Native Orchid Conservation on the Florida Panther National Wildlife Refuge:

A Review of Cooperative Research, Posed Questions, and Recommendations

2011 Florida Orchid Conservation Conference

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This workbook is an updated edition of "Native Orchid Conservation on the Florida Panther National Wildlife Refuge: Cooperative Research Funding Projects on *Eulophia alta, Cyrtopodium punctatum, Spiranthes odorata,* and *Bletia purpurea*" from 7 March 2007 prepared by SL Stewart, D Dutra, P Kauth, M Kane, T Johnson, N Philman, and L Richardson.

INTRODUCTION

The orchid family (Orchidaceae) contains an estimated 17,000-35,000 species (Dressler, 1993). Orchids exist as epiphytes, terrestrials, lithophytes, and aquatics making conservation a concern when trying to protect this family. While the largest diversity and distribution of orchids is concentrated in tropical regions, both sub-tropical and temperate regions of the world are home to orchid species. North America is home to approximately 250 species of orchids, with terrestrial, epiphytic, and aquatic representatives (Brown, 2003; Luer, 1975).

The Florida orchid flora possesses the largest number of orchids in North America, possessing approximately 120 species, natural hybrids, and color forms (Brown, 2002, 2005). Florida's high orchid diversity is due to the wide range of habitats within the state – temperate coastal plains in the panhandle, temperate oak and pine forests in north and central Florida, and subtropical/tropical wetlands in south Florida. Currently, many problems raise orchid conservation concerns such as habitat loss due to agricultural and residential land use, exotic plant and pest invasions, poaching, and habitat mismanagement. These have all contributed to the decline of Florida's native orchid populations. Although no Florida orchid is listed as federally endangered or threatened, many of the state's orchids face the immediate threat of extinction if conservation efforts are not initiated appropriately and timely.

In response to the immediate need for practical orchid species-specific conservation of Florida's native Orchidaceae, the Florida Panther National Wildlife Refuge (FPNWR), the University of Florida's Environmental Horticulture Department, and the Illinois College Orchid Recovery Program have partnered to undertake the necessary research. This partnership blends the conservation practitioners from the U.S. Fish and Wildlife Service with renowned academic orchid researchers from the University of Florida and Illinois College. Through investigations on orchid plant ecology, biology, propagation, pollination biology, and genetics, this conservation partnership has begun to establish effective and efficient means of orchid conservation using the orchid flora of the FPNWR as model systems. These model systems should be applied to orchid species and populations well beyond the borders of the FPNWR and southwest Florida. Hopefully, these systems are adopted by orchid researchers and conservationists worldwide to collaboratively protect and restore endangered and threatened orchid species.

Very few, if any, conservation programs currently exist in the United States that combine a proactive and integrated approach to species-specific orchid conservation with the expertise of conservation practitioners and two highly regarded research programs. Current studies should continuously be evaluated to determine new research opportunities to provide new conservation strategies for orchid preservationists.

2011 Florida Orchid Conservation Conference Objectives

I. Assess current orchid conservation measures
II. Identify current research strategies
III. Design research questions for your area
IV. Apply scientific results to field conservation
V. Create an orchid research network for large-scale conservation measures

COMMONLY USED APPROACHES FOR ORCHID CONSERVATION RESEARCH

Research conducted at the University of Florida's Environmental Horticulture Department and the Illinois College Orchid Recovery Program includes various techniques and tools that are commonly used. An abridged list of techniques and tools used by these institutions are described below. This list is to help further understand the research presented in this workbook that is conducted in laboratory orchid protocols.

ASYMBIOTIC vs. SYMBIOTIC SEED GERMINATION

It is well established that both epiphytic and terrestrial orchids utilize mychorrizal fungi *in situ* as a source of carbon to initiate seed germination (Zettler et al., 2006). When mychorrizal fungi are used to germinate orchid seeds, this is known as symbiotic seed germination. This germination technique has been used *in vitro* with great success, usually leading to an increased germination percentage or seedling development (Johnson et al., 2007). This technique usually requires a mycobiont (a fungus associated with orchid roots *in situ*) to be isolated and identified before it can be inoculated with seeds. However, orchid seeds can be germinated without a mychorrizal fungus, asymbiotic seed germination, when the medium contains all of the nutrients that a fungus would provide to the seed. Whichever technique is utilized, both can help us determine what the *in situ* plant responses would be to certain environmental conditions and help us understand basic information on the orchid's early plant growth and development (Dutra et al., 2008).

