IN VITRO ECOLOGY OF *Calopogon tuberosus* var. *tuberosus*: A NEW CONCEPT IN SPECIES CONSERVATION

By

PHILIP KAUTH

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2009

© 2009 Philip Kauth

To Meredith, who else…

ACKNOWLEDGMENTS

I thank Dr. Michael Kane for his dedication, passion, enthusiasm, and encouragement, which in turn fueled my passion and excitement. I thank my co-chair, Dr. Wagner Vendrame for his support and encouragement from a distance. I also thank Dr. Carrie Reinhardt-Adams, Dr. Debbie Miller, and Dr. Thomas Sheehan for serving on my supervisory committee and sharing their knowledge, ideas, and excitement.

Over the last several years I had the opportunity to share a work environment with great people. I could not have shared space, ideas, and knowledge over the years with better colleagues and friends than Tim Johnson, Daniela Dutra, Scott Stewart, Xiuli Shen, and Carmen Valero-Aracama. I especially thank Tim for the hours of brainstorming, talking, watching the fights, and trips to the Refuge. I thank Nancy Philman not only for her incredible friendship, but also for much of my development (even though she probably will deny her involvement). This research could not have been conducted without the assistance from many that have either collected seed, issued permits, served as field trip guides, or provided thoughtful insights including Larry Richardson (USFWS-FPNWR), Mary Bunch (South Carolina Heritage Preserves-SCDNR), Jim Fowler (Greenville, South Carolina), Howard Lorenz (Wisconsin), and Doug Goldman (Harvard University). I thank Kip Knudson not only for collecting seed and leading me through Carney Fen numerous times, but also for influencing me more than me takes credit for. I also thank Dr. Hector Perez and Dr. Charles Guy for use of their incubators and growth chambers.

I thank my parents for their endless support, love, and encouragement throughout my LONG educational process. I thank my two beautiful dogs, Mya and Ladybug, for providing hours of laughter, and for showing me that life is a true blessing and that everyone deserves a second chance at life. Finally I thank the love of my life, Meredith. I could not have done this without her love, sacrifice, and support and for that I am forever grateful.

TABLE OF CONTENTS

page

LIST OF TABLES

LIST OF FIGURES

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

IN VITRO ECOLOGY OF *Calopogon tuberosus* var. *tuberosus*: A NEW CONCEPT In SPECIES CONSERVATION

By

Philip Kauth

December 2009

Chair: Michael Kane
Cochair: Wagner Vendrame
Major: Horticultural Science

The importance of ecotypic differentiation has recently been highlighted for plant conservation purposes, but use of local plant material for orchid restoration has been largely ignored. Using local plant material is necessary to maintain ecosystem and plant population stability, but little is known regarding the degree of ecotypic differentiation necessary to maintain stability. Ecotypic differentiation among geographically distant populations of the orchid, *Calopogon tuberosus,* was examined using *in vitro* ecology methods. Comparative effects of photoperiod, germination media, temperature, and chilling on asymbiotic *in vitro* seed germination and seedling development of *C. tuberosus* populations were examined. Seedling biomass allocation and corm dormancy were also examined. Regardless of germination treatment, Michigan seeds germinated and developed rapidly, while south Florida seeds germinated and developed slowly. This was likely due to the faster onset of winter conditions following seed dispersal in more northern environments. Photoperiod did not significantly influence seed germination and development. Different germination media significantly influenced germination and development depending on source due to soil nutrient variation among populations. Higher temperatures promoted increased germination percentages in South

Carolina seeds, while Michigan and south Florida seed germination were higher at lower temperatures. Chilling seeds effectively increased germination in all populations, but seed germination in northern populations exceeded embryo viability in longer chilling treatments. This was reflected by the thicker testae required to protect seeds of northern populations form harsher winter climates. During a detailed timecourse seedling development study, Michigan seedlings allocated more biomass to corms, and developed corms faster than all other populations. Higher corm biomass allocation in northern populations correlated strongly with a shortened growing season. The rapid corm formation and biomass allocation in seedlings from more northern populations represented an adaptive response to a shorter growing season to increase survival. All populations required a chilling period to break corm dormancy, but longer chilling periods promoted faster and a higher percentage regrowth. Cutting corms also broke dormancy, but Michigan corms responded more rapidly compared to other populations. In conclusion, *in vitro* techniques were found to be appropriate to differentiate ecotypes by detecting their unique ecological growth strategies. Ecotypic differentiation influenced by growing season length, temperature, and soil nutrient availability is occurring in *C. tuberosus*.

CHAPTER 1
LITERATURE REVIEW

Introduction

The Orchidaceae is one of the largest and most diverse family of angiosperms with an estimated 17,000 to 35,000 species (Dressler, 1993). All orchids share at least six common features including zygomorphic flowers, a gynandrium (column), a rostellum, pollinia, a labellum, and microscopic seeds (Sheehan and Black, 2007). Seventy percent of all orchid species are epiphytes, but terrestrial, aquatic, lithophytic, and underground species exist as well (Dressler, 1993). The Orchidaceae has evolved highly specialized associations with pollinators and mycorrhizal fungi that allow species to occupy and survive various habitats (Otero *et al.*, 2004; Jersáková *et al.*, 2006). The highly diverse floral array is a direct result of the specific pollination mechanism of each species (Tremblay, 1992).

Civilization has been fascinated with orchids since antiquity. Early references to orchids can be found in ancient Chinese literature dating back to 800 B.C., in which Confucius lauded the orchid for its fragrance (Berliocchi, 2000). Orchids owe much of their appeal to the ancient Greeks who associated orchids with passion and fertility, and created many myths concerning the origin of orchids. These myths often involved devious characters and death, thus setting the future for these highly desired plants. The name orchid is derived from the Greek word for testicles, *orchis*, first used by Theophrastus in scientifically describing orchid plants.

Great scientists and writers have popularized orchids. Charles Darwin was captivated by their diverse pollination mechanisms. *Angraecum sesquipedale,* an orchid with a 30 cm nectar spur, is often referred to as Darwin's orchid since he hypothesized that a moth was the potential pollinator. Although Darwin was criticized, 40 years later a sphinx moth with a 30 cm proboscis was discovered in the same habitat as the orchid. Writers including Shakespeare, H.G. Wells,

and Thoreau included orchids in many of their writings (Berliocchi, 2000). In the James Bond novel and film, Moonraker, an orchid was the source of a deadly nerve toxin. The book Orchid Thief and the movie based on the book, Adaptation, popularized the famous and sought after *Dendrophylax lindenii*, the ghost orchid. As orchids gain fame and popularity an opportunity exists to educate the public about conservation of orchids.

Although the Orchidaceae is diverse and widespread, the family is critically imperiled. Over-collecting, pollinator decline, habitat conversion and loss, and habitat mismanagement have caused a drastic reduction in wild orchid populations (Koopowitz *et al.*, 2003). Many organizations and individuals are associated with restoring orchid populations to historic numbers by propagating orchids from seed. Large amounts of information exist regarding the propagation science of orchids for conservation and reintroduction purposes. However, large gaps in propagation science still exist since the first orchid seeds were successfully germinated asymbiotically *in vitro*. A major omission in propagation science of orchids is the use of *in vitro* conditions to study ecological factors that promote seed germination and seedling development. This area of propagation science has the potential to assist in orchid conservation, as well as further the scientific knowledge of orchids.

The Orchid Seed

Orchid seeds are exceedingly diverse in shape, size, and pattern. They are microscopic and range from 0.05 to 6 mm in length, 0.01 to 0.93 mm in width, and weigh 0.3 to 14 µg (Arditti, 1967; Arditti and Ghani, 2000). Seed capsules may hold anywhere between 1,300 to 4 million seeds (Arditti, 1967). Shapes are also various including filiform, fusiform, clavate, and ellipsoidal seeds (Molvray and Kores, 1995).

Orchid seeds share a common characteristic of a reduced embryo and the absence of endosperm or a cotyledon (Prutsch *et al.*, 2000) with the exception of *Sobralia* and *Bletilla* seeds

that have a rudimentary cotyledon (Arditti, 1967). Various surface depressions and patterns in the testa increase air resistance and allow seeds to remain air- and water-borne for long periods (Arditti and Ghani, 2000; Prutsch *et al*., 2000). The testa is normally derived from the outer integument, but as in the case of *Paphiopedilum delenatii* the testa is derived from both the inner and outer integument (Molvray and Kores, 1995; Lee *et al.*, 2006). In most species the testa is usually one cell thick, but made up of 20 to 600 cells (Molvray and Kores, 1995; Prutsch *et al*., 2000).

The embryo is attached to the testa by several cells, contains dense cytoplasm, and is made up of as few as ten cells (Stoutamire, 1964). At early globular stages, plastids with starch are visible, but soon disappear during the mature globular stage (Lee *et al*., 2006). At the mature globular stage, starch is replaced by lipid and protein bodies (Lee *et al*., 2006). Cuticular substances appear in the surface wall cells of the embryo during the early globular stage, but are not found in the suspensor region (Lee *et al*., 2006). The suspensor serves as a channel for free movement of nutrients and water as well as a food storage site for the embryo (Yeung *et al.*, 1996). The two-cell thick inner integument dehydrates and compresses around the embryo at full maturity (Lee *et al.*, 2005). A layer inside the inner integument becomes cutinized and a layer outside the inner integument becomes lignified at seed maturity (Yamazaki and Miyoshi, 2006). The lignification and cutinization process strengthens the carapace, which restricts embryo growth through mechanical or chemical means (Yamazaki and Miyoshi, 2006).

Orchid seeds contain an undifferentiated embryo lacking enzymes to metabolize polysaccharides (Manning and van Staden, 1987; Molvray and Kores, 1995). Sugars present in orchid embryos include sucrose, fructose, maltose, rhamnose, and glucose, but these are either utilized fully prior to germination or are present in insufficient quantities to support and sustain

germination (Manning and van Staden, 1987). Although lipids are used as a major nutrient source, embryos lack enzymes to convert lipids to soluble sugars (Manning and van Staden, 1987). Given that orchid seeds can not metabolize polysaccharides and lipids, they utilize a mycorrhizal relationship with compatible fungi (i.e. mycobiont) during germination and early development (Rasmussen *et al.*, 1990*b*). This mycorrhizal relationship is critical during germination and seedling development (Zettler, 1997). Following penetration, embryos digest the fungi providing water, carbohydrates, minerals, and vitamins (Rasmussen, 1992; Yoder *et al.*, 2000). The digestion of the mycorrhizal fungi in turn stimulates glucose and enzyme production, reserve mobilization, and post-germination nutrient support (Manning and van Staden, 1987).

Orchid Seed Germination

History and Background

Interest in orchid seed germination began in the 1800s. Early attempts to initiate germination involved sowing seeds onto organic substances such as sphagnum moss, bark, or leaf mold, but this often proved unsuccessful (Arditti, 1967). Growers also attempted to germinate seeds at the base of potted wild-collected mother plants. Bernard and Burgeff were the first to recognize the role of fungi in orchid seed germination and co-cultured fungi with orchid seeds (Bernard, 1899; Burgeff, 1909). They experimented with symbiotic seed germination, the co-culture of fungi with orchid seeds. Although seeds did not germinate readily, they concluded that orchid seeds could only germinate *in vitro* in the presence of an appropriate mycorrhizal fungus (Knudson, 1922).

Based on initial experiments by Bernard and Burgeff, Lewis Knudson further examined orchid seed germination. Using his knowledge and interest in the effects of sugars on plants and enzyme production in fungi, Knudson recognized that mycorrhizal fungi hydrolyzed starch and cellulose into usable simple sugars (Arditti, 1967). Using a nutrient solution supplemented with

1% sucrose, Knudson (1922) successfully germinated *Cattleya* seeds. From these initial experiments Knudson demonstrated asymbiotic *in vitro* germination, and developed Knudson Solution B with 2% sucrose or glucose. Knudson eventually improved Solution B and developed Solution C, which is widely used as an asymbiotic germination medium (Knudson, 1946).

Asymbiotic germination represents an ideal system for studying the growth and development of orchid seeds and seedlings. While asymbiotic germination is often the more popular *in vitro* technique, symbiotic seed germination has recently gained popularity for conservation and restoration projects. Factors such as photoperiod, temperature, culture media, and seed dormancy may influence both asymbiotic and symbiotic germination.

Symbiotic Orchid Seed Germination

The Orchidaceae have evolved a unique relationship with mycorrhizal fungi (mycobionts). Bernard in 1899 first recognized the role of mycobionts in orchid seed germination, and attempted to co-culture orchid seeds with mycobionts (Hadley, 1982). Burgeff, who was a contemporary to Bernard, not only studied the relationship between mycobionts and orchid seeds, but also isolated and identified many fungi in culture (Hadley, 1982). Although early attempts at symbiotic germination were often not successful, Bernard and Burgeff created the foundation to further study orchid seed germination.

The focus of symbiotic seed germination research changed throughout the 20th century. After Knudson (1922) discovered asymbiotic germination, little research on symbiotic germination continued until the problematic relationship of mycobiont specificity was examined (Curtis, 1939), and a more efficient symbiotic germination technique was developed (Downie, 1940). The nutritional requirements of mycobionts and the movement of nutrients between seeds and mycobionts were studied thoroughly in the 1960s and 1970s (Smith, 1966, 1967; Hijner and

Arditti, 1973; Hadley and Purves, 1974; Blakeman *et al.*, 1976; Hadley and Ong, 1978). In the late 1980s, symbiotic techniques were again refined (Clements *et al.*, 1985; Dixon, 1987).

Until the 1990s, the environmental cues that affected symbiotic seed germination were not studied as intensively as asymbiotic germination. Photoperiod and temperature affects on symbiotic seed germination were studied in depth (Rasmussen *et al.*, 1990*a*, *b*; Rasmussen and Rasmussen, 1991; Rasmussen, 1992; Zettler and McInnis, 1992, 1994; Zettler and Hofer, 1997, 1998). Recently, the focus has once again turned to refining symbiotic techniques to increase seedling acclimatization survival for reintroduction purposes (Brundrett *et al.*, 2003; Batty *et al.*, 2006*a*; Scade *et al.*, 2006). The role of fungal specificity has once again been revisited (Otero *et al.*, 2004, 2005; Stewart and Kane, 2006*b*) as well as molecular identification and mycobiont diversity (Taylor *et al.*, 2003; McCormick *et al.*, 2004; Shefferson *et al.*, 2005, 2007). The mutualistic/parasitic relationship between orchids and mycobionts has also been recently studied (Cameron *et al.*, 2006, 2007, 2008 Shimura *et al.*, 2007).

An important study involved the propagation of the federally endangered, *Platanthera holochila*, from Hawaii (Zettler *et al.*, 2005*a*). The mycobiont isolated from Hawaiian plants did not support germination; however, the mycobiont isolated from *Spiranthes brevilabris* from Florida supported germination raising concerns of the ecological importance of local adaptation. Introducing plants harboring the Florida mycobiont may adversely affect not only the *P. holochila* population, but also the entire ecosystem. Different strains within a fungal species may damage isolated ecosystems or other rare and endangered plants (Zettler *et al*., 2005*a*). For this reason, considering differences in mycobionts not only among but also within species is crucial.

Asymbiotic Orchid Seed Germination

Since Knudson (1922) showed that orchid seeds could be germinated *in vitro* without mycobionts, much information has been published on asymbiotic germination. Asymbiotic

germination is used more often than symbiotic techniques since asymbiotic methods are generally less complicated. Asymbiotic methods are ideal means to study physiological effects of photoperiod, temperature, and mineral nutrition on germination and subsequent development. Replicating the environmental conditions found *in situ* under *in vitro* conditions may lead to further insight into how environmental cues trigger germination.

A major difference between symbiotic and asymbiotic techniques is the germination medium. Because mycobionts are not utilized to provide nutrients to developing embryos, asymbiotic germination media is often supplemented with various sources and types of carbohydrates, nitrogen, vitamins, and undefined organic additives. Since Knudson demonstrated the feasibility of asymbiotic germination, the role of mineral nutrition has been researched extensively. Many different culture media have been developed since Knudson's original formula was published. Although many of these media have only minor differences in composition, growth and development of species may be significantly affected. More recently the role of individual media components have not been as extensively investigated, but rather commercially prepared media are often used to conduct screens to obtain satisfactory germination. Such studies also focused on characterizing the growth and development of embryos and seedlings to more precisely examine growth and development.

Nitrogen has long been considered an important component in the germination of orchid seeds. Recent reports have shown that different media may better support initial germination compared to advanced seedling development, and vice versa. The higher seed germination percentages of *Encyclia boothiana* var. *erythronioides* and *Calopogon tuberosus* on Knudson C. were attributed to high ammonium content, which can be utilized by seeds during early germination and development (Stenberg and Kane, 1998; Kauth *et al.*, 2006). Seedling fresh

weight of *Cattleya* and *Cymbidium* hybrids was greater when grown on a medium with a high ratio of ammonium to nitrate (Spoerl and Curtis, 1948).

While Knudson C also promoted seedling development of *E. boothiana* (Stenberg and Kane, 1998), *C. tuberosus* seedlings developed to more advanced stages on P723 Orchid Seed Sowing Medium (*Phyto*Technology Laboratories, Inc., Shawnee Mission, KS) (Kauth *et al.*, 2006). The limited development of *C. tuberosus* on Knudson C was attributed to a high nitrate concentration and the inability of the embryos to utilize nitrates during early growth and development (Raghavan and Torrey, 1964). Peptone, an organic nitrogen source present in P723, may have contributed to the increased seedling development by supplying auxin-like compounds or various amino acids (Curtis, 1947; Kauth *et al.*, 2006). However peptone responses may be species specific. Seed germination percentages of *Paphiopedilum insigne* and *P. hirsutissimum* were approximately 30% higher with peptone than without peptone, and seedlings were more uniform with peptone (Curtis, 1947). Adversely, seed germination of *Phaius grandiflorus* and *Platanthera clavellata* was hindered in the presence of peptone.

Although ammonium was found beneficial in asymbiotic germination of *E. boothiana* var. *erythronioides* and *C. tuberosus*, seed germination of other terrestrial orchids may be inhibited by it. Germination and growth of *Dactylorhiza incarnata* seeds, a European terrestrial orchid, were reduced in the presence of ammonium (Dijk and Eck, 1995*b*). As nitrogen concentration increased, embryo weight decreased in two species of *Dactylorhiza* (Dijk and Eck, 1995*a*). Likewise, a high ratio of ammonium to nitrate reduced the germination of *Vanda tricolor* (Curtis and Spoerl, 1948).

Amino acids have also been used as a substitute nitrogen source. Raghavan (1964) reported that only certain amino acids increase seed germination of *Cattleya*. Glycine, the

simplest amino acid, decreased overall germination of *Cattleya* seeds from 53% to 41%. However, germination in the presence of arginine, proline, and glutamine was similar to that with ammonium nitrate (Raghavan, 1964). Spoerl and Curtis (1948) also reported that glycine significantly reduced germination of *Cattleya* seeds after 2 months when compared with other amino acids. However, after 5 months germination in the presence of glycine increased from 22.5% to 64%. Amino acid enzyme systems within developing embryos change over time. Amino acids may not be available as initial nitrogen sources, but may be metabolized after a certain period of time (Spoerl and Curtis, 1948). Various orchid species respond differently to various amino acids during germination, and therefore further investigation should be carried out. Since not all amino acids are beneficial for seed germination, combinations of amino acids may increase germination (Spoerl and Curtis, 1948).

Edamin, a lactalbumin hydrolysate with peptides and 18 amino acids, increased the germination of a *Cattleya* × *Laelia* hybrid (Ziegler *et al.*, 1967). Embryos became green faster and seedling dry weight was greater with Edamin than seedlings cultured without Edamin. Tissue analysis of seedlings cultured on Edamin yielded showed increased levels of glutamine, asparagine, and gamma amino butyric acid. Although these amino acids were not found in Edamin, complex organic nitrogen sources, such as Edamin, may be important for the synthesis of amino acids (Ziegler *et al.*, 1967).

Nitrogen in the form of amino acids may be more readily available to germinating seeds or developing embryos than inorganic nitrogen (van Waes and Debergh, 1986*a*; Malmgren, 1993; Anderson, 1996; Malmgren, 1996; Stewart and Kane, 2006*a*). Majerowicz et al. (2000) reported increased growth of *Catasetum fimbriatum* seedlings in the presence of glutamine compared to media containing ammonium or nitrate. Stewart and Kane (2006*a*) reported improved

germination and subsequent development of *Habenaria macroceratitis* on Malmgren Modified Terrestrial Orchid Medium, which contains glycine as the sole nitrogen source. When inorganic nitrogen, such as ammonium, is utilized by germinating seeds, the nitrogen is converted to amino acids (Majerowicz *et al.*, 2000). Using amino acids as the sole nitrogen source may lead to more efficient nitrogen assimilation by avoiding certain nitrogen conversion steps.

Since orchid seeds have minimal carbohydrate reserves, an exogenous source of carbohydrates is required for *in vitro* orchid seed germination. Two sources of carbohydrates are available to the germinating embryo during the first stages of development in nature: minimal carbohydrates in the embryo, and those obtained from mycobionts (Rasmussen, 1995). Some orchid seeds contain glucoproteins that may release glucose upon hydrolyzation, explaining why some orchid seeds germinate in water (Rasmussen, 1995).

