of experiments to determine the breeding system of *Mystacidium*. #Fruit set differed significantly among treatments for all species, except *M. brayboniae*. However fruit set in selfed and outcrossed flowers does not differ significantly for any species (χ^2 test).

Species	Treatment	Flowers (n)	Fruit# set (%)	Average mass of fruit (mg ± SD)	Average fruit length (mm ± SD)	Average fruit width (mm ± SD)	Seeds with embryos (% ± SD)
M. brayboniae	Unmanipulated	8	0	0	0	0	0
	Selfed	7	86	0.18 ± 0.01	1.5 ± 0.08	0.7 ± 0.02	55.8 ± 3.8
	Outcrossed	7	71	0.28 ± 0.02*	1.8 ± 0.08*	0.8 ± 0.03	98.8 ± 0.7*
M. gracile	Unmanipulated	10	0	0	0	0	0
	Selfed	10	35	0.0001	6.6 ± 0.07	1.5 ± 0.08	35 ± 2.36
	Outcrossed	10	60	0.0001	7.2 ± 0.05*	2.1 ± 0.03*	96 ± 2.92*
M. capense	Unmanipulated	20	0	0	0	0	0
	Selfed	20	35	0.05 ± 0.002	13.5 ± 0.23	4.1 ± 0.07	44.3 ± 4.9
	Outcrossed	20	60	0.15 ± 0.002**	15.9 ± 0.11**	5.1 ± 0.08**	96.2 ± 1.74**
M. venosum	Unmanipulated	20	0	0	0	0	0
	Selfed	20	40	0.05 ± 0.01	11.8 ± 0.7	4.0 ± 0.1	37.1 ± 7.6
	Outcrossed	20	65	0.11 ± 0.01*	14.0 ± 0.6*	4.1 ± 0.1	99.0 ± 0.5**
M. aliceae	Unmanipulated	10	0	-	-	-	-
	Selfed	10	10	-	-	-	-
	Outcrossed	10	40	-	-	-	-
M. flanaganii	Unmanipulated	10	0	-	-	-	-
	Selfed	10	40	-	-	-	-
	Outcrossed	10	60	-	-	-	-
M. pusillum	Unmanipulated	10	0	-	-	-	-
	Selfed	10	50	-	-	-	-
	Outcrossed	10	60	-	-	-	-

*$P < 0.05$, **$P < 0.001$ (Mann Whitney U test comparing selfed and outcrossed treatments).

A noctuid moth carrying pollinaria of *M. pusillum* was caught in the light trap, which was left to run overnight on 12 July 2000, at Harold Johnson Nature Reserve. Four pollinaria were attached along the length of the moth's proboscis which was 2.9 cm long. The state of the moth made identification difficult, but it was possible to place it in the subfamily Plusiinae.

Two individual hawkmoths were observed foraging on flowers of *M. capense* at Bishopstowe on 12 December 2000 at 19h05 and at 19h16. Each moth visited up to 40 flowers in succession for approximately four seconds. Three hawkmoth foraging bouts on

M. capense were observed at Bishopstowe in December 2001 by S.D. Johnson (personal communication).

Despite frequent observations for pollinator activity, very few pollinators were caught. The low incidence of fruit set in 2000/2001 of *M. capense* at Bishopstowe (3.2 %) and at the Lion Park (2.7 %), and the fact that there was no fruit set of *M. pusillum* in 2001 at Harold Johnson Nature Reserve, probably reflects the low levels of pollinator activity at these sites in the 2000/2001 season.

No floral visitors were observed during two hours of evening observations of *M. tanganyikense* in Malawi by S. D. Johnson (personal communication).

Spur Length and Nectar Measurements

The only species of *Mystacidium* with spurs longer than 3 cm in length are *M. venosum* and *M. capense* (Table 5). The shorter-spurred species have spur lengths ranging from less than 1 cm to 2.56 cm, and *D. millarii* and *D. caffra* have spurs less than 2 cm long (Table 5).

The nectar sugar proved to be sucrose dominant in all the species sampled (sucrose / glucose + fructose > 0.999, Baker and Baker 1983). Amino acid content, as well as nectar sugar, was most concentrated in *M. flanaganii, M. gracile* and *M. pusillum*, and less so in the longer-spurred species (Table 4). The results obtained for *M. brayboniae* may be questionable as they were measured from plants cultivated in a greenhouse. The average nectar volume and concentration measurements of *M. brayboniae* taken in the morning and afternoon varied by only 0.01 µl and 0.3 % respectively, and were therefore combined to give final mean values.

Mystacidium capense and *M. venosum* exhibited very similar patterns in nectar fluctuation. Nectar volume, as well as the sugar concentration and sugar content slowly increased throughout the day, and began to peak from late afternoon until 20h00. From 20h00 to the early hours of the morning, nectar appeared to be reabsorbed (Figures 3.1 and 3.2. Also see appendix I). On the other hand, nectar properties of *M. gracile* remained fairly constant over a 24 hour period, peaking very slightly from the early evening until 04h00 (Figure 3.3).

Table 5. Average spur lengths and nectar properties of flowers of *Mystacidium*, *D. caffra*, *D. millarii* and *C. arcuata*. Means are followed by standard deviations. Values in brackets indicate sample size.

Species	Spur length (cm)	Nectar			
		Volume (µl)	Concentration (%)	Sugar ratio (fructose: glucose:sucrose)	Amino acid concentration (µmol/ml)
M. aliceae	0.93 ± 0.01 (30)	< 0.1	-	-	-
M. braybonіae	1.94 ± 0.04 (50)	0.59 ± 0.04 (50)	11.42 ± 0.42 (50)	2:0:98 (2)	0.34 (2)
M. capense	3.91 ± 0.04 (139)	2.51 ± 0.41 (30)	15.96 ± 0.87 (30)	8.5:3.5:88 (2)	0.49 (2)
M. flanaganii	1.96 ± 0.01 (35)	0.24 ± 0.02 (25)	23.60 ± 0.40 (25)	23:19:58 (1)	1.36 (2)
M. gracile	2.56 ± 0.05 (67)	0.77 ± 0.04 (20)	18.25 ± 0.37 (20)	14:15:71 (1)	1.56 (2)
M. pusillum	2.04 ± 0.01 (30)	0.20 ± 0.01 (30)	21.46 ± 0.50 (30)	20:24:56 (1)	1.56 (2)
M. venosum	4.70 ± 0.30 (70)	1.80 ± 0.95 (22)	16.00 ± 2.90 (22)	10:3:87 (2)	0.49 (2)
M. tanganyikense	1.68 ± 0.30 (24)	0.29 ± 0.02 (11)	-	12:8:80 (1)	-
D. caffra	1.40 ± 0.01 (30)	0.40 ± 0.02 (30)	14.86 ± 0.5 (30)	10:9:81 (1)	0.29 (2)
D. millarii	1.81 ± 0.01 (25)	0.50 ± 0.20 (15)	13.42 ± 0.6 (15)	-	-
C. arcuata	3.3 ± 0.3 (25)	2.1 ± 0.8 (25)	16.65 ± 2.60 (25)	-	-

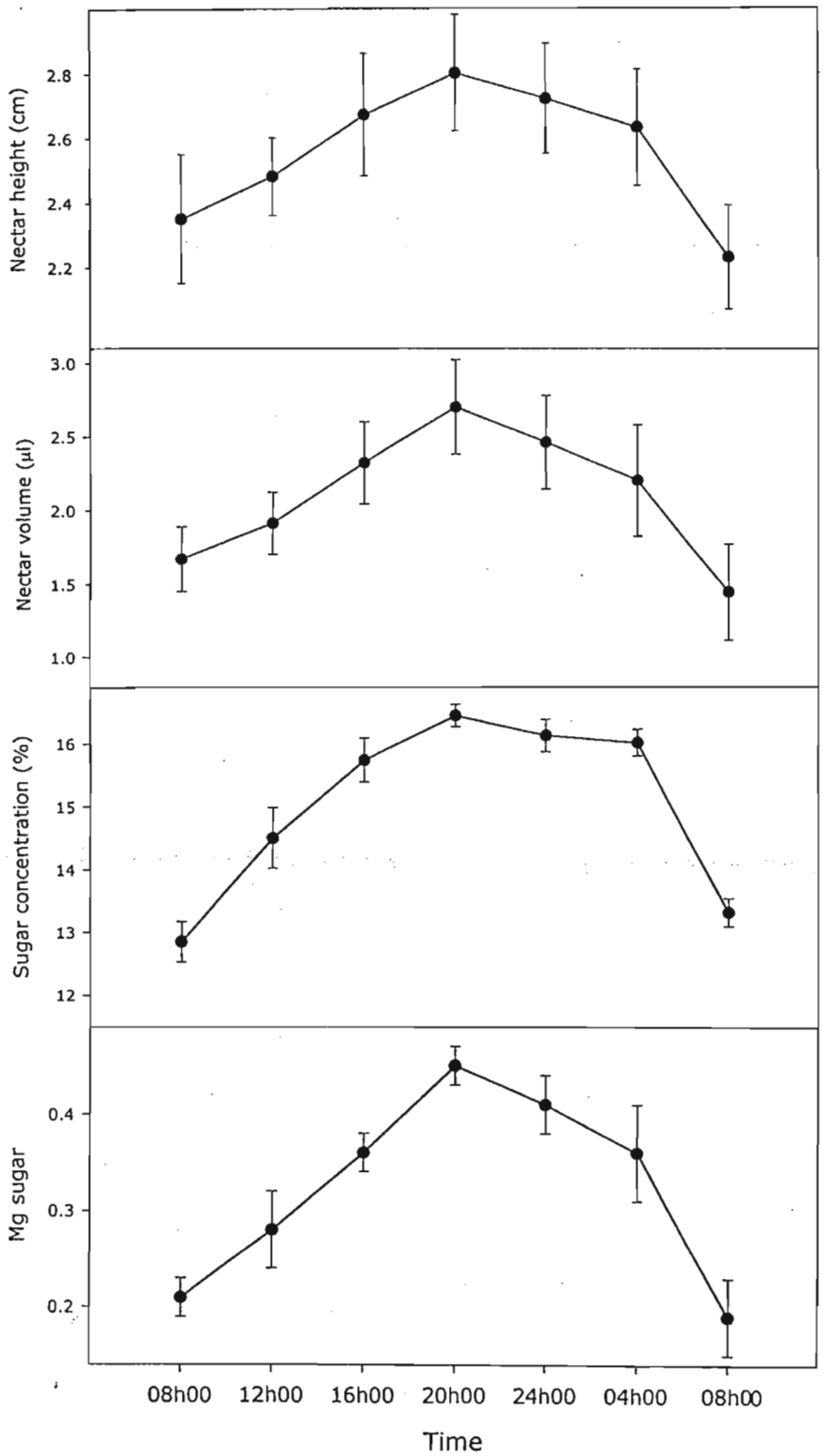


Figure 3.1. Fluctuation of nectar properties over 24 hours in flowers of *M. capense*. Error bars = standard deviation.

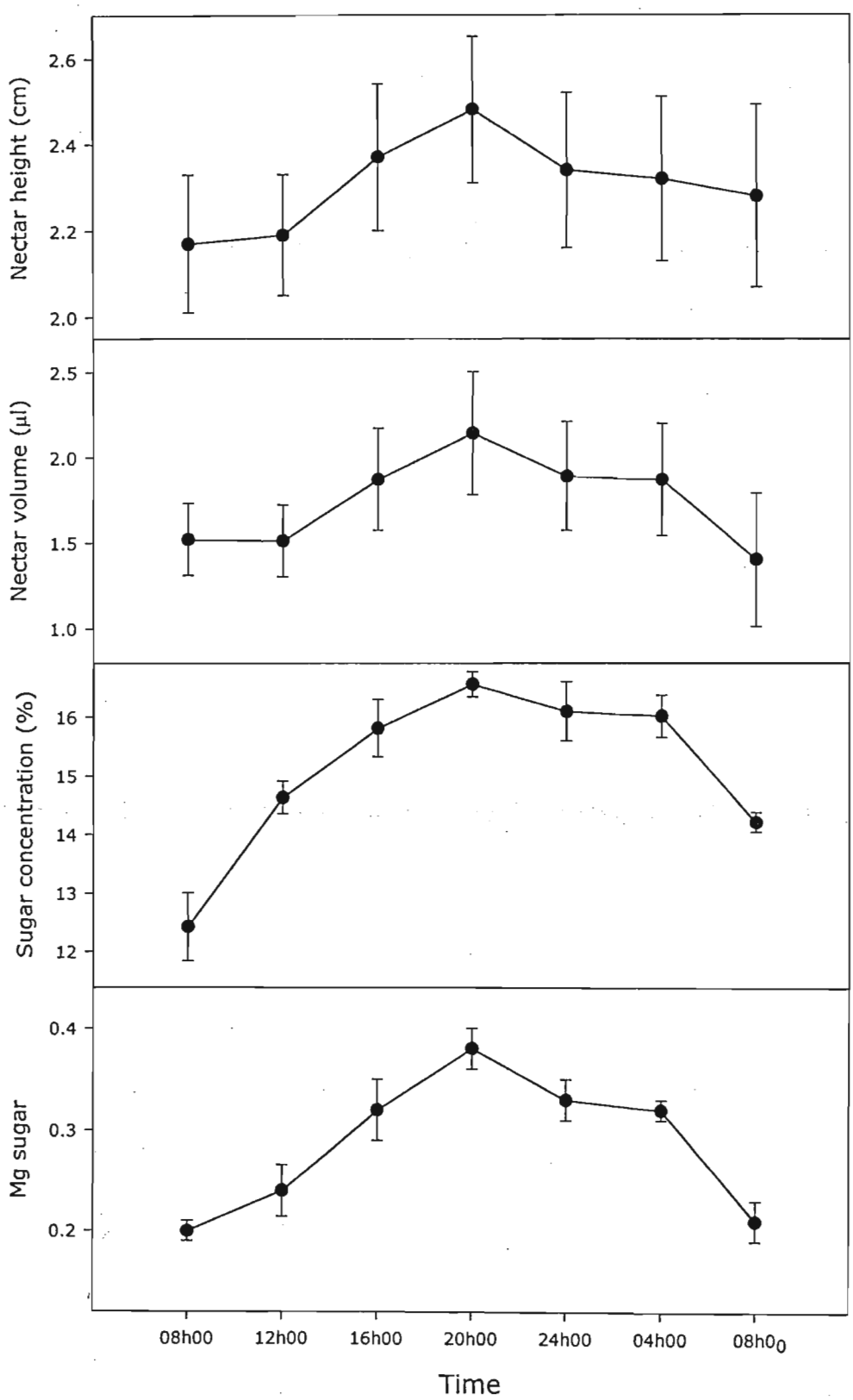


Figure 3.2. Fluctuation of nectar properties over 24 hours in flowers of *M. venosum*. Error bars = standard deviation.

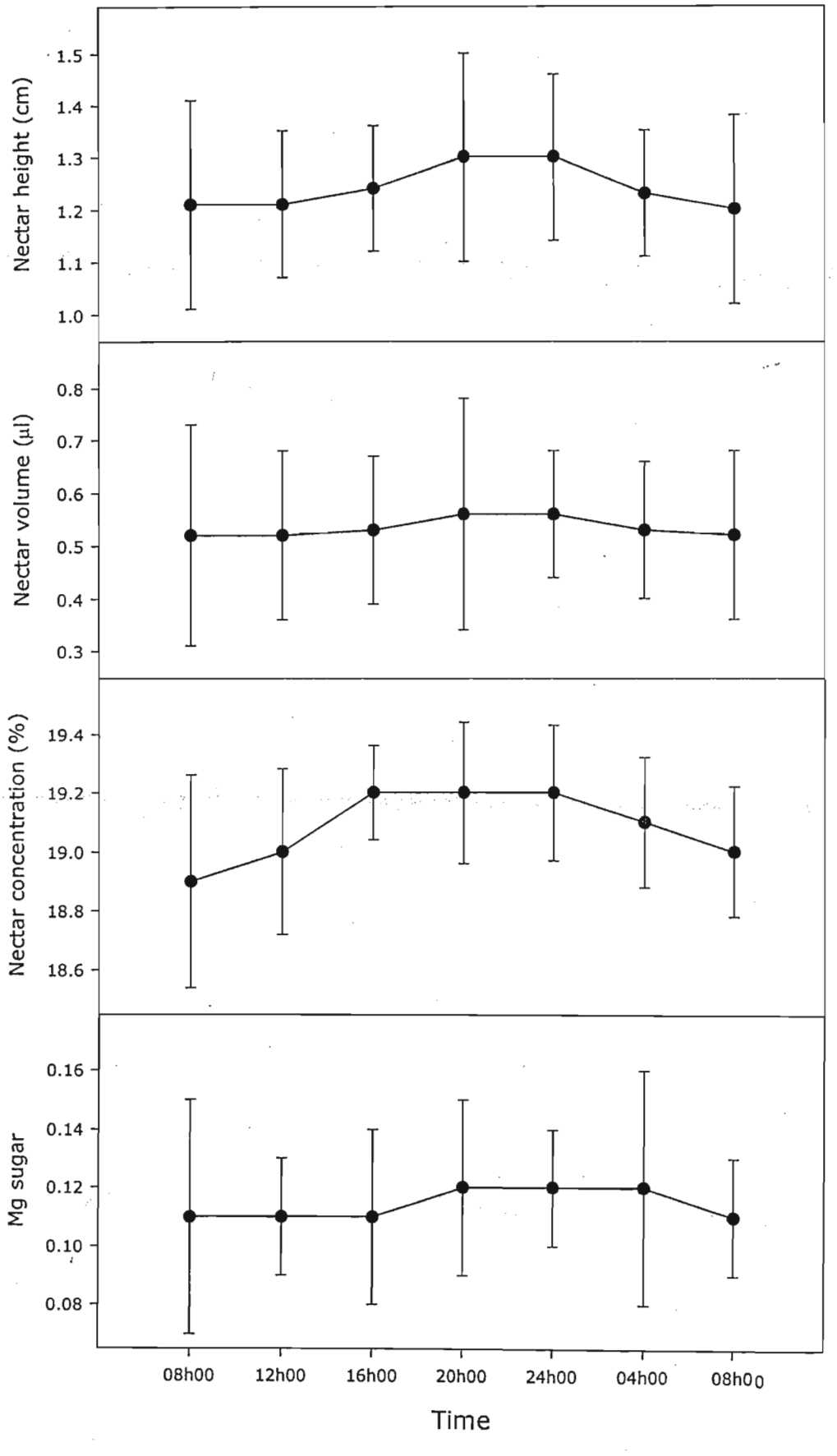


Figure 3.3. Fluctuation of nectar properties over 24 hours in flowers of *M. gracile*. Error bars = standard deviation.

Scent Chemistry

Nocturnal emission of scent occurred in all species except *M. alicea, M. braybonіae* and *D. caffra*, which were apparently unscented. The flowers of *M. capense* and *M. venosum,* both pollinated by hawkmoths, were heavily scented from dusk until about 21h00, producing a very sweet, jasmine-like fragrance. The scent of the noctuid-pollinated species, *M. gracile* and *M. pusillum*, remained intense from dusk until about 22h00. The scent of both species was also very sweet, but not jasmine-like, and the fragrance emitted by *M. gracile* could be described as having a spicy undertone. Curiously, out of four separate populations of *M. flanaganii,* only a single plant was found to emit a subtle yet sweet scent in the evening.

The scent analyses identified a variety of volatile compounds, which included representatives of the following biosynthetic classes : isoprenoids (monoterpenoids and sesquiterpenoids), fatty acid derivatives, benzoids, phenylpropanoids, nitrogen-bearing compounds, and lactones (Table 6). Monoterpenes and benzoids were common constituents in all the species sampled, with the hydrocarbon monoterpene α-Pinene, and benzaldehyde (benzoid) present in all four species. The monoterpene that occurred in the largest amount was linalool, an acyclic oxygenated monoterpene, constituting 33% and 5% of the fragrances emitted by *M. gracile* and *M. venosum* respectively. Oxygenated monoterpenes were present in all the species except *M. capense*, and were the major constituents of the scent chemistry of *M. gracile*, making up 33.6% of the total fragrance collected, with linalool being the dominant volatile compound. Benzoids constituted a large proportion of the scent chemistry of *M. gracile* (23%), particularly benzaldehyde (8%) and the aromatic ester benzyl benzoate (9%). The scent chemistry of *M. pusillum* was dominated by benzoids, with benzaldehyde, benzyl alcohol, and the aromatic ester benzyl acetate constituting 39.4% and 60.3% of the two samples collected. Benzoids were present in comparitively low amounts in the fragrances of the hawkmoth pollinated species *M. venosum* and *M. capense*. Sesquiterpenes were found only in the scent of *M. venosum* (constituting 10% and 15% of the fragrance). Lactones, which were absent in the noctuid-pollinated species *M. gracile* and *M. pusillum*, dominated the scent of the two hawkmoth pollinated species. Jasmine lactone constituted 75% of the fragrance of *M. capense*, and between 65% and 67.8% of that of *M. venosum*. Even though fatty acid derivatives were found in the scent chemistry of all the species examined, they were present in very low amounts, especially in the hawkmoth-pollinated species *M. venosum* and *M. capense* (making up less than 1% of the fragrance). Phenyl propanoids were also major constituents in the scent of *M. gracile* (33.1%), with the major phenolic compounds being Eugenol (19%) and (E)-cinnamic alcohol (12.5%). Eugenol and vanilline were minor constituents of the scent of *M. capense*. Nitrogen-containing compounds were found in low amounts, and were absent in the scent of *M. capense* and *M. pusillum*.

Table 6. Chemical composition of the floral scent of four *Mysatcidium* species. Total amount and relative amounts (in %) of floral volatiles emitted by *M. venosum* (M.ven 1 and 2), *M. capense* (M.cap), *M. gracile* (M.gra) and *M. pusillum* (M.pus 1 and 2).