SEEDLING DEVELOPMENT SCALES

Many protocols utilize a scale-based tool to help convey what the researchers are reporting in a more definitive approach by describing seed germination in "stages." These scales have been modified and added to throughout the years but they have many similarities. Published seed germination and development scales have ranged from having Stage 0 – "Hyaline embryo, testa intact" (Johnson et al., 2009) to Stage 6 – "Emergence of third leaf" (Johnson et al., 2011). However, readers should be cautious when a protocol defines a scale-based tool because these tools are not always consistent between protocols. Some scales have Stage 1 as the "intact testa" stage (Dutra et al., 2009) whereas Stage 5 has been defined as "Elongation of shoot and further development," "Elongation of first leaf and further development," and "Elongation of second leaf" (Dutra et al., 2009; Johnson et al., 2007; Johnson et al., 2011, respectively). Likewise, different scales determine when germination has occurred at different stages. Dutra et al. (2008) determined that germination occurred at Stage 1 when the embryo had swollen, but Johnson et al. (2011) determined that germination occurred at Stage 2 when the embryo had ruptured through the testa. Below are generic seedling developmental stages taken from Johnson et al. (2011) and corresponding photographs (Fig. 1):

Stage	Description
0	Hyaline embryo, testa intact
1	Embryo swollen
2	Embryo ruptured through testa (=germination)
3	Appearance of first leaf
4	Emergence of second leaf
5	Elongation of second leaf
6	Emergence of third leaf

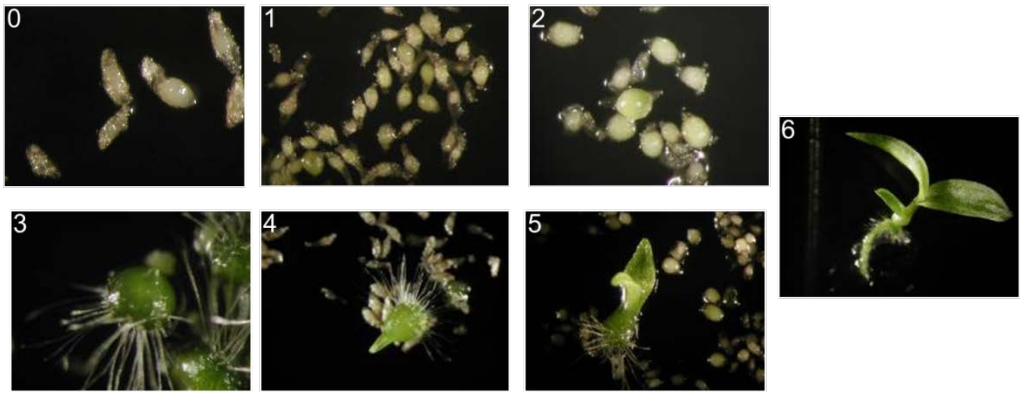

Figure 1: Orchid seedling development stages (Stage 0-6).

POLLINATION STUDY TECHNIQUES

Orchid conservation research must include the investigation of many different facets of an orchid's biology which should include understanding the breeding system. Two orchids, *Cyrtopodium punctatum* and *Eulophia alta*, whose reproductive biology has been studied utilized specific techniques on how to determine the species' reproductive mechanisms. Main assessments that are conducted with these different techniques evaluate if an orchid requires a pollinator to reproduce, and if so, to what geographic distance can the pollinia originate (e.g., the same flower or from distant populations). A table of pollination treatments from Johnson et al. (2009) is listed below with some modifications from Dutra et al. (2009).

Pollination treatment	Pollinators excluded	Pollinia removed	Pollen treatment	Assessment
Control (open pollination)	No	No	No pollination	Natural conditions
Agamospermy	Yes	Yes	No pollination	Non-sexual capsule set
Spontaneous autogamy	Yes	No	No pollination	Need for pollinators
Induced autogamy	Yes	Yes	Same flower	Self-compatibility of flowers
Artificial geitonogamy	Yes	Yes	Different flower, same plant	Self-compatibility of plants
Artificial xenogamy	Yes	Yes	Distant population	Potential out-breeding at long distances
Induced xenogamy	Yes	Yes	Same population, different plant	Potential our-breeding at short distances

PRESENTATION ABSTRACTS

Calopogon tuberosus Research on the Florida Panther National Wildlife Refuge

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Calopogon tuberosus is a terrestrial orchid found throughout eastern North America from Florida to Canada, and east to the Mississippi River. Two varieties of *C. tuberosus* exist: var. *tuberosus* and var. *simpsonii*, which are restricted to south Florida. The population on the Florida Panther National Wildlife Refuge (FPNWR) is unique because both varieties are found in several areas. Several areas of research were conducted. The seed germination ecology and biology of the FPNWR population was studied and compared to populations from north Florida to northern Michigan. These experiments revealed that the requirements to promote seed germination varied greatly among populations. A small reintroduction study was also conducted over a two year period on the FPNWR using only plants derived from FPNWR seeds. Because environmental conditions were harsh during the reintroduction study, plant survival was poor. In addition, a study to distinguish var. *tuberosus* from var. *simpsonii* was also conducted, but further analysis is needed to help distinguish these varieties.