The role of carbohydrates in asymbiotic germination has often been contradictory. In an early study on the germination of *Cattleya* seeds using several carbohydrate sources, d-mannose supported the highest germination percentage, while pentose sugars such as xylose supported no germination (Wynd, 1933). Conversely, Ernst (1967) reported that xylose proved effective in supporting germination and development of *Phalaenopsis* seeds. Both concluded that fructose did allow for moderate to exceptional germination. Several sugars are translocated to embryos from their mycobiont including trehalose, glucose, and mannitol (Smith, 1973). Trehalose was found to be suitable for several species, but to lesser extents than other sources (Ernst, 1967; Ernst *et al.*, 1971; Smith, 1973).

The role of carbohydrates is also crucial for seedling development. *Phalaenopsis* seedlings developed best on media with glucose, fructose, and oligosaccharides containing glucose or fructose (Ernst *et al.*, 1971). However, polysaccharides proved unsuccessful in germinating and

sustaining seedlings. The benefits of using glucose as the lone carbohydrate source were recently reported (Wotavová-Novotná *et al.*, 2007). Using lower concentrations of sucrose promotes shoot development, while higher concentrations promote root growth (Yates and Curtis, 1949).

Ernst and Arditti (1990) reported that *Phalaenopsis* seedlings developed in the presence of many carbohydrate sources including glucose, a simple sugar, and maltoheptaose, a long chain sugar. Germination percentage and seedling development was highest on glucose, with fewer seeds germinating on maltooligosaccharides. Although embryos did not develop further without sugar or at least a low concentration, endogenous carbohydrates must have been present to support early germination and development. After 6 months culture, seedlings cultured on glucose had higher fresh weights and survival than seedlings cultured on long-chain carbohydrates. The lower fresh weight of *Phalaenopsis* seedlings cultured with long-chain carbohydrates may be caused by insufficient enzymes responsible for breaking bonds in these carbohydrates (Ernst and Arditti, 1990).

Factors Effecting Orchid Seed Germination

Photoperiod

Although the effects of photoperiod have been widely researched, the results are often contradictory. Incubating terrestrial orchid seeds in complete darkness is often recommended while light incubation is epiphytic seeds is recommended for epiphytic seeds. Several explanations have been offered regarding this relationship. Upon dehiscence, seeds of terrestrial orchids may not germinate until buried (Rasmussen and Rasmussen, 1991). Many terrestrial orchids also grow in more shaded environments than their epiphytic counterparts (Rasmussen, 1995), and light may not penetrate the canopy and reach soil as readily (Rasmussen and Rasmussen, 1991).

A small increase in light intensity from complete darkness to 1.2 µmol m^{-2} s^{-1} reduced germination of European terrestrial orchids (van Waes and Debergh, 1986*b*). Seed germination of *Cypripedium acaule* was lower under a16/8 h photoperiod (6.7% germination) compared to complete darkness (96.7%) (St-Arnaud *et al.*, 1992). In addition, all embryos developed leaves in darkness, but only 60% of the embryos in the 16/8 h photoperiod developed leaves (St-Arnaud *et al.*, 1992). Zettler and Hofer (1997) reported a significant decrease in germination when *S. odorata* seeds were exposed to a brief period of illumination. Germination in complete darkness for 3 weeks was greater than germination of seeds exposed to either 7 days of an 8/16 h or 14/10 h photoperiod, and then placed in darkness for 2 weeks. Stewart and Kane (2006*a*) reported that light inhibited asymbiotic germination and development of *Habenaria macroceratitis*. Although embryos developed to a leaf-bearing stage in all photoperiod treatments, over 90% of the embryos developed leaves in complete darkness (Stewart and Kane, 2006*a*).

Embryos cultured in complete darkness often produce more rhizoids than those in light (Stewart and Kane, 2006*a*). Rhizoids, which are sites of fungal infections, may not be produced until seeds/embryos are buried and likely to encounter fungal mycobionts (Rasmussen, 1995). Rhizoid inhibition under light conditions may prevent embryo death by preventing the mobilization of valuable energy reserves prior to encountering conditions of likely mycorrhizal infection (Stewart and Kane, 2006*a*).

Stoutamire (Stoutamire, 1974) suggested that bog-inhabiting North American terrestrial orchids that are adapted to an open canopy are less sensitive to light. Kauth *et al.* (2006) found evidence for this with seeds of *Calopogon tuberosus* var. *tuberosus*, a North American terrestrial orchid. *Calopogon tuberosus* not only inhabits bogs, but also grows in areas of full sun such as open prairies and pine flatwoods. Although asymbiotic germination in complete darkness was

generally greater than germination in a 16/8 h photoperiod, seedling development was superior in a 16/8 h photoperiod. No embryos developed to an advance leaf-bearing stage under complete darkness, but over 20% of the embryos on P723 culture medium developed to advanced leaf-bearing stages in the 16/8 h photoperiod (Kauth *et al.*, 2006). Similar results were obtained with asymbiotic germination of *Bletia purpurea*, a terrestrial orchid that grows in prairies and under open canopies in south Florida (Dutra *et al.*, 2008). Germination and subsequent development under long day conditions may be an adaptation to shallow seed burial or germination above the substrate.

Several researchers reported that germination increases with brief periods of illumination. Rasmussen *et al.* (1990*a*) reported 75% germination of *Dactylorhiza majalis* seeds when illuminated for 10 days prior to dark incubation. This was a significant increase from 45% germination under continual darkness. Zettler and McInnis (1994) reported similar results with symbiotic germination of *Platanthera integrilabia*. Germination increased from 20% under complete darkness to 44% when seeds were exposed to 7 days under a 16/8 h photoperiod prior to dark incubation. While the exact function of light pretreatment is not understood, mycorrhizal fungi may benefit from brief periods of illumination (Zettler and McInnis, 1994).

While photoperiod has been studied extensively in orchid seed germination, light quality and quantity has been generally neglected. Fukai et al. (1997) examined the role of light quality on asymbiotic seed germination of the hybrid *Calanthe* Satsuma. After 4 months germination percentage was highest in complete darkness (57.7%) compared to 40.2% and 1.3% germination under red and blue light, respectively. Germination was also low (12.4%) under a combination of red and blue light as well as fluorescent lights (13.2%). Blue light, although inhibitory to germination, promoted a high level of embryo development (Fukai *et al.*, 1997). Blue light has

been shown to be important in photomorphogenesis as well as chlorophyll accumulation in non-orchid species (Kamiya *et al.*, 1981). Likewise, red light proved beneficial for asymbiotic seed germination of *Goodyera pubescens*, while blue and far red inhibited germination (McKinley and Camper, 1997). Approximately 33% germination was reported under red light and fluorescent light, while germination under blue light, UV light, and complete darkness was approximately 20%.

Rasmussen and Rasmussen (1991) studied the effects of light quality and quantity on symbiotic germination of *D. majalis*. Under a low white light intensity of 13 W m^{-2} (ca. 60 µmol m^{-2} s^{-1}), germination decreased from 20% in complete darkness to less than 5% (8/16 h photoperiod) and 0% (16/8 h photoperiod). Green or red light illumination before white light decreased germination to less than 10%. However, red light followed by dark incubation increased germination to 17%.

Red light, which is physiologically active, promotes germination; however, canopies absorb red light. Red light stimulation may be an adaptation for *D. majalis* growing in open areas (Rasmussen and Rasmussen, 1991). The role of phytochrome and red/far-red light has not been fully investigated in orchid seeds. Experiments with non-orchid seeds may be useful as models for future orchid seed research regarding phytochrome and light quality. Although only a few published articles exist that examine light quality on orchid seed germination, more research is required on more species in order to find a definitive role of phytochrome and light quality.

Temperature

The effects of temperature on orchid seed germination and development have been largely ignored compared to the effects of photoperiod and germination media. Temperatures are often selected with no justification or reference to those found in nature. Temperature is a major factor responsible for the onset and breaking of physiological seed dormancy (Baskin and Baskin,

2004). The lack of understanding regarding orchid seed germination and temperature may simply be due to many studies focusing on refining methods of existing germination protocols, as well as understanding the nutrient requirements of symbiotic and asymbiotic germination.

Several studies that provide insight into the relationship between orchid seed germination and temperature are available. As with many other species, orchid seeds germinate within a range of temperatures, but maximum germination is achieved only in a narrow range. Although *Dactylorhiza majalis* seeds germinated between 10 and 30°C, germination decreased below 15°C and above 27°C while the optimum range was between 23 and 24.5°C (Rasmussen *et al.*, 1990*a*; Rasmussen and Rasmussen, 1991). At higher temperatures, rhizoid formation was inhibited, which may be due to the lack of mycorrhizal colonization. Since rhizoids are the primary site of mycorrhizal infection, the lack of rhizoids may cause reduced mycorrhizal infections (Rasmussen *et al.*, 1990*b*).

Several thermo-inductive treatments are effective at breaking dormancy in mature orchid seeds including cold-stratification. The use of cold-stratification to break dormancy in orchid seeds is often used for difficult-to-germinate genera such as *Cypripedium*, *Epipactis*, and *Dactylorhiza* (Rasmussen, 1995). However, there is limited information on the exact mechanism by which cold-stratification promotes orchid seed germination. Cold temperatures may decrease enzymatic reactions, slow metabolic processes, or change enzyme production and concentration, and thus promoting germination (Bewley and Black, 1994).

However, variable results have been reported not only between species, but also within the same species. Ballard (1990) reported a maximum germination in *Cypripedium calceolus* of 16% after 4 months of cold-stratification at 5°C, while Coke (1990) reported 50% germination after 5 months cold-stratification. After 160 days incubation, germination of *C. calceolus* increased to

over 90% after cold-stratification at 5°C for 8 weeks (Chu and Mudge, 1994). Pretreatment of *C. calceolus* seeds at 6°C for 8 weeks reduced germination to 0.8% when incubated (van Waes and Debergh, 1986*b*). De Pauw and Remphrey (1993) reported higher germination for *C. candidum* seeds after two months. Germination of *Dactylorhiza lapponica* increased over 80% after three months chill at 3-4°C (Øien *et al.*, 2008). Different capsule ripening conditions and seed age may have caused the different results. van Waes and Debergh (1986*b*) used fully mature seeds collected from dehisced capsules, while Chu and Mudge (1994) used non-dehisced mature seeds. Dehisced seeds may need a longer period of cold-stratification than van Waes and Debergh (1986*b*) provided. Since Chu and Mudge (1994) cultured seeds in complete darkness while van Waes and Debergh (1986*b*) cultured seeds under a 14/10 h photoperiod, differences in germination may be attributed to other culture conditions.

The length of cold-stratification (chilling) is also an important factor to consider, and may be species specific. Rasmussen (1992), Tomita and Tomita (1997), and Miyoshi and Mii (1998) reported higher germination percentages when seeds of *Cypripedium macranthos*, *C. candidum*, and *Epipactis palustris*, respectively, were cold-stratified for 8 to 12 weeks. Zettler *et al.* (2001) found that germination percentage of *Platanthera leucophaea* increased after two cold-stratifications for 11 months as well as 107 days at 6°C following 95 days at 23°C. Sharma *et al.* (2003) reported a higher germination percentage of *Platanthera praeclara* after 6 months of cold-stratification compared to 0 and 4 months. Shimura and Koda (2005) reported the importance of fungal inoculation corresponding to cold-stratification on symbiotic germination of *C. macranthos*. A higher germination percentage was reported when seed cultures were inoculated with fungi after a 12 week cold-stratification compared to inoculation before or

several weeks after the cold-stratification. This might suggest that fungal infection in nature takes place after winter and prior to germination in early spring (Shimura and Koda, 2005).

Cold-stratification in orchid seeds has several ecological functions and effects. If seed dispersal occurs in fall, seed germination may be delayed until the next growing season when conditions are more favorable for growth and development. A low temperature requirement for long periods of time may prevent seeds from germinating immediately after dispersal (Rasmussen, 1995). The effects of chilling and thawing may cause degradation of the testa, which could lead to leaching of germination inhibitors, imbibition, and fungal infection (Rasmussen, 1995). Chilling also promotes the growth of rhizoids, which are important for the uptake of water and nutrients as well as establishing the mycorrhizal fungal relationship (Rasmussen, 1992).

Ecotypes

Introduction

The term *ecotype* was first defined by Turesson (1922*b*) in describing alpine plant populations. Ecotypes were defined as ecological sub-units of a species resulting from genotypic responses to a particular habitat (Turesson, 1922*a*). The term ecotype was preceded by several other terms that were established in debating the species concept. The *coenospecies* was considered the complete combination of genotypes within a complex, and *ecospecies* the genotypic sub-units of a coenospecies (Turesson, 1922*b*). Gregor *et al.* (1936) stated that coenospecies and ecospecies were distinguished by morphological, physiological, or cytological characters, while cytological components were not characteristic of the ecotype. In addition, ecotypes could not interbreed with other ecotypes of the ecospecies (Gregor *et al.*, 1936). Clausen *et al.* (1939) defined the coenospecies as a species-complex incapable of interbreeding. The ecospecies was considered a distinct species that may produce hybrid generations, and the

ecotype a subspecies of the ecospecies that could produce viable hybrids (Clausen *et al.*, 1939). Gregor (1939) further defined coenospecies as populations that were unable to exchange genes and ecospecies as populations with low gene exchange with other populations of a coenospecies. Gregor (1939) also introduced the term cline as any "gradation in measurable characters", and ecocline as a cline correlated with an ecological gradient. In short, an ecotype was defined as a "particular range on an ecocline" (Gregor, 1939). Daehler *et al.* (1999) defined ecotypes as subpopulations with genetic differences in morphology, physiology, and life history. However, this definition did not recognize the role of environment in ecotypic differentiation. Hufford and Mazer (2003) stated that ecotypes are "distinct genotypes or populations within a species, resulting from adaptation to local environmental conditions; capable of interbreeding with other ecotypes of the same species." This is a complete definition since the role of genetics and environmental conditions are recognized.

Importance of Ecotypes to Plant Conservation

A major concern in conservation and reintroduction programs is the source of plant material. Many species are adapted to local environmental conditions and selection pressures, thus the use of locally adapted material is essential. However, using local ecotypes for restorations is expensive and difficult to maintain and verify because collecting plant material directly from native habitats is often unregulated and guidelines vary (Smith *et al.*, 2007). Debate exists concerning collecting plant material from single or multiple sources for use in restoration projects. Sanders and McGraw (2005) found that populations of *Hydrastis canadensis* established more readily from single sources at the restoration site, but recommended using multiple sources over multiple sites to increase compatibility to source-sites. Regardless, introducing non-local plant material to naturally occurring populations might reduce fitness, fecundity, and ecosystem functions (Linhart, 1995; Hufford and Mazer, 2003; McKay *et al.*,

2005). These factors may be affected by heterosis, outbreeding, inbreeding, founder effects, and genetic swamping (Hufford and Mazer, 2003; McKay *et al.*, 2005). Numerous studies have shown a home-site advantage for local populations possibly due to habitat-specific selection pressures influenced by genetic adaptation to environmental conditions (van Tienderen, 1992; Kindell *et al.*, 1996; Nagy and Rice, 1997; Keller and Kollmann, 1999; Bischoff *et al.*, 2006).

Finding the correct balance between inbreeding and outbreeding depression of introduced populations is difficult yet essential (McKay *et al.*, 2005). Inbreeding depression occurs when populations become relatively small increasing the loss of genetic diversity (McKay *et al.*, 2005). Inbreeding depression may be the result of founder effects as reported for the Mauna Kea silversword. A restored population of the silversword was generated from two founders and a loss of genetic diversity resulted (Robichaux *et al.*, 1997; Friar *et al.*, 2000). The adverse consequences of inbreeding depression may be overcome by careful plant material collections (Williams, 2001).

Outbreeding depression often results from non-locally adapted populations producing viable hybrids (McKay *et al.*, 2005). These hybrids, in turn, can express lower fitness levels by increasing the numbers of unfit offspring (Hufford and Mazer, 2003; McKay *et al.*, 2005). Conversely, heterosis or hybrid vigor may result from outbreeding depression. This adversely affects restorations since non-local vigorous hybrids may cause genetic swamping and loss of diversity in native populations (McKay *et al.*, 2005).

Ecotypic differentiation may also be a consequence of primary productivity. Ecotypes from colder climates may not respond to increased temperatures as efficiently as southern ecotypes when transplanted to warmer climates (Shaver *et al.*, 1986). Leaf senescence of *Eriophorum vaginatum* ecotypes from northern latitudes occurred earlier than southern ecotypes when

transplanted to warmer climates. These ecotypes represented a genetic limitation on primary productivity, which could adversely affect restoration projects (Shaver *et al.*, 1986; Fetcher and Shaver, 1990).

Ecotype Development and Differentiation

The evolution and selection of ecotypes occurs over time and space, but development is not limited to single biotic or abiotic pressures (Linhart and Grant, 1996). Ecotypic development occurs from differences in many environmental characteristics such as photoperiod (Howe *et al.*, 1995; Kurepin *et al.*, 2007), temperature (Campbell and Sorensen, 1973; Downs and Bevington, 1981; Li *et al.*, 2005), altitude or elevation (Turesson, 1922*a*; Clausen *et al.*, 1941; Li *et al.*, 2005), water availability (Peñas-Fronteras *et al.*, 2009), and soil nutrient availability (Grześ, 2007; Macel *et al.*, 2007; Sambatti and Rice, 2007). Although generally more difficult to detect, biotic pressures such as pollination vectors (Robertson and Wyatt, 1990; Johnson, 1997) and grazing or herbivory (Suzuki, 2008) also influence ecotypic differentiation. Many of these pressures can be selected for or differentiated experimentally.

Common garden studies

Local adaptation has been studied in numerous species including both plants and animals (Nuismer and Gandon, 2008). Common garden and transplant studies are often used to detect local adaptation. Common garden studies test local adaptation and fitness of individuals from local or distant habitats in a common environment. These studies minimize the environmental impacts on fitness and may better identify the role of genetics. Transplant studies may better estimate the role of environmental variation on fitness since individuals are transplanted to foreign habitat (Nuismer and Gandon, 2008). Collecting plant material from natural populations and planting under controlled conditions often excludes maternal effects (Volis *et al.*, 2002).

Several techniques exist for conducting common garden and transplant studies. Controlled environments include greenhouses, growth chambers, field sites, and outdoor plots (Gallagher *et al.*, 1988; Howe *et al.*, 1995; Majerowicz *et al.*, 2000; Suzuki, 2008). Mature or juvenile plant material including seeds can be collected and grown. Also, using seeds from the F1 generation for transplant studies can reveal natural selection strategies (Volis *et al.*, 2002).

When using controlled environments, the natural conditions such as photoperiod and temperature found *in situ* can be replicated. Photoperiod is essential for the induction of dormancy and flowering (Howe *et al.*, 1995; Kurepin *et al.*, 2007). The critical photoperiod that induces flowering, germination, or dormancy in ecotypes is often a main focus. Critical photoperiodic responses in plants are classified as long-day requiring long days and short nights, short-day requiring short days and long nights, and day-neutral plants (Kurepin *et al.*, 2007). Under growth chamber conditions, the critical photoperiod can be tested by controlling the photoperiod. Northern ecotypes of many tree species often require longer critical photoperiods to maintain growth (Downs and Bevington, 1981) and initiate bud set (Howe *et al.*, 1995). The ability of plants to flower under sun or shade can also be analyzed. Alpine ecotypes of *Stellaria longipes*, growing in full-sun, were classified as day-neutral while prairie ecotypes, growing in shaded habitats, required long days to flower (Kurepin *et al.*, 2007).

When using field or outdoor plots in a common garden study, environmental conditions can not be controlled as they are in greenhouses or growth chambers. Field plot experiments are useful to study population variation. Ecotypic responses to grazing or herbivory can be studied efficiently. Dwarf ecotypes of *Persicaria longiseta* and *Spartina alterniflora* remained in their dwarf form regardless of grazing or fertilizing nutrients, respectively (Daehler *et al.*, 1999; Suzuki, 2008). The dwarf form of *P. longiseta* and *S. alterniflora* were found to be more

influenced by genetics than environmental conditions. Soil type and nutrient availability can influence ecotypic differentiation (Grześ, 2007; Sambatti and Rice, 2007). Soil was collected from two California sites where *Helianthus exilis* ecotypes were found, and used in a common garden study (Sambatti and Rice, 2007). Although gene flow did occur between populations, local adaptation to soil was detected. Ecotypes from riparian area were not adapted to water deficits in serpentine soils, and extending growth for longer periods led to higher mortality (Sambatti and Rice, 2007).