Compound	M.ven 1	M.ven 2	M.cap	M.gra	M.pus 1	M.pus 2
Isoprenoids						
Monoterpene hydrocarbons/irregular terpenes						
α-Pinene	0.2	-	0.1	0.1	0.5	0.5
β-Pinene	-	-	0.1	0.05	0.4	0.2
Myrcene	0.1	-	-	0.1	-	-
Limonene	0.3	1.2	0.1	0.4	-	-
(E)-Ocimene	-	1.0	-	3.5	-	-
Sabinene	-	-	-	-	2.0	1.9
6-Methyl-5-hepten-2-one	-	-	0.05	0.2	-	-
(E)-Geranylacetone	-	-	0.2	-	-	-
sum	**0.6**	**2.2**	**0.55**	**4.35**	**2.9**	**2.6**
Oxygenated monoterpenes						
Geraniol	-	1.3	-	0.3	-	-
Eucalyptol	-	-	-	0.1	-	-
Linalool	5.0	0.4	-	33.0	1.5	3.5
trans-Linalool oxide (Furanoid)	0.1	-	-	0.1	-	-
cis-Linalool oxide	-	-	-	0.1	-	-
sum	**5.1**	**1.7**	-	**33.6**	**1.5**	**3.5**
Oxygenated sesquiterpenes						
(E, E)-Farnesol	12.5	7.5	-	-	-	-
(E)-Nerolidol	2.5	3.2	-	-	-	-
sum	**15.0**	**10.7**	-	-	-	-
Fatty acid derivatives						
Octanol	-	-	-	0.2	-	-
Heptanal	-	-	-	0.05	-	-
Octanal	-	-	-	0.2	0.3	0.3
Nonanal	-	-	0.2	0.2	0.6	0.3
Decanal	-	-	-	0.3	0.4	0.2
(Z)-3-Hexanol	-	0.1	-	-	-	-
(Z)-3-Hexenyl acetate	-	0.1	-	-	-	-
(E)-2-Hexanal	-	-	-	-	0.2	0.1
Hexanal	-	-	-	0.1	-	-
(Z)-3-Hexenyl tiglate	-	-	-	-	0.3	0.1
sum	-	**0.2**	**0.2**	**1.05**	**1.8**	**1.0**
Benzoids						
Benzaldehyde	-	0.5	0.1	8.0	11.0	16.9
Benzyl acetate	0.2	0.3	-	0.2	28.0	23.0
Benzyl alcohol	0.5	-	-	2.5	0.4	20.4
Phenylethyl alcohol	0.3	0.5	2.0	0.1	-	-
Methyl salicylate	-	-	-	0.2	-	-
Benzyl tiglate	-	-	-	0.3	-	-
Benzyl benzoate	-	-	-	9.0	-	-
Phenylacetaldehyde	-	-	2.05	-	-	-
Phenol	-	-	-	0.1	-	-
Para-cresol	-	-	-	2.6	-	-
sum	**1.0**	**1.3**	**4.15**	**23.0**	**39.4**	**60.3**
Phenyl propanoids						
Eugenol	-	-	-	19.0	-	-
(E)-Isoeugenol	-	-	1.0	-	-	-
Vanillinie	-	-	0.03	0.3	-	-
Creosol	-	-	-	0.1	-	-
Cinnamic aldehyde	-	-	-	0.8	-	-
3-Phenylpropanol	-	-	-	0.05	-	-
Methyl (E)-cinnamate	-	-	-	0.05	-	-
(E)-cinnamyl acetate	-	-	-	0.3	-	-
(E)-cinnamic alcohol	-	-	-	12.5	-	-
sum	-	-	**1.03**	**33.1**	-	-
Nitrogen bearing compounds						
Methyl nicotate	0.2	-	-	-	-	-
Phenylacetonitrile	-	2.7	-	-	-	-
Indole	0.7	1.5	-	3.0	-	-
sum	**0.9**	**4.2**	-	**3.0**	-	-
Lactones						
Jasmine lactone	65.0	67.8	75.0	-	-	-
Delta octalactone	-	0.7	0.1	-	-	-
Delta decalactone	1.5	0.5	0.3	-	-	-
sum	**66.5**	**69.0**	**75.4**	-	-	-
Total	89.1	89.3	81.78	98.1	45.6	67.4

Selection on Floral Spur Length

There was no evidence for directional selection on spur length in either *M. venosum*, *M. capense* or *M. gracile*. Analysis using a normal approximation to the Mann Whitney U test (Zar 1984), indicated that the spur lengths of pollinated and unpollinated flowers were not significantly different in any of the three species (Table 7).

Fruit set was particularly low in 2000 at both the Lion Park site (2.7%) and at Bishopstowe (3.2%), and as a result it was not possible to obtain results from the experiments in which the length of the spurs of flowers of *M. capense* at both these sites were artificially manipulated.

Table 7. Relationship between spur length and pollination success in *M. venosum, M. capense* and *M. gracile*

Species	Site	Flowers (*N*)	Spur length (cm) $\bar{x}$ ± S.D.	Spur length of pollinated flowers (cm) $\bar{x}$ ± S.D.	Spur length of unpollinated flowers (cm) $\bar{x}$ ± S.D.	*Z*	*P*
M. venosum	Verulam	70	4.7 ± 0.3	4.73 ± 0.03	4.69 ± 0.14	0.28	NS
	Yellowwood	115	4.6 ± 0.7	4.40 ± 0.2	4.69 ± 0.6	0.26	NS
	Baynesfield	30	4.4 ± 0.3	4.30 ± 0.04	4.30 ± 0.16	0.61	NS
M. capense	Ixopo	109	3.9 ± 0.3	3.82 ± 0.21	3.91 ± 0.29	0.35	NS
M. gracile	Bulwer	47	2.7 ± 0.1	2.75 ± 0.04	2.72 ± 0.1	0.66	NS

Reproductive Success

Pollinaria removal and pollinia receipt can be used as a measure of male and female reproductive success (Nilsson 1992). Pollinia insertions were less frequent than pollinaria removals in all three species (Table 8). However, the percentage of pollinaria removed that reached stigmas was comparatively high (35-52%).

Hand pollinations significantly increased fruit set in populations of *M. capense* in 1999 at Bishopstowe and in 2000 at the Lion Park site (Bishopstowe: $Z = 0.03$, $P < 0.05$; Lion Park: $Z = 0.03$, $P < 0.05$; Figure 2.4). Further analysis with a two-way ANOVA revealed that there was a significant difference in the results from the two sites and that the difference between hand- and natural pollinations was highly significant (Table 9). Failure of outcrosses by hand to reach 100% fruit set may be related to resource partitioning or limitation.

Table 8. Reproductive success of *M. venosum*, *M. capense* and *M. gracile*. *N* = number of flowers observed.

Species	Site	Flowers with pollinia on stigmatic cavity (%)	Flowers with at least one pollinarium removed (%)	*Removed pollinia reaching stigmatic cavity (%)
M. venosum	Verulam (*N* = 66)	32	67	-
	Yellowwood (*N* = 111)	23	40	49.2
M. capense				
	Ixopo (*N* = 109)	19	20	52.1
M. gracile				
	Bulwer (*N* = 47)	9	21	35.3

*Total number of pollinaria removed divided by the total number of pollinated flowers in a population

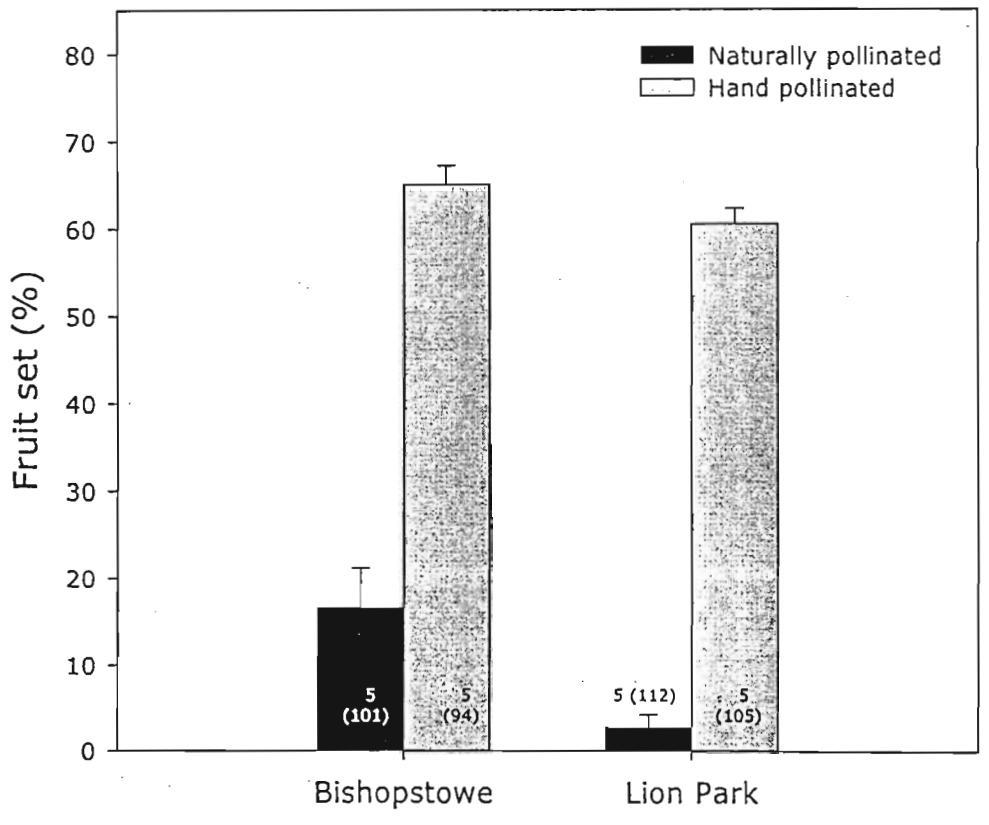


Figure 3.4. Difference in fruit set of hand- and naturally-pollinated plants of *M. capense* at Bishopstowe and at the Lion Park site. Numbers refer to number of plants. Numbers in brackets refer to number of flowers.

Table 9. Two way ANOVA of fruit set of hand- and naturally pollinated plants of *M. capense* at Bishopstowe and at the Lion Park

Source of variation	*F*	*df*	*P*
Site	8.452	1	0.01
Treatment	290.611	1	0.00
Site × treatment	1.366	1	0.26

Flower and Pollen Longevity

Flower longevity of *M. venosum* was significantly affected by both pollinaria removal and pollinia insertion (See appendix II). The average lifespan (± S.D.) of an unmanipulated flower was 24 days (± 0.7). Pollination shortened the flower lifespan to an average of 5 days (± 0.2), while the effect of pollinaria removal was weaker, shortening the flower lifespan to 19 days (± 1.6). The estimated lifespan of unvisited flowers of *M. aliceae, M. brayboniae, M. gracile, M. flanaganii* and *M. pusillum* was between 16 and 18 days, and approximately 25 to 28 days in *M. capense*.

Pollen of *M. venosum* remains fully viable for up to 10 days, and then appears to deteriorate. Nevertheless, some 20 day old pollinaria kept under outdoor conditions produced fruits with 80% seed set (See appendix II). Flower age (up to 10 days) did not appear to have any influence on fruit set.

DISCUSSION

Mystacidium generally shows a suite of floral adaptations to moth pollination. The flowers are white or pale green, nectar is concealed in spurs, and fragrance emission occurs in the evening in all species except *M. brayboniae* and *M. aliceae*. These characters are consistent with the syndrome of moth-flowers proposed by Faegri and van der Pijl (1979).

The essential distinction between flowers pollinated by settling and hawkmoths, respectively, is in the length of the nectary or spur. Flowers pollinated by settling moths generally have nectar concealed at a functional flower depth of less than 3 cm, whereas the nectar of flowers with a functional depth greater than 3 cm is available exclusively to long-tongued hawkmoths (Knudsen and Tollsten 1993). The length of the spurs of *M. venosum* and *M. capense* are 4.7 cm and 3.91 cm respectively, whereas the spur lengths of *M. tanganyikense, M. flanaganii, M. pusillum* and *M. gracile* range from 1.68 cm in *M. tanganyikense* to 2.56 cm in *M. gracile* (Table 5). The floral features of *M. venosum* and

M. capense are consistent with many other hawkmoth-pollinated orchids in Madagascar (Nilsson *et al.* 1985, Nilsson *et al.* 1992), and the results of this study show that these features directly indicate specialization to hawkmoth pollination. This was confirmed by observations of hawkmoths feeding on the flowers of both species, and by the capture of a hawkmoth carrying pollinaria of *M. venosum*. Despite frequent observations of hawkmoths foraging on the flowers of *M. venosum*, catching them proved to be difficult as the flowers grow high up in the canopy. Similarly, the hawkmoths observed feeding on flowers of *M. capense* were also out of reach. Light-trapping proved ineffective, possibly because hawkmoth activity occurred mainly at dusk when natural light levels were still relatively high. The fact that hawkmoths were rarely seen while observing the flowers of *M. capense* is consistent with the low incidence of fruit set in 2000/2001. The characteristics of *M. gracile, M. pusillum* and *M. tanganyikense* clearly represent adaptations to pollination by settling moths. Although pollinators of *M. tanganyikense* were not observed, the prediction that *M. gracile* and *M. pusillum* conform to the "syndrome" of settling-moth flowers proposed by Faegri and van der Pijl (1979) was confirmed in this study. A settling moth carrying pollinaria of *M. gracile*, and one carrying pollinaria of *M. pusillum* was caught. Again, despite many hours spent observing the plants for pollinators, and running a light trap to attract them, only the two settling moths were caught. The spring-flowering species, *M. gracile*, grows in mistbelt forest, therefore the wet weather conditions were rarely optimal for observing moths. In *M. pusillum*, there was no fruit set (80 plants examined) at the site of observation in 2001, which might explain why no pollinators were observed in that season. Yet, even though the observations of pollinators were rare, they are nevertheless consistent with the prediction that *M. venosum* and *M. capense* are hawkmoth-pollinated, and that *M. gracile* and *M. pusillum* are pollinated by settling moths.

The nectar of *Mystacidium* is sucrose dominant. The sucrose-dominant nectar of *M. venosum, M. capense, M. gracile* and *M. pusillum* corroborates other findings that the nectar of moth-pollinated flowers is sucrose rich or dominant (Baker and Baker 1982, Kevan and Baker 1983). The relatively dilute sugar concentration of the nectar of *M. venosum* (16 %) and *M. capense* (15.96 %) is slightly lower than the mean values of 19 % and 21.3 % reported by Pyke and Waser (1981) and Cruden *et al.* (1983), respectively, but is consistent with studies of *Aerangis ellisii* (Nilsson and Rabakonandrianina 1988) and *Angraecum arachnites* (Nilsson *et al.* 1985), which are hawkmoth-pollinated and had nectar concentrations of 16 % and 13.3 % respectively. The slightly more concentrated nectar of *M. gracile* (18.25 %), *M. flanaganii* (23.6 %) and *M. pusillum* (21.46 %) is consistent with the mean sugar concentration of nectar of settling moth flowers reported by Pyke and Waser (1981) and Baker and Baker (1983), which were 22 % and 18 % respectively. The nectar concentration of *M. brayboniae*

(11.42 %) reported here is likely to be inaccurate as measurements were taken from plants grown in a greenhouse. Cruden and Hermann (1983) found that the nectar of plants of *Hippobroma longiflora* grown in a greenhouse was half the concentration of the nectar of plants in a natural habitat. According to Baker and Baker (1982) and Kevan and Baker (1983), nectars of flowers visited by settling moths are richer in amino acids than the nectars of those pollinated by hawkmoths. Amino acid concentration in the nectar of *M. venosum* and *M. capense* would therefore be expected to be lower than that of the shorter-spurred species. The amino acid concentration in the nectar of both *M. venosum* and *M. capense* (0.49 µmol/ml) was lower than that of *M. gracile* (1.56 µmol/ml), *M. pusillum* (1.56 µmol/ml) and *M. flanaganii* (1.36 µmol/ml). These values are very close to those reported by Baker and Baker (1983) for the nectar of hawkmoth and settling moth-pollinated flowers, which were 0.54 µmol/ml and 1.06 µmol/ml respectively. Although hawkmoth flowers generally produce relatively low amino acid concentrations, hawkmoths imbibe large quantities of nectar in order to obtain adequate sugar to support costly hovering flight. Hawkmoth flowers have therefore apparently evolved a low amino acid concentration to keep amino acids taken in by the moths below a toxic level (Boggs 1987).

A flower generally begins to secrete nectar a few hours prior to the onset of pollinator activity, since a reasonable reward encourages repeat visits by a pollinator (Cruden *et al.* 1983) This is evident in both *M. capense* and *M. venosum* (Figures 3.1 and 3.2). Both species begin to secrete nectar from about 15h00, approximately 2 - 4 hours before hawkmoth activity. Cessation occurred once nectar production had reached a maximum at 20h00. The decrease in the quantity of nectar sugars from 20h00 demonstrates that nectar is reabsorbed (Refer to appendix I). The nectar dynamics of *M. venosum* and *M. capense* therefore suggest specialization for pollination early in the evening, and qualitative assessment of scent supports this, as scent production in both species was at its maximum from dusk until approximately 21h00. In contrast to hawkmoth activity which occurs for a brief period at dusk, noctuid moths are active and feed from dusk until late at night, and sometimes throughout the night. The nectar dynamics of *M. gracile* (Figure 3.3) show that the sugar content of the nectar increases slightly from 16h00 until 04h00 and that nectar volume remains fairly constant over a 24 hour period. This indicates that nectar is probably secreted prior to moth activity, and at a constant rate throughout the night, while settling moths remain active through the night. The very slight decrease and high variation in nectar volume, concentration and sugar content after 04h00 suggests that cessation of nectar production occurs diurnally as opposed to reabsorption as in the case of *M. venosum* and *M. capense*. The scent of *M. gracile* (assessed qualitatively) is at a maximum from 18h00 until 22h00, which also suggests adaptation to nocturnal pollination.

According to Kaiser (1993), moth-pollinated flowers generally have a "white flower" scent which is characterised by acyclic terpene alcohols such as linalool and farnesol, the corresponding hydrocarbons, and simple aromatic alcohols such as benzyl alcohol and phenylethyl alcohol, and their esters. The fragrances of *M. venosum, M. capense, M. pusillum* and *M. gracile* were similar in containing isoprenoids, benzoids and fatty acid derivatives, and the general trend in the composition of the floral scent of all these species was consistent with the basic framework for the scent of moth-pollinated flowers presented by Kaiser (1993).

The composition of moth-pollinated flowers often varies between a combination of benzoid compounds and compounds of isoprenoid origin, with either the benzoids or the terpenes being dominant (Kaiser, personal communication). For example, *Nicotiana sylvestris* is dominated by benzyl alcohol (Loughrin *et al.* 1990), and *Lonicera caprifolium* is dominated by linalool (Joulain 1986). The benzoids present in the scent composition of the species sampled in this study, such as benzaldehyde and benzyl alcohol, are common constituents of floral fragrances (Kaiser 1993, Knudsen *et al.* 1993), and aromatic esters such as benzyl acetate and methyl salicylate are common components in the scent blend of moth-pollinated flowers (Knudsen and Tollsten 1993). Linalool, reported here as the dominant compound in the scent of *M. gracile*, and also present in the scent of *M. venosum* and *M. pusillum*, is a monoterpene which is a universal constituent of floral scents (Knudsen and Tollsten 1993). Geraniolic compounds, on the other hand, are much less common monoterpenes and are found especially in *Rosa* species (Knudsen and Tollsten 1993). In this study, geraniol was present, albeit in a small amount, in the floral scent of *M. gracile* which is pollinated by settling moths, whereas Knudsen and Tollsten (1993) reported in their findings that geraniolic compounds were found only in the scent of hawkmoth-pollinated flowers, and were absent in settling-moth pollinated species. The low amounts of fatty acid derivatives present in the scent analyses in this study, corroborate the findings of Knudsen and Tollsten (1993) and Miyake *et al.* (1998), who reported that fatty acid derivatives are generally present in small amounts in the scent of moth-pollinated flowers.

In addition to the similarities in the chemical composition of the fragrances of the species reported here, there are also species-specific compounds, or compounds shared by closely related species that distinguish their floral scents from one another. For example, jasmine lactone was found to be the chief component in the scent chemistry of *M. capense* and *M. venosum*. This lipid metabolite has rarely been recorded as the dominant compound in floral scent, and probably plays an important role in the characteristic sweet scent of the two species (Kaiser, personal communication).The phenolic compounds eugenol and cinnamic alcohol are primarily responsible for the

'spicy-floral' scent of the terrestrial orchid *Gymnadenia conopsea* (Kaiser 1993). The slightly 'spicy' undertone in the scent of *M. gracile* is therefore probably attributable to these two compounds which were present in relatively high concentrations. (E, E) Farnesol occurred only in the hawkmoth-pollinated *M. venosum*. This is consistent with Knudsen and Tollsten's (1993) findings that oxygenated sesquiterpenes were not present in the floral scent of "phalaenophilous" flowers.

Although it is not clear how insects respond to specific floral volatiles, they can distinguish between complex floral scent blends (Pellmyr and Thien 1986, Dudareva and Pichersky 2000). It appears that floral scent is selected by groups of pollinators with affinities for certain scent blends, and that the presence of a combination of compounds such as terpenes and aromatic esters, combined with certain species-specific volatiles that contribute to the nature of each blend, are attractive to moths. The volatile compounds emitted by moth-pollinated flowers have been shown to ellicit search behaviours in hawkmoths, and serve as nectar guides and landing stimuli in settling moths (Brantjes 1973, 1978).

Although particular combinations of floral morphology and scent and nectar properties ("syndromes") have been reported to characterize plants that are serviced by different pollinator groups (Faegri and van der Pijl 1966), flowers of a given syndrome often lack one or more of the expected features (Opler 1983). As Johnson and Steiner (2000) pointed out, phylogenetic constraints on floral design may actually pose a limit on the convergence of floral traits. The traditional view that pollination systems tend toward specialization has been challenged by Waser *et al.* (1996), who claim that pollination systems are more often generalized. According to Chittka (1997), the role of colour in specifying plant pollination systems has been overemphasized, and Waser *et al.* (1996) showed that flower colour alone is not significantly correlated with pollination systems. Johnson and Steiner (2000) emphasize the need for pollination syndromes to be more critically examined using field experiments. As this study shows, *M. venosum, M. capense, M. gracile* and *M. pusillum* clearly conform to the distinctive "moth flower" syndrome, however the syndrome of *M. brayboniae* and *M. aliceae* is more difficult to characterise. The flowers are white and pale green respectively, produce nectar concealed in narrow spurs, but are not scented. *Mystacidium flanaganii* exhibits all the traits indicative of pollination by settling moths, yet it was also found to be scentless (apart from one individual). Observations of pollinators are needed in order to understand the pollination systems of these three species.

The pollinaria of *M. venosum, M. gracile* and *M. pusillum* were found to be attached along the length of the proboscides of the captured moths. This corroborates the suggestion by

Nilsson *et al.* (1992), that close viscidia on a small column results in the placement of pollinaria on the proboscis, whereas viscidia that are well separated on a large column is often indicative of deposition on the eyes of the pollinator. Unlike the orchids that attach polliniaria to the base of the pollinator's proboscis (Nilsson *et al.* 1985, Maad 2000), or to the eyes of the pollinator (Nilsson *et al.* 1992, Johnson and Liltved 1997), it is not essential for the spurs of *M. venosum, M. gracile* or *M. pusillum* to exceed the length of the proboscis for pollination to occur. The mean spur length of *M. venosum* (4.4 - 4.7 cm) was shorter than the pollinia-bearing tongue of the hawkmoth *Nephele a. accentifera*. Similarly, the mean spur lengths of *M. gracile* (2.56 cm) and *M. pusillum* (2.04 cm) were shorter than the pollinaria-bearing proboscides of the settling moths, which were 3.5 cm and 2.9 cm respectively.