Reproductive Biology and Asymbiotic Seed Germination of *Cyrtopodium punctatum*, an Endangered Florida Orchid

MICHAEL KANE and DANIELA DUTRA
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Cyrtopodium punctatum Lindley is an endangered epiphytic orchid restricted in the United States to southern Florida. Over the past 10 years, careful observations of plant populations in the Florida Panther National Wildlife Refuge (FPNWR) indicated limited seed production in the remaining plants. Consequently, the long-term sustainability of remaining populations is in question. In order to develop a conservation plan for the species, a reproductive biology study was conducted by Daniela Dutra. Information regarding the breeding strategies and pollination mechanisms in *C. punctatum* Florida populations is critical to develop strategies for recovery of the species. *C. punctatum* was found to be self-compatible and not autogamous. A pollinator is needed for seed production in the wild. Results of volatile floral fragrance analysis reveal that *C. punctatum* relies on a deceit pollination system using aromatic compounds to attract insect pollinators. The genetic diversity of *C. punctatum* of the existing populations on the FPNWR is currently being determined using genetic markers. An efficient protocol for the asymbiotic production of *C. punctatum* seedlings is being used in a reintroduction project involving this endangered species.

The Pollination Biology of *Eulophia alta*

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Environmental Horticulture Department, University of Florida

Eulophia alta is a terrestrial orchid with a distribution from Africa to south America and north to south Florida. Due to the large percentage of capsule formation, *E. alta* has previously been reported as an auto-pollinated orchid. Over several years, the breeding system of *E. alta*, located on the Florida Panther National Wildlife Refuge (FPNWR), was studied along with seed viability and germinability of the resultant seed. Spontaneous autogamy in *E. alta* was rare, and only a small percentage of flowers formed capsules. Hand pollination resulted in significantly greater capsule

formation when flowers were subjected to self-pollination or various types of cross-pollination. Pollen source had little effect on seed viability and germinability or seedling growth. However, seed resulting from spontaneous autogamy developed more slowly than seed originating from other treatments. Spontaneous autogamy is rare in *E. alta* found on the FPNWR, and naturally forming capsules may be the result of unobserved pollination events.

Orchid Floral Scents and Their Niche in Conservation

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The ghost orchid, *Dendrophylax lindenii*, is one of North America's rarest and well-known orchids. The species frequents shaded swamps in S Florida as a leafless epiphyte where its stunning floral often attracts the attention of poachers. Surprisingly, little information exists on *D. lindenii*'s biology *in situ*, raising conservation concerns. During the summer of 2009 in Collier Co., FL, a substantial number (ca. 13) of plants initiated anthesis offering a unique opportunity to study this species *in situ*. Flowers were sampled on-site during 2 nights using adsorbent connected to a battery-powered vacuum pump. Samples were then analyzed in the laboratory via gas chromatography/mass spectrometry (GC/MS analysis). Eight volatile compounds were identified as the primary constituents with a large abundance of a farnesene-based compound known to be linked to pestiferous insects (Hemiptera). Most of the volatile compounds reported herein are common floral fragrance constituents; however, additional studies are needed to determine what role (if any) these insects may have had on our analysis. A highlight of this study was the technique used to trap volatiles *in situ* with minimal disturbance to this rare orchid.

The Orchid Recovery Program at Illinois College – Who We Are and What We Do

LAWRENCE W. ZETTLER and LAURA COREY
Orchid Recovery Program, Illinois College

Established in 1996, the Orchid Recovery Program at Illinois College blends undergraduate student learning with the conservation of North America's rarest orchids. The program instills in its students an appreciation for our planet's natural resources and provides the basic tools for lifelong learning, stewardship and service. During the past 15 years, 58 IC undergraduate students have conducted research in the program, and several have since pursued graduate degrees. In 2002, one of our graduates, Dr. Scott L. Stewart, initiated a collaborative venture with Larry Richardson at the Florida Panther NWR with a vision for conserving S Florida's rare orchids through research. Nearly a decade later, the FPNWR has emerged as an important, internationally-recognized "living laboratory" for the study of terrestrial and epiphytic orchids alike involving a network of specialists. An important catalyst for epiphytic orchid research has been the establishment of a summer internship program that allows two IC students to work at the FPNWR each summer collecting data. This talk will provide a summary of the previous and ongoing studies carried out in the FPNWR by students and specialists affiliated with Illinois College and other institutions. Among the topics presented include using mycorrhizal fungi to cultivate *Epidendrum nocturnum* from seed leading to its successful reintroduction, and first documentation of epiphytic orchid germination *in situ* assisted by seed baiting on arboreal substrates. This talk will also present an update on ongoing work using molecular markers to identify fungi associated with seed germination, as well as the recent discovery of exotic pestiferous scales on native orchids in Collier Co., including Fakahatchee Strand.

BLETIA PURPUREA, PINE-PINK ORCHID

Bletia purpurea, a terrestrial orchid species, is restricted to Florida in the USA where it is listed as state threatened largely due to the threats from habitat alteration. It is found in several different habitats in Florida, such as mesic habitats, along pinelands, on floating logs, and along highly disturbed areas.

Study #1: *B. purpurea* asymbiotic seed germination, *in vitro* development, and *ex vitro* acclimatization (Dutra et al., 2008)

Six asymbiotic orchid seed germination media were examined for their effectiveness in promoting orchid seed germination under light and dark culture conditions. The six media were *Phyto*Technology Orchid Seed Sowing Medium (P723), Knudson C, Malmgren Modified Terrestrial Orchid Medium, Vacin & Wen Modified Orchid Medium (VM), BM-1 Terrestrial Orchid Medium and ½-strength Murashige & Skoog. Two photoperiods (0/24 h and 16/8 h L/D) were examined for their effectiveness in promoting seed germination and protocorm development.