While common garden studies can detect genetic components of ecotypes, reciprocal transplant studies may more efficiently detect environmental components of ecotypic differentiation. Fang *et al.* (2006) conducted a reciprocal transplant with dwarf and normal-stature pitch pines from Long Island, New York. Over a six year period, dwarf plants did not retain their dwarf stature when planted at non-dwarf sites, and normal stature plants became dwarf when planted at dwarf sites. Plants in dwarf sites also reproduced slowly and had lower survival. Fang *et al.* (2006) recommended preserving the dwarf-site habitat since the dwarf ecotype could not be preserved *ex situ*. While plants are generally transplanted among sites, soils can also be transplanted to detect soil and climate adaptation. Macel *et al.* (2007) transplanted *Holcus lanatus* and *Lotus corniculatus* and local soils between three sites, and plants were grown in native or foreign soils. Over two years, *H. lanatus* exhibited home-site advantage with no adaptation to local soil conditions indicating adaptation to climate. Conversely, *L. corniculatus* showed slight adaptation to soil conditions and no adaptation to climate. These results show that local adaptation is likely influenced by complex interactions among climate, soil nutrients, and soil biota (Macel *et al.*, 2007).

Seed germination ecology

When mature plants are not available for common garden or transplant studies, studying seed germination ecology can be used to differentiate ecotypes. Optimal germination conditions often differ among seed lots, populations, or sources, and sampling from one population or populations within the same vicinity may not sufficiently determine optimum conditions for germination (Quinn and Colosi, 1977). However, using seeds collected across a population may provide a representation of population adaptation. To determine whether optimum conditions for germination, seed from several populations should be collected (Nelson *et al.*, 1970; Seneca, 1974). However, controversy exists whether seeds should be germinated directly from plants in nature or plants grown under uniform conditions for several generations (Quinn and Colosi, 1977).

Using wild-collected seeds may not be appropriate for germination ecology studies because they may have environmental preconditioning that masks the effects of genetics (Nelson *et al.*, 1970). Growing plants under uniform conditions and then germinating second generation seeds can successfully determine whether germination ecotypes exist (Nelson *et al.*, 1970; Baskin and Baskin, 1973). However, limiting seed germination to non-wild grown seed ignores all adaptations and ecological influences are diminished (Quinn and Colosi, 1977). Baskin and Baskin (1973) suggested growing plants under uniform conditions for several generations before genetic differences can be allocated. Growing plants for several and not just one generation under uniform conditions may remove previous environmental influences (Baskin and Baskin, 1973; Linhart, 1996). Growing plants for one generation under uniform conditions and then germinating seeds may be an appropriate measure (Nelson *et al.*, 1970; Quinn and Colosi, 1977). Germination between first and second generation seeds should be comparatively studied (Baskin and Baskin, 1973; Quinn and Colosi, 1977).

Regardless of the argument concerning whether first or second seed generations should be used for germination ecology studies, these studies are powerful ways to differentiate ecotypes. Three sea oats eco-regions were identified through germination studies (Seneca, 1972). The first group included Virginia and North Carolina populations whose seeds required chilling and seedlings expressed intermediate vegetative growth. The second group included Atlantic Florida populations that did not require seed chilling and had low capacity for vegetative growth. The third region was the Gulf coast whose seeds had a chilling response and high potential for vegetative growth (Seneca, 1972). Probert *et al.* (1985*b*) found that northern European *Dactylis glomerata* ecotypes required both light and alternating temperatures for germination, while Mediterranean ecotypes germinated in continual darkness and constant temperatures. More northern ecotypes may have a deeper dormancy condition and therefore, have a more narrow range of germination conditions (Seneca, 1972; Probert *et al.*, 1985*a*).

Meyer (1992) and Mondoni *et al.* (2008) correlated habitat and local adaptation to ecotypes of *Penstemom eatonii* and *Anemone nemerosa*, respectively. *Penstemon eatonii* ecotypes from sites with colder winters required longer chill periods before germination began, and were slower to germinate than seeds from sites with warmer winters (Meyer, 1992). The cold stratification requirement for *A. nemorosa* germination was expressed more in mountain ecotypes, and radicles emerged one month earlier at lower temperatures compared to lowland ecotypes (Mondoni *et al.*, 2008).

Biomass allocation

Differences in biomass allocation and storage organ formation are strong indicators of ecotypic differentiation. The differential development may be influenced by growing season length. In *Sagittaria latifolia*, Rhode Island ecotypes formed corms two months before South Carolina ecotypes when grown in a common garden in Florida (Kane *et al.*, 2000). The

differences in timing of corm formation was likely influenced by a shorter growing season in Rhode Island, and quicker corm formation that favored winter survival (Kane *et al.*, 2000).

Biomass accumulation has also been correlated with differences in reproductive strategy. While many instances of differences in biomass allocation were due to genetics, others are plastic responses to environmental cues (Abrahamson, 1979). Larger allocation to underground storage organs promoted vegetative or clonal reproduction, while allocation to flower and seed organs promoted sexual reproduction (Abrahamson, 1975, 1979; Sun *et al.*, 2001; Thompson *et al.*, 2001). In *Scirpus mariqueter* ecotypes, reproductive strategy switched from lower elevational marsh locations to higher locations (Sun *et al.*, 2001). Higher biomass allocation to corms and rhizomes occurred in lower elevation ecotypes, thus allowing for more efficient colonization. Also, higher allocation to corms also provided efficient strategies to survive under disturbances such as tidal current or flooding (Sun *et al.*, 2001). Lowland ecotypes of *Cyperus rotundus* had larger tubers with higher non-structural carbohydrate contents (Peñas-Fronteras *et al.*, 2009). The allocation of *C. rotundus* followed similar patterns to *S. mariqueter* in that lower elevational ecotypes were better able to tolerate flooding and avoid starvation and seedling death (Peñas-Fronteras *et al.*, 2009)

Ecotypes of *Rubus hispidus*, wildflower species, and *Spartina anglica* responded similarly (Abrahamson, 1975, 1979; Thompson *et al.*, 1991). Higher biomass allocation to seed organs was observed in ecotypes from younger successional habitats, while those in mature habitats allocated more biomass to leaves and underground organs. This switch in reproductive strategy related to different rates of succession. In later successional habitats, woodlands species were found to be k-strategists with higher rates of clonal and vegetative growth. In earlier successional habitats, such as fields, species were reported to be r-strategists with higher rates of seed

production and sexual reproduction (Abrahamson, 1975, 1979; Thompson *et al.*, 1991). Abrahamson (1975) also noted that pollinators were less abundant in mature habitats compared to the earlier successional habitats.

In vitro ecology

Common garden and transplant studies, while useful, are generally limited to non-rare species since obtaining permits to collect and transplant rare species, such as orchids, is often difficult. Alternatively, seeds can be used to either produce mature plants for common garden studies or study the germination ecology of ecotypes. Unfortunately, collecting and germinating orchid seeds *in situ* is not an easy task. Orchids often require four or more years to flower from initial seed germination (Stoutamire, 1964), and *in situ* orchid seed germination is difficult and time consuming since germination is often low (Brundrett *et al.*, 2003; Zettler *et al.*, 2005*b*; Diez, 2007). Collecting native soils for germination purposes under greenhouse settings is not efficient either (Tim Johnson, unpublished data).

Using *in vitro* techniques to study orchid seed germination and seedling development is more feasible since orchid seeds germinate *in vitro* more readily than *in situ* (Dijk and Eck, 1995*b*; Kauth *et al.*, 2008). Many *in vitro* culture techniques can be grouped under the discipline of *in vitro* ecology. *In vitro* ecology has been previously defined to include exogenous factors (i.e., temperature, light, gas phase, culture media, photoautotrophy) that affect *in vitro* growth and development (Hughes, 1981; Williams, 2007).

In vitro ecology can also be used to identify, propagate, evaluate, and select plant genotypes and ecotypes for ecological purposes. Specifically, environmental and genetic variables that affect plant growth and development *in vitro* with ecological factors affecting growth and development *in situ* can be studied. *In vitro* ecology could also be used to assess ecotypic differentiation for habitat restoration and plant reintroduction programs by conducting

in vitro common garden studies or reciprocal transplant studies under controlled environmental conditions. While genotypic and ecotypic selection from tissue culture has been previously attempted, the validity of the system must be verified. Also, the ecological strategies of plants have not been thoroughly studied *in vitro*. *In vitro* conditions are different than those conditions found *in situ*. Conducting common garden studies *in vitro* may exclude possible environmental interactions that can influence gene expression. First generation seeds may also have environmental preconditioning that effect *in vitro* ecology studies (Seneca, 1974).

Wetland and marsh plants have been regenerated through micropropagation for restoration purposes (Kane, 1996; Rogers, 2003). Li *et al.* (1995) successfully regenerated callus cultures of *Spartina patens* and subsequently studied its salt tolerance. Tissue culture generated plants have also been used to block invasive species (Wang *et al.*, 2006). A more controversial technique is to engineer plants *in vitro* via somaclonal variation, and subsequently selecting genetically superior genotypes (Seliksar and Gallagher, 2000; Wang *et al.*, 2007). While this provides a major breakthrough for producing plants, introducing bioengineered plants to native habitats may lead to genetically altering the composition of other native plants due to hybridization.

A major concern with developing plants through micropropagation is the difficulty of acclimatizing plants to *ex vitro* conditions. Difficult-to-acclimatize sea oats genotypes were found to utilize leaf carbohydrate reserves less efficiently *in vitro*, and contained a lower photosynthetic capacity (Valero-Aracama *et al.*, 2006). Adjusting the *in vitro* environment also provided successful protocols for acclimatizing sea oats (Valero-Aracama *et al.*, 2007). By providing optimum growing conditions for all genotypes, restoration studies can be successful by incorporating numerous local genotypes.

Ecotypic Differentiation in Orchids

Research on ecotypic differentiation in plant species is abundant with the exception of orchids. Only a few articles exist on identifying pollinator ecotypes in two orchid species (Robertson and Wyatt, 1990; Johnson, 1997). Numerous authors that have investigated morphological or genetic diversity in orchids have come short of stating whether ecotypes exist (Dijk and Eck, 1995*b*; Goldman *et al.*, 2004*a*; Pillon *et al.*, 2007; Swarts, 2007). Also the issue of seed source has been addressed, but not in detail regarding ecotypic differentiation. As interest in orchid conservation continues to grow, research on ecotypic differentiation in orchids should be of concern. However, many organizations involved with orchid conservation are not informed about ecotypes or are content to reintroduce plants without knowing the source.

Seed source

One purpose of orchid seed germination is to provide plants for species-level conservation and reintroduction. However, populations of one species may inhabit different habitats across a geographic range. Differences in habitats may alter the genotypic and/or phenotypic compositions producing distinct ecotypes adapted to local environmental conditions (Hufford and Mazer, 2003). Introducing inappropriate ecotypes into a particular habitat could not only lead to the death of transplanted individuals, but loss of genetic diversity, as well as population degradation. With an increasing interest in orchid-species conservation, care must be taken to use local seed.

Zettler and McInnis (1992) reported germination differences between seed sources of *Platanthera integrilabia*. The highest germination percentage and seedling establishment was observed in seeds from the largest population of *P. integrilabia*, while smaller populations had lower seed germination and seedling establishment. Inbreeding depression in smaller populations could lead to differences in germinability, low viability, or reduced vigor (Zettler and McInnis,

1992). Zettler and Hofer (1998) reported differences in germination among populations of *Platanthera clavellata*. Although seed originating from Georgia had lower germination than other sources, seedling development was superior with Georgia seeds. Since *P. clavellata* is an auto-pollinated species, it may be likely that small differences in seed viability or genetic diversity would occur between populations (Zettler and Hofer, 1998). Although habitat conditions were not incorporated, the size of the populations and apparent isolation may have caused genetic differences in seed germination.

Recently the symbiotic germination between two populations of *Epidendrum nocturnum* was examined (Zettler *et al.*, 2007). Seed germination from Fakahatchee Strand State Preserve plants was 55.7%, but 12.7% from the plants located at the Florida Panther National Wildlife Refuge (FPNWR). However, seeds from the FPNWR had a viability of 79.7% compared to a viability of 72.6% for Fakahatchee seeds. Although seed handling and age may have contributed to these differences (Zettler *et al.*, 2007), the self-pollinating breeding system may have also contributed to the germination and viability differences.

Dijk and Eck (1995*b*) investigated the role of *in vitro* seedling mineral nutrition between coastal and inland populations of *Dactylorhiza incarnata* in the Netherlands. Major differences in seed germination responses to nitrogen type and population location were noted. Seedlings from coastal areas grew faster *in vitro* and were more tolerant of exogenous ammonium and nitrate, while the inland seedlings were more sensitive to both ammonium and nitrate. However, seedlings from both populations were more sensitive to high concentrations of exogenous nitrogen. Since the coastal seedlings developed quickly, they were also able to assimilate nitrate more efficiently. Both populations inhabit calcareous areas where high nutrient levels are found due to the introduction of fertilizers and poor drainage. These soil conditions have led to

decreased *D. incarnata* plant numbers. Increased nitrogen mineralization inland may have caused increased nitrogen sensitivity of these plants. Although Dijk and Eck (1995*b*) were uncertain whether habitat influenced developmental differences, habitat differences seem to have influenced the ecotype differentiation as shown by the observed differences in seedling development.

Pollination ecotypes

The only reports of ecotypes in the Orchidaceae have identified pollinator ecotypes. Ecotypic development influenced by pollinators may be due to colonization of the species into new habitats where more effective or different pollinators exist (Robertson and Wyatt, 1990; Johnson and Steiner, 1997). The short-spurred mountain ecotypes of *Platanthera ciliaris* from South Carolina were pollinated by the short-tongued *Papilio troilus*, while the long-spurred coastal ecotype was pollinated by the long-tongued *P. palamedes* (Robertson and Wyatt, 1990). Johnson and Steiner (1997) reported that *Disa draconis* from South African mountain sites was pollinated by horseflies while plants in sandplains were pollinated by tanglewing flies. Johnson (1997) also reported that short-spurred coastal ecotypes of *Satyrium hallackii* were pollinated by carpenter bees while long-spurred ecotypes from grasslands were pollinated by hawkmoths.

The differentiation of ecotypes was likely due to pollinator availability. Robertson and Wyatt (1990) reported that *P. troilus* was scarce in coastal areas, but *P. palamedes* was absent from mountain populations. Likewise, carpenter bees were rare in South African grasslands and hawkmoths were scarce in coastal habitats (Johnson, 1997). Johnson and Steiner (1997) noted that both short- and long-spurred plants in mountainous regions in South Africa were pollinated by short-tongued horseflies. They concluded that climate did not influence spur length since both ecotypes inhabit similar montane environments. Selection favored long-spurred plants in the sandplains since longer spurs ensured pollinaria attachment. Artificially shortening long-spurred

ecotypes did not decrease pollinaria removal, but did decrease pollination levels (Johnson and Steiner, 1997).

Plant of Study

Calopogon tuberosus (L) Britton, Sterns, and Poggenberg is a widespread terrestrial orchid of eastern North America. *Calopogon tuberosus* is distributed from Newfoundland, Canada to Cuba, and west to Texas (Luer, 1972). *Calopogon tuberosus* is an unusual orchid species given that it occupies diverse habitats including alkaline prairies, pine flatwoods, mesic roadsides, fens, and sphagnum bogs (Luer, 1972). The generic name *Calopogon*, meaning beautiful beard, is derived from the Greek words *kalos* (beautiful) and *pogon* (beard). The species name, *tuberosus*, is derived from the Latin word tuberosus, which inaccurately refers to the corms of the species (Luer, 1972). Nonresupinate pink flowers, corms, and grass-like leaves are characteristic of *Calopogon* species.

Two varieties of *Calopogon tuberosus* are recognized: var. *tuberosus* and *simpsonii* (Small) Magrath. Variety *tuberosus* is 10-75 cm in height with 2-17 flowers per stem and 1 or 2 leaves (Luer, 1975). Individual flowers with yellow trichomes on the floral lip are 2-3.5 cm in diameter (Brown, 2002). Although pink is the most common color, the rarer color form, *albiflorus* PM Brown, is often scattered among populations. The flowering period of var. *tuberosus* is March in southern states to August in northern states and Canada (Luer, 1975). Variety *tuberosus* grows in full sun and commonly inhabits sphagnum bogs and swamps in the north, as well as wet meadows, pine flatwoods, and sandy roadsides in the south (Brown, 2002; Luer, 1972). Plants readily form large populations with scattered plants, but occasionally large groups form. In a northern Michigan bog, Case (1987) observed 1,000 plants in less than one square meter.

Variety *simpsonii* is one of the rarest orchids in the United States being restricted to rocky prairies and marls of 4 counties in South Florida (Brown, 2002). Plants are larger than var. *tuberosus* with inflorescences up to 120 cm and 5-25 flowers (Brown, 2002). This variety also has 4-5 slender leaves, and pink to white trichomes on the white to light pink floral lip. Flowers are commonly pale pink, but a white flower form also exists (forma *niveus* P.M. Brown). The flowering period is earlier than var. *tuberosus* with plants initiating flowers in late December and continuing through June (Brown, 2002).

Historically two other varieties have been described, but recent work relegated these varieties to var. *tuberosus*. Variety *nanum* was first described as *Limodorum tuberosum* var. *nanum* in 1913 as small plants with 2-3 purple flowers from Newfoundland, Canada (Nieuwland, 1913). However, this variety was never officially recognized. While var. *tuberosus* can be 75 cm in height, var. *nanum* was a maximum of 13 cm in height. St. John first described var. *latifolius* as *Calopogon pulchellus* forma *latifolius* in 1921 from Sable Island, Nova Scotia, Canada. Fernald (1946) elevated the form to varietal level, and Boivin (1967) published the variety as *Calopogon tuberosus* var. *latifolius*. The plants were originally separated from var. tuberosus as short plants with wide leaves and larger corms. The morphological characteristics of var. *latifolius* are found in plants inhabiting coastal bogs of eastern Maine and the Canadian Maritime (Goldman *et al.*, 2004*a*).

Diversity within *Calopogon tuberosus*

Recently the genetic and morphological diversity within *Calopogon tuberosus* has been examined. With a large range of distribution, morphological and genetic traits varied between *C. tuberosus* var. *tuberosus*, *C. tuberosus* var. *simpsonii*, and *C. tuberosus* var. *latifolius* (Goldman *et al.*, 2004*a*, *b*; Trapnell *et al.*, 2004). Although var. *latifolius* is currently not recognized as an official variety, Goldman *et al.* (2004*b*) included plants expressing traits of this variety in his

publications. However, the authors did not state whether ecotypes existed within the *C. tuberosus* complex.

In a morphometric treatment of *Calopogon*, Goldman *et al*. (2004*a*) sampled *C. tuberosus* from the following areas of the species range: southwest, southeast including var. *simpsonii*, and north including var. *latifolius*. The northern range extending into southern Canada was divided from the southern range according to the Wisconsin glaciation, and the eastern range was separated from the western range by the Mississippi embayment. The morphological separation between southern plants and northern plants was evident by the larger size of southern plants. Based on three tests (Principle component analysis-PCA, average Euclidean distance, average squared Mahalanobis distance) for diversity, southern plants were more diverse morphologically than northern plants, with var. *simpsonii* being more diverse than both the northern and southeastern plants. Although southwest plants were larger than the rest of the species, morphologically they were similar to southeast plants. Interestingly, southwest plants were found to be the most diverse in the average Euclidian distance test, but least diverse in the squared Mahalanobis distance test. The reason for this may be due to isolation and unsuitable habitat in the Mississippi embayment. Since var. *latifolius* did not form a distinct group and was similar to the northern var. *tuberosus*, Goldman *et al.* (2004*a*) chose not to recognize var. *latifolius*.

In an earlier morphological study, Catling and Lucas (1987) relegated var. *latifolius* to var. *tuberosus*. Plants from Sable Island (var. *latifolius*) as well as eastern Ontario (var. *tuberosus*) were cultivated under uniform conditions. Both varieties expressed plasticity year to year. A plant referred to as var. *latifolius* with leaves 14 mm wide and 10 cm long became more tuberosus-like the second year of cultivation with leaves 9.5 mm wide and 10 cm long. Another

plant referred to as var. *latifolius* had leaves 13 mm wide and 9 cm long the first year and 10 mm wide and 14.5 cm long leaves the second year. Although these plants expressed phenotypic plasticity between years, several plants referred to as var. *latifolius* became even more extreme with 30 mm wide leaves. Catling and Lucas (1987) reported that var. *latifolius* was the end of a continuum of variation among the plants of the northeast, and plants were not discrete individuals in nature. Likewise, since the characteristics of var. *nanum* were observed also throughout the northeastern range, the variety was not officially recognized.

Using AFLP analysis, Goldman *et al.* (2004*b*) reported that var. *latifolius* was genetically similar to var. *tuberosus*, and not recognized as a distinct variety. Also based solely on genetic analysis, var. *simpsonii* formed a coherent group and was recognized as a distinct variety. Although Goldman *et al.* (2004*a*) reported high morphological diversity of var. *simpsonii*, Trapnell *et al.* (2004) found that var. *simpsonii* had low genetic diversity. However, Trapnell *et al.* (2004) sampled only one population of var. *simpsonii*. Goldman *et al.* (2004*a*) noted that var. *simpsonii* is often self-pollinated due to a short rostellum, possibly contributing to the low genetic diversity among the population sampled by Trapnell. Goldman *et al.* (2004*a*) sampled large plants of var. *simpsonii* from Florida and smaller plants of var. *simpsonii* from the Bahamas, contributing to the diversity in morphology.