It has been suggested that pollen-limitation should lead to selection on characters that influence the probability of pollen receipt (Johnston 1991, Johnson and Steiner 1997). Natural fruit set in *M. capense* in 2000/2001 was 2.7 % and 3.2 % at the Lion Park site and Bishopstowe respectively, and 16.5 % in 1999 at Bishopstowe. Hand pollinations significantly increased fruit set at both sites, indicating high levels of pollen limitation. Studies by Johnson and Steiner (1997) and Nilsson (1988) showed that selection favoured long spurs in the sandplain complex of *Disa draconis* and *Platanthera* respectively. Alexandersson and Johnson (2002) also found that selection favours long spurs in the hawkmoth-pollinated flowers of *Gladiolus longicollis*, which exhibit natural phenotypic variation in flower-tube length. However, studies on the relationship between spur length and pollination success in *M. venosum, M. capense* and *M. gracile* showed that no selection was taking place. Unfortunately, experiments to determine whether spur length affects fruit set in *M. capense* failed due to lack of pollination visits.

According to Johnson and Steiner (2000), selection on floral traits is less likely to occur in plants with generalist pollination systems, unlike those with relatively specialized systems (Nilsson 1988). However, Herrera (1996) found no significant selection on spur length in the day-flying hawkmoth pollinated *Viola cazorlensis*. The low percentage of pollinaria observed on the stigmatic cavities of plants of *M. venosum, M. capense* and *M. gracile* relative to pollinaria removal (Table 8) is not uncommon in orchids (Nilsson and Rabakonandrianina 1988, Ackerman and Montalvo 1990), and reproductive success in orchids is often limited by pollinators (Johnson and Bond 1992, Burd 1994). The finding that it is not essential for the spurs of *M. venosum* and *M. gracile* to exceed the length of the pollinators' proboscides for pollination to occur, and that no selection for spur length is taking place, may indicate that the plants rely on a variety of moths (with varying proboscis lengths) for pollination. According to Petterson (1991), long spurs are favoured if low reproductive success is due to strongly skewed morphological interactions with

long-tongued moth pollinators, whereas diverse interactions result in the evolution of traits allowing a diversity of pollinators. Having longer spurs results in the placement of pollinaria closer to the base of a pollinator's proboscis. While this may increase male fitness by reducing the loss of pollinaria (Johnson and Steiner 1997), pollinaria will not reach the stigmas of short-spurred plants. On the other hand, female fitness is increased through selection for longer spurs, since pollinia from both long and short spurs will reach the stigmas of long-spurred plants. Since *M. venosum, M. capense* and *M. gracile* do not exhibit selection for long (or short) spurs, it would seem that a 'balancing' of male and female function allows these species to function with different species of moths.

Very often, pollen produced by outcrossing species may never reach stigmas, since it is either not removed or is lost during transport. In orchids, failure of pollen removal is often due to insufficient visitation, since the aggregation of pollen into pollinia determines the number of effective visits required to remove a flower's pollen (Harder 2000). Frequent visits by pollinators however, may still result in the failure of pollen removal and deposition due to visitors functioning poorly as pollinators. At Verulam in 1998, frequent visits of hawkmoths to flowers of *M. venosum* were observed, yet only *c.*30 % of the flowers observed received pollinia whereas 70 % had pollinaria removed. It was suggested that low pollen receipt was a result of 'wastage' following removal (see appendix II). However, Harder (2000) found that orchid pollen is more successfully dispersed than species with granular pollen, and that despite a high pollen removal failure, the loss of pollen incurred during transport is reduced in orchids compared to species with granular pollen. Harder (2000) attributes the low transport loss of pollen in orchids to the nature of the pollinaria, which adhere to the pollinators. He showed that the percentage of the pollen removed that reached stigmas of orchids was higher than other abiotic and animal-pollinated monocotyledons. Pollen removal in *M. capense* and *M. gracile* was much lower than that of *M. venosum* and was likely due to insufficient visitation. The high percentage of removed pollinaria that reach the stigmas in *M. venosum* (49.2 %), *M. capense* (52.1 %) and *M. gracile* (35.3 %) may explain the success of these orchids, despite low rates of visitation.

Another aspect that compensates for low-visitation, is flower longevity. According to Arditti (1979), tropical orchids are characterised by having long-lived flowers in the absence of pollination. Unvisited flowers of *Mystacidium* have a lifespan ranging from ±16 - 28 days. Stigmatic pollen deposition may trigger early flower senescence (Gregg 1991, Aizen 1993, Clayton and Aizen 1996), which is a likely adaptation to promote visitation to other unpollinated flowers (Arditti 1976, Gori 1983), and to minimize geitonogamy (Harder and Johnson, unpublished data). The results for *M. venosum* are consistent with those of Clayton and Aizen (1996), which showed that the flower lifespan

of *Chlorae alpina* was strongly affected by pollinia insertion, and that pollinaria removal shortened the lifespan in unpollinated flowers, but to a much lesser extent. As with *C. alpina*, pollen removal in *M. venosum* does not always coincide with pollen deposition and many pollinaria are 'wasted' rather than being successfully exported to other flowers. Clayton and Aizen (1996) thus suggested that a longer subsequent flower lifespan could therefore be predicted for an unpollinated flower which has its pollinaria removed, than for a pollinated flower which has its pollinaria in place.

CONCLUSION

The floral traits and nectar properties of *Mystacidium* species have most likely been moulded by the requirements of their respective pollinators. Long-lived flowers and pollen, and the high incidence of removed pollen reaching stigmas are traits that ensure the success of these orchids, despite very low levels of pollinator visitation and severe pollen limitation of fruiting success.

CHAPTER 4
PHYLOGENY

Cladistic analyses of *Mystacidium* based on morphological and anatomical characters indicated that the genus may not be monophyletic. *Diaphananthe,* represented here by *D. caffra* and *D. millarii*, appears to be nested within the genus. The cladogram addresses the problem of morphological separation of *M. venosum* and *M. capense*, and of *M. flanaganii* and *M. pusillum*. The phylogeny indicates that the white, long-spurred flowers of *M. capense* and *M. venosum* are basal within the genus. The short-spurred species, and those with green flowers, are derived. The phylogeny presented here is not well supported, though it probably represents a near complete analysis of the available morphological and anatomical characters. The use of molecular characters may be required to improve the resolution of the phylogeny.

INTRODUCTION

Phylogenetic approaches to analysing character evolution can provide new insights into the evolutionary history of a genus. Information obtained from phylogenetic hypotheses combined with ecological data can be used to infer relationships and examine evolutionary pathways (Armbruster 1992, McDade 1992). The tremendous radiation in angiosperms has partly been attributed to adaptations to pollinators (Stebbins 1970). Mapping pollination systems onto phylogenies is a way of elucidating how floral traits evolve if shifts in pollination systems have taken place (Armbruster 1992, Johnson *et al.* 1998, Johnson *et al.* 2002). Furthermore, character mapping is a useful means of interpreting convergent evolution, reversals and pre-adaptation (Weller and Sakai 1999). The use of morphological data for the construction of a phylogeny may often lead to robust phylogenetic hypotheses (Weller and Sakai 1999), however the reliance on floral traits may result in a circular relationship between evolutionary interpretation and phylogeny estimation (Armbruster 1992). Although molecular data are often viewed as having a higher reliability as they are less likely to show convergence (Chase and Hills 1992), morphological data provide an independent data set and generally allow for more complete taxon sampling.

A phylogenetic analysis of the genus *Mystacidium* has not been carried out before due to the limited information on anatomy and floral morphology. The taxonomy of the genus is therefore unsatisfactory, with certain species being differentiated only according to flowering time (refer to Chapter 1). The aim of this study was therefore to construct a phylogeny of *Mystacidium* using morphological and anatomical characters (Chapter 1) in order to clarify relationships within the genus, and to determine the pathway of floral evolution and pollination systems. *Cyrtorchis arcuata* was considered as a potential outgroup, and *Diaphananthe caffra* and *D. millarii* were included because these species

were historically placed in *Mystacidium* and bear strong similarities to *Mystacidium* in their overall floral construction.

MATERIALS AND METHODS

A cladistic analysis of *Mystacidium* was carried out to establish the relationships among the eight species in the genus. *Cyrtorchis arcuata* was used as an outgroup to root the tree on the grounds that it shares features with *Mystacidium* that may be indicative of an evolutionary relationship. The genus *Diaphananthe* is closely related to *Mystacidium*, and *D. caffra* and *D. millarii* were recently transferred from *Mystacidium* to *Diaphananthe* (Linder 1989). These two species were therefore also included in the analysis. From the morphological and anatomical studies reported in Chapter 1, a total of 26 potentially phylogenetically informative characters were selected for the analyses, 23 of which were morphological and three were anatomical. The complete character list is given in Table 10, and the distribution of characters in Table 11. Anatomical features that were excluded from the cladistic analysis because discrete states could not be absolutely defined, included the number of xylem/phloem groups in roots, the shape of and differences between abaxial and adaxial leaf epidermal cells, and the extent of sclerenchyma fibres encompassing the phloem of main vascular bundles in leaves.

Initial analyses were performed using the ie* procedure in Hennig86 (Farris 1988), which produces a complete set of most parsimonious trees (Platnick 1989), and the graphics were produced using CLADOS (Nixon 1993). Parsimony analyses were also conducted using PAUP* (Swofford 1998). In order to explore as much 'tree space' as possible, an "island search" was conducted using 1000 RANDOM replicates employing NNI branch swapping, and holding 5 trees per replicate. This approach resulted in a large pool of starting trees, the shortest of which were subsequently analysed more rigorously using TBR branch swapping with MULTREES. Relative support for the nodes recovered was assessed using bootstrap (Felsenstein 1985), jackknife (Miller 1974) and decay (Bremer 1994) analyses. Bootstrap analyses construct a given number of "pseudo-replicate" data sets equivalent in size to the original data set by random character sampling with replacement. Jackknife analyses involve sampling characters from the original data set without replacement to simulate a data set that has fewer characters than the original. Recent empirical studies have indicated that

estimates of support obtained by jackknife and bootstrap analyses are very similar (Mort *et al.* 2000). The bootstrap analyses (Felsenstein 1985) were conducted using 500 replicates with 10 replicates of RANDOM stepwise addition, employing the TBR branch-swapping algorithm with MULTREES. The jackknife analyses (Miller 1974) were also conducted using 500 replicates and TBR branch swapping, with 33% character deletion. Bremer support (Bremer 1994), which is the increase in tree length required to lose a node, and branch lengths were also obtained using PAUP* (Swofford 1998). Constraint trees can be constructed to test various hypotheses of phylogenetic relationships. In order to explore the hypothesis that the two *Diaphananthe* species included in the cladistic analyses are embedded in *Mystacidium*, a constrained search using PAUP* was implemented by placing *caffra* and *D. millarii* as a sister group to *Mystacidium* and assessing the cost in parsimony.

To further explain the relationship between *Mystacidium* and *Diaphananthe*, a further two *Diaphananthe* species, *D. fragrantissima* and *D. xanthopollinia*, each belonging to two different *Diaphananthe* sections, were also included in a subsequent analysis. As these two species had not been studied in detail, anatomical features had to be scored as missing characters. Cladistic analyses were carried out as described above for the first data set.

Table 10. The character list used for the analysis of the morphological and anatomical characters of *Mystacidium*. Bold print refers to changes made for subsequent analysis with *D. fragrantissima* and *D. xanthopollinia*.

1.	Viscidia adjacent (0)/apart (1)
2.	Viscidia one (0)/viscidia two (1)
3.	Outer rostellum lobes glaborous (0)/slightly papillose (1)/hairy (2)
4.	Central rostellum lobe same width as outer lobes (0)/ broader (1)
5.	Outer rostellum lobes shorter than central lobe (0)/same length (1)/longer (2)
6.	**Central rostellum lobe bifid (0)/not bifid (1)**
7.	Central rostellum lobe papillose (0)/glaborous (1)
8.	Anther cap beaked (0)/smooth (1)/notched (2)
9.	Anther cap not bright green (0)/bright green (1)
10.	Inflorescence lax (0)/crowded (1)
11.	Flower scented (0)/not scented (1)
12.	Flowers white (0)/pale green (1)
13.	Petals lanceolate (0)/oblanceolate (1)/ovate (2)
14.	Sepals lanceolate (0)/oblanceolate (1)/ovate (2)
15.	Dorsal sepal tip acute (0)/rounded (1)
16.	Side lobes of lip absent (0)/present (1)
17.	Lip ecallose (0)/callus present (1)
18.	Spur tip pointed (0)/rounded (1)
19.	Spur orientation pendant (0)/horizontal (1)
20.	Spur length > 30 mm (0)/spur < 30 mm long (1)
21.	Leaves bilobed (0)/not bilobed (1)
22.	V-shape of leaf in transection broad and rounded (0)/acute (1)
23.	Roots along stem of host (0)/tangled mass (1)
24.	Velamen more than 3-layered (0)/3-layered (1)/one-layered (2)
25.	Cells of outer velamen layer: not larger than other layers (0)/much larger (1)
26.	**Mid rib bundle sheath complete (0)/incomplete (1)**
27.	**Outer rostellum lobes absent (0)/present (1)**
28.	**Stem >5 cm long (0)/stem < 5 cm long (1)**

Table 11. Distribution of characters among *Mystacidium*, *D. caffra*, *D. millarii* and *C. arcuata*. Bold print refers to changes made for subsequent analyses with *D. fragrantissima* and *D. xanthopollinia*. Character 6 in *M. tanganyikense* was scored as '1' in the subsequent analysis (see text).

C. arcuata	000?0	00000	00000	00000	00000	**000**
M. aliceae	11100	11101	11010	10001	01020	**011**
M. brayboniae	01111	10001	10210	10011	00011	**011**
M. capense	01202	10000	00000	10000	00011	**111**
M. flanaganii	11110	10000	?1110	10001	00010	**111**
M. gracile	01112	11100	01110	10001	00110	**111**
M. pusillum	01111	11000	01111	00001	10010	**111**
M. venosum	01202	10000	00000	10000	01010	**011**
M. tanganyikense	11211	00100	01000	10001	00020	**011**
D. caffra	11010	11211	10220	01111	00020	**011**
D. millarii	11010	10211	10221	01011	0?0??	**?11**
D. fragrantissima	**000?0**	**01100**	**01000**	**11101**	**010??**	**?00**
D. xanthopollinia	**11010**	**11100**	**01220**	**01101**	**0?0??**	**?10**

RESULTS

The analyses of the data set for *Mystacidium* using both Hennig86 and PAUP* yielded a single tree. The tree length in Hennig86 was 56 steps (consistency index [CI] = 0.57 and retention index [RI] = 0.61) (Figure 4.1), and in PAUP* it was 53 steps (CI = 0.60 and RI = 0.63) (Figure 4.2). The trees in the two programs differed in the placement of *M. tanganyikense*. In order to explore whether forcing *M. tanganyikense* as a sister to the *M. aliceae* clade in PAUP* would affect parsimony, a constrained analysis was implemented, which resulted in an increase in only one step. The less than one percent increase in parsimony, and the poor support for the *M. tanganyikense* node (Figure 4.2) denotes that there is no substantial difference in the topology of the two trees.

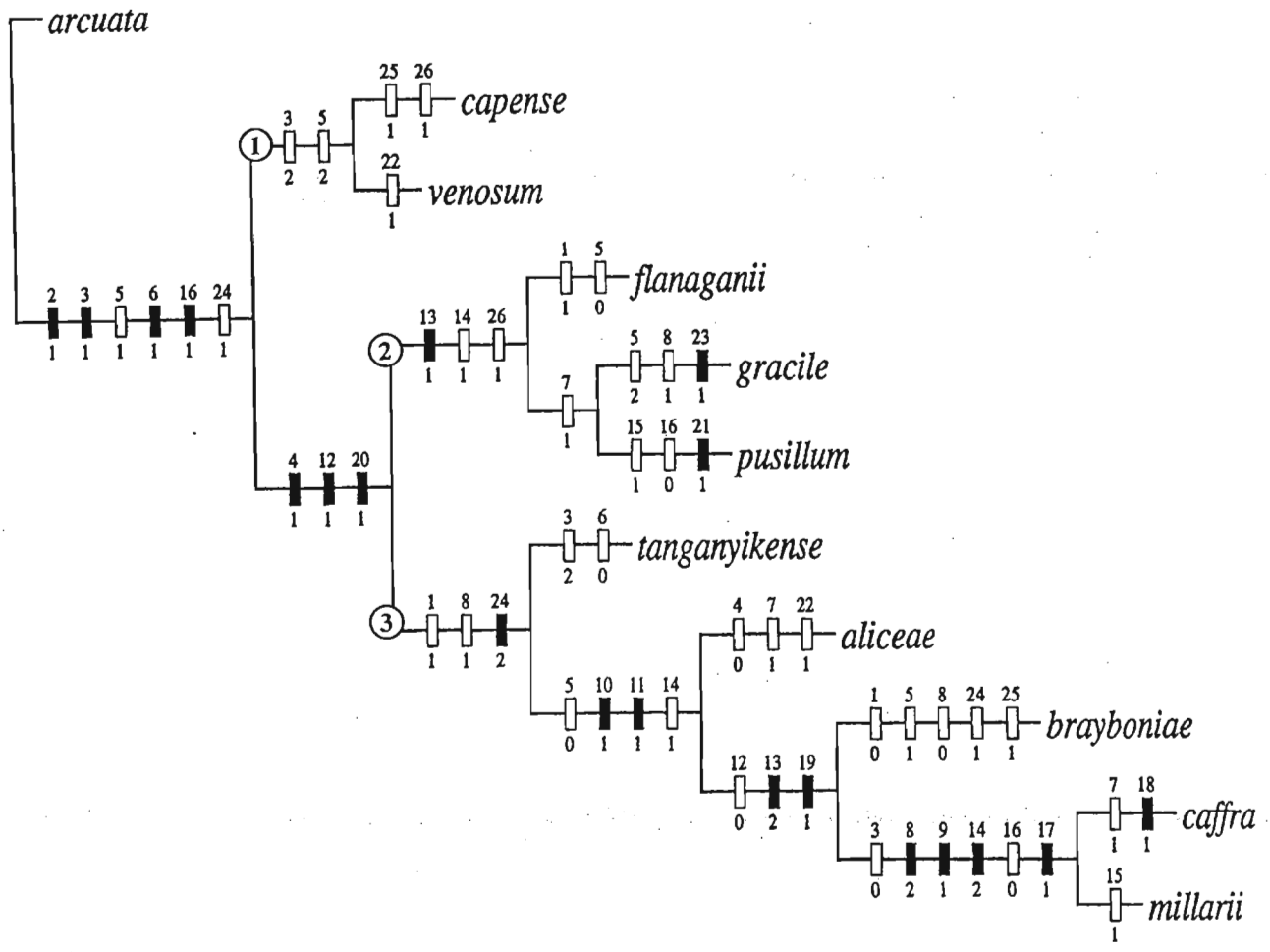


Figure 4.1. Cladogram of *Mystacidium* recovered from Hennig86 (Farris 1988). Solid bars represent unique apomorphies, and hollow bars represent characters that are homoplasious (ie. appear to have evolved more than once).The number above each bar refers to the character in Table 10, and the number below the bar refers to the character state. The clades are numbered 1-3 to facilitate reference in the text.

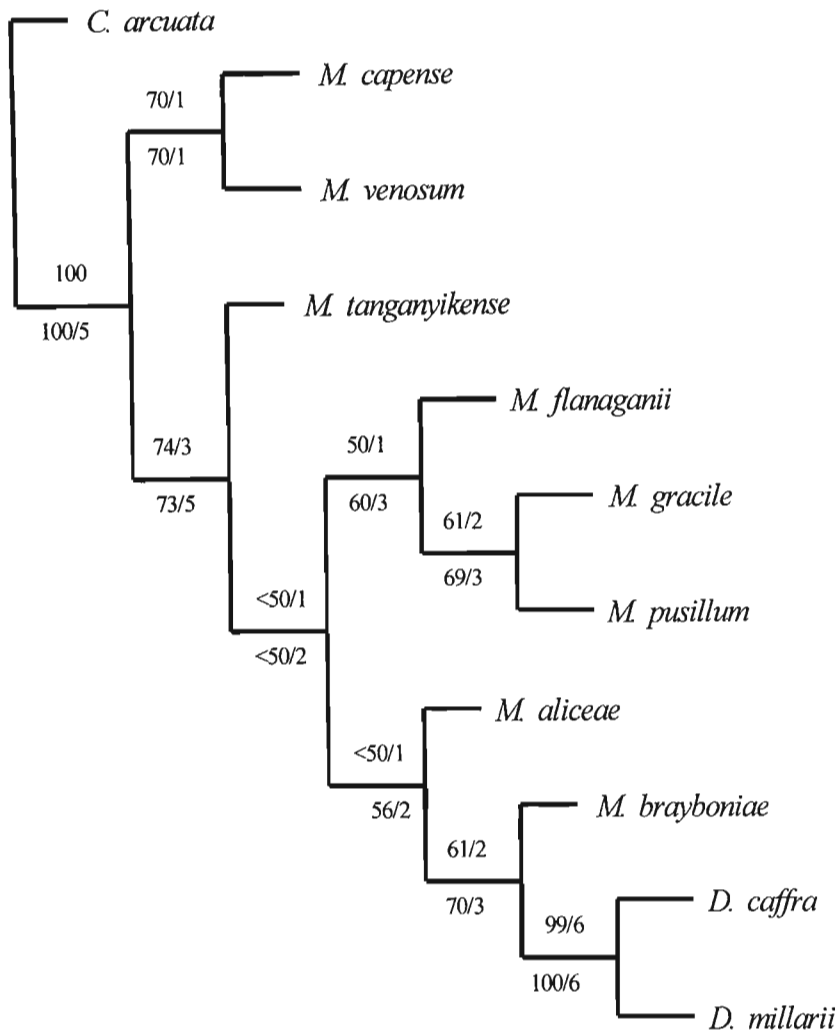


Figure 4.2. The single most parsimonious tree recovered from parsimony analysis using PAUP* (Swofford 1998) for *Mystacidium*. Numbers above each branch are bootstrap/decay values; numbers below the lines are jackknife percentiles/branch lengths.