After 5 weeks, germination occurred regardless of medium or photoperiod treatment (Fig. 2). However, advanced seedling development to stage 6 only occurred on VM in the 16/8 h L/D photoperiod.

To investigate the effects of potting media on greenhouse seedling acclimatization, *Sphagnum* moss, Fafard Mix 2, Fafard Mix 2:sand (1:1 v:v) and Fafard Mix 4 were evaluated. After 15 weeks, all potting media demonstrated high survival rates (98.8% total survivorship). However, the only significant difference between the treatments was that seedlings acclimatized in Fafard Mix 4 displayed significantly longer root lengths (41.4mm).

Because of the similarities found between these treatments, it was suggested that this species has adapted to a wide variety of conditions for seed germination and seedling development.

<u>Recommendation</u>: Future research on symbiotic seed germination of this species is recommended to determine *in situ* requirements.

Study #2: *B. purpurea* seed germination and development examining light, nutrients, and carbohydrates (Johnson et al., 2011)

The effects of carbohydrate presence, carbohydrate source, nutrient availability, and illumination on seed germination and early development were investigated using asymbiotic seed germination. Seed germination was limited (>20%) when seeds were cultured in darkness without sucrose but had higher germination under illumination without sucrose. Germination percentage was enhanced when mineral nutrients were incorporated into the media. Therefore, carbohydrate presence is necessary when cultured in darkness and increased nutrient availability increased germination.

To determine if carbohydrate source affected seed germination, six difference types were used at two different concentrations, 10 and 50 mmol. Sucrose, fructose, glucose and trehalose enhanced germination and development while mannitol and sorbital did not. The greatest observed development was obtained for seedlings cultured under illuminated conditions with 10 mmol sucrose. Therefore, it can be suggested that carbohydrates, from photosynthesis, mychorrizal fungi, or exogenously applied, play a vital role in regulating seed germination.

Recommendation: Genetic or molecular approaches should complement current germination and early development studies.

Recommendation: Using asymbiotic techniques, studies of how symbiosis is affected by light quality, photoperiod, substrate, nutrients, temperature, and fungal species can further explain orchid seed physiology.

Study #3: *B. purpurea* seed germination and development examining effects of light and temperature (Johnson and Kane, 2011)

The effects of simulated south Florida temperatures and illumination (dark and 12 h photoperiod) were investigated for their role in regulating seed germination and development. Temperature has been found to have an effect on the light sensitivity of seeds to regulate seasonal germination. These treatments were applied under asymbiotic culture conditions.

Simulated seasonal temperatures were the following oscillating temperatures: 22/11, 29/19, 33/24, and 27/15°C (representing winter, spring, summer, and autumn temperature extremes in south Florida, respectively). An additional 25/25°C constant temperature was also screened due to its use in many germination studies. Seeds were then treated under continuous darkness or 12/12 h (L/D) photoperiod with this various temperatures.

After 6 weeks in culture, seeds exhibited high germination percentages (nearly 100%) regardless of treatment. However, low temperatures with illumination delayed both germination and development. This delay in germination and development could be an adaptation to delay germination during winter when frosts are possible. It was also found that temperature, illumination, and the interaction between these factors had significant effects on the percentage of seedlings in developmental stages. Seedlings cultured in the dark at 22/11°C resulted in more seedlings with only one leaf (~60%), whereas seedlings cultured in 33/24°C resulted in more seedlings with two leaves (~80%). Finally, the greatest percentage of seedlings developing three leaves (the highest developmental stage) was observed at a constant 25°C with illumination.

Recommendation: Further examination of the roles that soil water potential, light quality, and the season on symbiotic fungal activity may allow prediction of when germination will most likely occur *in situ*.

CALOPOGON TUBEROSUS, COMMON GRASS-PINK ORCHID

Calopogon tuberosus, a terrestrial orchid species, is widespread throughout eastern North America. It is found in several different habitats, such as bogs, marl prairies, and mesic roadsides. There have been three specific geographic areas described: northern plants in glaciated areas; southwest plants west of the Mississippi Embayment, and southeast plants east of the Mississippi River and south of the glaciated zone. These specific areas are identified through various morphological differences such as labellum apex shape, flower size, and inflorescence height.

Study #1: *C. tuberosus* ecotypic differences in asymbiotic germination responses (Kauth et al., 2008)

Ecotypic differentiation was examined using seeds collected from six different locations (south Florida, north central Florida, the upper Michigan peninsula, and three locations in South Carolina). Using asymbiotic seed germation, effects of three photoperiods (8/16, 12/12, 16/8 h L/D) were examined. Additionally, six culture media were evaluated for seed germination and development. The six media were *Phyto*Technology Orchid Seed Sowing Medium (P723), Knudson C, Malmgren Modified Terrestrial Orchid Medium, Vacin & Wen Modified Orchid Medium, BM-1 Terrestrial Orchid Medium and ½-strength Murashige & Skoog.