Floral Biology of *Calopogon tuberosus*

The biology and flower orientation of *Calopogon tuberosus* provide a unique pollination mechanism. Orchid flowers are normally non-resupinate, but *Calopogon* flowers are non-resupinate with the lip petal the uppermost (Robertson, 1887). The gynandrium protrudes from the base of the labellum, and the stigma and anther is found at the apex of the column (Robertson, 1887). Since the flowers are nectarless, pollination occurs by deceit or mimicking other rewarding orchids in close proximity (Firmage and Cole, 1988). However, *Calopogon*

flowers may also mimic non-orchids such as *Rhexia* species (pers. obs.). Yellow trichomes resembling pollen are found at the peak of the labellum (Firmage and Cole, 1988). The flowers and trichomes reflect ultra-violet light, which attracts potential pollinators to the uppermost portion of the labellum (Thien and Marcks, 1972). Upon landing on the hinged labellum, the pollinator is dropped onto the column where the pollinia attach to the top of the abdomen (Thien and Marcks, 1972; Proctor, 1998).

Several bee species have been reported to pollinate *C. tuberosus* including *Bombus* (Thien and Marcks, 1972), halictid bees (Firmage and Cole, 1988), and *Xylocopa* (Dressler, 1981). However, Thien and Marcks (1972) observed that queen bumblebees and carpenter bees were too large to effectively pollinate flowers because they were strong enough to leave the flower before falling on the pollinia. Both carpenter and honeybees were observed visiting *C. tuberosus* flowers at Goethe State Forest, Levy County, Florida; however, neither effectively removed the pollinia. Although the weight of the carpenter bee caused the labellum to collapse onto the column, the bee escaped. The honeybee was too small to collapse the labellum onto the column. Smaller worker bees are likely more efficient pollinators than queen bumblebees (Thien and Marcks, 1972). Other visitors to *Calopogon* flowers include beetles, flies, and small butterflies, but are not likely pollinators (Firmage and Cole, 1988; Proctor, 1998).

Seed Germination of *Calopogon tuberosus*

Conditions for *in vitro* seed germination of native orchids have long been debated, and one protocol may not be appropriate for germination of another species (Arditti *et al.*, 1985). While one study might recommend dark incubation, another study may recommend light incubation. The optimum temperature for seed germination is also variable between studies. Although these parameters are essential for seed germination, germination comparisons between genotypes or populations of native orchids have not been studied. The following studies demonstrate

differences between seed germination for *C. tuberosus*. Interestingly, only one study cited the source of seeds (Kauth *et al.*, 2006).

Whitlow (1996) outlined a commercial seed culture protocol for *C. tuberosus*. No germination media was cited, although *C. tuberosus* germinated on various culture media (Whitlow, pers. comm.). Cultures were incubated under cool-white fluorescent lights (no intensity or photoperiod cited). After development for 5 months, seedlings were placed in cold storage in darkness for 3 months at 4^{o}C, and subsequently placed under fluorescent lights. Although not all seedlings required chilling, seedlings were 'programmed' on the same developmental schedule after chilling. After 5 months development under fluorescent lights, seedlings were taken out of culture and placed under cold storage for an additional 3 months. Following the cold treatment seedlings were planted in a 1:1 mixture of sand and peat moss. Although no quantitative germination rates were reported, Whitlow stated that seeds of *C. tuberosus* germinated readily.

Henrich *et al.* (1981) and Myers and Ascher (1982) followed similar protocols for *C. tuberosus*. Both used a culture medium developed by Norstog for barley embryos. Henrich *et al.* (1981) stored cultures in polyethylene bags in the dark at 25^{o}C for 6 months. After 29 days culture, germination was 29%. Myers and Ascher (1982) also stored cultures in the dark at 25^{o}C. After embryos developed, embryos were transferred to MS medium and stored in the dark at 25^{o}C. When leaf expansion occurred, seedlings were placed under fluorescent lights for 1-3 weeks. Seedlings were then transferred to glass jars with MS medium and placed under lights. When at least one leaf fully expanded and dark green roots formed, the plantlets were removed from flask and potted in sphagnum moss (Myers and Ascher, 1982).

Of the studies published on seed culture of *C. tuberosus*, Anderson (Anderson, 1990) directly compared seed germination under continual darkness and light incubation. Cultures were placed in both light and darkness at 22^{o}C. Burgeff N_3f, Knudson C, and modified Lucke's medium were compared for germination. Leaf development was more efficient on Lucke's medium with leaves reaching 5 cm in 8 weeks. Seedlings incubated under fluorescent lights were larger than seedlings developed in darkness, but no statistics were reported. Within 2 months, light-grown seedlings produced small corms, 5 cm long leaves, and roots. Unlike Whitlow, who allowed the seedlings to end their growth cycle *in vitro*, Anderson (1990) acclimatized 2 month-old seedlings.

In a comparative study between culture medium and light treatment of seeds from Florida, Kauth *et al.* (2006) found that seeds germinated under both continual darkness and a 16 h photoperiod, but light incubation was superior for seedling development. Seed germination on Knudson C (Knudson, 1946) was higher compared to seeds germinated on Malmgren modified terrestrial orchid medium (Malmgren, 1996) and *Phyto*Technology orchid seed sowing medium (*Phyto*Technology Laboratories, LLC, Shawnee Mission, KS). However, seedling development was superior on *Phyto*Technology orchid seed sowing medium.

Habitat Descriptions of *Calopogon tuberosus*

The populations studied were chosen on several criteria including their widespread distribution, varying habitats, and proximity to local individuals who collected seed on my behalf. The populations were located at the following sites: 1) Florida Panther National Wildlife Refuge, Collier County, FL; 2) Goethe State Forest, Levy County, FL; 3) Ashmore Heritage Preserve, Greenville County, SC; 4) Site C: Greenville County, SC; 5) Eva Chandler Heritage Preserve, Greenville County, SC; and 6) Carney Fen, Menominee County, MI. The following

paragraphs describe each sites and *C. tuberosus* populations using previously published documents as well as personal observations.

Habitat and environmental conditions at each site are vastly different (Table 1-1). The populations in South Carolina are found where the Blue Ridge Escarpment begins about 300-500 m above sea level. The Michigan population is the only population from the glaciated area. Due to latitudinal differences, maximum and minimum day lengths differ from almost 16 h photoperiods during Michigan summers to fewer than 14 h photoperiods during south Florida summers. Average yearly precipitation is great in Florida, about double that in Michigan (excluding snow levels). Mean monthly temperature averages (Figure 1-1) also differ greatly leading to differences in growing season lengths (Table 1-1).

Carney Fen

Carney Fen (designated as Michigan) is located on the upper Michigan peninsula (Figure 1-2E) in Menominee County and is part of the Escanaba State Forest Management Unit. The 2,485 acre parcel of land is the newest addition to the Michigan State Natural Areas Program as of June 2009. Carney Fen is classified as a northern fen (Figure 1-2D), which is characterized as a graminoid dominated wetland with neutral to moderate alkaline soils (Kost *et al.*, 2007). Northern fens occur in flat or depressed areas in glacial lakeplains, outwashes, or kettle depressions (Cohen and Kost, 2008). The poorly drained soils are composed of peat or marl with a high nutrient availability due to groundwater rich in calcium and magnesium (Cohen and Kost, 2008). The groundwater provides a stable hydroperiod year-round, but water-levels normally do not exceed a few centimeters (Kost *et al.*, 2007). Northern fens rich in nutrients support rich species diversity of both plants and animals, including many rare species (Cohen and Kost, 2008).

Carney Fen proper is divided into two distinct parcels separated by County Road 374 and north of the Wiregrass Lake wetland complex. The fen is surrounded by a conifer swamp in the low areas and hardwood forests in the upland areas. The area north of CR 374 transitions into a tamarack savanna with a fen understory with open canopy that provides sufficient sunlight. The savanna transitions into a conifer swamp with a canopy of white cedar, tamarack, and black spruce, and in recent years the tree species have colonized the savannah area (personal observation). The area south of CR 374 occurs on sedge peat with a higher pH that supports more plant species.

The *C. tuberosus* population at Carney Fen is distributed throughout the area including north and south areas. Where it is not growing in direct full-sun, it occupies canopy gaps where sunlight penetrates the forest floor. In all cases, *C. tuberosus* is found at the top of sphagnum hummocks often at the base of trees that can be colonized by more than 30 plants (Figure 1-2C). *Calopogon tuberosus* generally flowers from late June to early July. Vegetative or juvenile plants have been observed in early June.

Carney Fen is dominated by rushes, sedges, and grasses. While sphagnum mosses (*Sphagnaceae*) are present, brown mosses (*Amblystegiaceae*) are more common. Northern fens support a high diversity of plant species due to the rich minerotrophic conditions and microtopography. Peat mounds and hummocks are found scattered throughout Carney Fen creating micro-environments consisting of more acidic-loving sphagnum mosses and plants (Amon *et al.*, 2002).

Common graminoids found at Carney Fen include (Kost *et al.*, 2007; Cohen and Kost, 2008): *Calamagrostis canadensis* (bluejoint grass), *Calamagrostis stricta* (reed grass), *Deschampsia cespitosa* (hair grass), *Panicum lindheimeri* (panic grass), *Muhlenbergia*

glomarata (marsh-wild timothy), *Carex lasiocarpa* (wiregrass sedge), *Carex aquatilis* (water sedge), *Cares livida* (livid sedge), *Carex limosa* (mud sedge), *Carex sterilis* (dioecious sedge), *Scirpus cespitosa* (tufted bulrush), and *Eleocharis elliptica* (golden-seeded spike rush).

Herbs and forbs commonly found include (Kost *et al.*, 2007; Cohen and Kost, 2008): *Aster borealis* (rush aster), *Campanula aparinoides* (marsh bellflower), *Euthamia graminifolia* (grass leaved goldenrod), *Solidago uliginosa* (bog goldenrod), *Iris versicolor* (blue-flag iris), *Iris lacustris* (dwarf lake iris), *Lysimachia terrestris* (swamp candles), *Potentilla palustris* (marsh cinquefoil), *Menyanthes trifoliate* (bog buckbean), *Lobelia kalmii* (Kalm's lobelia), *Drosera rotundifolia* (round-leaved sundew), *Sarracenia pupurea* (purple pitcher plant), *Utricularia intermedia* (flat-leaved bladdwort), and *Parnassia glauca* (grass-of-Parnassus). Carney Fen also has approximately 26 species of terrestrial orchids many of which are state protected and threatened.

A few common shrubs and trees include (Kost *et al.*, 2007; Cohen and Kost, 2008): *Potentilla futicosa* (shrubby cinquefoil), *Myrica gale* (sweet gale), *Salix pedicellaris* (bog willow), *Vaccinium* sp. (blueberry), *Vaccinium oxycoccos* (cranberry), *Adromeda glaucophylla* (bog rosemary), *Ledum groenlandicum* (Labrador tea), *Kalmia polifolia* (bog laurel), and *Chamaedaphne calyculata* (leather leaf), *Larix larnicina* (tamarack), *Thuja occidentalis* (white cedar), and *Picea mariana* (black spruce).

Ashmore Heritage Preserve

Ashmore Heritage Preserve (designated as South Carolina 1) is located in northern Greenville County, South Carolina, and is part of Caesar's Head State Park and the Mountain Bridge Wilderness Area, and is part of the South Carolina Heritage Preserve program (Figure 1-2E). The preserve is a total of 1,125 acres, and is host to a variety of habitats (South Carolina Department of Natural Resources). A 1.6 km long hiking trail encompasses the preserve starting

along Persimmon Ridge Road. This particular area of the preserve is approximately 400 m in elevation, descending to 320-340 m. At the center of the preserve is the 53 acre Lake Wattacoo, which is fed by Wattacoo Creek (Figure 1-3D) that falls 45 m down a granitic cliff (South Carolina Department of Natural Resources). A bog-like area with a sphagnum moss floor is found on the north and east side of the lake where rare plants grow (Figure 1-3C). This area is not a true bog because Wattacoo Creek provides a supply of flowing water, which is characteristic of a fen.

Calopogon tuberosus is found along Lake Wattacoo growing in the fen areas. The area supports a rather large number of plants with approximately 50-100 in the flowering stage. Plants flower from mid-May to early June, but the majority flower toward the end of May. Plants not only grow directly into the sphagnum moss, but also in areas of bare soil. Because the area is inundated with water, several plants grow in standing water.

The fen area is dominated by sphagnum mosses and several graminoid genera such as *Andropogon*, *Rhynchospora*, and *Fimbristylis* (Nelson, 1986). Due to the lack of prescribed fire, the area is slowly being colonized by tag alder species. Other plants found in the fen area at Ashmore Preserve include *Amianthium muscitoxicum* (flypoison), *Rhexia virgnica* (common meadow beauty), *Sarracenia jonesii* (mountain sweet pitcher plant), *Sarracenia rubra* (sweet pitcher plant), *Sarracenia purpurea* (purple pitcher plant), *Drosera rotundifolia* (round-leaved sundew), *Osmunda regalis* (royal fern), *Pogonia ophioglossoides* (rose pogonia orchid), *Aletris farinosa* (white colicroot), and *Lycopodium* sp (clubmoss).

Site C

Site C (designated as South Carolina 2) is not recognized as a preserve, but is in close proximity to Eva Chandler and Ashmore Heritage Preserves in Greenville County, South Carolina (Figure 1-4E). The site has characteristics of both high elevation seeps and granitic

flatrocks with a gradual slope (Figure 1-4B, C, D). Granitic flatrock communities do not have a year-round supply of water, which determines plant diversity (Porcher and Rayner, 2001). Large graminoid-dominated areas are found at the perimeter (Figure 1-4D), and small micro-islands habitats are found toward the base of the slope (Figure 1-4B, C). The soil depth within the micro-island habitats at Site C is extremely shallow and no more than 4 cm deep.

The *C. tuberosus* population at Site C is larger than the other South Carolina populations, with as many as 100 flowering plants. Many plants are in the juvenile stage and do not flower, therefore more than 500 plants may be found in the area. The plants are found both in the grassy perimeter areas as well as the micro-islands, where they grow directly on top of granite surrounded by the shallow soil.

The vegetation is dominated by graminoid species including many *Juncus* (rush), *Andropogon* (bluestem), and *Calamagrostis* (reed grass) spp. (Nelson, 1986; Porcher and Rayner, 2001). Herbs and forbs found at Site C include: *Krigia montana* (dwarf mountain dandelion), *Oenothera perennis* (little evening primrose), *Castilleja coccinea* (Indian paintbrush), *Helenium autumnale* (common sneezeweed), *Crotonopsis elliptica* (outcrop rushfoil), *Talinum teretifolium* (Appalachian fameflower), *Senecio tomentosus* (woolly ragwort), *Hypericum gentianoides* (pineweed), and *Tiarella cordifolia* (heartleaf foamflower).

Eva Chandler Heritage Preserve

Eva Chandler Heritage Preserve (designated as South Carolina 3) is part of the South Carolina Heritage Preserve program. The preserve is located in northern Greenville County, South Carolina, and is next to Caesars Head State Park (Figure 1-5E). The 253 acre preserve, which is approximately 500 m in elevation, contains several communities including a pine/hardwood ecosystem and granite outcrop (South Carolina Department of Natural Resources). A small stream originating from a floodplain flows down the outcrop, and supplies a

constant flow of water (Figure 1-5D). The granite outcrop area has elements of a granitic dome and flatrock, high elevation seep, acidic cliff, and spray cliff. Spray cliffs differ from high elevational seeps by a constant supply of flowing water, and vascular plants are restricted to edges of the water flow (Nelson, 1986). Acidic cliffs differ from granitic domes and flatrocks by a more sloped rock surface and lack of a canopy and woody species (Nelson, 1986). Granitic domes have a sloping terrain compared to granitic flatrock and often have sliding water (Nelson, 1986).

Regardless of the granite outcrop classification, Eva Chandler Preserve contains several seepage communities called cataract bogs (Porcher and Rayner, 2001). Although the term bog is used, the communities are actually fens because their main water source is seepage and not rainwater (Porcher and Rayner, 2001). Micro-island habitats form along edges of streams where organic matter accumulates in cracks and fissures in the granite outcropping (Figure 1-5 B, C). While the soil is saturated throughout the year, the availability of flowing water changes seasonally (Nelson, 1986). The underlying soil is shallow leading to slow succession, but woody species can colonize the deeper soils (Porcher and Rayner, 2001). Approximately eight cataract bogs are found at Eva Chandler Heritage Preserve varying in size from 30 cm to more than 2 m in diameter. The soil in the cataract bogs are a few cm to about 25 cm deep in the largest bogs, and a distinct layering exists of leaf litter, soil, and then granite.

The *C. tuberosus* population is rather large with more than 200 plants among the eight bogs; however; more plants may exist since many are in the juvenile stage. The plants are found at the periphery of the cataract bogs where woody species do not colonize. Due to the slightly higher elevation, the flowering period is a few days later than the other South Carolina

populations. The plants at this location are diverse in color with most plants a paler shade of pink.

The vegetation in the surrounding area is very diverse including xeric species such as *Opuntia compressa* (eastern prickly pear cactus), graminoids, herbs, and small trees (Nelson, 1986; Porcher and Rayner, 2001). Small trees that colonize larger cracks and fissures in the granite include *Alnus serrulata* (tag alder), *Acer rubrum* (red maple), and *Pinus virginiana* (Virginia pine). Carnivorous plants growing alongside *C. tuberosus* include *Drosera rotundifolia* (round-leaved sundew), *Untricularia cornuta* (horned bladderwort), and *Sarracenia jonesii* (mountain sweet pitcher plant). Other herbs and forbs growing with or near *C. tuberosus* include *Spiranthes cernua* (nodding ladies' tresses orchid), *Pogonia ophioglossoides* (rose pogonia orchid), *Platanthera integrilabia* (white fringeless orchid), *Parnassia grandifolia* (large leaf grass-of-Parnassus), *Achillea millefolium* (thousand-leaved groundsel), *Helenium autumnale* (common sneezeweed), and *Castilleja coccinea* (Indian paintbrush). Graminoids found in the area include *Carex prasina* (drooping sedge), *Carex biltmoreana* (stiff sedge), *Danthonia spicata* (poverty wild-oat grass), *Dicahnthelium acuminatum* (tapered rosette grass), and *Calamagrostis* spp (reed grass).

Goethe State Forest

Goethe State Forest (designated as north central Florida) is located in Levy County, Florida, and is part of the state forest system of Florida (Figure 1-6E). The area encompasses more than 53,000 acres with approximately 15 different natural communities including flatwoods, dome and basin swamps, sandhills, and ruderal sites including mesic to wet roadways (Florida Division of Forestry, 2005). Goethe State Forest also holds one of the largest old-growth longleaf pine flatwoods in Florida (Florida Division of Forestry, 2005).

Calopogon tuberosus is found along a contiguous 3 km stretch of County Road 336. The areas where *C. tuberosus* grows is a mesic roadside with sandy soils at the edge of a longleaf pine flatwoods (Figure 1-6C, D). The roadside is slightly sloped with a seasonally wet sphagnum moss slough. Plants are scattered across both sides of CR 336, and are found in both the higher road and slough areas. The population of *C. tuberosus* is large (> 1000) with a mix of blooming and juvenile plants. Plants flower from late April to mid June, with peak flowering in mid May. Of all the populations, this is the only location where *C. tuberosus* is found in large groups, possibly due to a high percentage of vegetative growth via corms (Figure 1-6B). The population is morphologically diverse containing numerous color forms including the pale and alba form of *C. tuberosus* (Figure 1-6B). Hybridization may also be occurring with other *Calopogon* species including *C. pallidus*.

Compared to the other sites, the vegetation along the roadside is dominated more by forbs and herbs rather than graminoids. Several commonly found graminoids include *Rhynchospora colorata* (star rush white-top), *Xyris* spp. (yelloweyed grass), *Juncus effusus* (soft rush), *Andropogon virginicus* (broom sedge bluestem), and *Schizachyrium scoparium* (little bluestem). Herbs and forbs found are as follows: *Coreopsis* spp. (tickseed), *Mimosa nuttallii* (sensitive briar), *Aletris lutea* (yellow colic root), *Aletris obovate* (southern colic root), *Rhexia* spp. (meadowbeauty*), Asclepias tuberosa* (butterfly weed), *Helinathus* spp. (sunflower), *Eriocaulon decangulare* (tanangle pipewort), *Pogonia ophioglossoides* (rose pogonia orchid), *Spiranthes vernalis* (grass-leaved ladies' tresses orchid), *Hypericum myrtifolium* (myrtleleaf St. John's wort), *Sabatia campanulata* (slender rose gentian), *Erigeron* sp. (fleabane), *Baluduina angustifolia* (coastalplain honeycomb head), *Polygonella polygama* (October flower), and *Solidago odora* var. *chapmanii* (Chapman's goldenrod). Larger shrubs are scarce but include

Serenoa repens (saw palmetto) and *Vaccinium* sp. (blueberry). Several carnivorous plants are found including *Drosera* spp. (sundew) and *Pinguicula* spp. (butterwort).