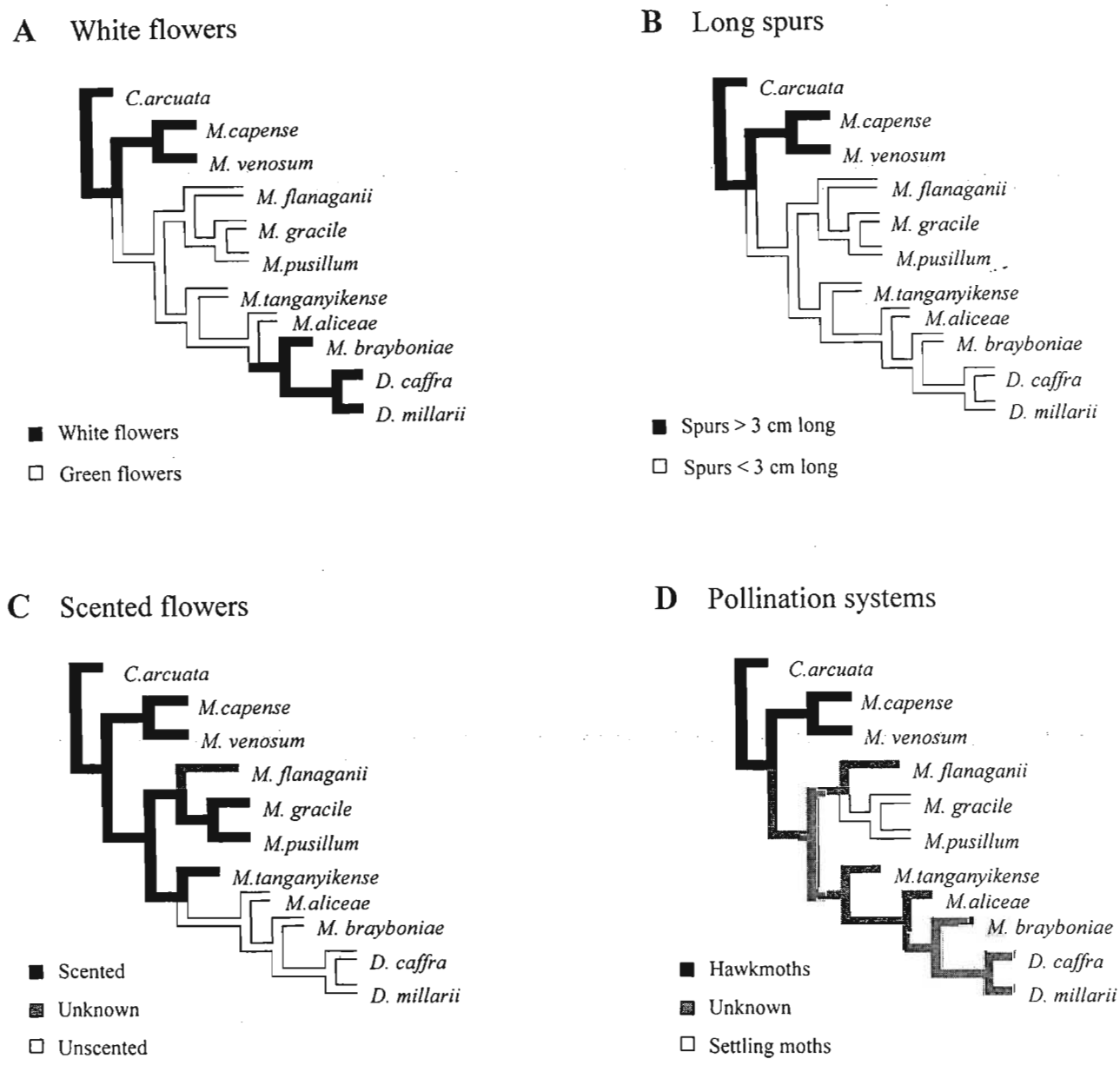


Figure 4.3. Trends in floral evolution and pollination systems in *Mystacidium*. Traits mapped are derived from the cladogram in Figure 4.1.

Some of the trends in the evolution of floral morphology and pollination systems in *Mystacidium* are summarized in Figure 4.3. White flowers evolved twice (Figure 4.3 A). White flowers with long spurs, which are pollinated by hawkmoths (Figures 4.3 A, B, D) are basal in the genus. *Mystacidium gracile* and *M. pusillum*, which are pollinated by settling moths (Figure 4.3 D), probably evolved from long-spurred ancestors that were pre-adapted to hawkmoth pollination.

The basic column of *Mystacidium* is three-lobed, with the outer rostellum lobes being well developed and slightly hairy, and two pollinaria are attached to separate viscidia. Very slight modifications of the column have occurred in the genus, and variation in the column of the two *Diaphananthe* species from the basic *Mystacidium* column structure is more pronounced. Separate viscidia (viscidia lying apart, character 1) evolved in the ancestor of *M. flanaganii*, as well as in clade 3 (Figure 4.1). However, *M. brayboniae* exhibited a reversal to adjacent viscidia. Very hairy outer rostellum lobes (character 3) evolved in clade 3. Hairs were reduced to papillae in clades 2 and 3, and completely lost in *D. caffra* and *D. millarii*. A broad central lobe (character 4) evolved in clades 2 and 3, except for *M. aliceae*, which exhibited a reversal to a narrow central lobe. Long outer lobes (character 5) evolved in clade 1 and in *M. gracile*, whereas *M. flanaganii*, *M. aliceae*, *D. caffra* and *D. millarii* exhibited reversals to short outer lobes. *Mystacidium tanganyikense* exhibited a reversal to a bifid rostellum (character 6), and a glabrous central lobe (character 7) evolved independently in *M. gracile*, *M. pusillum*, *M. aliceae* and *D. caffra*.

Both *Mystacidium* and *Diaphananthe* bear a removable anther cap. A round, or smooth-rimmed anther cap (character 8) evolved in *M. gracile*, and in *M. tanganyikense* and *M. aliceae*. In clade 3, this character state was reversed in *M. brayboniae*, which has a beaked anther cap, while notched anther caps evolved in *D. caffra* and *D. millarii*.

Green-coloured flowers (character 12) evolved in clades 2 and 3, however *M. brayboniae*, *D. caffra* and *D. millarii* underwent reversals to white-coloured flowers. A rounded sepal tip (character 15) evolved independently in *M. pusillum* and *D. millarii*, and side lobes of the lip (character 16) evolved in all clades, however reversals occurred in *M. pusillum*, *D. caffra* and *D. millarii*, where side lobes are absent.

An acute V-shape of the leaf in transverse section (character 22) evolved independently in *M. venosum* and *M. aliceae*. A three-layered velamen (character 24) evolved in clades 1, 2 and in *M. brayboniae*, whereas a one-layered velamen evolved in *M. tanganyikense*,

M. aliceae and *D. caffra*. The outer layer of the velamen evolved large cells (character 25) in *M. capense* and *M. brayboniae*, and an incomplete bundle sheath (character 26) evolved in clade 2 and in *M. capense*.

The cladograms indicate that the two *Diaphananthe* species included in the analyses are embedded in *Mystacidium*. Constraining *D. caffra* and *D. millarii* as a sister group to *Mystacidium* resulted in an increase in tree length of 4 steps, or 7%. Although the analyses are hypotheses, the monophyly of *Mystacidium* needs to be reconsidered. In order to further test the hypothesis that *Diaphananthe* may be nested in *Mystacidium*, two more species, *D. fragrantissima* and *D. xanthopollinia*, were added to the data set and slight changes were made to the original character matrix. *Mystacidium tanganyikense* was originally scored as having a bifid central rostellum lobe (character 6) since the notch at the apex of the rostellum distinguishes it from the remaining *Mystacidium* species. In the subsequent analysis, the scoring of *M. tanganyikense* was changed, since *D. fragrantissima* exhibits a deeply bifid rostellum similar to that of *C. arcuata*, and unlike the slight bifid nature of the central rostellum lobe of *M. tanganyikense*. Character 26 was omitted from the original matrix for the subsequent analysis due to the absence of anatomical information for *D. fragrantissima* and *D. xanthopollinia*. Finally, two more characters, the absence of outer rostellum lobes (character 27) and stem length (character 28), were added to the data set. Parsimony analysis, using the ie* procedure in Hennig86, also yielded a single tree (Figure 4.4) of 65 steps (CI = 0.50, RI = 0.60). However, bootstrap and decay indexes revealed that few nodes were supported. The cladogram (Figure 4.4) illustrates that *D. fragrantissima* is excluded from *Mystacidium*, however there was very little bootstrap support (less than 50%) for that placement. The phylogenetic position of *D. fragrantissima* therefore remains uncertain.

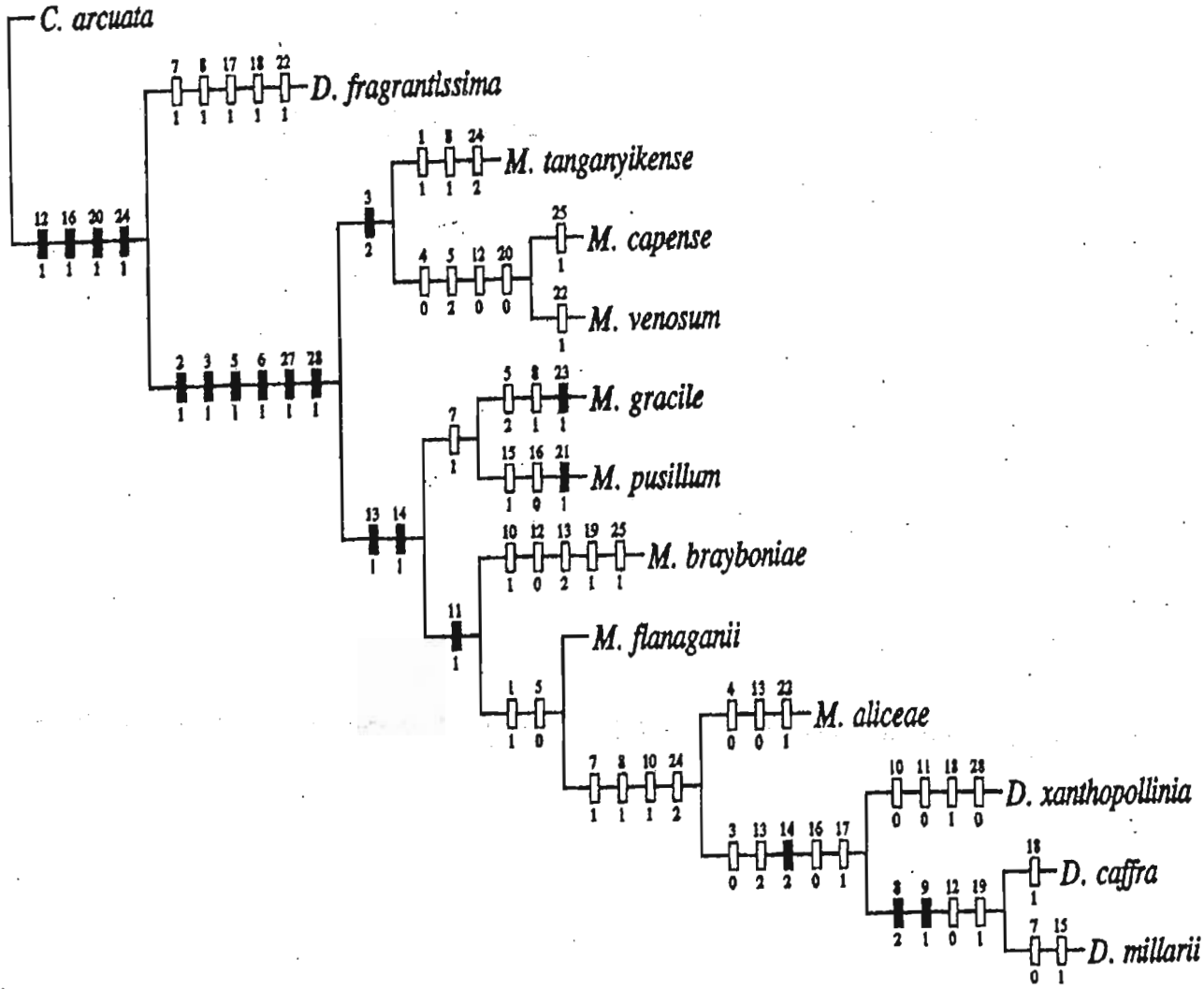


Figure 4.4. Cladogram of *Mystacidium* recovered from Hennig86 (Farris 1988), with *D. fragrantissima* and *D. xanthopollinia* included in the data set.

DISCUSSION

The phylogenetic analyses presented here suggest that *Mystacidium* is not a monophyletic genus. The traditional notion that well-developed lateral rostellum lobes characteristic of *Mystacidium*, with their "curious bearded appendages", indicate monophyly in the group needs to be re-evaluated. Although the cladogram (Figure 4.1) suggests that *D. caffra* and *D. millarii* are embedded in *Mystacidium*, the two species exhibit a number of unique attributes, including the glabrous outer rostellum lobes (character 3), a notched anther cap (character 8), a green anther cap (character 9), ovate sepals (character 2) and the presence of a pencillate callus (character 17) in the throat of the spur (*D. caffra*) or at the throat of the spur (*D. millarii*). Further analyses including *D. xanthopollinia* and *D. fragrantissima*, suggest that *D. millarii*, *D. caffra* and *D. xanthopollinia*, which all belong to *Diaphananthe* sect. R*hipidoglossum*, may be embedded in *Mystacidium*, whereas *D. fragrantissima*, which belongs to *Diaphananthe* sect. *Diaphananthe*, is not. Thus the monophyly of *Diaphananthe* is also open to question. There has been much confusion surrounding the original reviews of *Diaphananthe* and *Mystacidium* (Bolus 1896, 1905, Summerhayes 1960, Cribb 1989). In order to make an informed taxonomic statement regarding *Mystacidium*, and to argue against the current inclusion of *D. millarii* and *D. caffra* in *Diaphananthe*, it would be necessary to examine *Diaphananthe* as a whole. The morphology of the genus is quite variable, and as Linder (1989) stated, it has a "variable rostellum and spur configuration".

Diaphananthe, which consists of approximately 42 species, is a widespread genus occurring throughout tropical Africa, with only four species found in South Africa. *Mystacidium* on the other hand, is biogeographically localised, with seven of the eight species being confined to southern Africa, suggesting that *Mystacidium* is a more recent and specialized group. It is thus odd that the cladogram suggests that *Mystacidium* is a basal group in which *Diaphananthe* has evolved as a specialized clade. The basal position of *D. fragrantissima* in the subsequent analysis may imply that only some of the southern African *Diaphananthe* species have evolved within *Mystacidium*, and that the rest of *Diaphananthe* is sister to *Mystacidium*.

The cladogram (Figure 4.1) indicates that the long-spurred, white flowers of *M. venosum* and *M. capense* are a basal group within the genus, and that pollination by

hawkmoths is likely to be ancestral to pollination by settling moths. The evolution of white flowers in *M. brayboniae*, *D. caffra* and *D. millarii* (Figure 4.3 A), which lack a scent and have horizontally orientated spurs, may be a result of a shift from moth to long-proboscid fly pollination. Although there were no pollinators observed for these three species, flowers pollinated by long-proboscid flies are known to lack a scent, have elongated perianth tubes or spurs, and have brightly coloured flowers (Vogel 1954, Johnson 1992, Manning and Goldblatt 1996, Johnson and Steiner 1995). Furthermore, Johnson and Steiner (1995) observed that long-proboscid flies visited blue, white and pink flowers with horizontally orientated spurs. Evidence exists for shifts between hawkmoth and long-proboscid fly pollination in *Disa* (Johnson *et al.* 1998), *Lapeirousia* (Manning and Goldblatt 1996) and *Zaluzianskya* (Johnson *et al.* 2002). If *M. brayboniae*, *D. caffra* and *D. millarii* are pollinated by long-proboscid flies, a shift from settling moth pollination is likely to have followed this line of least resistance (Stebbins 1970).

The cladogram (Figure 4.1) addresses the problem of morphological separation of *M. pusillum* and *M. flanaganii,* which have been distinguished only according to flowering time (Linder and Kurzweil 1999). *Mystacidium flanaganii* differs from *M. pusillum* in the position of the viscidia (character 1), the length of the outer rostellum lobes (character 5), the papillate central lobe (character 7), the shape of the sepal tip (character 15), the presence of side lobes of the lip (character 16), and the bilobed leaves (character 21). It is clear from the distribution of characters on the cladogram that *M. flanaganii* and *M. pusillum* are two separate species (Figures 4.1 and 4.2).

Mystacidium capense and *M. venosum* have also been distinguished entirely according to flowering time, and although it appears that these two closely related species do not differ within external morphology, the V-shape of the leaves in transverse section of *M. capense* is broader than in *M. venosum* (character 22). Anatomically, *M. venosum* and *M. capense* differ in the size of the cells of the outer velamen layer (character 24), which are much larger than those of the inner layer in *M. capense*, and in the midrib bundle sheath (character 26), which is incomplete in *M. capense*.

CONCLUSION

In order to clarify the relationship of *Mystacidium* and *Diaphananthe*, further anatomical and morphological studies of *Diaphananthe* are necessary, and it would also be worthwhile to experiment with different outgroups in order to unequivocally establish polarity in the data set. If the appropriate outgroup is identified, the correct polarization of characters within lineages may allow evolutionary relationships among various characters to be established with clarity (Weller and Sakai 1999). Molecular analyses are currently being undertaken in collaboration with Dr. M.E. Mort (University of Kansas). Using molecular data to reconstruct the phylogeny of *Mystacidium* will hopefully improve on the morphological phylogeny presented here.

CONCLUSIONS

The morphology, colour and scent (advertising features) of flowers of *Mystacidium,* and the nectar characteristics (rewarding features), are closely associated with their pollinators. Diversified floral form and vegetative uniformity in *Mystacidium* suggests that pollination drives evolutionary processes in the genus. The different species occupy a variety of habitats, and as Stebbins (1970) stated, floral traits have probably been moulded through selection by the most efficient pollinators in a particular habitat. The adaptive nature of floral traits in *Mystacidium* is evident in that the flowers attract and accommodate specific pollinators. However, observations of moths proved difficult, and much remains unknown about the pollination of *Mystacidium*. The sweet evening-scented, white flowers of *M. venosum* and *M. capense* are attractive to hawkmoths, and the concealed sucrose-rich nectar in the long spurs are accessible exclusively to these long-tongued pollinators. The utility of long spurs in moth pollination systems is a "line of least resistance", explaining the shift to settling moth pollination evident in *M. gracile* and *M. pusillum*. Physiological properties associated with pollination systems are reflected in the fluctuation of nectar properties over a 24 hour period in *M. venosum* and *M. capense*, where volume and peak sugar content coincide with the brief feeding period of their hawkmoth pollinators.

Pollination efficiency (pollen removal relative to pollen receipt) appears to be limited in *Mystacidium*, since hand-pollinations increased fruit set. However, the success of these orchids may be related to their long-lived flowers and pollen, as well as the high incidence of removed pollen reaching stigmas in a given population. Ecologically, the ability of the species of *Mystacidium* to perform CAM photosynthesis ensures that they have the ability to withstand the harsh microclimates in which they occur. Nocturnal nectar secretion often occurs in CAM plants (Bùrquez and Corbet 1991), and this may have facilitated the evolution of moth pollination in the genus.

The cladistic analysis clarified that *M. pusillum* and *M. flanaganii*, and *M. capense* and *M. venosum*, respectively, are distinct species that differ morphologically and anatomically. Although it appears that *Mystacidium* may not be monophyletic, the phylogeny of the group using additional character sets, including those based on DNA sequences, requires further investigation.

REFERENCES

Ackerman J.D. and Montlavo A.M. (1990) Short- and long-term limitations to fruit production in a tropical orchid. *Ecology* 71: 263-272.

Aizen M.A. (1993) Self-pollination shortens flower lifespan in *Portulaca umbraticola* (Portulacaceae). *International Journal of Plant Sciences* 154: 412-415.

Alexandersson R. and Johnson S.D. (2002) Pollinator-mediated selection on floral tube length in a hawkmoth-pollinated Gladiolus (Iridaceae). *Proceedings of the Royal Society B.* 269:631-636.

Arditti J. (1976) Post-pollination phenomenon in orchid flowers. *Orchid Review* 84: 261 - 268.

Arditti J. (1979) Aspects of the physiology of orchids. *Advances in Botanical Research* 7: 421-697.

Arditti J. (1992) Fundamentals of Orchid Biology. J. Wiley and Sons, New York. pp. 444-451.

Armbruster W.S. (1992) Phylogeny and the evolution of plant-animal interactions. *BioScience* 42: 12-20.

Baker H.G. (1977) Non-sugar chemical constituents of nectar. *Apidologie* 8: 349-356.

Baker H.G. and Baker I. (1975) Studies of nectar constitution and pollinator-plant coevolution. In: Gilbert L.E. and Raven P.H. (eds.). Animal and Plant Coevolution. University of Texas Press, USA. pp 100-140.

Baker H.G. and Baker I. (1982) Chemical constituents of nectar in relation to pollination mechanisms and phylogeny. In: Nitecki M.H. (ed.). Biochemical aspects of evolutionary biology. Proceedings of the 4th annual spring systematics symposium. University of Chicago Press, Chicago. pp 131-171.

Baker H.G. and Baker I. (1983) Floral nectar constituents in relation to pollinator type. In: Jones C.E. and Little R.J. (eds.). Handbook of Experimental Pollination

Biology. New York, Van Nostrand-Reinhold. pp 117-141.

Barrett C.H., Harder L.D. and Worley A.C. (1996) The comparative biology of pollination and mating in flowering plants. *Philosophical Transactions of the Royal Society of London B.* 351: 1271-1280.

Barthlott W. (1976) Morphologie der Samen von Orchideen im Hinblick auf Taxonomische und Funktionelle Aspekte. Proceedings of the Eighth World Orchid Conference, 444-453.

Bell G. (1985) On the function of flowers. Proceedings of the Royal Society of London, Series B. *Biological Sciences* 224: 223-265.

Benzing D.H. and Otto D.W. (1981) Vegetative reduction in epiphytic Bromeliaceae and Orchidaceae: its origin and significance. *Biotropica* 13: 131-140.

Boggs C.L. (1987) Ecology of nectar and pollen feeding in Lepidoptera. In: Slansky F. and Rodriguez J.G. (eds.). Nutritional Ecology of Insects, Mites, Spiders and Related Invertebrates. John Wiley and Sons, New York.

Bolus H. (1896) *Icones Orchidearum Austro-Africanarum Extra-Tropicarum* t. 7. Orchids of South Africa v. 1.

Bolus H. (1896) *Icones Orchidearum Austro-Africanarum Extra-Tropicarum* t. 55. Orchids of South Africa v. 1.

Bolus H. (1905) Contributions to the African flora. *Transactions of the Southern African Philosophical Society* XVI: 147.

Bolus H. (1911) *Icones Orchidearum Austro-Africanarum Extra-Tropicarum* t. 6B. Orchids of South Africa v. 2

Bond W.J., Stock W.D. and Hoffmann M.T. (1994) Has the Karoo spread - a test for desertification using stable carbon isotopes from soils. *South African Journal of Science* 90: 391-397.