Short days promoted the highest germination for north central Florida (60.2%) and south Florida (48.5%) populations. Germination of South Carolina seeds was higher on media containing higher calcium and magnesium concentrations. After 16 weeks, south Florida seedlings were small and did not form corms, whereas north central Florida seedlings were larger and formed corms. Finally, seed germination and corm formation was rapid in Michigan seeds across all treatments and allocated more biomass to corms compared to the other sources. Rapid seed germination, seedling development, and corm formation in northern populations may be adaptive to ensure rapid corm development during short northern growing seasons to increase winter survival.

Following a screen of the six media, P723 was adequate for germinating Florida and Michigan seeds. However, seeds germinated on Vacin & Wen Modified Orchid Medium and Knudson C media, which contain higher concentrations of calcium and magnesium, resulted in higher germination for the South Carolina sources. This may be due to the high calcium and magnesium content in the *in situ* conditions of these sources.

<u>Recommendation</u>: Studying the varying degrees of testa permeability or hardness could help determine if longer pretreatments are needed to weaken the testa for higher germination percentages.

<u>Recommendation</u>: Soil analysis from each population may provide insight into differences in soil nutrient availability, and ultimately what is necessary for optimal seed germination rates *in vitro* and *in situ*.

Study #2: *C. tuberosus* ecotypic differences in *in vitro* ecology (Kauth and Kane, 2009)

After 20 weeks, the timing of corm formation and biomass allocation was evaluated *in vitro* from seeds from Michigan, South Carolina, and Florida. Corm formation for Michigan seedlings was evident by week 8, week 10 for South Carolina, week 14 for north central Florida seedlings, and week 18 for south Florida seedlings. Shoot senescence began with Michigan seedlings after 16 weeks and continued south in latitude until south Florida seedlings senesced after 38 weeks. Likewise, corm biomass allocation followed the same north to south trend with approximately 97% of plant biomass being allocated to corms in Michigan seedlings compared to only 7% of biomass in south Florida seedlings. Correlation analysis revealed that as percent corm biomass allocation increased, the length of the growing season decreased.

These adaptations are likely influenced by environmental responses at each site such as photoperiod, temperature, and growing season length.

Recommendation: Combining *in situ* with *in vitro* studies may provide better insight into the ecotypic differentiation of *C. tuberosus*.

Study #3: *C. tuberosus* field establishment techniques (Kauth et al., 2010)

Using corms and seedlings transplanted to either unburned or burned areas, seedling survival was studied to help prescribe better field establishment techniques. Propagule type did not influence survival (6.25% for corms and 8.33% for seedlings), however the date of the field establishment did. A higher percentage of propagules survived when they were planted during the early growing season. Although more seedlings actively grew in the burned plot, seedlings produced more shoots in the unburned plot. This could be due to the unburned site providing shading to the seedlings to decrease seedling desiccation compared to those in the burned site.

Recommendation: Observations over many growing seasons could provide information on sustained survival.

Recommendation: Studies evaluating seedling survival should use symbiotically grown seedlings and/or inoculation of the soil with mycorrhizal fungi.

Study #4: *C. tuberosus* corm chilling relieves dormancy in distant populations (Kauth et al., 2011a)

To investigate the relationship between ecotypic differentiation and chilling requirements, seeds from south Florida, north central Florida, South Carolina, and Michigan were used. After 20 weeks in *in vitro* culture, corms were chilled for 0, 2, 4, 6, and 8 weeks. After 16 weeks under *ex vitro* conditions in a growth chamber following the chilling period, shoot length, leaf number, leaf width, root number, root length, and corm diameter were measured. Longer chilling periods (6 and 8 weeks) broke corm dormancy more effectively than shorter treatments regardless of population.

Corms from all populations sprouted after 6 and 8 weeks of chilling. Less than 20% shoot emergence was observed in unchilled and 2-week chilled corms among all populations. However, a higher percentage of shoots sprouted on corms after 8 weeks of chilling. South Carolina and south Florida corms chilled longer than 6 weeks had the quickest shoot emergence. Michigan corms required 4 weeks or longer for quickest shoot emergence. Shoots emerged faster when north central Florida corms were chilled for 8 weeks. Following 16 weeks, north central Florida and South Carolina seedlings were larger than Michigan and south Florida seedlings.

Shoot emergence was lower for Michigan plants than all other populations after an 8-week chilling treatment and could be due to Michigan's longer winters. Therefore, these plants may require a chill period longer than 8 weeks for maximum shoot emergence. However, the longer chilling requirement for Florida plants was unexpected and could be to ensure they do not initiate shoot emergence until the threat of freezing temperatures is surpassed. Although timing of shoot emergence was different, clear differences in growth and development of seedlings were not evident.

CYRTOPODIUM PUNCTATUM, CIGAR ORCHID

Cyrtopodium punctatum, an epiphytic orchid species, is restricted to Florida in the USA. It is listed as endangered in the state of Florida due to over-collecting during the past century and habitat loss through cypress logging. This species produces impressive pseudobulbs, which are indicative to its common name. With careful observations, there is limited seed production in the remaining small population. Therefore, the long-term sustainability of remaining populations is of great importance.