Florida Panther National Wildlife Refuge

The Florida Panther National Wildlife Refuge (FPNWR; designated as south Florida) is located 20 miles east of Naples, FL along Interstate 75, and consists of 26,400 acres within the Big Cypress Basin (U.S. Fish and Wildlife Service, 2009). The FPNWR (Figure 1-7E) was established in 1989 in order to protect the Florida panther and the mosaic of habitats located throughout. As a national wildlife refuge, the FPNWR actively manages the area with invasive plant removal, prescribed burning, native plant propagation, and restoration activities.

The FPNWR is divided into 50 fire-management units. *Calopogon tuberosus* is currently found in units 23, 24, 25 where wet prairies are located (Figure 1-7C, D). Wet prairies are included under the flatwoods marsh habitat classification, and both are included under the larger freshwater marsh ecosystem (Kushlan, 1990). Wet prairies have the shortest hydroperiods of freshwater marshes of approximately 50-170 days (Duever *et al.*, 1986; Kushlan, 1990; Rutchey *et al.*, 2006). Wet prairies dry during low rainfall and are only inundated during seasonal rains (Kushlan, 1990), explaining the dry conditions often encountered in units 23, 24, and 25 at the FPNWR. During the wet season water levels are normally below 20 cm (Duever *et al.*, 1986). Sandy/clay mineral soils with low organic matter, high fire frequency of 2-4 years, and diverse vegetation dominated by graminoids are found are characteristic of wet prairies (Davis, 1943; Florida Natural Areas Inventory, 1990; Kushlan, 1990; Rutchey *et al.*, 2006).

The prairies at the FPNWR are found between pine flatwoods dominated by *Pinus elliotii*, and are dominated by a short grass and sedge (less than 1 m) flora (Davis, 1943; Duever *et al.*, 1986). The population of *C. tuberosus* is morphologically diverse, because both var. *tuberosus* and *simpsonii* (Figure 1-7A, B) are found together. Possible interbreeding between the two

varieties exists. The largest population of *C. tuberosus* is found in unit 23 where several hundred plants flower from March through May with peak flowering season in mid April.

The vegetation is dominated by graminoids with a few forbs and herbs. Herbs and forbs include *Crinum americanum* (swamp lily), *Helenium nuttallii* (sneezeweed), *Oxypolis filiformis* (water carrot/cowbane), *Hypericum fasciculatum* (St. John's wort), *Dyscheriste oblongifolia* (twinflower), and *Polygala incarnata* (procession flower). *Serenoa repens* (saw palmetto) and *Sabal palmetto* (cabbage palm) are found in very low numbers mainly due to colonization of nearby pine flatwoods. Graminoids commonly found in wet prairies include (Davis, 1943; Duever *et al.*, 1986): *Aristida virgata* (switch grass), *Aristida affinis* (poverty grass), *Cladium jamaicensis* (saw grass), *Rhynchospora tracyi* (Tracy's beak-rush), *Schoenus nigricans* (black sedge), Muhlenbergia spp. (muhly grass), *Schizachyrium scoparium* (little bluestem), *Panicum hemitomom* (maidencane), *Dichromena colorata* (white-top sedge), and *Hypoxis juncea* (yellow-star grass).

Table 1-1. Summary of *Calopogon tuberosus* populations studied.

Population	Coordinates	Habitat	Elevation[a]	Growing Season[b]	Day Length[c] Max (h)	Min	Precipitation[b]
Michigan	45° 34' 47" N 87° 39' 38" W	Northern fen	240 m	125 days	15.7	8.8	740 mm
South Carolina 1	35° 05' 12" N 82° 34' 47" W	Fen	340 m	210 days	14.5	9.8	800 mm
South Carolina 2	35° 05' 06" N 82° 35' 43" W	Seepage	450 m	210 days	14.5	9.8	800 mm
South Carolina 3	35° 04' 28" N 82° 36' 19" W	Cataract bog	500 m	210 days	14.5	9.8	800 mm
North Central Florida	29° 09 18" N 82° 37 12" W	Road	12 m	270 days	14.1	10.3	1400 mm
South Florida	26° 10' 06" N 81° 21' 51" W	Prairie	4 m	365 days	13.8	10.5	1450 mm

[a]Elevational data from Google Earth last retrieved 18 Aug 2009. [b]Data from the National Climactic Data Center (2009). Growing season length is the number of days between the last spring frost (above 0°C) and first fall frost (below 0°C). [c]Photoperiod from National Weather Service (http://www.weather.gov/) last retrieved 18 August 2009.

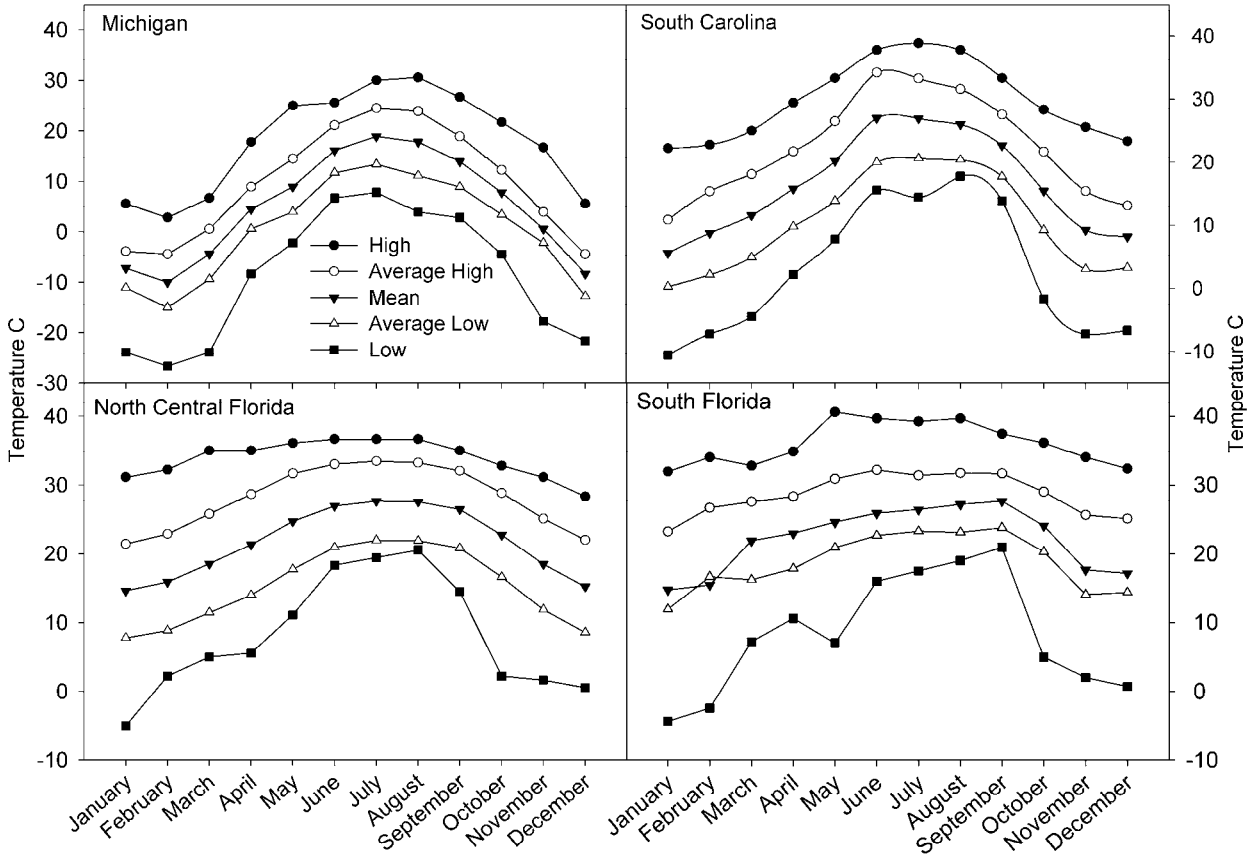

Figure 1-1. Monthly temperatures at population locations studied. Temperature data except South Florida were obtained from the closest city within 50 km to specific sites. A) Michigan: Escanaba, MI. B) South Carolina: Greenville, SC. C) North Central Florida: Ocala, FL. D) South Florida temperature data collected on the Florida Panther National Wildlife Refuge with a HOBO H8 Pro series weather station. Data from National Weather Service (http://www.weather.gov/) last retrieved Feb 18, 2009.

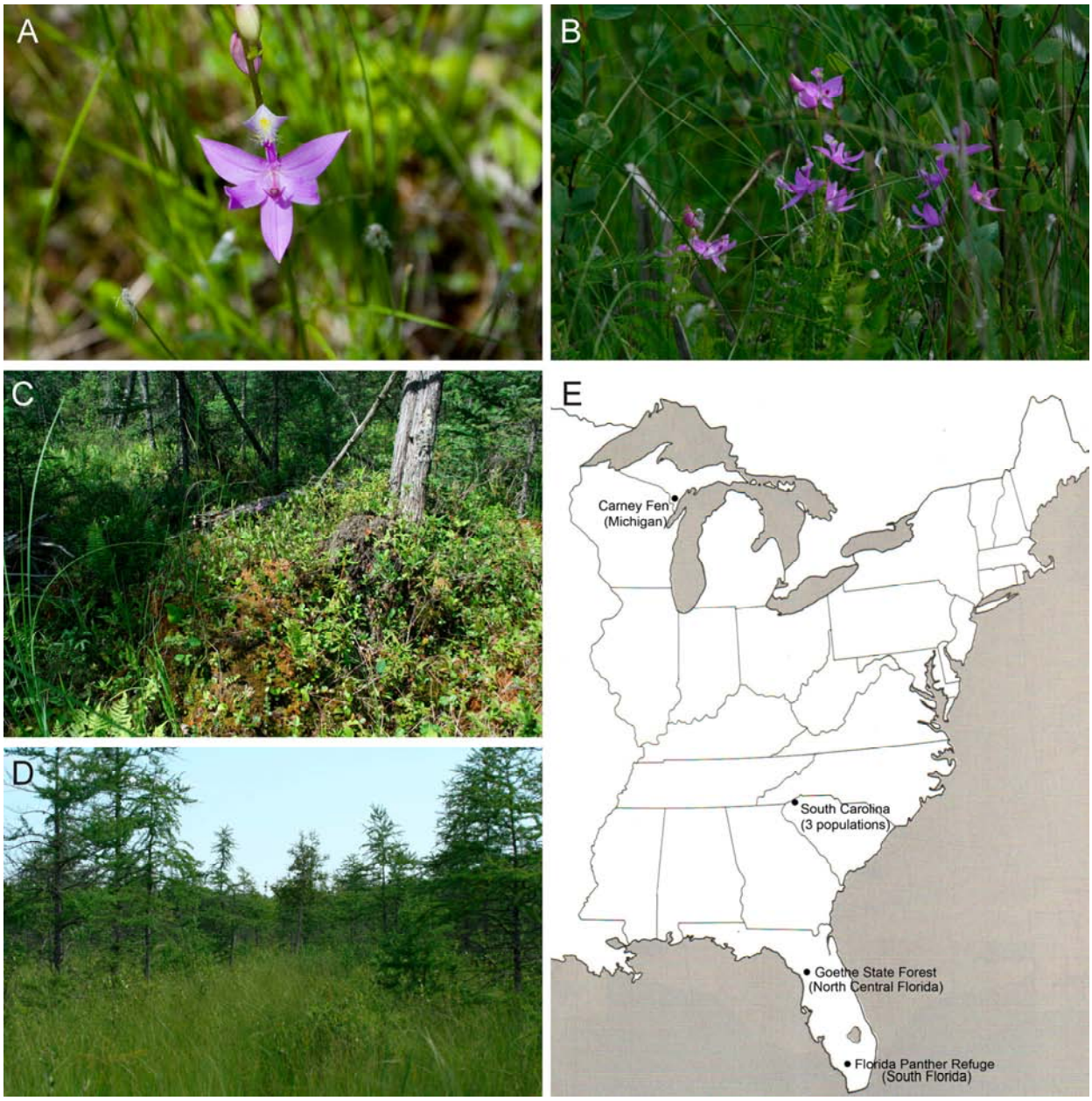

Figure 1-2. Carney Fen, Michigan. A) Individual flower – note the strong apex on the labellum. B) Multiple plants growing in close proximity. C) Sphagnum hummock containing numerous *C. tuberosus* plants. D) Northern fen habitat. E) Location of Carney Fen in the upper Michigan peninsula.

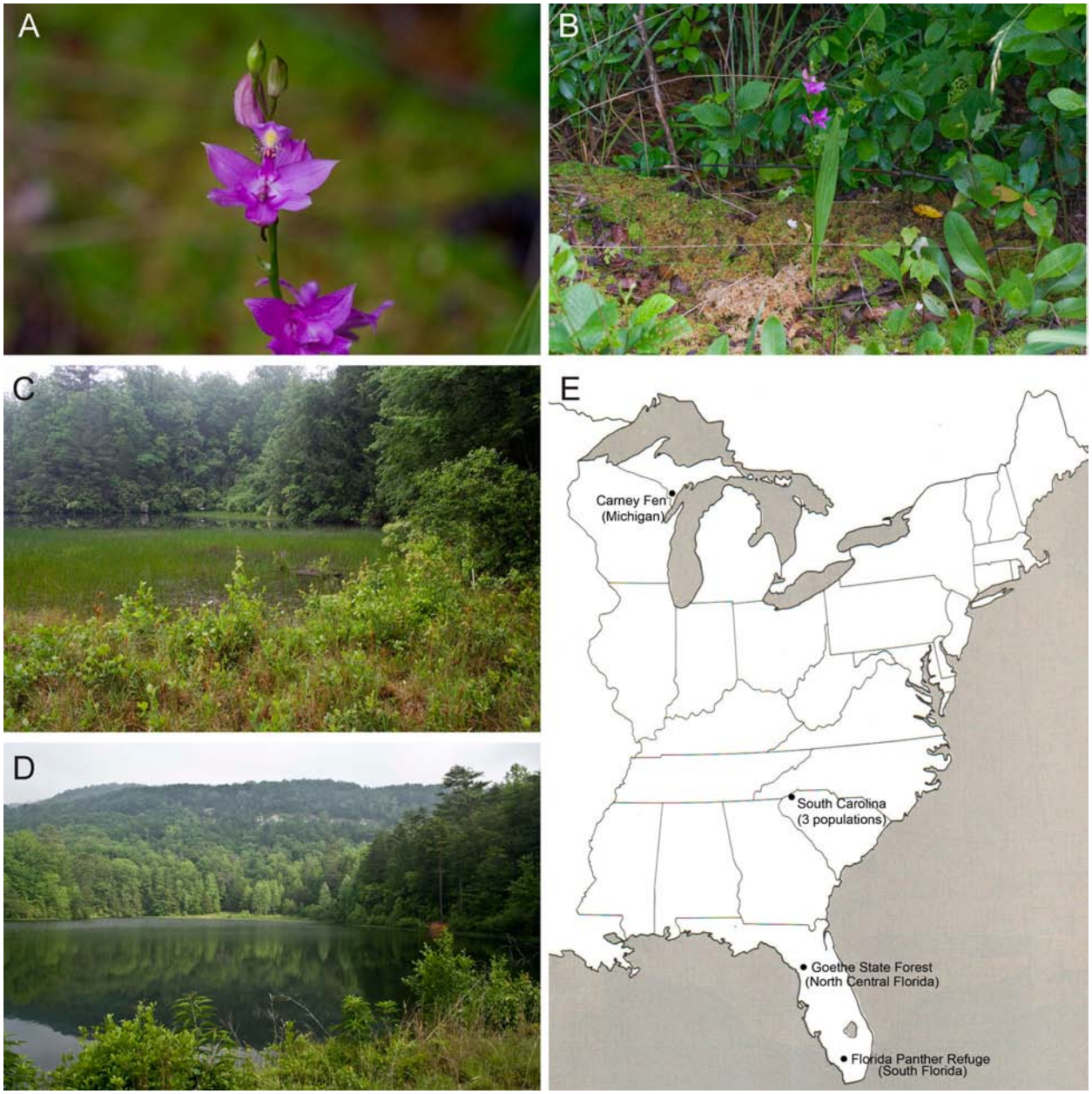

Figure 1-3. Ashmore Heritage Preserve, South Carolina. A) Individual flower. B) *C. tuberosus* plant growing in sphagnum moss– note the wide leaf. C) Area containing *C. tuberosus* population around the lake edge. D) Habitat at Ashmore Heritage Preserve. E) Location of South Carolina populations in the upstate.

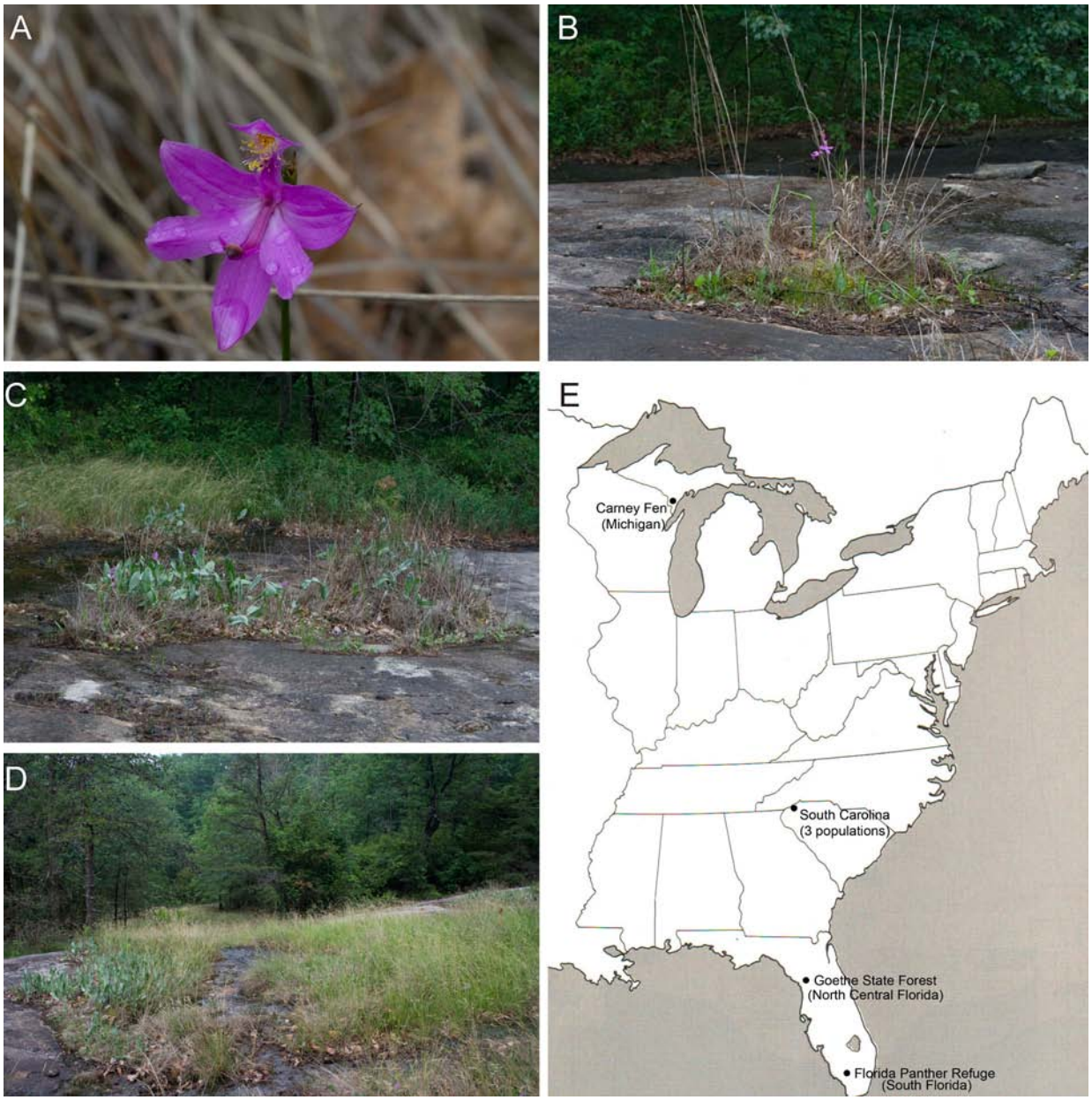

Figure 1-4. Site C, South Carolina. A) Individual flower. B) Small micro-island habitat. C) Large micro-island habitat. D) Seepage area with flowing water. E) Location of South Carolina populations in the upstate.