Brantjes N.B.M. (1973) Sphingopholous flowers, function of their scent. In: Brantjes

N.B.M, Linskens H.F. (eds.). Pollination and Dispersal. Nijmegen, Publ. Dept. Botany. pp 27-46.

Brantjes N.B.M. (1978) Sensory responses to flowers in night flying moths. In: Richards A.J. (ed.). The pollination of flowers by insects. Linnean Society Symposium Series No.6. Academic Press, London, pp. 13-19.

Bremmer K. (1994) Branch support and tree stability. *Cladistics* 10: 369-372.

Burd M. (1994) Bateman's principle and plant reproduction: the role of pollen limitation in fruit and seed set. *Botanical Review* 60: 89-137.

Bùrquez A. and Corbet S.A. (1991) Do flowers reabsorb nectar ? *Functional Ecology* 5: 369-379.

Calvo R.N. and Horvitz C.C. (1990) Pollinator limitation, cost of reproduction, and fitness in plants: a transition matrix demographic approach. *American Naturalist* 136: 499-516.

Chase J.M. and Hills H.G. (1992) Orchid phylogeny, flower sexuality, and fragrance seeking. *Bioscience* 42:43-49.

Chittka L. (1997) Bee colour vision is optimal for coding flower color, but flower colors are not optimal for being coded - why ? *Israel Journal of Plant Sciences* 45: 115 - 127.

Clayton S. and Aizen M.A. (1996) Effects of pollinia removal and insertion on flower longevity in *Chloraea alpina* (Orchidaceae). *Evolutionary Ecology* 10: 65-660.

Cox P.A., Elmqvist, T., Pierson E.D. and Rainey W.E. (1991) Flying foxes as strong interactors in South Pacific island ecosystems: a conservation hypothesis. *Conservation Biology* 5: 448-454.

Cribb P.J. (1989) Orchidaceae. *Flora of Tropical East Africa*. Part 3.

Cruden R.W. and Hermann S.M. (1983) Studying nectar ? Some observations on the art. In: Bentley B. and Elias T. (eds.) The Biology of Nectaries. Columbia University

Press, New York. pp 223-241.

Cruden R.W., Hermann S.M. and Peterson S. (1983) Patterns of nectar production and plant-pollinator coevolution. In: Bentley B. and Elias T. (eds.) The Biology of Nectaries. Columbia University Press, New York. pp 82-125.

Dafni A. (1992) Pollination Ecology: A Practical Approach. Oxford University Press, New York.

Darwin C.H. (1862) On the various contrivances by which British and foreign orchids are fertilized by insects. -John Murray, London (2nd edition).

Dobson H.E.M. (1994) Floral volatiles in insect biology. In: Bernays E.A. (ed.). Insect-Plant Interactions vol V. CRC Press, Boca Raton. pp 47-81.

Dodson C.H. (1962) The importance of pollinators in the evolution of the orchids of tropical America. *American Orchid Society Bulletin* 31: 525-534.

Dressler R.L .(1993) The Orchids: Natural History and Classification. Harvard University Press, USA.

Dudareva N. and Pichersky E. (2000) Biochemical and molecular genetic aspects of floral scents. *Plant Physiology* 122: 627-633.

Earnshaw M.J., Winter K., Ziegler H., Stichler H., Cruttwell N.E.G., Kerenga K, Cribb P.J., Wood J., Croft J.R., Carver K.A. and Gunn T.C. (1987) Altitudinal changes in the incidence of crassulacean acid metabolism in vascular epiphytes and related life forms in Papua New Guinea. *Oecologia* 73: 566-572.

Edwards G. and Walker D.A. (1983) C_3, C_4: Mechanisms, and Cellular and Environmental Regulation, of Photosynthesis. Blackwell Scientific Publications, Oxford.

Faegri K. and van der Pijl L. (1966) The Principles of Pollination Ecology. Pergamon Press, Oxford, London, Edinburgh, New York, Paris, Braunschweig.

Faegri K. and van der Pijl L. (1979) The Principles of Pollination Ecology. 3rd edition.

Pergamon Press, Oxford, New York, Toronto, Sydney, Paris, Frankfurt.

Farris J.S. (1988) Hennig86 reference. Published privately.

Felsenstein J. (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39: 783-791.

Gasson P. and Cribb P.J. (1985) The leaf anatomy of *Ossiculum aurantiacum* Cribb & Van der Laan (Orchidaceae-Vandoideae). *Kew Bulletin* 41: 827 - 832.

Gerstberger, P. & Leins, P. (1978) Rasterelektronen-mikroskopische Untersuchungen an Blutenknopsen von Physalis philadelphica (Solanaceae). *Berichte der deutschen botanischen Gesellschaft* 91: 381-387.

Goh J.C. and Kluge M. (1989) Gas exchange and water relations in epiphytic orchids. In: Lüttge U. (ed). Vascular Plants as Epiphytes - Evolution and Ecophysiology, Ecological Studies, Vol. 76. Springer-Verlag, Berlin. pp. 139-166.

Gori D.F. (1983) Post-pollination phenomena and adaptive floral changes. In: Jones C.E. and Little R.J. (eds.). Handbook of Experimental Pollination Biology. Van Nostrand Reinhold, New York. pp 31-49.

Grant V. and Grant K.A. (1965) Flower Pollination in the Phlox Family. Columbia University Press, New York.

Gregg K.B. (1991) Reproductive strategy of *Cleistes divaricata* (Orchidaceae). *American Journal of Botany* 78: 350-360.

Griffiths H. (1989) Carbon dioxide concentrating mechanisms and the evolution of CAM in vascular epiphytes. In: Lüttge U. (ed). Vascular Plants as Epiphytes - Evolution and Ecophysiology, Ecological Studies, Vol. 76. Springer-Verlag, Berlin. pp. 42-86.

Harder L.D. (2000) Pollen dispersal and the floral diversity of monocotyledons. In: Wilson K.L. and Morrison D.A. (eds.). Monocots: Systematics and Evolution. CSIRO: Melbourne.

Herrera C.M. (1996) Floral traits and plant adaptation to insect pollinators: a devil's advocate approach. In: Lloyd D.G. and Barrett S.C.H. (eds.). Floral Biology: Studies on Floral Evolution in Animal-pollinated Plants. Chapman and Hall. pp 65 - 87.

Heyneman A.J. (1983) Optimal sugar concentrations of floral nectars - dependence on sugar intake efficiency and foraging costs. *Oecologia* 60: 198-213.

Hodges S.A. (1997) Floral nectar spurs and diversification. *International Journal of Plant Sciences* 158: 81-88.

Johnson S.D. (1992) Plant animal relationships. In: Cowling R.M. (ed.). The Ecology of Fynbos. Cape Town, Oxford University Press. pp 175-204.

Johnson S.D. (1995) Observations of hawkmoth pollination in the South African orchid *Disa cooperi. Nordic Journal of Botany* 15: 121-125.

Johnson S.D. (1997) Pollination ecotypes of *Satyrium hallackii* (Orchidaceae) in South Africa. *Botanical Journal of the Linnean Society* 123: 225-235.

Johnson S.D. and Bond W.J. (1992) Habitat dependent pollination success in a Cape orchid. *Oecologia* 91: 455-456.

Johnson S.D. and Steiner K.E. (1995) Long-proboscid fly pollination in orchids. *Plant Systematics and Evolution* 195: 169-175.

Johnson S.D. and Liltved W.R. (1997) Hawkmoth pollination of *Bonatea speciosa* (Orchidaceae) in a South African coastal forest. *Nordic Journal of Botany* 16: 5-10.

Johnson S.D. and Steiner K.E. (1997) Long-tongued fly pollination and evolution of floral spur length in the *Disa draconis* complex (Orchidaceae). *Evolution* 51: 45-53.

Johnson S.D., Linder H.P. and Steiner K.E. (1998) Phylogeny and radiation of pollination systems in *Disa* (Orchidaceae). *American Journal of Botany* 85: 402-411.

Johnson S.D. and Edwards T.J. (2000). The structure and function of orchid pollinaria.

Plant Systematics and Evolution 222: 243-269.

Johnson S.D. and Steiner K.E. (2000) Generalization versus specialization in plant pollination systems. *Trends in Ecology and Evolution* 15: 140-143.

Johnson S.D., Edwards T.J., Carbutt C. and Potgieter C. (2002) Specialization for hawkmoth and long-proboscid fly pollination in *Zaluzianskya* section *Nycterinia* (Scrophulariaceae). *Botanical Journal of the Linnean Society* 138: 17-27.

Johnston M.O. (1991) Natural selection on floral traits in two species of Lobelia with different pollinators. *Evolution* 45: 1468 - 1479.

Joulain D. (1986) Study of the fragrance given off by certain springtime flowers. In: Brunke E.J. (ed.). Progress in Essential Oil Research. Walter de Gruyter & Co, Berlin. pp 57-67.

Kaiser R.A.J. (1993) The scent of orchids: olfactory and chemical investigations. Amsterdam, Elsevier.

Kaiser R.A.J. (1993) On the scent of Orchids. In: Teranishi R., Buttery R.G. and Sugisawa H. (eds.). Bioactive Volatiles in Plants. American Chemical Society, Washington, DC.

Kaiser R.A.J. and Tollsten L. (1995) An introduction to the scent of Cacti. *Flavour and Fragrance Journal* 10: 153-164.

Kearns C.A. and Inouye D.W. (1993) Techniques for Pollination Biologists. University Press of Colorado, Colorado.

Kevan P.G. and Baker H.G. (1983) Insects as flower visitors and pollinators. *Annual Review of Entomology* 28: 407-453.

Kluge M. and Fisher K. (1967) Über Zasammenhänge zwischen dem CO_2-Austausch und der Abgade von Wasserdampf durch *Bryophyllum daigremontianum* Berg. *Plantum* 77: 212-223.

Kluge M. and Ting I.P. (1978) Crassulacean acid metabolism. Analysis of an ecological

adaptation. Ecological Studies, Vol 30. Springer-Verlag, Berlin.

Kluge M., Vinson B. and Ziegler H. (1997) Ecophysiological studies on orchids of Madagascar: incidence and plasticity of crassulacean acid metabolism in species of the genus *Angraecum* Bory. *Plant Ecology* 135: 43-57.

Knudsen J.T. and Tollsten L. (1993) Trends in floral scent chemistry in pollination syndromes: floral scent composition in moth-pollinated taxa. *Botanical Journal of the Linnean Society* 113:263-284.

Knudsen J.T. and Tollsten L. and Bergström L.G. (1993) Floral scents - a checklist of volatile compounds isolated by head-space techniques. *Phytochemistry* 433: 253-280.

Knudsen J.T. and Tollsten L. (1995) Floral scent in bat pollinated plants: a case of convergent evolution. *Botanical Journal of the Linnean Society* 119: 45-57.

Koopowitz H. and Marchant T.A. (1998) Postpollination nectar reabsorption in the African epiphyte *Aerangis verdickii* (Orchidaceae). *American Journal of Botany* 85: 508-512.

Kurzweil H. (1991) A method to illustrate the delicate flower structures of the genus *Disperis* (Orchidaceae). *Electron Microscopy Society of South Africa - Cape Town* 21: 25-26.

la Croix I. and Cribb P.J. (1998). Orchidaceae. In: Pope G.V. (ed.). *Flora Zambesiaca* vol 11, part 2. Royal Botanic Gardens, Kew, London.

Levin R., Raguso R.A. and McDade L.A. (2001) Fragrance chemistry and pollinator affinities in Nyctaginaceae. *Phytochemistry* 58: 429-440.

Linder H.P. (1989) Notes on Southern African angraecoid orchids. *Kew Bulletin* 44: 317-319.

Linder H.P. and Kurzweil H. (1999) Orchids of Southern Africa. Rotterdam, Balkema.

Lindley J. (1836) Notes upon some genera and species of Orchidaceae in the collection

formed by Mr. Drége, at the Cape of Good Hope. In: Hooker W.J. (ed.). *Companion to Curtis' Botanical Magazine* v 2: 206.

Loughrin J.H., Hamilton-Kemp T.R., Andersen R.A. and Hildebrand D.F. (1990) Volatiles from flowers of *Nicotiana sylvestris*, *N. otophora*, and *Malus domestica*: headspace components and day/night changes in their relative concentrations. *Phytochemistry* 29: 2473-2477.

Lüttge U. (1987) Vascular epiphytes. Setting the scene. In: Lüttge U. (ed). Vascular Plants as Epiphytes - Evolution and Ecophysiology, Ecological Studies, Vol. 76. Springer-Verlag, Berlin. pp. 1-14

Luyt R. and Johnson S.D. (2001) Hawkmoth pollination of the African epiphytic orchid *Mystacidium venosum*, with special reference to flower and pollen longevity. *Plant Systematics and Evolution* 228: 49-62.

Luyt R. and Johnson S.D. (2002) Postpollination nectar reabsorption and its implications for fruit set in an epiphytic orchid. *Biotropica*. In press.

Maad J. (2000) Phenotypic selection in hawkmoth-pollinated *Platanthera bifolia*: targets and fitness surfaces. *Evolution* 54: 112-123.

Manning J.C. and Goldblatt P. (1996) The *Prosoeca peringueyi* (Diptera: Nemestrinidae) pollination guild in southern Africa: long-tongued flies and their tubular flowers. *Annals of the Missouri Botanical Gardens* 83: 67-86.

McDade L.A. (1992) Pollinator relationships, biogeography, and phylogenetics. *BioScience* 42: 21-26.

Miller R.G. (1974) The jackknife - A review. *Biometry* 61: 1-15.

Miyake T., Yamaoka R. and Yahara T. (1998) Floral scents of hawkmoth pollinated flowers in Japan. *Journal of Plant Research* 111: 199-205.

Mort M.E., Soltis D.E., Soltis P.S. and Mabry M.L. (2000) An empirical test of three methods for estimating internal support on phylogenetic trees. *Systematic Biology* 49: 160-171.

Neiland M.R.M. and Wilcock C.C. (1994) Maximization of reproductive success by European Orchidaceae under conditions of infrequent pollination. *Protoplasma* 187: 39-48.

Nepi M., Guarnieri M. and Pacini E. (2001) Nectar secretion, reabsorption, and sugar composition in male and female flowers of *Cucurbita pepo*. *International Journal of Plant Sciences* 162: 353 - 358.

Neurenbergk E.L. (1963) On the CO_2 metabolism of orchids and its ecological aspect. In: Proceedings of the 4th World Orchid Conference. Straits Times Press, Singapore. pp 158-169.

Nilsson L.A. (1978) Pollination ecology and adaptation in *Platanthera chlorantha* (Orchidaceae). *Botaniska Notiser* 131: 35-51.

Nilsson L.A. (1988) The evolution of flowers with long corolla tubes. *Nature* 334: 147-149.

Nilsson L.A. (1992) Orchid pollination biology. *Trends in Evolution and Ecology* 7: 255-259.

Nilsson L.A., Jonsson L., Rason L. and Randrianjohany E. (1985) Monophily and pollination mechanisms in *Angraecum arachnites* Schltr. (Orchidaceae) in a guild of long-tongued hawk-moths (Sphingidae) in Madagascar. *Botanical Journal of the Linnean Society* 26: 1-19.

Nilsson L.A. and Rabakonandrianina E. (1988) Hawkmoth scale analysis and pollination specialization in the epilithic Malagasy endemic *Aerangis ellisii* (Reichenb. fil.) Schltr. (Orchidaceae). *Botanical Journal of the Linnean Society* 97: 49-61.

Nilsson L.A., Rabakonandrianina E., Razananaivo R. and Randriamanindry J. (1992) Long pollinia on eyes: hawkmoth pollination of *Cynorkis uniflora* Lindley (Orchidaceae) in Madagascar. *Botanical Journal of the Linnean Society* 109: 145-160.

Nixon K.C. (1993) *DADA*. Published privately.

Nseya A.M. and Arends J.C. (1995) Contribution á l'étude anatomique et caryologique des Orchidaceae: le genre *Cyrtorchis* Schltr. *Bulletin. Museum National d'Histoire Naturelle* 17: 75 - 93.

O'Connel L.M. and Johnston M.O. (1998) Male and female pollination success in a deceptive orchid, a selection study. *Ecology* 79: 1246 - 1260.

O'Leary M.H. (1981) Carbon isotope fractionation in plants. *Phytochemistry* 20: 553-567.

Opler P.A. (1983) Nectar production in a tropical ecosystem. In: Bentley B. and Elias T. (eds.) The Biology of Nectaries. Columbia University Press, New York. pp 30-79.

Osmond C.B., Ziegler H., Stichler W. and Trimborn P. (1975) Carbon isotope discrimination in alpine succulent plants supposed to be capable of crassulacean acid metabolism (CAM). *Oecologia* 18: 209-217.

Osmond C.B. (1978) Crassulacean acid metabolism: a curiosity in context. *Annual Review of Plant Physiology* 29: 379-414.

Pellmyr O. and Thien L.B. (1986) Insect reproduction and floral fragrances: keys to the evolution of the angiosperms. *Taxon* 35: 76-85.

Petterson M.W. (1991) Pollination by a guild of fluctuating moth populations: option for unspecialization in *Silene vulgaris*. *Journal of Ecology* 79: 591 - 604.

Pfitzer E. (1889) Orchidaceae. In: Engler A and Prantl K. (ed.). *Die natürlichen Pflanzenfamiien* II. 6. 214, 216.

Platnick N.I. (1989) An empirical comparison of microcomputer parsimony programs. II. *Cladistics* 5: 145-161.

Porembeski S. and Barthlott W. (1988) Velamen radicum micromorphology and classification of Orchidaceae. *Nordic Journal of Botany* 8: 117-137.

Primack R.B. (1985) Longevity of individual flowers. *Annual Review of Ecological Systematics* 16: 15-37.

Proctor M., Yeo P. and Lack A. (1996) The Natural History of Pollination. Timber Press, Oregon.

Pyke G.H. and Waser N.M. (1981) The production of dilute nectars by hummingbird and honeyeater flowers. *Biotropica* 13: 260-270.

Raguso R.A. (2001) Floral scent, olfaction, and scent-driven foraging behaviour. In: Chittka L. and Thomson J.D. (eds.). Cognitive Ecology of Pollination. Animal Behaviour and Floral Evolution. Cambridge University Press, Cambridge. pp 83-105.

Robertson J.L. and Wyatt R. (1990) Evidence for pollination ecotypes in the yellow fringed orchid, *Platanthera ciliaris*. *Evolution* 44: 121-133.

Rolfe R.A. (1898) Orchideae. In: Thistelton-Dyer W.T. (ed.). *Flora of Tropical Africa* 7: 133, 169.

Rolfe R.A. (1912) Orchideae. In: Thistelton-Dyer W.T. (ed.). *Flora Capensis* 5, 3: 79.

Schlechter R. (1914) Die Orchideen: 593.

Schlechter R. (1918) Versuch einer natuerlichen Neuordnung der afrikanischen Angraekoiden Orchideen. *Botanisches Centralblatt. Beihefte* 36: 62-181.

Scoble, M.J. (1992) The Lepidoptera: Form, Function and Diversity. Oxford University Press, New York.

Simpson B.B. and Neff J.L. (1983) Evolution and diversity of floral rewards. In: Jones C.E. and Little R.J. (eds.). Handbook of Experimental Pollination Biology. New York, Van Nostrand-Reinhold. pp 142-159.

Southwick E.E. (1990) Floral nectar. *American Bee Journal* 130: 517-519.

Stebbins G.L. (1970) Adaptive radiation of reproductive characteristics in Angiosperms, 1: pollination mechanisms. *Annual Review of Ecology and Systematics* 1: 307-326.

Steiner K.E. and Whitehead V.B. (1990) Pollination adaptation to oil-secreting flowers - *Rediviva* and *Diascia*. *Evolution* 44: 1701-1707.

Stewart J., Linder H.P., Schelpe E.A. and Hall A.V. (1982) Wild Orchids of Southern Africa. Macmillan, Johannesburg.

Summerhayes V.S. (1945) *Botanical Museum Leaflets of Harvard University* 12: 113.

Summerhayes V.S. (1949) African orchids XIX. *Kew Bulletin* 3: 442.

Summerhayes V.S. (1960) African orchids XXVII. *Kew Bulletin* 14: 126-160.

Swofford D.L. (1998) PAUP* 4.0: phylogenetic analysis using parsimony (and other methods) beta vers 4.0. Sinauer, Sunderland, Massachusetts, USA.

Teerie J.A., Tonsor S.J. and Turner M. (1981) Leaf thickness and carbon isotope composition in Crassulaceae. *Oecologica* 50: 367-369.

Torres C. and Galetto L. (1998) Patterns and implications of floral nectar secretion, chemical composition, removal effects and standing crop in *Mandevilla pentlandiana* (Apocynaceae). *Botanical Journal of the Linnean Society* 127: 207-223.

van Wyk, B-E. (1983) Nectar sugar composition in Southern African Papilionoideae (Fabaceae). *Biochemical Systematics and Ecology* 21(2): 271-277.

van der Pijl L. (1961) Ecological aspects of flower evolution. II. Zoophilous flower classes. *Evolution* 15: 44-59.

van der Pijl L. and C.H. Dodson (1966) Orchid Flowers: Their Pollination and Evolution. University of Miami Press, Florida.

Varassin I.G., Trigo J.R. and Sazima M. (2001) The role of nectar production, flower pigments and odour in the pollination of four species of *Passiflora* (Passifloraceae) in south-eastern Brazil. *Botanical Journal of the Linnean Society* 136: 139-152.

Vogel S. (1954) Blütenbiologische Typen als Elemente der Sippengliederung. *Botaniese*

Studien 1 - Jena: Fischer.

Wasser N.M. (1983) Competition for pollination and floral character differences among sympatric plant species: a review of evidence. In: Jones C.E. and Little R.J. (eds.). Handbook of Experimental Pollination Biology. New York, Van Nostrand-Reinhold. pp 277-293.

Waser N.M., Chittka L., Price M.V., Williams N. and Ollerton J. (1996) Generalization in pollination systems, and why it matters. *Ecology* 77: 1043-1060.

Wasserthal L.T. (1997) The pollinators of the Malagasy star orchids *Angraecum sesquipedale, A. sororium* and *A. compactum* and the evolution of extremely long spurs by pollinator shift. *Botanica Acta* 110: 343-359.

Weller S.G. and Sakai A.K (1999) Using phylogenetic approaches for the analysis of plant breeding system evolution. *Annual Review of Ecological Systematics* 30: 167-199.

Winter K., Wallace B.J., Stocker G.C. and Roksandic Z. (1983) Crassulacean acid metabolism in Australian vascular epiphytes and some related species. *Oecologia* 57: 129-141.

Zar J.H. (1984) Biostatistical Analysis (2nd edition). Prentice Hall, USA.