Study #1: *C. punctatum* asymbiotic seed germination (Dutra et al., 2009a)

Five asymbiotic orchid seed germination media were examined for their effectiveness in promoting orchid seed germination under light and dark culture conditions. The five media were *Phyto*Technology Orchid Seed Sowing Medium (P723), Knudson C, Malmgren Modified Terrestrial Orchid Medium, Vacin & Wen Modified Orchid Medium and ½-strength Murashige & Skoog.

After 10 weeks, regardless of media, the highest germination was achieved under complete darkness. The protocorms that had reached advanced seedling development stages in the dark were achlorophyllous with numerous rhizoids but became chlorophyllous soon after exposure to light. Following greenhouse acclimatization, seedlings exhibited 90% survival.

The use of manual pollination to promote seed capsule formation combined with this asymbiotic seed culture protocol and the subsequent re-introduction of seedlings provides a means to increase *C. punctatum* populations and perhaps increase the genetic diversity in these limited populations.

Recommendation: Future research should explore if these responses suggest that photoperiod might play a role in seed germination *in situ*.

Study #2: *C. punctatum* pollination biology (Dutra et al., 2009b)

Pollinator identification was conducted by observing, photographing, collecting, and identifying flower visitors. Flower visitors capable of pollinating the flowers was determined by either direct observation of pollination or by detection of pollinia attached to the visitor's body. Breeding system determination used seven artificial, hand-pollinated treatments. After treatments were applied, entire inflorescences were bagged with small pore cloth mesh to prevent pollinator interaction (Fig. 3).

Figure 3: Mesh bags encased inflorescences to exclude pollinators.

Fruit formation occurred in four of the seven pollination treatments, which indicated that *C. punctatum* is self-compatible and not autogamous. This demonstrates the need for an insect pollinator for reproduction. Surprisingly, there was no evidence with this study that open pollination (control) resulted in capsule formation. This could suggest that the current small and fragmented populations are being pollinated at a very small rate since capsules have been seen to form without hand-pollination. Finally, seed viability and germinability were determined for each treatment and neither were found to be significant.

Four bee species were observed visiting the flowers: *Apis mellifera, Megachile xylocoides, Xylocopa virginica*. *X. virginica* were frequent visitors to the flowers (Fig. 4). Additionally, nine fragrance compounds were identified. Understanding a plant's breeding system and pollinator diversity is essential for conservation and management strategies.

Figure 4: *X. virginica* were frequent visitors.

Recommendation: A more detailed study on the insect populations of the area should be conducted, decreasing the closeness of the orchid populations to agricultural fields to minimize pesticide externalities.

Recommendation: A complete genetic analysis of the fragmented *C. punctatum* populations to determine current diversity for developing strategies to guide future reintroductions.

DENDROPHYLAX LINDENII, GHOST ORCHID

Dendrophylax lindenii, an epiphytic orchid species, is restricted to Florida in the USA where it is listed as state endangered largely due to poaching and habitat loss. This epiphyte is one of only a few leafless orchids in Florida which leads to its majestic appeal. This orchid is usually found deep within the interiors of cypress hammocks and is difficult to reach, raising conservation concerns for understanding the *in situ* biology and ecology. Although it is hypothesized that this orchid is pollinated by the giant sphinx moth, no pollination studies have been conducted to support this.

Study #1: *D. lindenii* floral fragrance extraction and analysis (Sadler et al., 2011)

Using a new technique aimed at capturing floral fragrance, eight volatile compounds were found associated with the floral fragrance of *D. lindenii*. The most abundant compounds when regarding the percentage of the total fragrance analysis were (E,E)-α-farnesene (71%), (E)-β-ocimene (9%) and methyl salicylate (8%). Other compounds were linalool (5%), sabinene (4%), (E)-α-bergamotene (2%), α-pinene (1%), and 3-carene (1%). All compounds, excluding (E,E)-α-farnesene, were common floral scent constituents in other angiosperms. All but one (3-carene) have been reported from moth-pollinated Orchidaceae. This suggests that this new technique, which did not impose physical harm to the orchids, is successful in elucidating volatile compounds from orchids.

Interestingly, (E,E)-α-farnesene, the most abundant compound, is odorless to humans and has been linked with insects and insect-damaged plants. A similar compound, trans-β-farnesene has been found as an alarm pheromone in aphids. Indeed, scale insects were observed on the ovaries of flowers at the time of sampling.

Recommendation: Additional studies should explore whether the insects, the plant, or both are release (E,E)-α-farnesene.

Recommendation: Utilize this floral headspace collection method with other conservation programs involving other rare epiphytic orchids, as well as non-orchids, such as bromeliads and cacti.

Recommendation: This new technique can document changes in the blend of compounds emitted over time.

Recommendation: This technique might make it possible to monitor long-term changes in floral scent with the manipulation of substrate, fungi, or both.