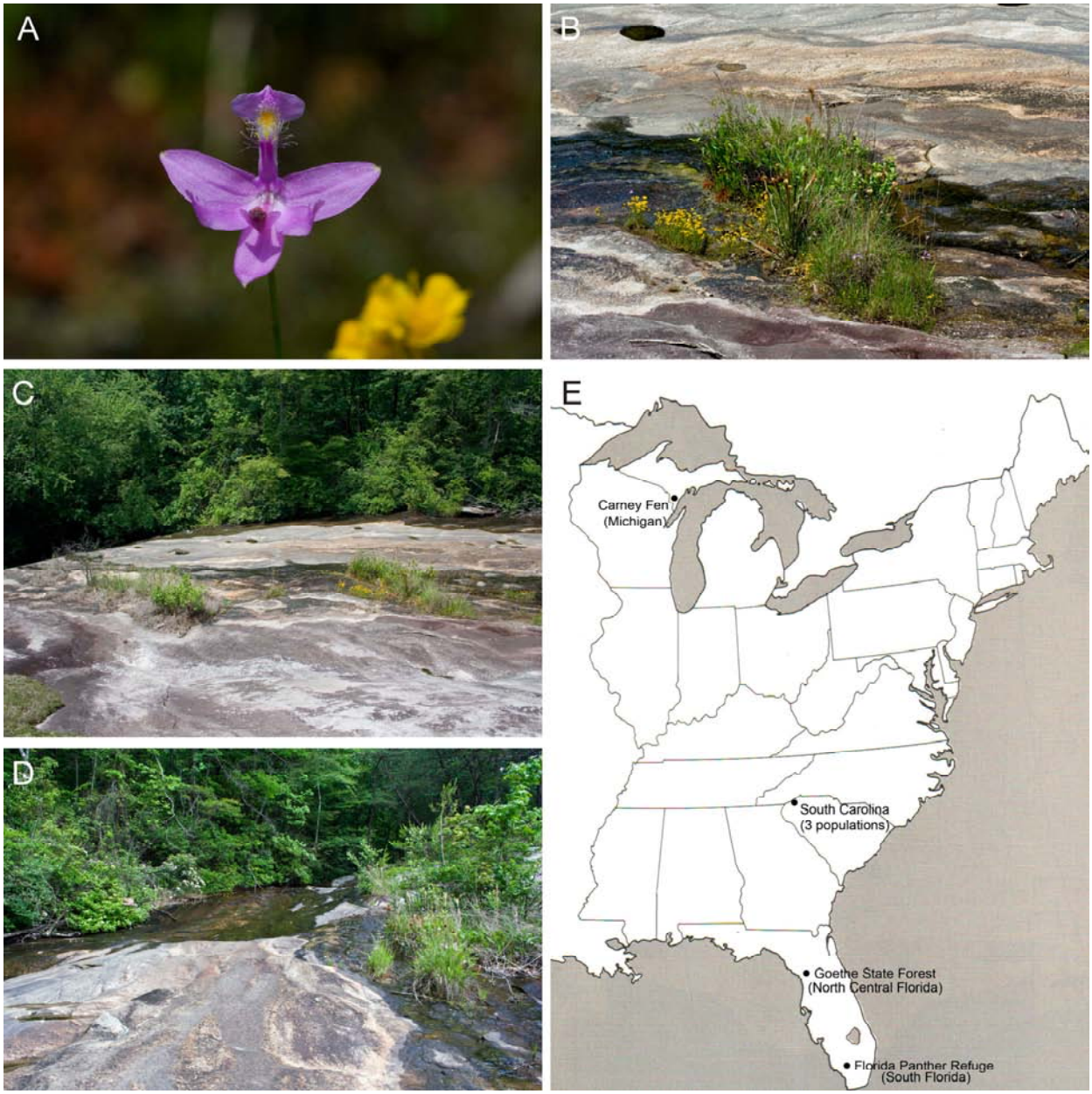

Figure 1-5. Eva Chandler Heritage Preserve, South Carolina. A) Individual flower. B) Cataract bog habitat. C) Multiple cataract bogs. D) Flowing water on granite outcropping. E) Location of South Carolina populations in the upstate.

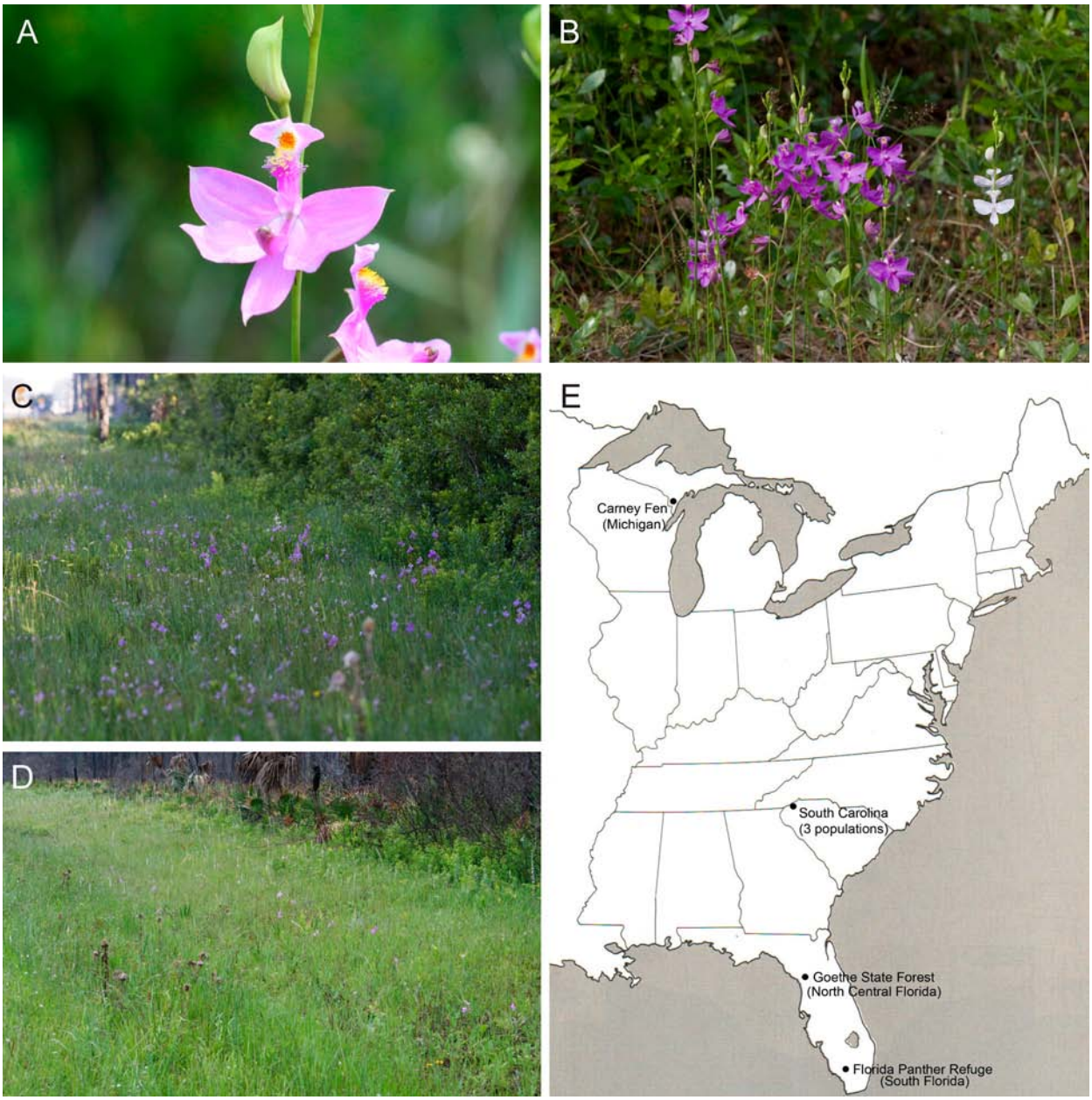

Figure 1-6. Goethe State Forest, Florida. A) Individual flower. B) Group shot with lighter pale flower. C) Roadside habitat. D) Roadside habitat. E) Location of Goethe State Forest in north central Florida.

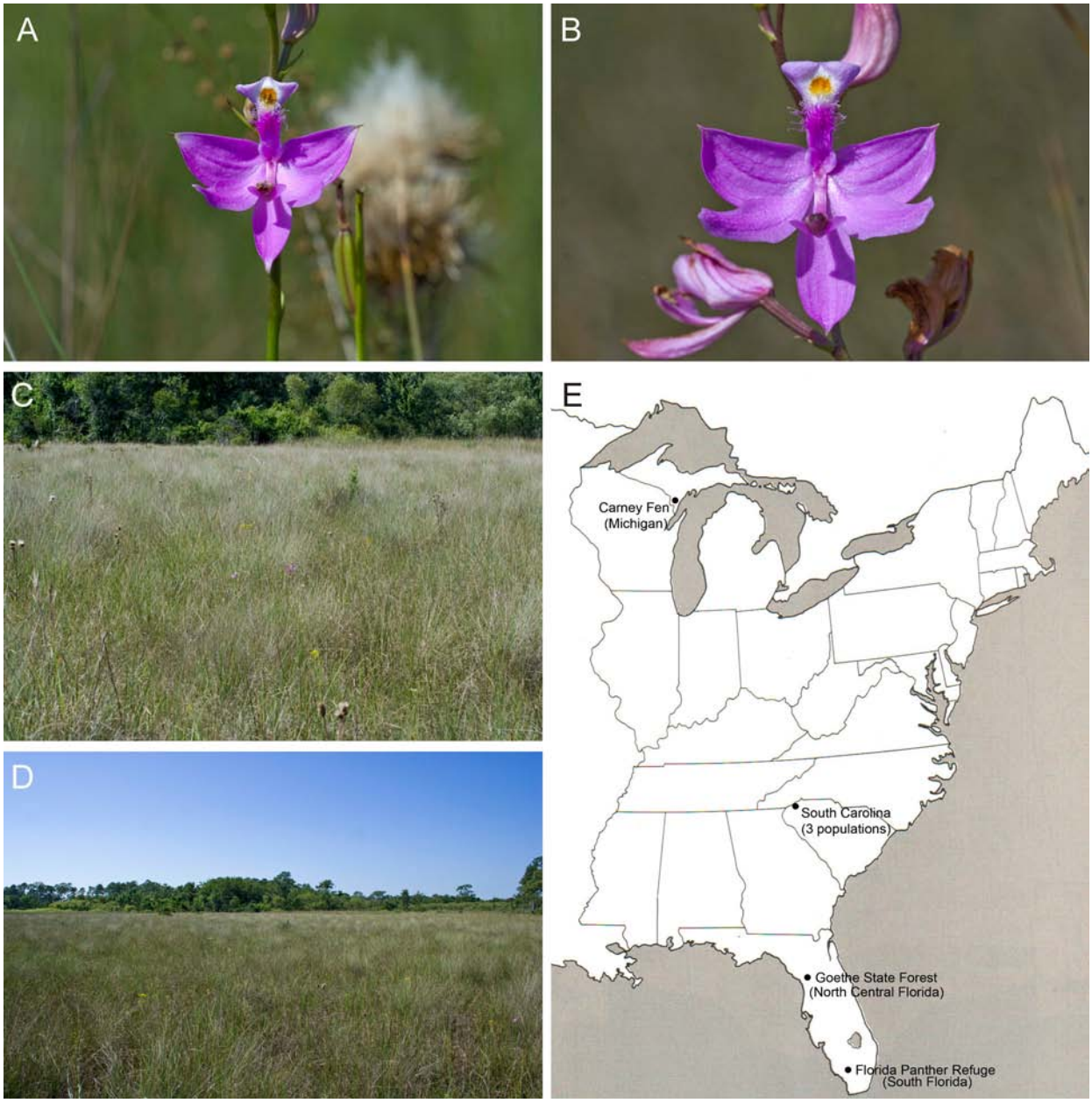

Figure 1-7. Florida Panther National Wildlife Reserve, Florida. A) Individual flower with crested labellum characteristic of *C. tuberosus* var. *tuberosus*. B) Flower with flat labellum and white trichomes characteristic of *C. tuberosus* var. *simpsonii*. C) Prairie habitat. D) Wet prairie habitat. E) Location of Florida Panther National Wildlife Refuge in south Florida.

CHAPTER 2
EFFECTS OF PHOTOPERIOD AND GERMINATION MEDIA ON *IN VITRO* SEED ECOLOGY OF *Calopogon tuberosus*

Introduction

Ecotypic differentiation has recently been recognized as an important issue in several plant sciences including conservation, restoration, and population genetics (Hufford and Mazer, 2003). Ecotypic differentiation enables species to survive diverse habitats and environmental conditions across their geographical range, but the specific functions they serve in ecosystems remain unclear (Seliksar *et al.*, 2002). For this reason, using local plant material for restoration purposes or reintroductions may be necessary to maintain ecosystem stability (Linhart, 1995). Introducing poorly adapted ecotypes into unsuitable habitats may lead to reduced plant population fitness (Hufford and Mazer, 2003; McKay *et al.*, 2005).

Common garden studies are often utilized to detect local adaptation (Sanders and McGraw, 2005), but obtaining permits to collect and transplant protected, rare, threatened, or endangered species is often difficult. Alternatively, studying the ecology and physiology of seed germination and seedling development from widespread populations may provide insight into the range of ecotypic differentiation (Singh, 1973). Studies of orchid seed germination ecology are needed to support reintroduction programs that typically use seed germination as a propagation tool. Studying the germination ecology of orchid seeds *in situ* is difficult and time consuming because orchid seeds are minute and germination is often low (Brundrett *et al.*, 2003; Zettler *et al.*, 2005*b*; Diez, 2007). Alternative to *in situ* germination, asymbiotic *in vitro* techniques were developed to germinate orchid seed (Kauth *et al.*, 2008). *In vitro* conditions can also be manipulated to, at best, mimic *in situ* conditions such as photoperiod and temperature.

Given that *Calopogon tuberosus* is a commonly recognized orchid in North America, information exists regarding ecology, pollination, and seed germination for this species.

However, information regarding seed germination is often conflicting. Different photoperiods were recommended for seed germination of *C. tuberosus* including complete darkness to light incubation (Stoutamire, 1974; Whitlow, 1996; Kauth *et al.*, 2006). Likewise, different germination media were also recommended (Henrich *et al.*, 1981; Arditti *et al.*, 1985; Anderson, 1990; Kauth *et al.*, 2006).

Differences in germination and seedling development might be the result of local adaptation to specific environmental conditions. Attributing ecotypic differentiation to germination differences is difficult since seed source is rarely reported in *C. tuberosus* seed germination studies, and basing recommendations for seed germination of *C. tuberosus* on one population is tenuous. Evaluation of *in vitro* seed germination from diverse populations may clarify whether ecotypic differentiation occurs among *C. tuberosus* populations. In this paper, the effects of photoperiod and culture media on asymbiotic seed germination and seedling development are compared among widespread populations of *C. tuberosus*.

The objectives of these experiments were: 1) Compare the effects of photoperiod on seed germination ecology among latitudinally widespread *C. tuberosus* populations; 2) Compare the effects of germination media on seed germination ecology among latitudinally widespread *C. tuberosus* populations; 3) Determine whether soil nutrients may contribute to differences in seed germination among latitudinally widespread *C. tuberosus* populations; and 4) Validate the use of *in vitro* studies in differentiating ecotypes of *C. tuberosus*.

Materials and Methods

Seed Source

Intact seed capsules (slightly yellow in color) were collected before dehiscence approximately 2 months after peak flowering. Capsules were collected from the Florida Panther National Wildlife Refuge (Collier County, Florida), Goethe State Forest (Levy County, Florida),

Ashmore Heritage Preserve (Greenville County, South Carolina), Eva Chandler Heritage Preserve (Greenville County, South Carolina), Site "C" near Eva Chandler Heritage Preserve (Greenville County, South Carolina), and Carney Fen (Menominee County, Michigan). Non-dehisced capsules were collected to reduce the potential for surface contamination of seeds. Upon collecting and receiving capsules, seeds were pooled according to source and stored at 23°C over silica desiccant for 2 weeks. After 2 weeks, seeds were removed from the capsules and stored over silica desiccant at -11°C until use.

Seed Viability Test

A viability test (Lakon, 1949) was performed on seed from all populations by staining embryos with 2, 3, 5 triphenyl tetrazolium chloride (TTC). Seeds were first scarified in an aqueous 5% $CaOCl_2$ solution for 0, 0.5, 1, 2, or 3 h. Two replications of approximately 100 seeds each were used per treatment. After scarification, seeds were rinsed twice in distilled-deionized (dd) water and suspended in sterile water for 24 h in darkness at 23˚ ± 2˚ C. Water was replaced with TTC and seeds were soaked for 24 h at 30˚ C in darkness. After the TTC soak, embryos were scored as viable if any degree of red staining was observed.

Media and Seed Preparation

Media were prepared in 1000 mL batches, and the pH was adjusted to 5.7 with 0.1N KOH prior to autoclaving for 40 min at 117.7 kPa and 121°C. Forty mL sterile medium was dispensed into square 100x15 mm Petri plates with a 36-cell bottom (Integrid™ Petri Dish, Becton Dickinson and Company, Franklin Lakes, NJ, USA). Seeds were surface sterilized in sterile scintillation vials for 3 min in a solution of 5 mL absolute ethanol, 5 mL 6% NaOCl, and 90 mL sterile dd water. Seeds were rinsed twice with sterile dd water after surface sterilization. Solutions were removed from the vials with sterile 5 mL glass Pasteur pipettes. Seeds were then placed on the surface of the germination media with a 10µL sterile inoculating loop. The interior

16 cells of the Petri plates were used for subreplications to avoid uneven media drying at the edges. Petri plates were sealed with one layer of Nescofilm (Karlan Research Products, Santa Rosa, CA, USA). Seed germination and seedling development (Table 2-1) were monitored weekly for 8 weeks according to the six developmental stages described by Kauth (2005).

Photoperiod Effects on Asymbiotic Germination and Early Seedling Development

The first experiment was a 6x3 factorial was designed with six seed sources and three photoperiods including a short day (SD; 8/16 h L/D), neutral day (ND; 12/12 h L/D), long day (LD; 16/8 h L/D). These photoperiods were chosen to represent commonly encountered photoperiods throughout the range of *C. tuberosus*. *Phyto*Technology Orchid Seed Sowing Medium (#P723; *Phyto*Technology Laboratories, Shawnee Mission, KS, USA) was used based on previous success with *C. tuberosus* seed germination and development (Kauth *et al.*, 2006). Ten replicate Petri plates with five randomly selected subreplications were used per seed source and photoperiod treatment. An average of 48.5 seeds was sown for each subreplication for an average of 242.5 seeds per replication and 2425 seeds per treatment. Each subreplication Culture vessels were placed under cool-white fluorescent lights (F96712, General Electric) at an average of 33.3 ± 7 (12/12 photoperiod), 31.6 ± 5 (8/16 photoperiod), and 31.6 ± 6 (16/8 photoperiod) µmol m^{-2} s^{-1} and incubated at 25 ± 0.4°C.

Photoperiod Effects on Advanced *In Vitro* Seedling Development

After 8 weeks, developing embryos and seedlings were transferred from Petri plates to 95x95x 100 mm *Phyto*Tech culture boxes (*Phyto*Technology Laboratories, Shawnee Mission, KS, USA) containing 100 mL P723 medium; seedlings were maintained in corresponding photoperiods. Ten seedlings were transferred to each culture vessel. After an additional 8 weeks and 16 weeks total, five culture vessels per treatment (50 total seedlings) were randomly selected. However, due to the lack of developed embryos 30 seedlings from South Carolina were

used. Seedling percent biomass allocation was determined by dividing corm, root, and shoot weights by the total seedling weight. Additional data recorded were leaf number, shoot length, root number, root length, and corm diameter. Culture conditions were the same as previously described.

Asymbiotic Germination Media Evaluation

A 6x6 factorial design with six germination media (Table 2-2) and six seed sources was used. Five media commercially prepared by *Phyto*Technology Laboratories were used: BM-1 Terrestrial Orchid Medium (BM-1; #B141; van Waes and Debergh, 1986*a*), Knudson C Orchid Medium (KC; #K400; Knudson, 1946), Malmgren Modified Terrestrial Orchid Medium (MM; #M482; Malmgren, 1996), Orchid Seed Sowing Medium (#P723), and Vacin and Went Modified Orchid Medium (VW; #V895; Vacin and Went, 1949). Murashige and Skoog Medium in half strength (MS; #M5524; Murashige and Skoog, 1962) was commercially prepared by Sigma-Aldrich (St. Louis, MO, USA). BM-1 and VW were further supplemented with 0.1% charcoal, KC was further supplemented with 0.1% charcoal and 0.8% TC® agar (*Phyto*Technology Laboratories), MM was further supplemented with 0.8% TC® agar, and ½ MS was further supplemented with 0.1% charcoal, 0.8% TC® agar, and organic supplements including peptone found in P723. All media contained 2% sucrose. Five replicate Petri plates with three randomly selected subreplications with an average of 62.6 seeds were used per treatment. An average of 187.8 seeds was sown per replicate plate for a total of 939 seeds per treatment. Germination and development were monitored biweekly for 8 weeks. Culture vessels were placed under ND conditions, cool-white fluorescent lights at 33.3 ± 7 µmol m^{-2} s^{-1} and 25 ± 0.4°C. Only one photoperiod was chosen as to not confound the experimental design.