Zimmerman J.K. and Aide T.M. (1989) Patterns of fruit production in a neotropical orchid: pollinator vs. resource limitation. *American Journal of Botany* 76: 67-73.

APPENDIX I

Postpollination nectar reabsorption and its implications for fruit quality in an epiphytic orchid

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ABSTRACT

We present evidence that pollination triggers nectar reabsorption in flowers of the epiphytic orchid *Mystacidium venosum.* The amount of sugar in nectar of *M. venosum* decreased significantly by more than 50% within 72 hours of pollination. Hand-pollinated flowers from which nectar was previously removed set significantly smaller fruits with a lower percentage of viable seeds than hand-pollinated flowers containing nectar, suggesting that resources reclaimed by nectar reabsorption are allocated to fruit production.

Key words: epiphyte; Mystacidium venosum; *nectar reabsorption; Ochidaceae*

NECTAR MAY BE ENERGETICALLY EXPENSIVE FOR A PLANT because of its sugar content, cost of initial secretion and later active maintenance at a volume and concentration that is attractive to the pollinators for which the plant is adapted (Cruden *et al.* 1983; Kevan & Baker 1983). Estimates of the cost of nectar production in terms of daily photosynthate vary from 3.3% in short-lived flowers to 30% in long-lived flowers (Southwick 1984; Harder & Barrett 1992). There is evidence that some plants are able to reabsorb sugar from nectar (Cruden *et al.* 1983; Búrquez & Corbet 1991). Several authors have shown movement of labelled sugars from nectar to the flower tissue (eg Bieleski & Redgwell 1980), but these studies do not demonstrate that there is net movement of solutes from nectar into the flower. More convincing evidence for reabsorption is obtained when it is shown that there is a net solute loss in unvisited flowers (eg Búrquez & Corbet 1991)

Several authors have argued that the function of reabsorption is to reduce the sugar concentration and thus viscosity of nectar in order to make it more attractive to pollinators. Nicolson (1995) demonstrated that the flowers of *Grevillea robusta* reabsorb nectar to maintain a constant low concentration in spite of evaporation. However, another function of nectar reabsorption may be for a plant to retrieve some of the resources from unused nectar in both unpollinated and pollinated flowers.

Koopowitz and Marchant (1998) showed that sugar concentration in the nectar of the

epiphytic orchid *Aerangis verdickii* drops significantly within 48 hours of pollination. While this provides some evidence for reabsorption of sugar, they did not measure post-pollination nectar volumes in the treated plants after 48 hours, and thus did not eliminate the possibility that the decreased sugar concentration was due to net movement of water into the nectar from drying floral tissues or from the atmosphere. To show nectar reabsorption following pollination, it is necessary to demonstrate an overall decline in nectar sugar weight (mg).

The study species *Mystacidium venosum* Harv. ex Rolfe (Aerangidinae) is an almost stemless epiphyte that bears up to ten semi-pendulous inflorescences, each with 4 to 10 flowers in an alternate arrangement. The white flowers are sweet-scented, 1.5 to 2 cm in diameter and pollinated by hawkmoths (Luyt & Johnson 2001). Narrow, tapering spurs, 2.5 to 5.5 cm in length, contain *c.* 1.8 µl of nectar with a sugar concentration of *c.* 16% (Luyt & Johnson 2001). Previous studies of this species show that the amount of sugar in the nectar of bagged flowers fluctuates on a daily basis (Luyt & Johnson 2001). Thus sugar reabsorption may be integral to the maintenance of nectar at optimal volume and concentration in this species.

The objectives of this study were (1) To determine whether *M. venosum* is capable of reabsorbing sugar following pollination; and (2) To determine if fruit quality is affected by the reabsorption of nectar.

METHODS

The study was conducted between June 1998 and November 1999. Plants of *M. venosum* were collected at the beginning of the flowering season in May/June 1998 from Verulam, 40 km north of Durban, Kwa-Zulu Natal, South Africa, and kept in the greenhouse of the Botany Departmental gardens of the University of Pietermaritzburg, for subsequent observation and experiments.

POST-POLLINATION NECTAR REABSORPTION. - In order to test whether *M. venosum* reabsorbs unused nectar following pollination, 42 pairs of flowers were randomly selected from c. 60 plants in the greenhouse. For each pair, one flower was cross-pollinated by hand and the other was maintained as an unpollinated control. Hand-pollinations were carried out in the late afternoon. The height of the nectar column, which was visible through the translucent spur tissue, of each flower was measured immediately. After 48 hours, 22 pairs were randomly selected, and the height of the nectar column and the sugar concentration of each flower was measured using callipers and an Atago N1 0-32% pocket refractrometer, respectively. After a further 24 hours (72 hours from the time of pollination), the remaining 20 pairs were measured in the same way. The nectar sugar concentration, nectar volume, and nectar sugar weight (calculated according to Dafni, 1992) of pollinated and unpollinated flowers were compared.

In order to derive an equation to estimate the nectar volume from the height of the nectar column, the height and volume of nectar in the spurs of 48 flowers were measured. The height of the nectar column was measured using a steel ruler, and the volume of the nectar in each flower was measured using a calibrated micropipette. The relationship between the height of the nectar column and nectar volume was determined using regression analysis (Zar 1984).

THE EFFECT OF NECTAR REMOVAL ON FRUIT SET. - Studies were carried out in June 1998 and 1999 in order to determine whether the reabsorption of nectar by *M. venosum* following pollination increases the resources available to developing seeds. In 1998, 42 pairs of flowers were randomly selected from different plants in the greenhouse. Both flowers in each pair, which were on the same inflorescence, were cross-pollinated by hand, and the nectar from one flower was subsequently removed. Since the spur entrance of *M. venosum* is extremely small and the spurs very slender, the only effective way of removing the nectar was to cut off the spur tip at the point of the highest level of nectar. The flowers were left to set fruit. After approximately 12 weeks from the time of pollination, the diameter and length of the fruits, as well as seed viability, within each pair were compared using the t-test for paired samples (Zar 1984). This experiment was repeated in the following season. The sample size was increased to 60 pairs of flowers. The diameter and length of the fruits, the seed viability, as well as the fruit weight within each pair was compared after approximately 20 weeks from the time of pollination.

RESULTS

POST-POLLINATION NECTAR REABSORPTION. - A best fit curve for the relationship between the height of the nectar column and the volume of nectar was obtained by exponential regression, using the equation $y = e^{(-1.62 + 0.80x)}$ ($R^2 = 0.81$, $P < 0.001$). The average nectar volume of unpollinated and pollinated flowers at the start of the experiment was not significantly different (unpollinated = 1.45 µl ± 0.17, pollinated = 1.49 µl ± 0.15; $t = 0.19$, $P > 0.5$). The nectar volume of pollinated flowers was higher than the nectar volume of unpollinated flowers after 48 hours, but the difference was not significant (Figure 1a). After 72 hours, the volume of nectar in the pollinated flowers had reduced significantly (Figure 1a).

The sugar concentration of nectar was significantly lower in the pollinated flowers than the unpollinated flowers after both 48 and 72 hours. (Figure 1b). There was no significant difference in the sugar weight of nectar between pollinated and unpollinated flowers measured after 48 hours ($Z = 0.6$, $P > 0.05$). However, the sugar weight of the nectar of the pollinated flowers was significantly lower than that of the unpollinated flowers measured after 72 hours (Figure 1c). Since the net reabsorption of nectar is more accurately measured by the change in the sugar weight of the nectar, it

can be inferred that nectar is reabsorbed within 72 hours. The significant reduction in the sugar concentration of the pollinated flowers after 48 hours may have been the result of water gain from the air or from the plant. It was accompanied by a slight increase in the volume of the nectar after 48 hours.

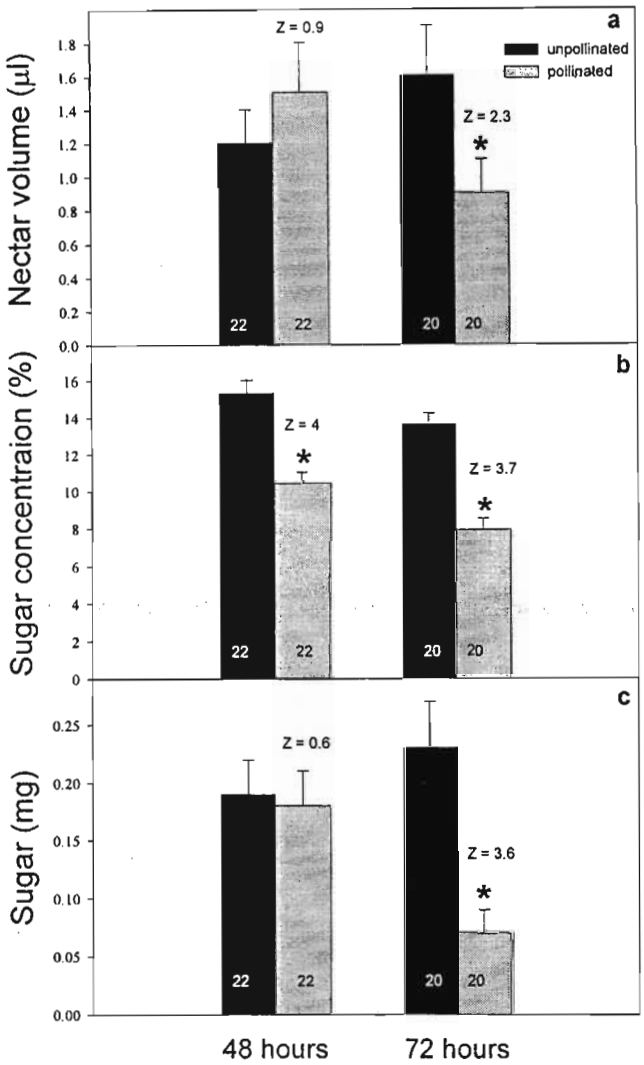

FIGURE 1. Changes in nectar sugar properties of unpollinated and hand-pollinated flowers of *M. venosum* 48 and 72 h following pollination. (a) Mean nectar volume (µl); (b) Mean sugar concentration (%); (c) Mean sugar content (mg). Numbers in bars indicate sample size. An asterisk denotes a significant difference (Normal approximation of the Mann Whitney U test).

DISCUSSION

Our results show that *M. venosum* reabsorbs unused nectar following pollination, and that fruit quality may be enhanced as a consequence. Koopowitz & Marchant's (1998) finding that unused nectar following pollination of *A. verdickii* is reabsorbed after 48 hours was based on a decrease in nectar concentration of pollinated flowers. Although the sugar concentration in flowers of *M. venosum* also declined significantly after 48 hours this was probably due to water gain, as the nectar sugar weight of pollinated flowers only showed a significant reduction after 72 hours (Figure 1c). The decrease in sugar content after 72 hours was accompanied by a significant decrease in nectar volume. This corroborates Koopowitz & Marchant's (1998) suggestion that transport of sugar takes place and water then follows the osmotic gradient.

Resource availability for the production of fruits and seeds may be relatively limited in pollinator-rewarding orchids compared to non-rewarding ones due to the energetically expensive task of nectar production (Meléndez-Ackerman *et al.* 2000). However, even though nectar may be energetically costly to produce, it may also be expensive to reabsorb. Búrquez & Corbet (1991) suggested that if carbon is a limiting resource, and the energetic cost of reabsorption is less than the energy present in the nectar, reabsorption can increase the resources available to developing seeds. The data on the effect of nectar removal on fruit set in the immediate flower (Table 1) suggest that this may be the case in *M. venosum*. However, energetically valuable sugars not utilized by the pollinator may be retrieved through reabsorption and allocated to other areas of the plant. Resources allocated to vegetative growth, such as leaf size, and reproductive growth, such as flower number, would increase reproductive success in the future season.

Cruden & Hermann (1983) suggested that water and the constituents of nectar in old or wilting flowers may diffuse into the drying tissue, thereby enabling the plant to reclaim energy rich compounds. These compounds could then be translocated to developing fruits elsewhere on the inflorescence. While unpollinated flowers did not exhibit any signs of nectar reabsorption after 72 hours, it is possible that reabsorption may have occurred at a later stage after senescence.

The results of this study suggest that nectar reabsorption is triggered by the pollination process in *M. venosum* (Figure 1). The advantage of pollination-triggered nectar-reabsorption may be that carbon-rich compounds are absorbed directly at the site of fruit production. Such a mechanism would be effective if some nectar is left behind after a moth has visited a flower, which could then be reabsorbed. However, even if moths do remove all the nectar, it may also be energetically worthwhile for the plant to reabsorb any nectar that is secreted following pollination.

The flowers of *M. venosum* are likely to have been shaped by the behaviour and morphology of hawkmoths. Hawkmoths require copious amounts of nectar to sustain their energetic requirements and the mechanism of post-pollination nectar reabsorption could offset this cost by retrieving resources for investment in seed production.

LITERATURE CITED

BIELESKI, R. L. AND R. J. REDGWELL. 1980. Sorbitol metabolism in nectaries from flowers of Rosaceae. Australian Journal of Plant Physiology 7: 15-25.

BÚRQUEZ, A. AND S. A. CORBET. 1991. Do flowers reabsorb nectar? Functional Ecology 5: 369-379.

CRUDEN, R. W. AND S. M. HERMANN. 1983. Studying nectar? Some observations on the art. -In: Bentley, B. and Elias, T. (eds.)., The Biology of Nectaries. Columbia University Press, New York.

CRUDEN, R. W., S. M. HERMANN, AND S. PETERSON. 1983. Patterns of nectar production and plant-pollinator coevolution. -In: Bentley, B. and Elias, T. (eds.)., The Biology of Nectaries. Columbia University Press, New York.

DAFNI, A. 1992. Pollination Ecology: A Practical Approach. Oxford University Press, New York.

DRESSLER, R. L. 1981. The Orchids: Natural History and Classification. Harvard University Press, USA.

HARDER, L. D. AND BARRETT , S. C. H. 1992. The energy cost of bee pollination for *Pontedria cordata* (Pontedriaceae). Functional Ecology 6: 226-233.

KEVAN, P. G. AND H. G BAKER. 1983. Insects as flower visitors and pollinators. Annual Review of Entomology 28: 407-453

KOOPOWITZ, H. AND T. A. MARCHANT. 1998. Postpollination nectar reabsorption in the African epiphyte *Aerangis verdickii* (Orchidaceae). American Journal of Botany 85(4): 508-512.

LUYT, R. AND S. D. JOHNSON. 2001. Hawkmoth pollination of the African epiphytic orchid *Mystacidium venosum*, with special reference to flower and pollen longevity. Plant Systematics and Evolution 228: 49-62.

MELÉNDEZ-ACKERMAN, E. J., J. ACKERMAN, AND J. A. RODRÍGUEZ-ROBLES. 2000. Reproduction in an orchid can be resource-limited over its lifetime. Biotropica 32: 282-290.

NICOLSON, S. W. 1995. Direct demonstration of nectar reabsorption in the flowers of *Grevillea robusta* (Proteaceae). Functional Ecology 9: 584-588.

SOUTHWICK, E. E. 1984. Photosynthate allocation to floral nectar: a neglected energy investment. Ecology 65: 1775-1779.

ZAR, J. H. 1984. Biostatistical Analysis (2nd edition). Prentice Hall, USA.

APPENDIX II

Plant Syst. Evol. 228: 49–62 (2001)

Plant Systematics and Evolution

Hawkmoth pollination of the African epiphytic orchid *Mystacidium venosum*, with special reference to flower and pollen longevity

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Abstract. The pollination biology of *Mystacidium venosum* Harv. ex Rolfe, an epiphytic orchid endemic to southern Africa, was investigated. The white flowers of this orchid have long spurs (*c* 4.5 cm) and contain dilute sucrose-dominated nectar which is secreted during the afternoon and early evening. Scent, dominated by Jasmine lactone and (E, E)-Farnesol, is emitted in the evening. A breeding system experiment established that self pollination results in inferior quality fruits. Field observations at three sites in Kwa-Zulu Natal, South Africa, in 1998 and 1999 showed that hawkmoths were frequent visitors to the orchid shortly after dusk, and carry pollinaria along the length of their tongues. No evidence for directional selection on spur length was found at any of the three sites. Results showed that flower longevity is strongly reduced by pollination, while pollinaria removal had only a small effect. Pollinia removed from flowers remained viable for up to 20 days under outdoor conditions.

Key words: *Mystacidium venosum*, Orchidaceae, epiphyte, flower longevity, hawkmoth, pollen longevity, pollination.

Flowers tend to exhibit features that can be related to the size, behaviour and energetic requirements of their pollinators. These pollination syndromes are manifestations of convergent evolution within the angiosperms (Proctor et al. 1996, Johnson and Steiner 2000). The adaptations involved in the pollination of orchids, in particular, are remarkably diverse, and occur mainly on the plant side in the asymmetric orchid-pollinator relationship (Nilsson 1992). Hawkmoth-pollinated flowers are usually characterized by long floral spurs, a white or pale-coloured perianth, and production of a relatively dilute, sucrose-rich nectar and a sweet scent in the evening (Haber and Frankie 1989). On the basis of floral syndromes, it has been predicted that 50% of the African orchids are pollinated by Lepidoptera (Dodson 1962), however the only studies on hawkmoth-pollinated orchids in Africa have been made on terrestrial species (Johnson 1995, 1997), although a number of studies have confirmed that hawkmoth pollination systems occur in the epiphytic orchids of Madagascar (Nilsson et al. 1985, Nilsson and Rabokonandrianina 1988, Nilsson et al. 1992, Wasserthal 1997).

Mystacidium Lindl. belongs to the subtribe Aerangidinae, which occurs in southern and tropical Africa. There has been no pollination research on this, or any other epiphytic orchid genus in southern Africa. Most of the species

in this subtribe conform to the syndrome of moth pollination (Dressler 1981). *Mystacidium* is a small genus consisting of approximately 10 epiphytic species, 7 of which occur in South Africa. There is considerable variation in the spur length among *Mystacidium* species, with *M. venosum* and *M. capense* having the longest spurs (>4 cm) in the genus. Hawkmoth-pollinated flowers have evolved long spurs, a trait that enables effective pollination by long-tongued insects. A mechanism for the evolution of floral spurs in plants when pollen is placed on the face of the pollinator was proposed by Darwin (1862), and later experimentally tested by Nilsson (1988). This model may not apply to plants such as *Mystacidium* which place pollen along part of the length of the tongue of the pollinator (this study, unpublished data).

Reproductive fitness in many orchids is pollinator-limited (Zimmerman and Aide 1989, Calvo and Horvitz 1990, Johnson and Bond 1997), resulting in strong potential for selection to favour floral structures and attractants that appeal to the most reliable pollinators. Adaptation to a different pollinator may result in new diagnosable taxa; for example, the shift from bee to hawkmoth pollination between subspecies of *Satyrium hallackii* Johnson (1997). These shifts in the floral syndrome may be governed by Stebbins' (1970) "most effective pollinator" principle, whereby the plant adapts to the most efficient pollinator in a particular habitat.

Pollinia may be transported by widely foraging insects, such as hawkmoths, which may only visit flowers intermittently. It may therefore be a number of days before the pollinia are transferred to the stigmatic surface of another orchid. Although orchid pollen longevity has been investigated in laboratory studies, there has been only one previous study that we are aware that deals with pollen longevity under outdoor conditions with natural fluctuations of temperature (Alexandersson 1999). Low pollinator visitation in orchids may be a selective factor that has led to long-lived flowers (Arditti 1979). Flower senescence triggered by pollination reduces the physiological costs associated with maintenance, and in some cases, similar, albeit weaker, adaptive responses occur when pollinia are removed from unpollinated flowers (Clayton and Aizen 1996).

The objectives of this study were to test the hypothesis that *M. venosum* is pollinated by hawkmoths, to describe in detail aspects of the floral biology (including nectar and scent chemistry, and pollen and flower longevity), and to determine, through comparison of spur lengths in pollinated vs. unpollinated flowers, if selection favours plants with longer spurs.

Materials and methods

Study species. *Mystacidium venosum* Harv. ex Rolfe is an almost stemless epiphyte. Up to ten semi-pendulous inflorescences, 4 to 9 cm in length, arise below the leaves. Each inflorescence usually bears 4 to 10 flowers in alternate arrangement. The white flowers are sweet-scented, 1.5 to 2 cm in diameter, and have narrow, tapering, nectariferous spurs 2.5 to 5.5 cm in length. *M. venosum* is widespread in a variety of habitats, usually in the warmer areas of bush and forest, and on a wide variety of host trees, for example *Ficus natalensis*, *Ekebergia capensis*, *Podocarpus latifolius* (personal observations), and on exotic species such as *Mangifera indica* and *Cupressus* and *Pinus* species. It occurs in both very exposed positions, and in shade. It has been recorded in the Eastern Cape, Kwa-Zulu Natal, Mpumalanga and Swaziland.

Study area. Observations were made at two separate study sites in 1998 and at one in 1999. In 1998 the first site was at a cemetery in Verulam, 40 km north of Durban, Kwa-Zulu Natal. At this site a population of approximately ten thousand individual plants of *M. venosum* grow on *Cupressus* trees. The second site was at a picnic spot in Yellowwood, Durban, Kwa-Zulu Natal. At this site, a population of about two thousand individual plants of *M. venosum* colonize three Mango trees (*Mangifera indica*). Plants of *M. venosum* also grow on indigenous trees in the adjacent forest of the Kenneth Stainbank Nature Reserve. In 1999 a population of about 80 individual plants growing on a large Fig tree (*Ficus*) was observed at Baynesfield Estate near Thorneville, Kwa-Zulu Natal. Plants were collected from the Verulam site and kept in the greenhouse of the Botany Department

gardens of the University of Natal, Pietermaritzburg, for subsequent observation and experiments.

Breeding system. To determine whether *M. venosum* is dependent on pollinators for fruit set, a breeding system experiment was carried out in a greenhouse from which insects were excluded. Individual flowers were randomly allocated to selfed and outcrossed treatments, until there were 20 representatives for each treatment. Selfed flowers were pollinated with pollinia from flowers on the same inflorescence, and outcrossed flowers were pollinated with pollinia from flowers from a different plant, while a further 20 flowers were not hand-pollinated to test for autogamy. The percentage of flowers which developed into fruits was determined after ± 5 months when the fruits were mature. Fruit weight, length and diameter, as well as the percentage of seeds with embryos, was measured.

Pollinator observations. Pollinator observations were made at the Verulam site on 1, 4, 5, 9, 10, 15, 18 and 19 June 1998, and were usually carried out between 17:00 hours and 18:30 hours. A total of approximately 10 hours was spent in evening observations. Observations were carried out at Baynesfield on 31 May, and 8, 10, 18, 21 and 30 June 1999. A torch was used to observe moths after dusk, and the time that each moth commenced its foraging bout was recorded. An insect net was used to capture moths foraging on the flowers of *M. venosum* wherever possible. The length of the moth's proboscis, as well as the distance of pollinaria attachment from the tip of the proboscis, was measured to the nearest 0.5 mm using a steel ruler.