EPIDENDRUM NOCTURNUM, NIGHT-FRAGRANT EPIDENDRUM ORCHID

Epidendrum nocturnum, an epiphytic orchid species, is listed as state endangered largely due to the threats from poaching and habitat alteration. Suggested by its common name, this striking white orchid has been described to be fragrant at night. Due to flowers not always opening, it has been suggested that it is capable of self-pollination.

Study #1: *E. nocturnum* symbiotic seed germination and reintroduction (Zettler et al., 2006)

Using *E. nocturnum*, a protocol was developed to propagate an epiphytic orchid with a mychorrizal associate that was useful for conservation. This was considered because the prevailing use of asymbiotic germination has raised conservation concerns, given the importance of fungi to orchids *in situ*.

To compare the effects of two commonly used symbiotic media, seeds were sown on standard oat medium (SOM) and modified oats medium (MOM-nitrogen containing medium). Seedlings cultured on SOM developed to stages 3 and 4, whereas seedlings cultures on MOM remained at stage 2. Seeds incubated in the absence of fungus (control) resulted in minimal germination (0.1%).

To compare the effects of initial light versus dark incubation on germination and development, half of the plates received a 14-h photoperiod for 7 days and the rest were in complete darkness. Seeds pretreated with light resulted in the highest percentage of stage 5 seedlings (MOM=5.1%, SOM=6.0%) compared to complete darkness (MOM=4.7%, SOM=2.4%).

Following *in vitro* conditions, stage 5 seedlings were greenhouse acclimatized. Seedlings were transferred to moistened *Sphagnum* moss with and without Miracle-Gro. The seedlings without Miracle-Gro displayed the highest survival rate (>90%), whereas those exposed to Miracle-Gro experienced lower survival (44%).

With this protocol, a total of 43 seedlings were placed *in situ* 16 months after sowing. When reintroducing an orchid germinated symbiotically, conservation efforts are urged to be careful when selecting fungi that are found in the geographic area of the site. These data suggest that using fungi could complement, not replace, existing asymbiotic techniques to benefit orchid conservation.

<u>Recommendation</u>: Future studies of *E. nocturnum* should determine if this species if capable of self-pollination.

<u>Recommendation</u>: Research is needed that addresses the effect of fertilizers on orchid-fungal symbiosis *in vitro* and *in situ*, and the role of potting media on acclimatization.

EULOPHIA ALTA, WILD COCO ORCHID

Eulophia alta, a terrestrial orchid species, is the only species in the genera present in North America. It is restricted to southern Florida near moderately wet roadsides or near the edges of forest sites. Although this orchid is currently not listed as a rare species, urban development throughout its range is threatening its habitat. It is also listed by the federal government as a wetlands indicator species.

Study #1: *E. alta* asymbiotic and symbiotic seed germination (Johnson et al., 2007)

Using collected intact root systems, 10 fungal associates were discovered. This process included 1) growing endophytic orchid mycobiont-infected cells on agar and 2) isolating the hyphal tips of the growing fungi. Three genera (*Sclerotinia*, *Fusarium*, and *Armillaria*) were not expected and are typically not associated with endophytic root-inhabiting orchid mycorrhizae.

Asymbiotic seed germination used five media types to assess the optimal germination conditions: *Phyto*Technology Orchid Seed Sowing Medium (P723), Knudson C, Malmgren Modified Terrestrial Orchid Medium, Vacin & Wen Modified Orchid Medium and ½-strength Murashige & Skoog. Symbiotic seed germination used eleven possible mycobionts for evaluation.

After 10 weeks of *asymbiotic* culture conditions, P723 medium supported the highest asymbiotic germination percentage with 56.1% and a higher percentage of protocorms in Stage 2 (54.7%) and Stage 3 (2.1%) than all other media. Overall, all treatments had Stage 3 after 14 weeks, but few seeds grew beyond this stage. A possible explanation for the improved germination on P723 medium is the presence of the organic nitrogen source: peptone.

After 6 weeks *symbiotic* culture only one mycobiont supported germination (70.1%) and development to Stage 3 (44.3%). After 18 weeks of dark culture, protocorms were too fragile to handle and were considered dead. This could be due to the lack of light for photosynthesis to initiate or the mycobiont nutrient stress resulting in fungal pathogenicity.

Recommendation: Further examine input of nitrogen quality and quantity to improve asymbiotic culture of *E. alta*.

Study #2: *E. alta* pollination treatments on seed formation and seed germination (Johnson et al., 2009)

Seven pollination treatments (open, agamospermy, spontaneous autogamy, induced autogamy, artificial geitonogamy, artificial xenogamy, and induced xenogamy) were tested to investigate if *E. alta* is an auto-pollinated species.

All treatments resulted in capsule formation, but capsules resulting from spontaneous agamospermy aborted before 6 weeks of development. After 10 weeks of the experiment, spontaneous autogamy (auto-pollination) was rare with 7.1% of observed flowers forming capsules. Greater capsule formation was found with induced autogamy (46.4%), artificial

geitonogamy (64.3%) and xenogramy at short (42.9%) and long (67.9%) distances. Therefore, spontaneous autogamy is rare in *E. alta* and naturally forming capsules may be the result of unobserved pollination events.