Soil Analysis

In order to determine if soil nutrient availability reflected germination media, a soil analysis from each site was conducted. Soil was collected from each study site during the 2009 growing season. Soil cores were taken with a 5.5 x 6 cm tulip bulb planter and stored at approximately 6°C until preparation. Because of the fragile nature of the cataract bogs at Eva Chandler Preserve (South Carolina 3), soil was collected from the forested area approximately 5 m from the cataract bog (see Figure 1-5D). Soil at Site C (South Carolina 2) was not collected from the micro-island habitats, but rather from the larger areas where *C. tuberosus* grows (see Figure 1-4D). Soils were dried at 30°C for 72 hours, ground with a mortar and pestle, and sieved through a No. 20 mesh screen. Samples were submitted to the University of Florida/Institute of Food and Agricultural Sciences Extension Soil Testing Laboratory. All soil tests were performed according to Environmental Protection Agency (EPA) standards.

Total Kjeldahl Nitrogen (TKN), which tests the sum of organic nitrogen, ammonia, and ammonium, was performed according to EPA 351.2. However, this test does not give the amount of available nitrogen to plants. Potassium, calcium, magnesium, phosphorus, and iron were extracted using the Mehlich 1 technique and analyzed using inductively coupled plasma spectrometry (ICP). ICP metals were tested according to EPA 200.7. Organic matter was tested using loss on ignition, and pH was tested according to EPA 150.1. For detailed testing methods see Mylavarapu and Kennelley (2002).

Statistical Analysis

Germination percentages were calculated by dividing the number of germinated seeds by the total number of seeds with an embryo in each subreplication. The percentage of embryos and seedlings in a developmental stage (Table 2-1) was calculated by dividing the number of seeds in

a stage by the total number of seeds. A developmental index of germinated seeds was modified from Otero *et al.* (2004):

$$DI = \frac{(N_1 + N_2*2 + N_3*3 + N_4*4 + N_5*5 + N_6*6)}{(N_1 + N_2 + N_3 + N_4 + N_5 + N_6)}$$

where N_1 is the number of seeds in stage 1, etc. Stage 0 ungerminated seeds were excluded since only germinated seeds were of concern. Data were arcsine transformed to normalize variation prior to analysis. Germination, seedling development, and embryo viability data were analyzed using general linear model procedures (proc glm) and least-square means (lsmeans) using SAS v9.1 (SAS Institute, 2003).

Results

Seed Viability

For all populations except south Florida (no difference in pretreatment time), the highest percentage of viable embryos was observed after 3 h of calcium hypochlorite pretreatment (Table 2-4). Maximum embryo viability from seed collected in 2006 was as follows: 85.4% south Florida, 66.7% north central Florida, 25.0% South Carolina 1, 38.1% South Carolina 2, 42.1% South Carolina 3, and 50.3% Michigan. Embryo viability was also tested for seeds collected in 2007, but seeds were only scarified in $CaOCl_2$ for 3 h. Embryo viabilities were as follows: 70.5% south Florida, 53.9% north central Florida, 25.0% South Carolina 1, 10.6% South Carolina 2, 30.3% South Carolina 3, 20.8% Michigan.

Photoperiod Effects on Germination and Early Development

ANOVA results indicate that photoperiod alone did not significantly influence germination ($F_2 = 0.58$, $p = 0.56$), but the interaction between photoperiod and source ($F_5 = 9.97$, $p < 0.0001$) as well as source alone ($F_{10} = 225.1$, $p < 0.0001$) were highly significant. However, photoperiod ($F_2 = 8.43$, $p = 0.0002$), source ($F_5 = 211.5$, $p < 0.0001$), and their interaction ($F_{10} = 6.31$, $p <$

0.0001) were highly significant for early embryo and seedling development during weeks 1-8. Seeds from Michigan germinated and developed more quickly compared to other populations. Imbibition occurred one week after seeds were inoculated onto the germination medium. However, total germination by week 8 was still low compared to Florida populations. Germination of Michigan seeds was similar regardless of photoperiod (Figure 2-1A). Embryos developed quickly with corms forming by week 6. Approximately 95% of the germinated seeds in all photoperiods developed to stage 6 (Figure 2-1A). The average developmental stage was between stage 5 and 6 with no difference among photoperiods (Figure 2-2A).

Germination of South Carolina seeds was the lowest of all populations (Figure 2-1). Germination percentages in all South Carolina populations did not exceed 4%. The highest germination was observed under long day conditions in South Carolina 3 seeds (Figure 2-1D). Although embryos did develop to stages 5 and 6 in all South Carolina populations (Figure 2-1B, C, D), development on average was low. South Carolina 1 (Figure 2-2B) and South Carolina 2 (Figure 2-2C) embryos developed slowly. The average developmental index for both South Carolina 1 and 2 was less than the stage 2. Development of South Carolina 3 (Figure 2-2D) embryos was more advanced than other South Carolina populations. Under long day conditions, South Carolina 3 embryo development was to stage 4.

Seeds from north central Florida germinated quickly and corms formed on seedlings after week 8 (Figure 2-1E). Greater than 16% of the protocorms in each photoperiod developed to an advanced leaf-bearing stage (Figure 2-1E). Total seed germination percentage was highest under short day conditions for both the north central Florida (60.2%) and south Florida (48.5%) populations (Figure 2-1E, F). Photoperiod did not significantly influence embryo development in either Florida population. However, development was more advanced in the north central Florida

population with most embryos developing to stage 4 by week 8 (Figure 2-2E). Development of north central Florida embryos was only exceeded by the Michigan population. Although South Florida seed germination was relatively high, development was low (Figure 2-2F). Fewer than 5% of the south Florida seeds under SD conditions developed past imbibition after 8 weeks culture (Figure 2-1F). Approximately 10% of the seeds under both ND and LD conditions developed past imbibition (Figure 2-1F). South Florida seeds germinated slowly and development was delayed compared to all other sources.

Photoperiod effects on Advanced Seedling Development

Advanced seedling development was monitored from weeks 8-16. After 16 weeks culture, Michigan seedlings began to senesce while South Carolina and Florida seedlings continued to grow (Figure 2-3). Corm formation was limited in south Florida seedlings, while Michigan, South Carolina, and north central Florida seedlings all formed corms (Figure 2-3). Biomass allocation was similar among photoperiods within each seed source (Figure 2-4). Maximum dry weight allocation to corms was observed in Michigan seedlings (Figure 2-4A). Although north central Florida seedlings formed large corms, the percent dry biomass allocation was more evenly distributed among shoots, corms, and roots than other populations (Figure 2-4C). The greatest seedling shoot biomass allocation was observed in South Carolina 3 and south Florida populations (Figure 2-4B, D).

After 16 weeks *in vitro* culture, few differences in seedling growth and development existed among photoperiod within each individual seedling source (Figure 2-5). Photoperiod had a significant influence on shoot length ($F_2 = 8.58$, $p = 0.0002$) and root length ($F_2 = 4.24$, $p = 0.02$), but not leaf number ($F_2 = 1.45$, $p = 0.23$), root number ($F_2 = 1.92$, $p = 0.15$), and corm diameter ($F_2 = 0.57$, $p = 0.57$). However, major differences were observed among seedling source for leaf number ($F_3 = 2.92$, $p = 0.03$), shoot length ($F_3 = 119.3$, $p < 0.0001$), root number

($F_3 = 46.7$, $p < 0.0001$), root length ($F_3 = 73.3$, $p < 0.0001$), and corm diameter ($F_3 = 20.8$, $p < 0.0001$). Michigan seedlings generally had the lowest shoot length, root number, and root length (Figure 2-5). North Central Florida seedlings had the longest shoot length, highest root number, longest roots, and largest corms. Within seed source, neutral day photoperiods generally promoted growth of south Florida seedlings further than short and long days (Figure 2-5). Photoperiod did not influence seedling growth of Michigan and north central Florida seedlings with the exception of corm diameter. Neutral day photoperiods promoted the largest corm growth (Figure 2-5E), while Michigan corms were the smallest in the short day photoperiod (Figure 2-5E).

Media Effects on Germination and Early Development

Germination media ($F_5 = 2.25$, $p = 0.05$), seed source ($F_5 = 375.2$, $p < 0.0001$), and the interaction between source and medium ($F_{25} = 4.95$, $p < 0.0001$) significantly influence germination. Early embryo and seedling development during weeks 1-8 were significantly influenced by source ($F_5 = 63.8$, $p < 0.0001$), medium ($F_5 = 4.73$, $p < 0.0001$), and their interaction ($F_{25} = 3.11$, $p < 0.0001$). Michigan seeds germinated and embryos developed quickly on all media compared to other sources. The highest germination percentage of Michigan seeds occurred on P723 and BM-1 (Figure 2-6A). Seed germination was still lower compared to Florida populations with germination remaining below 40%. A large percentage of embryos developed to stage 6. However, embryo development was lowest on KC and VW with the average stage of development remaining between 4 and 5, while embryos on all other media approached stage 6 (Figure 2-7A).

Seed germination for South Carolina 1 was highest on VW, but germination was only 4.9% (Figure 2-6B). No germination occurred on P723 (Figure 2-6B). Clear differences in germination were not evident in South Carolina 2 (Figure 2-6C), but germination on P723 was

significantly lower than all other media. Germination on KC and MS (no difference between MS and VW) was highest for South Carolina 3 seeds, while lowest germination occurred on P723 (Figure 2-6D). Although germination of South Carolina 1 and 2 seeds improved, germination was still lower than 5% on all media. Development among South Carolina populations was very different. No South Carolina 1 embryos developed to stage 6, and less than 1% developed to stage 5 (Figure 2-6B). Advanced stages of development (stages 4-6) were observed for both South Carolina 2 and 3 (Figure 2-7C, D). The average stage of development was lower than 3 for South Carolina 1 embryos, which was lower than near stage 6 development for South Carolina 2 and 3. However, examining development of South Carolina populations was deceptive since germination was very low. Development of South Carolina 2 embryos approached stage 6 (Figure 2-7C), but germination was lower than 5%. Development of South Carolina 3 embryos was highest on MS, VW, P723, and BM-1 (Figure 2-7D). However, more embryos development on MS, KC, and VW since germination was very low on P723 and BM-1.

In both Florida populations, few differences in total germination existed among media; at least four media provided high germination percentages (Figure 2-6E, F). Subsequent development differed greatly. For north central Florida, more advanced development (stage 4+) occurred on BM-1, MS, and P723 (Figure 2-7E). High germination percentages of north central Florida seeds were observed on MM, but the majority of seeds remained in stage 1 (Figure 2-6E). Only a small percentage of south Florida embryos developed to stage 6 (Figure 2-6F). Development was highest on P723, VW, and BM-1 for south Florida embryos with the average developmental stage of near 4 (Figure 2-7F). Germination percentages were high on KC and MM for south Florida seeds, but as with north central Florida seeds development was below an average stage of 3.

Media Effects on Advanced Seedling Development

Corm development was more advanced after 8 weeks on BM-1, MS, and P723 compared to development on ½ MS, KC, and VW (Figure 2-8). Seedling development of Michigan, South Carolina 3, and north central Florida seedlings was superior to other populations (Figure 2-8). However, development of Michigan, South Carolina 3, and north central Florida seedlings differed markedly. Corm formation was more pronounced in seedlings from northern latitudes. Thus by week 8, no corm formation was observed in Florida seedlings while early and advanced corm formation was observed in South Carolina and Michigan seedlings, respectively.

Soil Nutrient Analysis

Soil analysis revealed that nutrient availability differed among habitats. Overall, soil from Michigan was the richest in nutrients and poorest in south Florida with the exception of nitrogen and calcium. Northern fens generally have high levels of calcium and magnesium as a result of the mineral rich groundwater percolating to the surface (Amon *et al.*, 2002; Bedford and Godwin, 2003; Cohen and Kost, 2008). This was observed in Carney Fen (Michigan), which contained the highest amount of calcium and magnesium (Table 2-3). In addition, nitrogen, phosphorus, and potassium levels were also highest at Carney Fen. Soil from south Florida was also high in calcium and total nitrogen, but lowest in phosphorus and iron. Iron levels were highest in soils from South Carolina 1 and 3 as well as north central Florida. Total nitrogen levels were similar in soils from South Carolina 1 and 3 as well as north central Florida, but much higher in South Carolina 2. Soil pH levels were alkaline in Michigan and south Florida, and acidic South Carolina and north central Florida. Total organic matter was exceedingly high (78.5%) in the Michigan soil, followed by South Carolina 2 and 3, south Florida, South Carolina 1, and north central Florida.

Discussion

Seed Viability and Quality

Differences in seed germination responses are often attributed to seed viability and quality. Comparisons of orchid seed germination among populations of the same species have been reported, but *C. tuberosus* has not been examined. Symbiotic germination and mycorrhizal specificity among populations rather than ecotypic differentiation were examined in these studies (Zettler and McInnis, 1992; Zettler and Hofer, 1998; Sharma *et al.*, 2003). However, differences in seed germination and viability among populations were described which might be accounted for by ecotypic differentiation.

Population size and inbreeding depression may influence low seed germination of several *C. tuberosus* populations as well as differences in seed viability. Lower germination percentages in small populations of *Platanthera integrilabia*, compared to larger populations, were attributed to lower seed viability (Zettler and McInnis, 1992). Similarly, *Platanthera clavellata* seed germination differences were attributed to inbreeding depression (Zettler and Hofer, 1998). Reduction in pollinator numbers at different sites may lead to seed viability differences in *C. tuberosus* as reported for *Platanthera leucophaea* and *P. praeclara* (Bowles *et al.*, 2002; Sharma *et al.*, 2003).

Another plausible explanation regarding differences in seed viability may be self-pollination. *Calopogon tuberosus* is a non-rewarding/out-crosser pollinated by *Bombus*, *Xylocopa*, and *Megachile* bees through deception (van der Pijl and Dodson, 1966; Thien and Marcks, 1972; Dressler, 1981). Self-pollination in *C. tuberosus* may be common as Firmage and Cole (1988) reported in Maine populations. Self-pollination in *Calypso bulbosa*, and likely *C. tuberosus*, was mediated by bumblebees since a mechanism for autogamy does not exist (Alexandersson and Agren, 2000). While fruit set is generally not affected by self-pollination,

reduced seed viability or embryo production can be reduced (Tremblay *et al.*, 2005). Low seed viability and germinability in certain *C. tuberosus* populations may be caused by higher levels of self-pollination; however, further investigation is warranted.

The correlation between TTC determined seed viability and the corresponding observed percent germination is often variable and species specific (van Waes and Debergh, 1986*a*; St-Arnaud *et al.*, 1992; Shoushtari *et al.*, 1994). van Waes and Debergh (1986*b*) reported various optimal pretreatment times from 45 min to 16 h in calcium hypochlorite in 31 species of terrestrial orchids, thus differences in *C. tuberosus* viability are not surprising. Tetrazolium testing can overestimate viability because this test does not detect inactive enzymes that may become active during germination (Lauzer *et al.*, 2007). For this reason, fluorescein diacetate (FDA) is used with results often correlating with germination (Pritchard, 1985; Vendrame *et al.*, 2007). Lower germination percentages compared to viability may reflect non-optimal temperatures with seeds from northern climates requiring cooler temperatures *in vitro* or stratification to germinate; these concerns are currently being addressed in separate experiments. In addition, seeds that do not germinate *in vitro* may have an intrinsic dormancy mechanism. Embryo damage during surface sterilization is also a likely scenario that may have reduced germination.

Photoperiod

No other published research exists that compare photoperiodic effects on North American orchid seed germination spanning several populations of the same species from distant geographic sources. For non-orchid ecotypes photoperiod was reported to be an important factor on germination (Singh, 1973; Probert *et al.*, 1985*b*; Seneca, 1974). Due to latitudinal differences in location, *C. tuberosus* populations experience different seasonal variations in photoperiod, temperature regimes, and growing season duration. *Calopogon tuberosus* flowers in early June to

mid-July in the north and mid-May to early June in the south (Luer, 1972). In Florida, seed capsules dehisce and seeds are disbursed in July when photoperiods are approximately 13-14 h. Short day conditions promoted the highest germination percentage for both Florida populations. At both Florida locations, the shortest natural photoperiods do not approach 8 h, but approximately 10 h. Whether Florida seeds are somewhat light sensitive during germination remains unclear without also conducting *in situ* germination studies.

Development of embryos in all three photoperiods for north central Florida was very rapid compared to south Florida embryos. A large percentage of south Florida seeds germinated only to the imbibition stage by week 8, perhaps due to the longer growing season in south Florida. In north central Florida, lower daily surface temperatures in winter can drop below the freezing point, while low daily winter temperatures in south Florida rarely drop below 5°C. The warmer climate in south Florida may allow slower embryo development without increased mortality, assuming that temperatures within the soil where embryos reside are also different. After 16 weeks culture, south Florida seedlings were small and did not form corms, while north central Florida seedlings were larger and had readily formed corms.

Although total germination was low in Michigan seeds, seedling development and corm formation was more rapid than those from the southern populations. Imbibition occurred after 1 week, and corm initiation began by week 6. Regardless of photoperiod, after 16 weeks culture the large seedling corm:shoot:root ratios generated in seedlings from the Michigan population suggest a high percentage of carbohydrates are allocated to corms. Rapid seed germination, seedling development, and corm formation in northern populations may indicate that seedlings do not respond to photoperiod ensuring rapid corm development prior to the shorter growing

seasons. Kane *et al.* (2000) similarly reported rapid corm development in northern ecotypes of the wetland non-orchid species *Sagittaria latifolia.*

Media Screen and Soil Nutrient Availability

P723 proved to be an adequate medium for germinating Florida and Michigan seeds, but discrepancies between germination and viability were perhaps caused by using a non-optimal medium for other seed sources. Abundant literature exists on the influence of media mineral nutrition for orchid seed germination and subsequent seedling development (Curtis, 1947; Spoerl and Curtis, 1948; Raghavan, 1964; van Waes and Debergh, 1986*a*; Kauth *et al.*, 2006). However, site-specific differences in soil nutrient availability could explain differences in germination and development as found in *Dactylorhiza incarnata* by Dijk and Eck (1995*b*). Seedlings from coastal areas grew faster *in vitro* and were more tolerant of exogenous ammonium and nitrate compared to seedlings from inland populations. Coastal populations inhabited calcareous areas where high nutrient levels were found due to the introduction of fertilizers and poor drainage (Dijk and Eck, 1995*b*).

The wide range in pH levels among the populations is not surprising. Sheviak (1983) found *C. tuberosus* in soils with a wide pH tolerance from 4.5 to 7.5, but few plants were found in soils with a moderate pH of 6.0-7.0. Sheviak (1983) noticed that nutrient availability was different with varying pH levels. Phosphorus was higher in soils with a pH from 6.0-7.0, iron was higher in more acidic soils, and potassium, calcium, and magnesium were higher in neutral to alkaline soils. The same trends were observed in the *C. tuberosus* soil samples. Michigan soil, with a pH of 7.0, contained higher phosphorus levels than acidic South Carolina soils and highly alkaline south Florida soils. The more acidic South Carolina and north central Florida soils contained more iron compared to the alkaline soils of Michigan and south Florida. Calcium levels were

higher in the alkaline soils of Michigan and south Florida, and magnesium and potassium levels were higher in Michigan soils.

Although soil from Michigan contained the highest amount of nitrogen and phosphorus, available nitrogen and phosphorus levels were reported in low levels in northern fens (Amon *et al.*, 2002; Bedford and Godwin, 2003). However, higher nitrogen levels can be present due to agricultural and septic tank drainage, and atmospheric nitrogen deposition (Bedford and Godwin, 2003). Carney Fen (Michigan) is in close proximity to agricultural areas (personal observation), which may be an influence on the higher nitrogen and phosphorus levels. The Kjedahl total nitrogen test includes organic nitrogen, ammonium, and nitrate, which may also influence the high nitrogen levels in Carney Fen.

The low phosphorus in south Florida soil was surprising since increased urbanization and agriculture in south Florida has increased phosphorus levels (Bruland *et al.*, 2006). Bruland *et al.* (2006) reported higher nutrient levels in south Florida soils near the Everglades compared to the soil from the Florida Panther National Wildlife Refuge (south Florida). This may be influenced by the higher nutrient load from Lake Okeechobee flowing through southeast Florida. Lower nutrient levels in soil on the Florida Panther National Wildlife Refuge might be explained by the lower development in southwest compared to southeast Florida, and is relatively isolated from the Lake Okeechobee flowage. Porcher and Rayner (2001) reported that soils in cataract bog areas in South Carolina were high in magnesium and calcium, but Michigan soil was higher in calcium and magnesium while south Florida soil was higher in calcium. Because soil analysis for reference habitats in South Carolina were not found, it is difficult to assess what Porcher and Rayner (2001) meant by high levels.

Assessing the nutrients and the appropriate concentrations from soil analysis to promote seed germination is difficult because nutrient levels in the soils and germination media are not equivalent. However, general conclusions can be made by comparing the two. Seeds from South Carolina 3 germinated better on media with higher mineral salt concentrations, calcium, and magnesium. High germination of Michigan seeds was observed on P723 containing peptone, a source of organic nitrogen. While ½ MS was also supplemented with peptone, it also contained higher levels of inorganic nitrogen. Due to the higher organic matter in the Michigan soil, the higher organic nitrogen in P723 may have increased germination of Michigan seeds. Florida soils were generally nutrient poor with the exception of calcium. Because few differences in germination were observed among media within each Florida population, this may indicate that Florida seeds will germinate well on any media with a necessary supply of nutrients. Further investigation is needed to address soil nutrient availability in order to develop germination media based on soil analysis.