Nectar and scent properties. The volume of nectar in the spurs of 22 unvisited flowers at the Verulam site was measured using a calibrated micropipette (Fisherbrand 1–5 µl), and the average nectar concentration was determined with an Atago N1 0–32% pocket refractrometer. Two nectar samples, taken from plants collected at the Verulam site, were applied as spots to Whatman no.1 filter paper. The nectar sugar composition was analysed by B-E van Wyk using basic HPLC techniques (van Wyk 1993). The nectar sugar composition in *M. venosum* was based on the average of the two samples, which were taken from ten flowers.

Timing of nectar production was determined by monitoring the change in nectar height, volume and concentration over a 24 hour period at 4 hour intervals. Nectar height measurements were taken from the same flowers at each 4-hourly interval. In order to measure nectar volume and concentration, flowers had to be destroyed, therefore measurements were obtained from different flowers every four hours. The conversion to nectar sugar weight (mg) was done according to Dafni (1992).

Scent production was qualitatively assessed in the greenhouse by sniffing several flowers at regular intervals throughout the day. In order to collect the scent of *M. venosum*, a trapping technique involving the adsorption on charcoal followed by solvent extraction (Kaiser 1993) was applied to flowers collected from the Verulam site. Two separate samples were collected, in which whole inflorescences were placed in a glass vessel and the scented air surrounding the flowers was drawn through an adsorption trap by a battery operated pump. The first sample was collected on 23 June 1999 between 17:30 hours and 19:30 hours at a flow rate of 144.5 ml/min, and the second sample was collected on 5 July 1999 from 17:45 hours to 22:30 hours at a flow rate of 194.5 ml/min. Both samples were analysed by Dr. Roman Kaiser.

Functional morphology. In order to document the floral mechanism of *M. venosum*, plant material collected from the Verulam site was dissected and the freshly cut columns were photographed using the standard SEM technique. The samples were sputter-coated with Au/Pd and viewed and photographed in a Hitachi-S570 scanning electron microscope at 10 kV.

Spur length and pollination success. The spur length of *M. venosum* was measured in one flower from each of 70 inflorescences at the Verulam site, 115 at the Yellowwood site, and 30 at Baynesfield. To establish the relationship between spur length and pollination, the flowers were examined for the presence of pollinia in the stigmatic cavity and removal of pollinaria from the anther. To determine whether the number of flowers per plant has an influence on fruit set, the number of scars (which indicated flowers which did not set fruit) and the number of fruits per inflorescence in 24 plants at the Verulam site were counted.

Flower and pollen longevity. An experiment was carried out in the greenhouse to determine the effect of pollinaria removal and pollination on the subsequent lifespan of flowers of *M. venosum*. Treatments were implemented at the time of flower

anthesis, and included: (1) unmanipulated flowers (control); (2) removal of both pollinaria only; and (3) pollination using pollinia from a separate plant. The flowers were monitored every day until the end of the flower lifespan, which was marked by the closing or "collapse" of the corolla.

To determine how long pollen can remain viable for under normal conditions, pollinaria were removed from flowers at flower anthesis and stored attached to insect pins after recording the date. The insect pins carrying the pollinaria were kept upright on a polystyrene board which was placed in an open box. The box was covered with insect netting and left outdoors to approximate field conditions. Flowers 1, 5 and 10 days old were cross-pollinated by hand using either 1, 5, 10, 15 or 20 day old pollinia. Percentage fruit set and the percentage of seeds with embryos was recorded ±5 months later when the fruits had fully matured.

Results

Breeding system. *M. venosum* is clearly dependent on pollinators for fruit set, as no fruits were produced by bagged, unmanipulated flowers (Table 1). The cross-pollinations resulted in 65% fruit set, while 40% of the self-pollinations were successful in producing fruit. The cross- and self-pollinated flowers did not differ significantly with respect to fruit set success ($X^2 = 0.07$, $P > 0.05$). However, fruit mass, fruit length, as well as the percentage of seeds with embryos in fruits was significantly lower in the selfed treatment, indicating that *M. venosum* is only partially self-compatible.

Pollinator observations. Hawkmoths were observed foraging on the flowers of *M. venosum* on each evening of the study at Verulam – a total of 17 visits were observed. At Baynesfield, hawkmoths were observed foraging on flowers on four separate occasions. At both sites, hawkmoth activity was confined to a short period after dusk, between 17:15 hours and 18:00 hours at the Verulam site, with peak activity occurring between 17:20 hours and 17:30 hours, and between 17:26 hours and 18:00 hours at Baynesfield (Fig. 1). At the Verulam site, individual hawkmoths visited up to thirty flowers in succession, with each visit lasting approximately 2 seconds. Although hawkmoths were frequently observed, they were difficult to capture owing to the height of the orchids in the trees. On June 9 1998, a hawkmoth foraging the flowers of *M. venosum* was caught at 17:24 hours. The hawkmoth was identified as *Nephele accentifera accentifera* (de Beauvois). Twenty pollinaria were attached along the proboscis which was 6.15 cm long (Fig. 3c–e). The placement of pollinaria on the proboscis ranged from 2.8 cm to 3.4 cm from the tip, with an average distance of 3.05 cm (S.D. = 0.15, $N = 20$). The captured hawkmoth was deposited in the Transvaal Museum, Pretoria, as a voucher specimen. At 17:30 hours on June 8 1999, a hawkmoth, *Hippotion eson* (Cramer), was captured at Baynesfield whilst feeding from *M. venosum*, however there were no pollinaria attached to its proboscis which was 5.2 cm long.

Nectar and scent properties. The average standing crop of nectar in flowers at the Verulam site at 17:00 hours on June 1 1998 was 1.8 µl (S.D. = 0.95, $N = 22$) with a nectar sugar concentration of 16% (S.D. = 2.9, $N = 22$). The nectar proved to be sucrose-rich,

Table 1. Results of an experiment to determine the breeding system of *M. venosum*

Treatment	Flowers (*n*)	Fruit set (%)	Average mass of fruit (mg ± SD)	Average fruit length (mm ± SD)	Average fruit width (mm ± SD)	Seeds with embryos (% ± SD)
Unmanipulated	20	0	0	0	0	0
Selfed	20	40	0.05 ± 0.01	11.8 ± 0.7	4.0 ± 0.1	37.1 ± 7.6
Outcrossed	20	65	0.11 ± 0.01*	14.0 ± 0.6*	4.1 ± 0.1	99.0 ± 0.5***

*$P < 0.05$, ***$P < 0.001$ (Mann Whitney U test comparing selfed and outcrossed treatments)

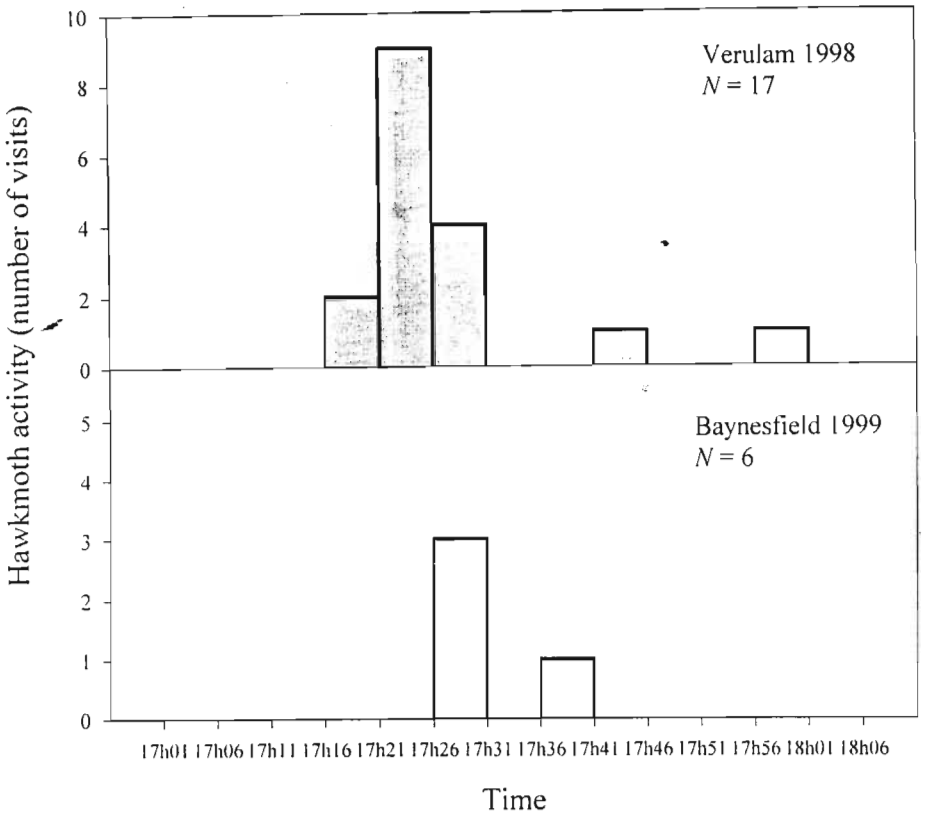

Fig. 1. Hawkmoth activity at *M. venosum* as recorded from observations during 1998 and 1999 at two different study sites

with a relative sugar composition (in percent) of 10:3:87 (fructose:glucose:sucrose). Hawkmoth activity coincided with the maximum production of nectar (Figs. 1, 2). Nectar volume, as well as the sugar percentage and the sugar content in the nectar, slowly increased throughout the day, and began to peak from early evening until 20:00 hours. From 20:00 hours to the early hours of the morning, nectar appeared to be reabsorbed (Fig. 2). Only a very slight floral scent could be detected during the daytime. From 17:00 hours a strong, sweet, jasmine-like scent began to emanate from the flowers, and by 17:30 hours it could be detected from several metres away. The scent began to dissipate from about 20:00 hours. The scent was composed chiefly of Jasmine lactone and (E, E)-Farnesol (Table 2).

Functional morphology. The column of *M. venosum* bears two pollinaria at its apex beneath a removable anther cap (Fig. 3b). The column (including the anther cap) is *c.* 1.6 mm tall. The two pollinia, which are *c.* 0.4 mm in diameter, are attached by the stipes to the viscidia. The viscidia are situated at the terminal end of the three-lobed rostellum, which protrudes downwards from the column. The outer lobes of the rostellum are minutely hairy. The stipes are *c.* 1.1 mm in length, and lie along the rostellum. The stigmatic cavity is notched at the apex and lies directly behind the rostellum.

The pollination mechanism of *M. venosum* was interpreted on the basis of floral morphology as follows. The small side-lobes of the three-lobed labellum flank the entrance to the spur, and the deflexed midlobe guides the proboscis into it. As the proboscis moves forward, it brushes the end of the rostellum and dislodges the two pollinaria which are attached via the stipes to the viscidia. The average distance from the tip of the proboscis to the place where

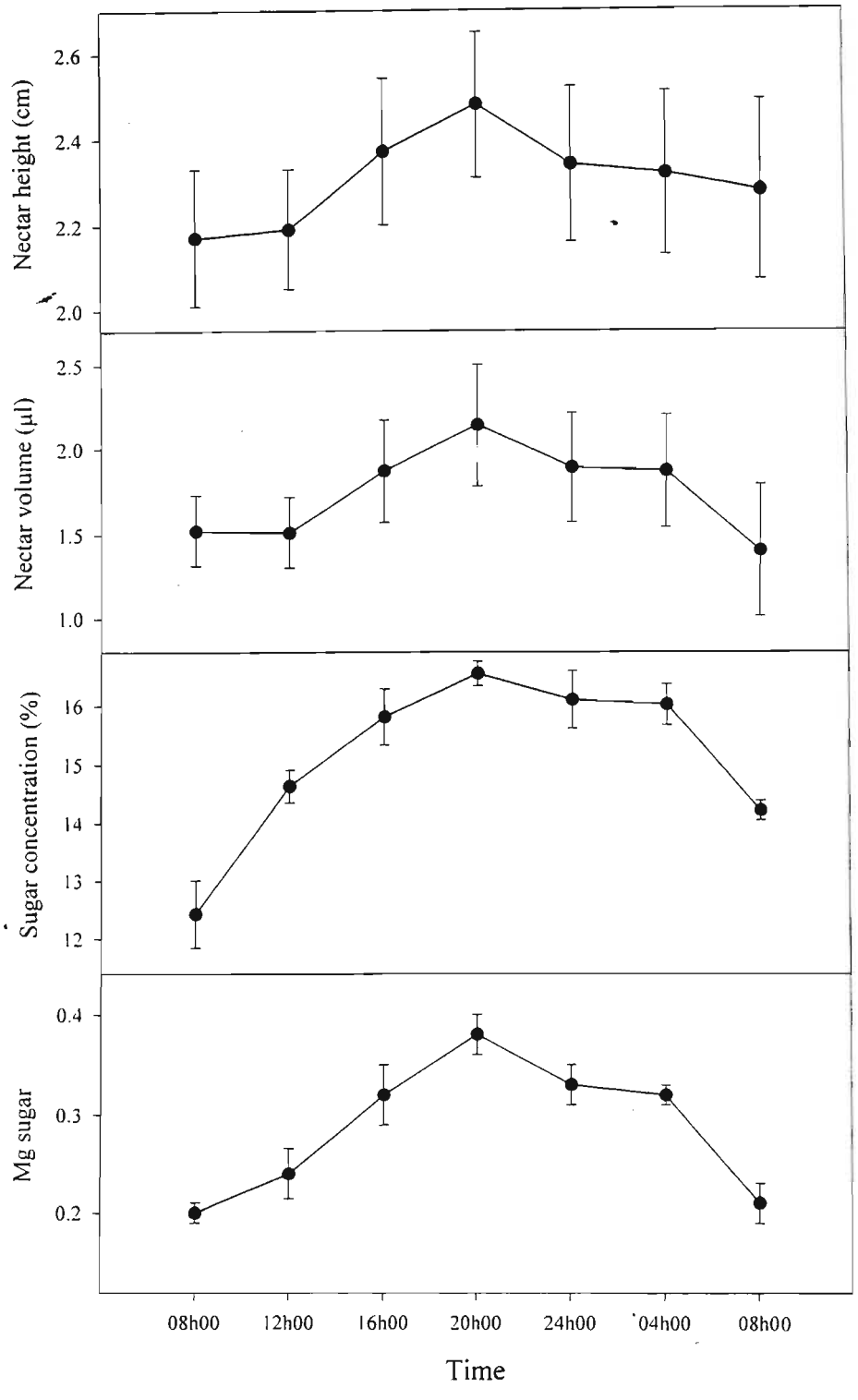

Fig. 2. Fluctuation of nectar properties over 24 hours in flowers of *M. venosum*

pollinaria were attached ($\bar{x} = 3.05$ cm) is less than the mean spur length (4.4–4.7 cm, see below), suggesting that effective contact between the proboscis and the viscidia occurs before the proboscis is fully inserted. Pollination occurs when pollinaria attached to the

Table 2. Composition of the trapped scent of *M. venosum*

Compound	Percent	
	Sample 1	Sample 2
Alpha-pinene	0.20	0.00
Myrcene	0.10	0.00
Limonene	0.30	1.20
(E)-Ocimene	0.00	1.00
(Z)-3-Hexenyl acetate	0.00	0.10
(Z)-3-Hexenol	0.00	0.10
Trans-linalool oxide (Furanoid)	0.10	0.00
Benzaldehyde	0.00	0.50
Linalool	5.00	0.40
Benzyl acetate	0.20	0.30
Methyl nicotate	0.20	0.00
Geraniol	0.00	1.30
Benzyl alcohol	0.50	0.00
Phenylethyl alcohol	0.30	0.50
Phenylacetonitrile	0.00	2.70
Delta-octalactone	0.00	0.70
(E)-Nerolidol	2.50	3.20
Delta-decalactone	1.50	0.50
Jasmine lactone	65.00	67.80
(E, E)-Farnesol	12.50	7.50
Indole	0.70	1.50
	89.10	89.30

proboscis contact the stigma situated behind the rostellum. The pollen masses stick to the receptive stigmatic surface, and as the moth leaves the flower, the notched stigma "scrapes" the pollinium off the proboscis.

Observations during hand-pollination of flowers of *M. venosum* showed that several minutes after removing the pollinaria with a dissecting needle, the stipes moved, or twisted, changing the orientation of the pollen masses so that they faced one another. The stipes also bent slightly, so that the pollen masses were orientated in a way that would enable them to become lodged in the notched stigma. Since these movements took several minutes, it is likely to be a mechanism that lowers the probability of geitonogamy (selfing between flowers on the same plant), as the pollen masses should only be orientated to strike a stigma by the time the moth has moved on to another plant (cf. Darwin 1862, Johnson and Edwards 2000).

Spur length and pollination success. The average length of the spurs of *M. venosum* measured was 4.7 cm (S.D. = 0.3, $N = 70$) at the Verulam site, 4.6 cm at the Yellowwood site (S.D. = 0.7, $N = 115$), and 4.4 cm at Baynesfield (S.D. = 0.3, $N = 30$). Of the 70 flowers examined at the Verulam site, four had begun to set fruit. Of the remaining 66 flowers, 32% had pollinia on the stigmatic cavity and 67% had at least one pollinarium removed. Four of the 115 flowers examined at the Yellowwood site were also setting fruit. Twenty-three percent of the other 111 flowers had pollinia on the stigmatic cavity, and 40% had at least one pollinarium removed. Analysis using a normal approximation to the Mann Whitney U test (Zar 1984), indicated that the spur lengths of pollinated and unpollinated flowers were not significantly different at any of the sites (Verulam: pollinated $\bar{x} = 4.73$, unpollinated $\bar{x} = 4.69$, $Z = 0.28$, $N = 70$; Yellowwood: pollinated $\bar{x} = 4.4$, unpollinated $\bar{x} = 4.69$, $Z = 0.26$, $N = 115$; Baynesfield: pollinated $\bar{x} = 4.3$, unpollinated $\bar{x} = 4.3$, $Z = -0.61$, $N = 30$).

The number of fruits per plant showed a highly significant positive correlation with the number of flowers ($r^2 = 0.59$, $P < 0.001$). However, the percentage fruit set per plant was only weakly positively correlated, albeit significantly, with the number of flowers per plant ($r^2 = 0.17$, $P < 0.05$).

Flower and pollen longevity. Flower longevity was significantly affected by both pollinaria removal and pollinia insertion (Fig. 4). The average lifespan of an unmanipulated flower was 24 days (±0.7). Pollination shortened the flower lifespan to an average of 5 days (±0.2), while the effect of pollinaria removal was weaker, shortening the flower lifespan to 19 days (±1.6).

Pollen of *M. venosum* remains fully viable for up to 10 days, and then appears to deteriorate. Nevertheless, some 20 day old pollinaria kept under outdoor conditions produced fruits with 80% seed set (Fig. 5).

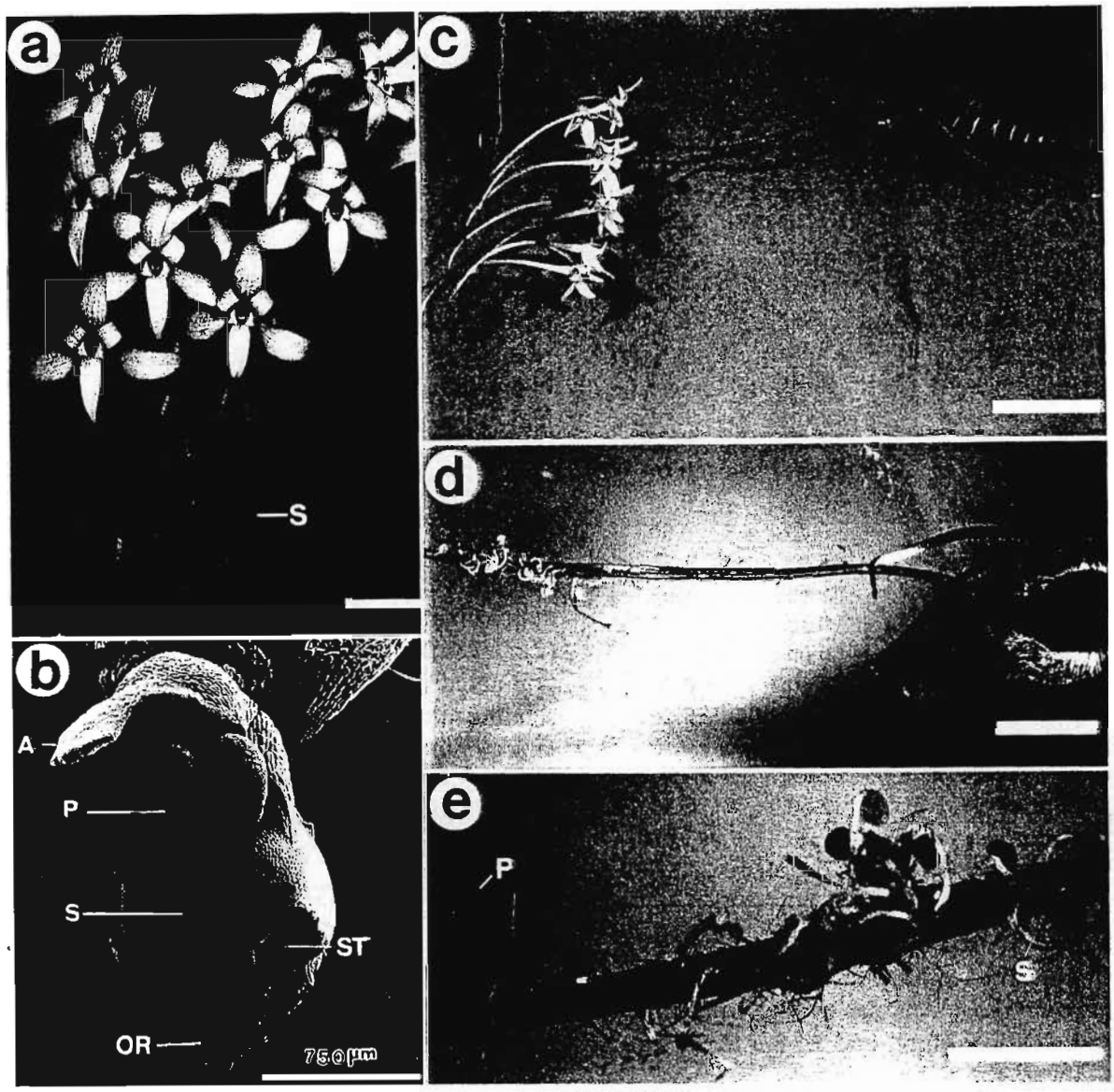

Fig. 3. Floral morphology of *M. venosum*, and the hawkmoth *Nephele accentifera accentifera*. **a** Inflorescences of *M. venosum*. *S* Spur. Scale bar = 10 mm. **b** SEM, front view of column. *A* anther cap. *P* pollinium. *S* stipe *OR* outer rostellum lobe *ST* stigma. **c** *N. accentifera accentifera* positioned in front of an inflorescence of *M. venosum*. Scale bar = 30 mm. **d** *N. accentifera accentifera* with pollinaria of *M. venosum* attached to the proboscis. Scale bar = 5 mm. **e** Close-up view showing final position of pollinaria. *P* pollinium. *S* Stipe. Scale bar = 2 mm

Flower age (up to 10 days) did not appear to have any influence on fruit set.