Seeds formed from the autogamous treatment exhibited a slower rate of germination and seedling development compared to the other pollination treatments suggesting that seeds of lower fitness may be produced.

Recommendation: Further study if autogamy of *E. alta* is common throughout its range or limited to certain populations.

Recommendation: A pollinator study should be conducted to determine whether a pollinator exists for *E. alta*.

NON-SPECIES SPECIFIC RESEARCH STUDIES

Study #1: Orchid distribution survey at McBride's Pond (Massey, 2010)

In an observational study, orchids were divided into three categories based on their morphological differences – leafless, orchids with leaves and pseudobulbs, and orchids with leaves but without pseudobulbs. The orchids were then further separated into their stages of development- seedling, juvenile, and mature plants.

After sampling 419 orchids, most of the orchids were found on pop ash (*Fraxinus caroliniana*) (371) with 100%, 89%, and 88% of them being leafless, leaves with pseudobulbs, and leaves without pseudobulbs, respectively. All of the observed seedlings were either on moss or a combination of moss and bark. A majority of leafless orchids were found on horizontal substrates, whereas orchids with leaves were affixed to the substrates at a 45 degree angle or a vertical position. Orchids were generally found facing in a northerly or northeasterly direction.

Recommendation: Future studies should survey the trees in the same area or analyze the bark of the tree species in the areas to better understand the *in situ* relationship between orchid and substrate.

Study #2: Comparative water balance profiles of orchid seeds (Yoder et al., 2010)

To investigate orchid seed morphology as it relates to ecology, testa porosity was studied for 2 epiphytic, 1 aquatic, and 7 terrestrial orchids. Water balance profiles including water content, water loss rate, activation energy, and equilibrium humidity were compared between: *Cleistes bifaria, Encyclia tampensis, Epidendrum nocturnum, Habenaria repens, Isotria medeoloides, Liparis elata, L. hawwiensis, Platanthera holochila, P. integrilabia,* and *P. leucophaea*. Using this information, habitat was considered to determine if there was a correlation between these data.

It was hypothesized that variations in seed size and testa characteristics could be linked to water loss rates. To test this hypothesis, seeds were placed in glass desiccators with different saturated salt solutions to adjust the relative humidity in each vessel. Although the seeds of the 10 taxa varied significantly with respect to fresh mass, dry mass, and water mass, no significant differences were detected in percentage of water content regardless of size, origin, and ecological niche. Likewise, all seeds displayed a proportionate, exponential pattern of water loss over time at 0% relative humidity. However, terrestrial orchids retained water more effectively than epiphytes.

Seeds did not easily gain water content from the atmosphere. This seems intuitive because orchid seeds are windborne for dispersal to new, suitable habitats. However, epiphytic seeds are more porous than terrestrial suggesting that they have adapted to quickly affix themselves to limited arboreal substrates; otherwise the seedlings would perish from lack of photosynthesis. Overall, that seeds of terrestrials are larger, lose water at a slower rate, and are less porous than the epiphytes.

Recommendation: Studies are needed to demonstrate the true relationship of the seed's morphology and their success in a given ecological niche.

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CONSERVATION STRATEGIES QUESTIONS TO CONSIDER

In what ways do you currently help protect native orchids?

What current policies, if any, are established in your area for orchid protection?

How can the current research literature help you and help the greater society?

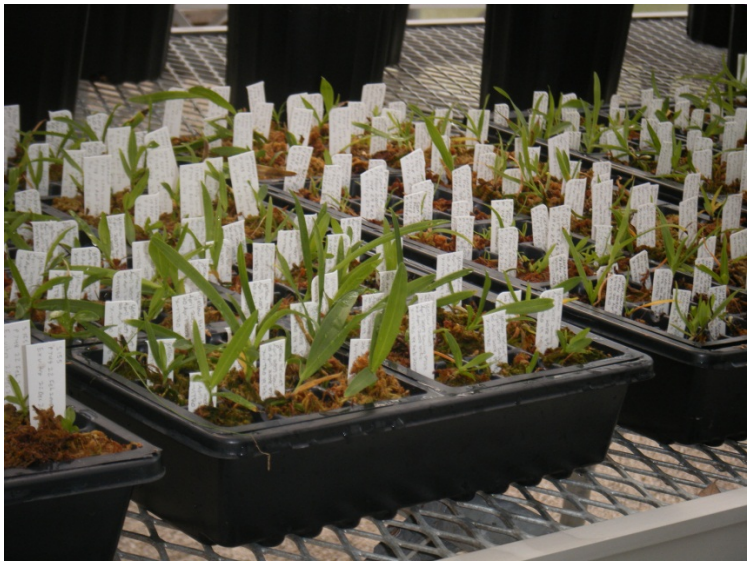
What are the biggest threats in your area for native orchids?

Do you, or the organizations you are involved with, help support the conservation and/or research of native orchids?

What research is currently being conducted in your area, if any?

How can we eliminate or decrease the occurrences of poaching native orchids?

What are local institutions you could partner with to help further orchid conservation?

What research could be conducted *in the next year* in your area regarding orchid conservation?

What research could be conducted *in the next five years* in your area regarding orchid conservation?