Discussion

Mystacidium venosum shows a clear suite of floral adaptations to hawkmoth pollination: the flowers are long-spurred; white in colour; and fragrance emission and nectar production is at a maximum in the evening. These floral characters are consistent with the currently accepted syndrome of hawkmoth pollination proposed by Faegri and van der Pijl (1978). Similar traits have been found in Malagasy orchids that are pollinated by medium sized hawkmoths (Nilsson et al. 1985, Nilsson and

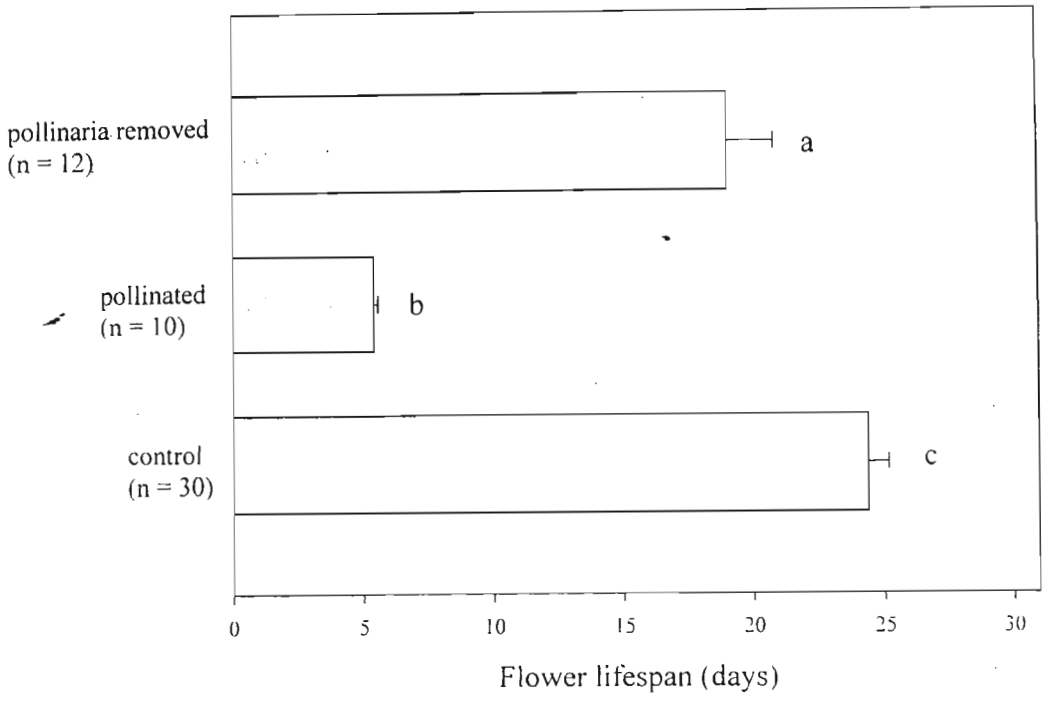

Fig. 4. Longevity of flowers of *M. venosum* in response to pollinaria removal and pollination (ANOVA, $F = 69.4$, $P < 0.001$). Letters indicate significant differences in mean flower lifespan

Rabakonandrianina 1988, Nilsson et al. 1992), as well as terrestrial hawkmoth-pollinated orchids in southern Africa (Vogel 1954, Johnson 1995, Johnson and Liltved 1997).

The relatively dilute sugar concentration in the nectar of *M. venosum* (16%) is slightly below the mean values of 19% and 21.3% reported for hawkmoth pollinated flowers in general by Pyke and Waser (1981) and Cruden et al. (1983), respectively, but is consistent with studies on *Aerangis ellisii* (Nilsson and Rabakonandrianina 1988) and *Angraecum arachnites* (Nilsson et al. 1985), which are hawkmoth pollinated, and had nectar sugar concentrations of 16% and 13.3%, respectively. The sucrose-rich nectar found in *M. venosum* is consistent with reports for many other hawkmoth-adapted flowers (Baker and Baker 1982, Kevan and Baker 1983).

Another characteristic of flowers adapted to hawkmoths, is the lack of a landing platform. In *M. venosum* the midlobe of the lip is deflexed (Fig. 3a), thereby facilitating pollination by hawkmoths which hover while feeding from the flower, and do not require support from the flower. Hawkmoth pollination probably also occurs in *Mystacidium capense*, which can very often only be discerned from *M. venosum* by flowering time. The morphology of other *Mystacidium* species, all of which have spurs less than 2 cm in length, indicates that different pollination mechanisms may be involved.

Hawkmoths are apparently attracted to flowers from a distance by scent, and as they approach the flowers, they are guided by both odour and visual cues (Brantjes 1978). Hawkmoth activity at flowers of *M. venosum* occurred strictly at dusk between 17:15 hours and 18:00 hours (Fig. 1). Scent production (assessed qualitatively), nectar volume, and nectar sugar content peaked from 17:00 hours to 20:00 hours (Fig. 2). This suggests that *M. venosum* is specialised for pollination early in the evening. This is consistent with *Cynorkis uniflora*, which was described as a "shortly-after-dusk" flower (Nilsson et al. 1992), and with a number of studies on South African

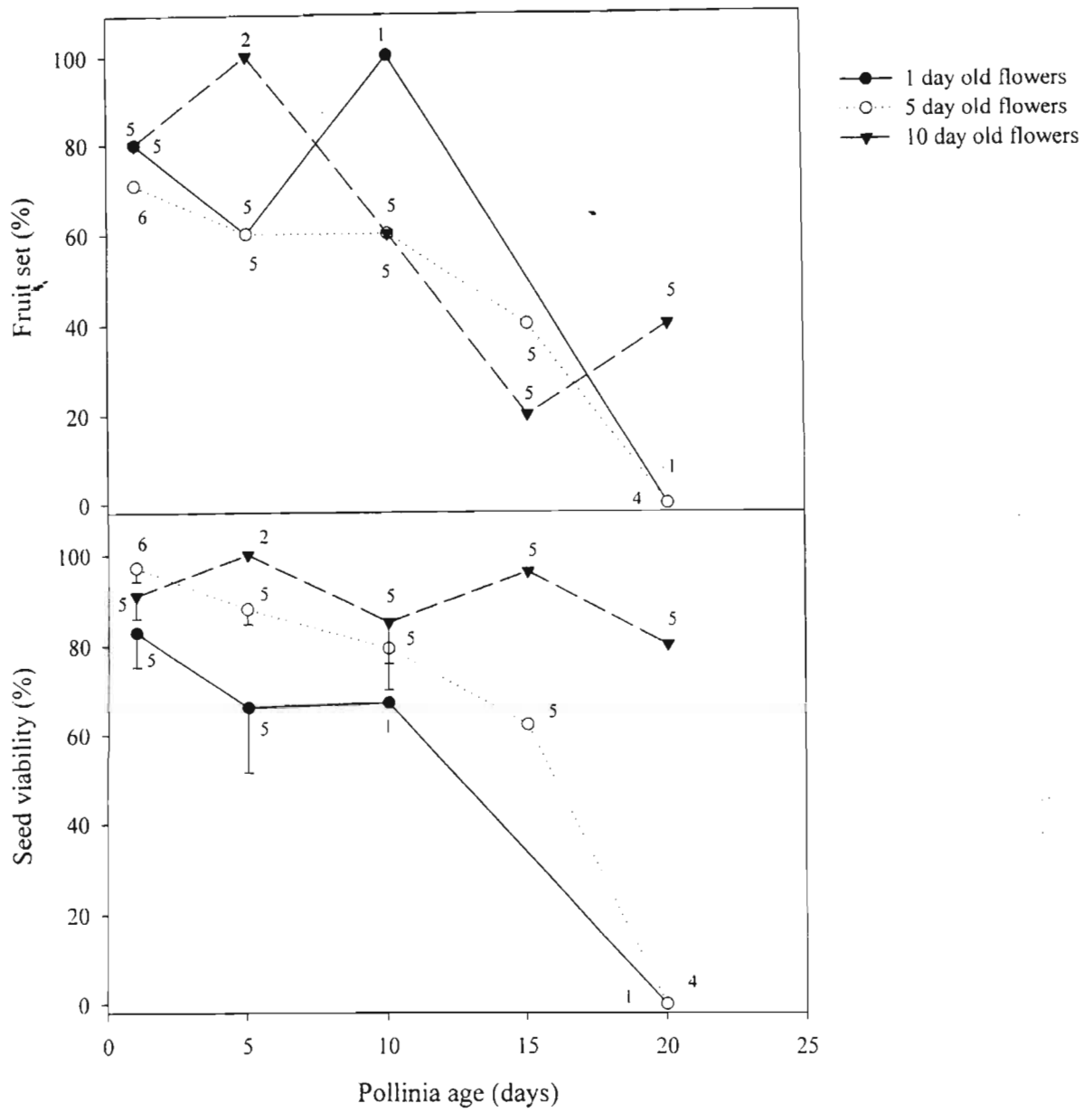

Fig. 5. Effect of pollinaria age on fruit set and seed viability following hand pollinations of flowers of *M. venosum*. Numbers indicate sample size (flowers per treatment)

terrestrial orchids that are pollinated by hawkmoths (Johnson 1995, Johnson and Liltved 1997). *Nephele a. accentifera*, here found to carry pollinia of *M. venosum*, and *Hippotion eson*, which was caught whilst feeding on flowers of *M. venosum*, have been reported to be guided largely by vision in their relationship with flowers, and are therefore active early in the evening when luminance levels still allow colour and contrast perception (Nilsson et al. 1992).

Jasmine lactone, a lipid metabolite, has rarely been recorded as the dominant compound in floral scent, and probably plays an important role in the characteristic sweet scent of flowers of *M. venosum* (Kaiser, personal communication). Acyclic terpene alcohols such as linalool and farnesol, together

with simple aromatic alcohols such as benzyl alcohol and phenylethyl alcohol, were also present in the floral scent of *M. venosum* (Table 2). These substances, and the esters derived from them, often form the basic framework for the scent of "white flowers" which are mostly pollinated by moths (Kaiser 1993).

The pollinaria of *M. venosum* were found to be attached along the length of the proboscis of *N. acentifera acentifera*. This is consistent with Nilsson's (1992) suggestion, that viscidia that are well separated on a large column is usually indicative of deposition of pollinaria on the eyes, whereas close viscidia on a small column indicates placement of pollinaria on the proboscis.

Orchid flowers generally have a long lifespan (Dressler 1990, Nilsson 1992, Clayton and Aizen 1996), and stigmatic pollen deposition may trigger early flower senescence (Gregg 1991, Aizen 1993, Clayton and Aizen 1996). The results for *M. venosum* correlate with those of Clayton and Aizen (1996), which showed that the flower lifespan of *Chlorae alpina* was strongly affected by pollinia insertion, while pollinaria removal shortened the lifespan in unpollinated flowers, but to a much lesser extent. As with *C. alpina*, pollen removal in *M. venosum* does not always coincide with pollen deposition and many pollinaria are 'wasted' rather than being successfully exported to other flowers. Clayton and Aizen (1996) thus suggested that a longer subsequent flower lifespan could therefore be predicted for an unpollinated flower which has its pollinaria removed, than for a pollinated flower which has its pollinaria in place.

Orchid pollen is expected to remain viable for long periods, since orchids are frequently spatially dispersed and often occur in low numbers, and their pollinators do not usually confine their visits to orchids alone. Several days may elapse between when a pollinarium is removed to when it is finally deposited on a stigma (Johnson and Edwards 2000). Therefore, low pollinator activity and long distances between plants should result in selection for greater pollen longevity (Dafni and Firmage 2000). This study showed that the pollen of *M. venosum* remained viable for up to 20 days after being exposed to outdoor conditions. Neiland and Wilcock (1995) showed that *Dactylorhiza purpurella* pollinia retained their viability for 51 days, but this was under laboratory conditions. Alexandersson (1999) showed that a labelled *Calypso bulbosa* pollinium produced a normal fruit 10 days after being removed from a flower by a bumblebee.

The mean spur length of *M. venosum* (4.4–4.7 cm) was shorter than the proboscis of the pollinia-bearing hawkmoth *Nephele a. accentifera accentifera*. Consequently, the proboscis is not fully inserted into the spur, and pollinaria are placed along its length. This plant-pollinator pattern does not conform to the model of flower depth evolution presented by Nilsson (1988), which predicts that successful import and export of pollen requires that the spur of the orchid must be longer than the proboscis of its pollinator. Unlike the orchids that attach pollinaria to the base of the pollinator's proboscis (Nilsson et al. 1985, Maad 2000), or to the eyes of the pollinator (Nilsson et al. 1992, Johnson and Liltved 1997), it is not essential for the spurs of *M. venosum* to exceed the length of the pollinator's proboscis for pollination to occur. This could explain the non-significant effect of spur length on pollination success in *M. venosum* in this study. Johnson and Steiner (1997), however, showed that selection favours long spurs in *Disa draconis* where pollinaria are placed along the length of the pollinator's proboscis. They attributed this to the fact that short-spurred flowers will only be pollinated by pollinaria from short-spurred flowers, whereas long-spurred flowers can be pollinated by pollinaria from long and short-spurred flowers.

Orchid floral traits influence both male and female reproductive success, which can be estimated by the removal and receipt of pollinaria, respectively (Nilsson 1992). The low percentage of pollinaria observed on the

stigmatic cavities of plants of *M. venosum* relative to pollinaria removal is not uncommon in orchids (Nilsson and Rabakonandrianina 1988, Ackerman and Montalvo 1990). Plants that successfully receive pollinia are fewer than those from which pollinaria are removed as loss of pollinaria may occur. Pollinaria removal is therefore only a remote estimate of the male role in reproductive success (Nilsson 1992). That almost 70% of the flowers of *M. venosum* observed at the Verulam site had pollinaria removed, and only *c.* 30% received pollinaria may very well be reflected in the haphazard arrangement of pollinaria observed on the proboscis of *Nephele a. accentifera accentifera* (Fig. 3c–e). As many as 20 pollinaria were indiscriminately arranged along the length of the proboscis, and yet successful capture of the pollinaria by the stigma requires precise orientation of the pollen masses. It is therefore possible that pollinaria were ineffective or 'wasted' following removal, resulting in low pollen receipt. Thus, the low level of fruit set observed at the Verulam site (19%) may be due to pollen limitation caused by inefficient pollen transfer, rather than the lack of visitation per se.

Observations at the Verulam site indicated that a larger number of flowers per plant increased the chances of fruit set. This correlates with a number of findings on orchids where an increase in the number of flowers per plant contributes to the attraction of pollinators, thereby increasing the chance of reproductive success (Nilsson 1992, Rodríguez-Robles et al. 1992).

The evolution of a floral trait is the result of antagonistic selective pressures (Schemske 1980). Selection will modify floral characters that will make them more attractive to the most efficient pollinators in the habitat (Stebbins 1970). The colour, morphology and daily fluctuation in sugar and nectar in flowers of *M. venosum* are likely to have been shaped by the morphology and tightly restricted foraging periods of hawkmoths. On the other hand, large inflorescences and long-lived flowers and pollen, are probably more general traits that compensate for the low density of orchid plants and frequent pollen-limitation of fruiting success.

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References

Ackerman J. D., Montalvo A. M. (1990) Short- and long-term limitations to fruit production in a tropical orchid. Ecology 71: 263–272.

Aizen M. A. (1993) Self-pollination shortens flower lifespan in *Portulaca umbraticola* H.B.K. (Portulacaceae). Int. J. Plant Sci. 154: 412–415.

Alexandersson R. (1999) Reproductive biology of the deceptive orchid *Calypso bulbosa*. PhD thesis, University of Umeå.

Arditti J. (1979) Aspects of the physiology of orchids. Adv. Bot. Res. 7: 421–697.

Baker H. G., Baker I. (1982) Floral nectar constituents in relation to pollinator type. In: Jones C. E., Little R. J. (eds.) Handbook of experimental pollination biology. New York, Van Nostrand-Reinhold.

Brantjes N. B. M. (1978) Sensory responses to flowers in night flying moths. In: Richards A. J. (ed.) The pollination of flowers by insects. Linnean Society Symposium Series No.6. Academic Press, London, pp. 13–19.

Calvo R. N., Horvitz C. C. (1990) Pollinator limitation, cost of reproduction, and fitness in plants: a transition matrix demographic approach. Amer. Nat. 136: 499–516.

Clayton S., Aizen M. A. (1996) Effects of pollinia removal and insertion on flower longevity in *Chloraea alpina* (Orchidaceae). Evol. Ecol. 10: 65–660.

Cruden R. W., Hermann S. M., Peterson S. (1983) Patterns of nectar production and plant-pollinator coevolution. In: Bentley B., Elias T. (eds.) The biology of nectaries. Columbia University Press, New York.

Dafni A. (1992) Pollination ecology: a practical approach. Oxford University Press, New York.

Dafni A., Firmage D. (2000) Pollen viability and longevity. Plant Syst. Evol. 222: 113–132.

Darwin C. H. (1862) On the various contrivances by which British and foreign orchids are fertilized by insects. 2nd edn. John Murray, London.

Dodson C. H. (1962) The importance of pollinators in the evolution of the orchids of tropical America. Amer. Orchid Soc. Bul. 31: 525–534.

Dressler R. L. (1981) The orchids: natural history and classification. Harvard University Press, USA.

Dressler R. L. (1990) The orchids. Harvard University Press, Cambridge, MA.

Faegri K., Pijl L. van der (1978) The principles of pollination ecology, 3rd edn. Pergamon, Oxford, New York, Toronto, Sydney, Paris, Frankfurt.

Gregg K. B. (1991) Reproductive strategy of *Cleistes divaricata* (Orchidaceae). Amer. J. Bot. 78: 350–360.

Haber W. A., Frankie G. W. (1989) A tropical hawkmoth community: Costa Rican dry forest Sphingidae. Biotropica 21: 155–172.

Johnson S. D. (1995) Observations of hawkmoth pollination in the South African orchid *Disa cooperi*. Nord. J. Bot. 15: 121–125.

Johnson S. D. (1997) Pollination ecotypes of *Satyrium hallackii* (Orchidaceae) in South Africa. Bot. J. Linn. Soc. 123: 225–235.

Johnson S. D., Bond W. J. (1997) Habitat dependent pollination success in a Cape orchid. Oecologia 91: 455–456.

Johnson S. D., Liltved W. R. (1997) Hawkmoth pollination of *Bonatea speciosa* (Orchidaceae) in a South African coastal forest. Nord. J. Bot. 16: 5–10.

Johnson S. D., Steiner K. E. (1997) Long-tongued fly pollination and evolution of floral spur length in the *Disa draconis* complex (Orchidaceae). Evolution 51: 45–53.

Johnson S. D., Steiner K. E. (2000) Generalization versus specialization in plant pollination systems. Trends Ecol. Evol. 15: 140–143.

Johnson S. D., Edwards T. (2000) The structure and function of orchid pollinaria. Plant Syst. Evol. 222: 243–269.

Kaiser R. (1993) The scent of orchids: olfactory and chemical investigations. Elsevier, Amsterdam.

Kevan P. G., Baker H. G. (1983) Insects as flower visitors and pollinators. Ann. Rev. Entomol. 28: 407–453.

Maad J. (2000) Phenotypic selection in hawkmoth-pollinated *Platanthera bifolia*: targets and fitness surfaces. Evolution 54: 112–123.

Neiland M. R. M., Wilcock C. C. (1995) Maximization of reproductive success by European Orchidaceae under conditions of infrequent pollination. Protoplasma 187: 39–48.

Nilsson L. A. (1988) The evolution of flowers with long corolla tubes. Nature 334: 147–149.

Nilsson L. A. (1992) Orchid pollination biology. Trends Ecol. Evol 7: 255–259.

Nilsson L. A., Rabakonandrianina E. (1988) Hawkmoth scale analysis and pollination specialization in the epilithic Malagasy endemic *Aerangis ellisii* (Reichenb. fil.) Schltr. (Orchidaceae). Bot. J. Linn. Soc. 97: 49–61.

Nilsson L. A., Jonsson L., Rason L., Randrianjohany E. (1985) Monophily and pollination mechanisms in *Angraecum arachnites* Schltr. (Orchidaceae) in a guild of long-tongued hawkmoths (Sphingidae) in Madagascar. Bot. J. Linn. Soc. 26: 1–19.

Nilsson L. A., Rabakonandrianina E., Razananaivo R., Randriamanindry J. (1992) Long pollinia on eyes: hawkmoth pollination of *Cynorkis uniflora* Lindley (Orchidaceae) in Madagascar. Bot. J. Linn. Soc. 109: 145–160.

Proctor M., Yeo P., Lack A. (1996) The natural history of pollination. Timber Press, Oregon.

Pyke G. H., Waser N. M. (1981) The production of dilute nectars by hummingbird and honeyeater flowers. Biotropica 13: 260–270.

Rodriguez-Robles J. A., Meléndez E. J., Ackerman J. D. (1992) Effects of display phenology, and nectar availability on effective visitation frequency in *Comparettia falcata* (Orchidaceae). Am. J. Bot. 79(9): 1009–1017 (Abstract).

Schemske D. W. (1980) Evolution of floral display in the orchid *Brassavola nodosa*. Evolution 34: 489–493.

Stebbins G. L. (1970) Adaptive radiation of reproductive characteristics in Angiosperms, I: pollination mechanisms. Ann. Rev. Ecol. Syst. 1: 307–326.

Vogel S. (1954) Blütenbiologische Typen als Elemente der Sippengliederung. Bot. Stud. 1: 1–338.

Wasserthal L. T. (1997) The pollinators of the Malagasy star orchids *Angraecum sesquipedale*, *A. sororium* and *A. compactum* and the evolution of extremely long spurs by pollinator shift. Botanica Acta 110: 343–359.

Wyk B-E. van (1993) Nectar sugar composition in Southern African Papilionoideae (Fabaceae). Biochem. Syst. & Ecol. 21(2): 271–277.

Zar J. H. (1984) Biostatistical Analysis, 2nd edn. Prentice Hall, USA.

Zimmerman J. K., Aide T. M. (1989) Patterns of fruit production in a neotropical orchid: pollinator vs. resource limitation. Amer. J. Bot. 76: 67–73.

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