Conclusions

The results presented provide insight into physiological and developmental aspects that are important aspects of ecotypic differentiation. Based on *in vitro* seed germination studies, ecotypic differentiation is likely occurring within *C. tuberosus* evident by rapid germination and subsequent seedling development, as well as immediate corm formation in northern populations. Rapid corm development in northern plants may be a consequence of the relatively shorter growing season experienced by these populations. Conversely, southern plants display greater shoot biomass allocation and a slower tendency to form corms.

Results indicate that photoperiod is not a strong selection pressure for seed germination and subsequent development, but germination media containing different nutrients is a strong selection pressure. The differences in germination media reflect the variation in soil type and

nutrient availability among *C. tuberosus* habitats, and the species ability to adapt to wide-ranging conditions. The ability of *Calopogon tuberosus* to adapt to different soil types is remarkable. These soils include the alkaline, peat-based, and nutrient rich soils of northern fens to the acidic, nutrient poor, sand-based soils in north central Florida.

Table 2-1. Six stages of orchid seed development.

Stage	Description
1	Imbibed seed, swollen and turning green
2	Enlarged embryo without testa
3	Presence of shoot apex
4	Elongated shoot apex and rhizoids
5	Emerging leaf and developing roots
6	Seedling with fully developed leaves and roots

Table 2-2. Comparative mineral salt content of orchid seed germination media. BM-1 Terrestrial Orchid Medium (BM-1), Knudson C (KC), Malmgren Modified Terrestrial Orchid Medium (MM), Murashige and Skoog (MS), *Phyto*Technology Orchid Seed Sowing Medium (P723), Vacin and Went (VW).

	MM	BM-1	P723	VW	KC	½ MS
Macronutrients (mM)						
Ammonium			5.15	7.57	13.82	10.31
Calcium	0.73		0.75	1.93	2.12	1.50
Chlorine		0.0021	1.50		3.35	3.10
Magnesium	0.81	0.83	0.62	1.01	1.01	0.75
Nitrate			9.85	5.19	10.49	19.70
Potassium	0.55	2.20	5.62	7.03	5.19	10.89
Phosphate	1.03	2.20	0.31	3.77	1.84	0.63
Sulfate	0.92	1.10	0.71	8.71	8.69	0.86
Sodium	0.20	0.20	0.10	0.20		0.10
Micronutrients (μM)						
Boron		161.7	26.7			50
Cobalt		0.105	0.026			0.053
Copper		0.10	0.025			0.5
Iron	100	100.2	50	100	90	50
Iodine			1.25			2.50
Manganese	10	147.9	25	30	30	50
Molybdenum		1.03	0.26			0.52
Zinc		34.8	9.22			14.95
Organics (mg l^{-1})						
Biotin	0.05	0.05				
Casein hybrolysate	400	500				
Folic acid	0.5	0.5				
L-Glutamine		100				
Glycine	2.0	2.0				
myo-Inositol	100	100	100			100
Nicotinic acid		5.0	1.0			1.0
Peptone			2000			2000
Pyridoxine HCl		0.5	1.0			1.0
Thiamine HCl		0.5	10			10
Total mineral salt	4.35	6.98	24.72	35.54	46.72	48.01
Total inorganic N	n/a	n/a	15.00	12.76	24.31	30.01

Table 2-3. Comparative soil nutrient analysis from the study sites. All units are in mg kg^{-1} with the exception of organic matter (OM) and pH.

	Michigan	South Carolina 1	South Carolina 2	South Carolina 3	North Central Florida	South Florida
Total N	12453	1603	5150	1639	1705	5729
P	21.7	3.58	2.89	3.30	4.28	0.08
K	226.6	45.5	93.5	124.1	20.1	23.2
Ca	20780	99.3	876.2	605.0	670.2	6515
Mg	4194	20.3	316.7	128.5	38.4	50.4
Fe	9.86	86.5	46.3	276.8	81.4	0.10
OM (%)	78.5	7.45	19.3	11.4	6.74	8.91
pH	7.00	4.90	5.50	5.00	5.00	8.00

Table 2-4. Effect of scarification time on embryo viability of *Calopogon tuberosus* seeds from populations studied. Seeds were scarified for 0, 0.5, 1, 2, and 3 h in a 5% aqueous $CaClO_2$ solution prior to staining in tetrazolium. Values represent the mean of two replications with 100 seeds each. Means with the same letter within populations are not significantly different at α=0.05. All values are percent viability.

Treatment Length	South Florida	North Central Florida	South Carolina 1	South Carolina 2	South Carolina 3	Michigan
Control	78.3a	41.1b	4.40b	5.40b	14.9b	24.1b
0.5 hour	85.4a	50.0b	5.85b	11.6b	20.4b	30.7b
1 hour	79.0a	66.1a	10.6b	6.30b	39.1a	32.5b
2 hours	84.9a	63.8a	15.0b	11.5b	41.1a	33.3b
3 hours	84.3a	66.7a	25.0a	38.1a	42.1a	50.3a

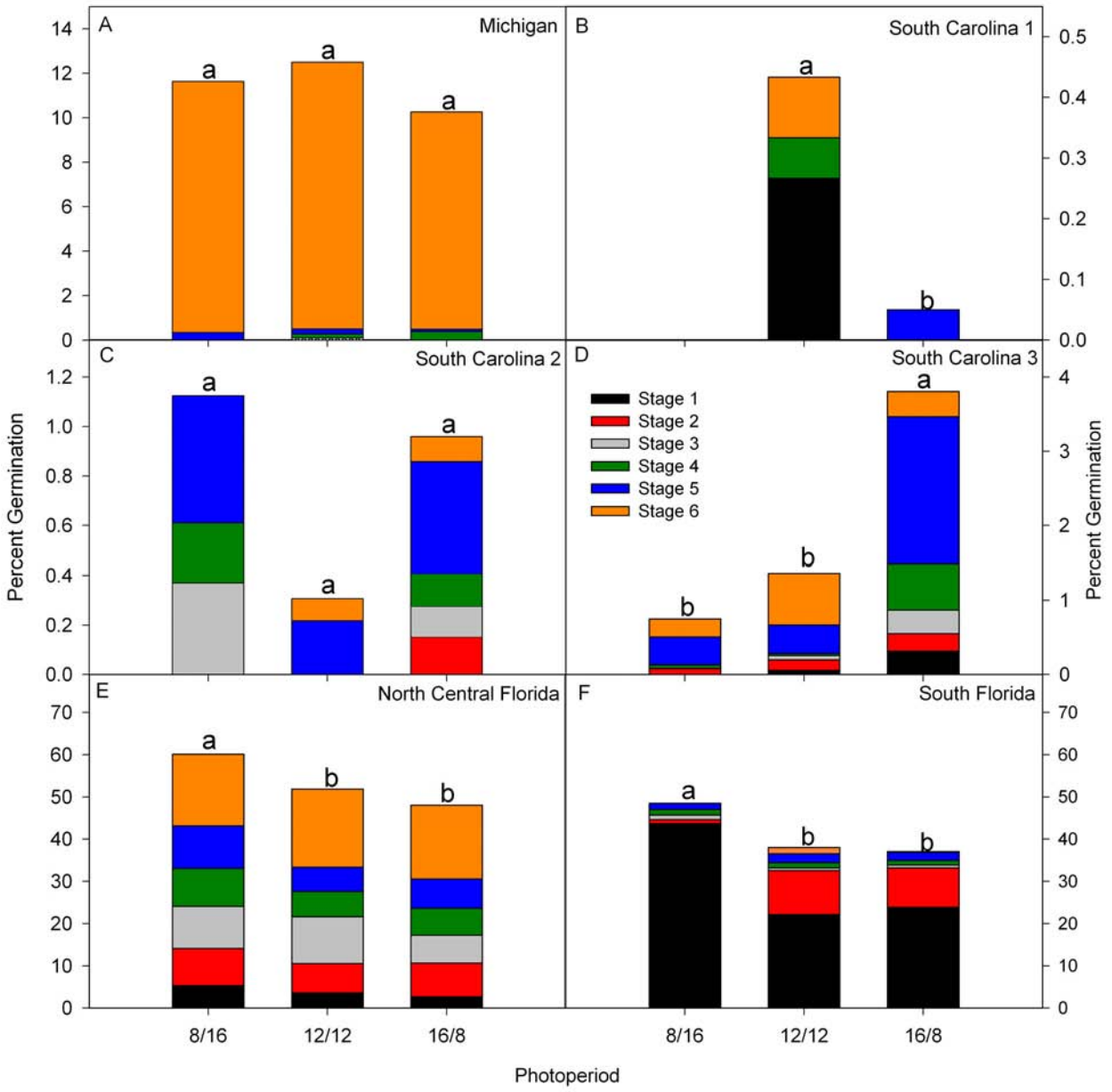

Figure 2-1. Photoperiod effects on *in vitro* seed germination and development of *Calopogon tuberosus* from widespread populations. Data was collected after 8 weeks culture on P723 medium. A) Michigan population from Carney Fen. B) South Carolina population from Ashmore Heritage Preserve. C) South Carolina population from Site C. D) South Carolina population from Eva Chandler Heritage Preserve. E) North central Florida population from Goethe State Forest. F) South Florida population from the Florida Panther National Wildlife Refuge. Histobars (mean response of 10 replications with 5 subreplications each) within each seed source with the same letter are not significantly different (α=0.05). See Table 2-1 for stages of development.

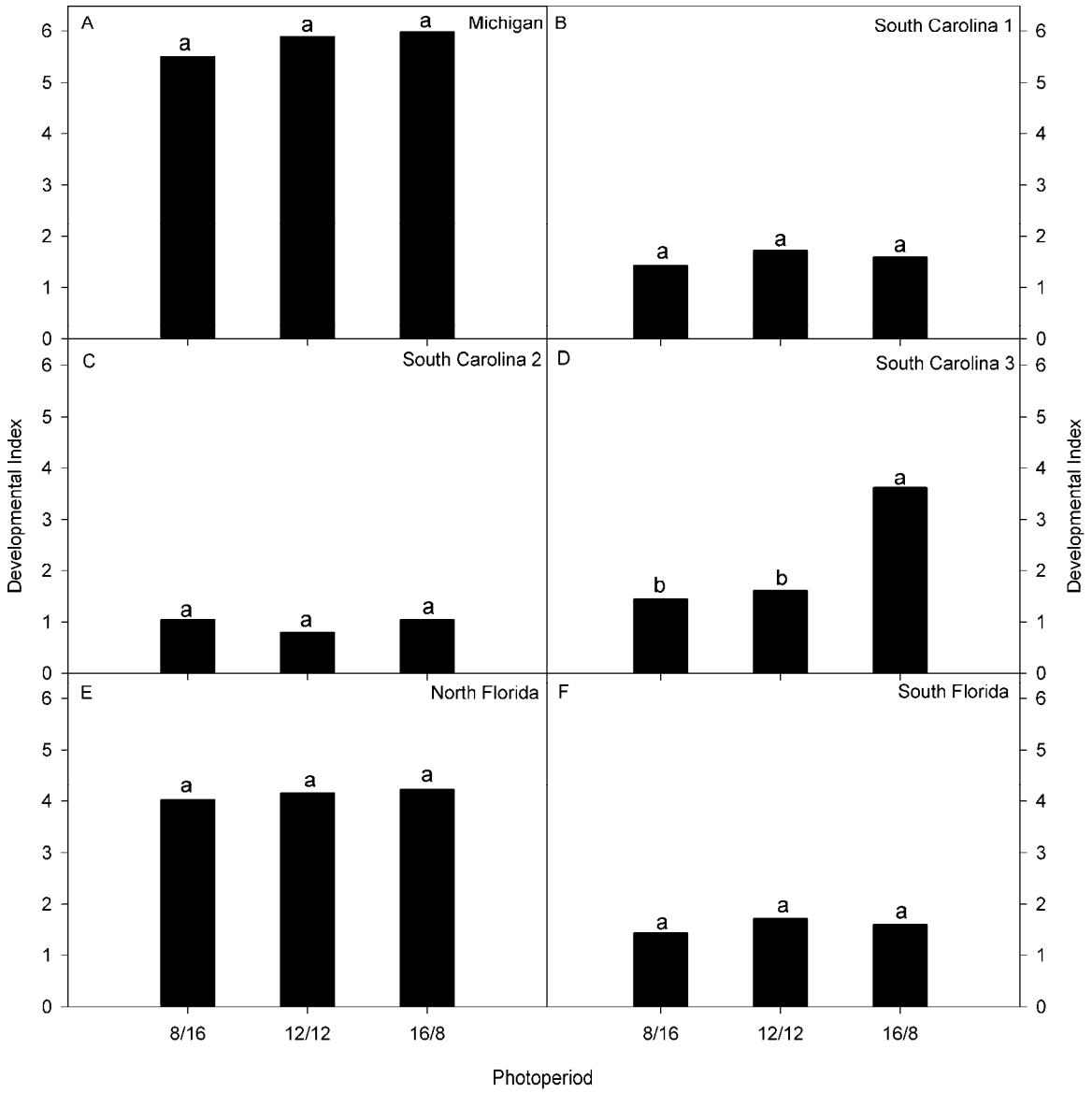

Figure 2-2. Photoperiodic effects on the developmental index of *Calopogon tuberosus* seedlings from widespread populations. Data was collected after 8 weeks culture on P723 medium. See Equation 3-1 for developmental index. A) Michigan population from Carney Fen. B) South Carolina population from Ashmore Heritage Preserve. C) South Carolina population from Site C. D) South Carolina population from Eva Chandler Heritage Preserve. E) North central Florida population from Goethe State Forest. F) South Florida population from the Florida Panther National Wildlife Refuge. Histobars (mean response of 10 replications with 5 subreplications each within each seed source with the same letter are not significantly different (α=0.05). Developmental index was calculated as follows: DI = (N_1 + N_2*2 + N_3*3 + N_4*4 +N_5*5 + N_6*6)/ (N_1 +N_2 + N_3 + N_4 + N_5 + N_6) where N_1 is the number of seeds in stage 1, etc.

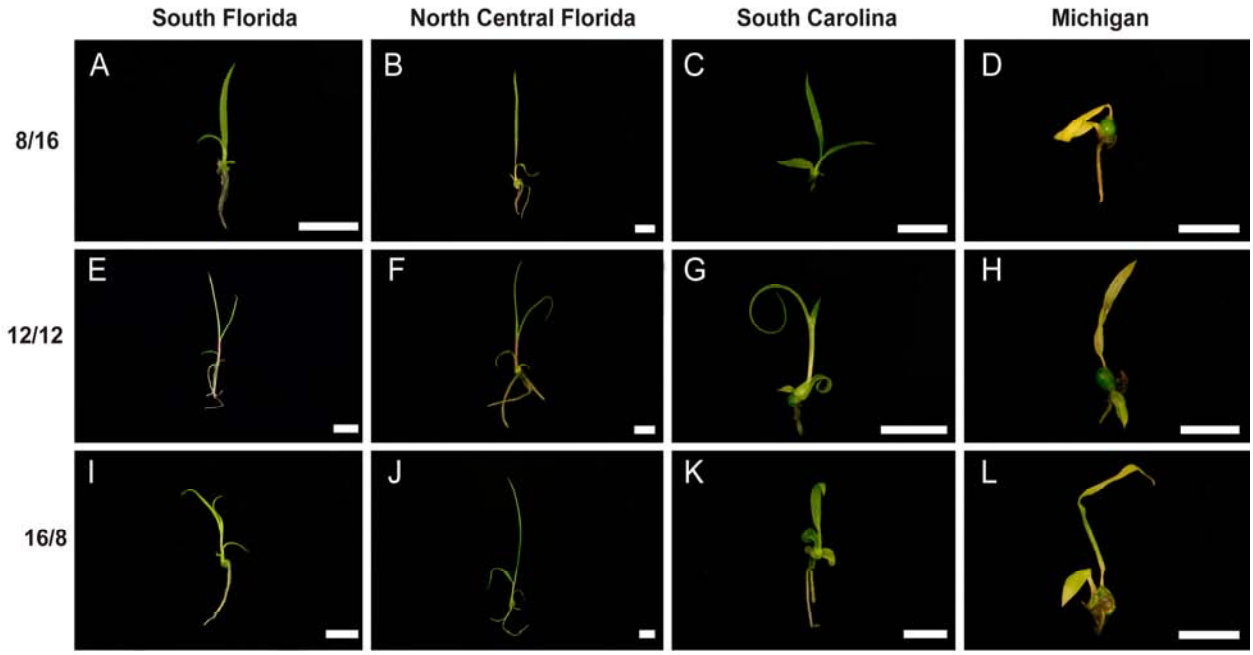

Figure 2-3. Effects of photoperiod on *in vitro* seedling development of *Calopogon tuberosus* from widespread populations. Seedlings represent average size after 16 weeks culture. A, B, C, D) Seedlings cultured under an 8/16 h L/D photoperiod. E, F, G, H) Seedlings cultured under a 12/12 h L/D photoperiod. I, J, K, L) Seedlings cultured under a 16/8 h L/D photoperiod. A, E, I) South Florida seedlings. B, F, J) North Central Florida seedlings. C, G, K) South Carolina seedlings. D, H, L) Michigan seedlings. Scale bars = 1 cm.

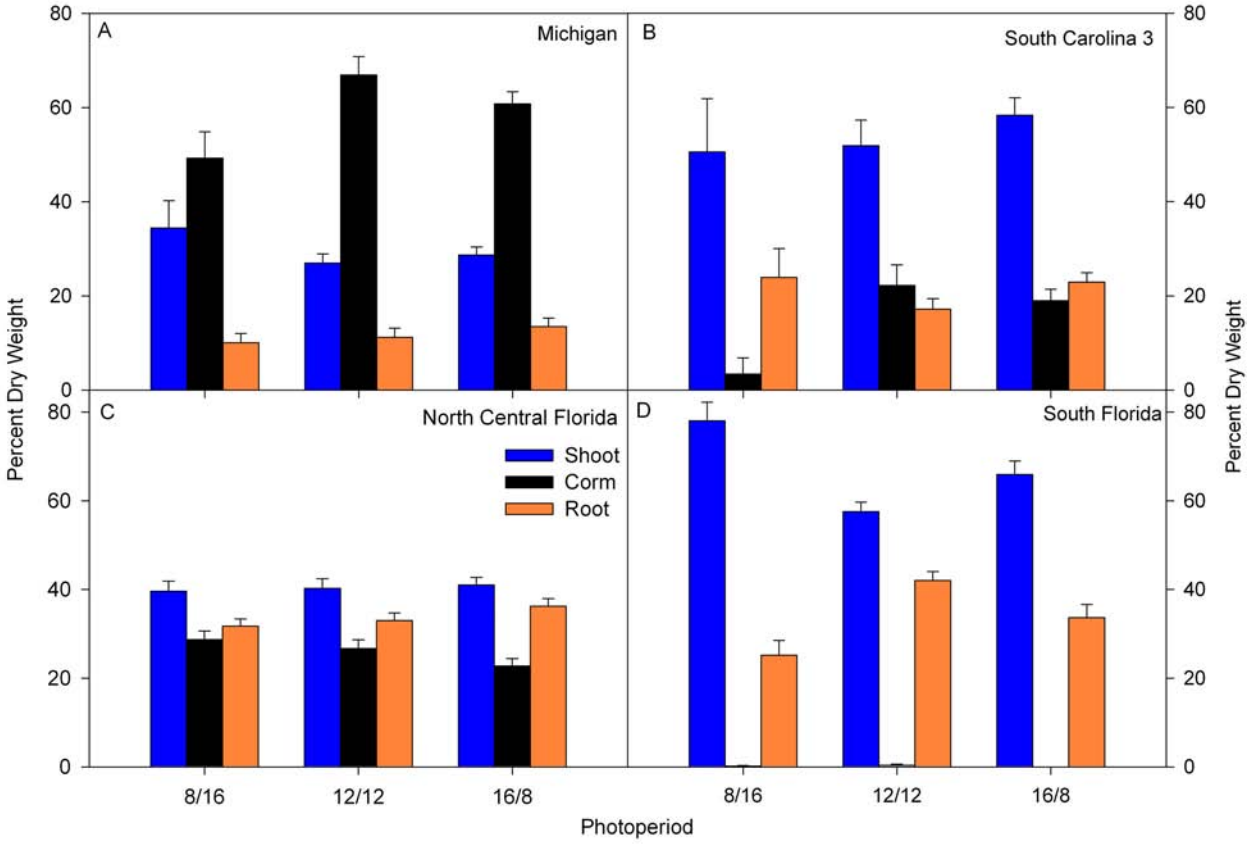

Figure 2-4. Percent dry weight biomass allocation in *Calopogon tuberosus* seedlings. Data was collected after 16 weeks *in vitro* culture. A) Michigan population. B) South Carolina population from Eva Chandler Heritage Preserve. C) North central Florida population. D) South Florida population. Histobars represent the mean response of 50 seedlings ± S.E.

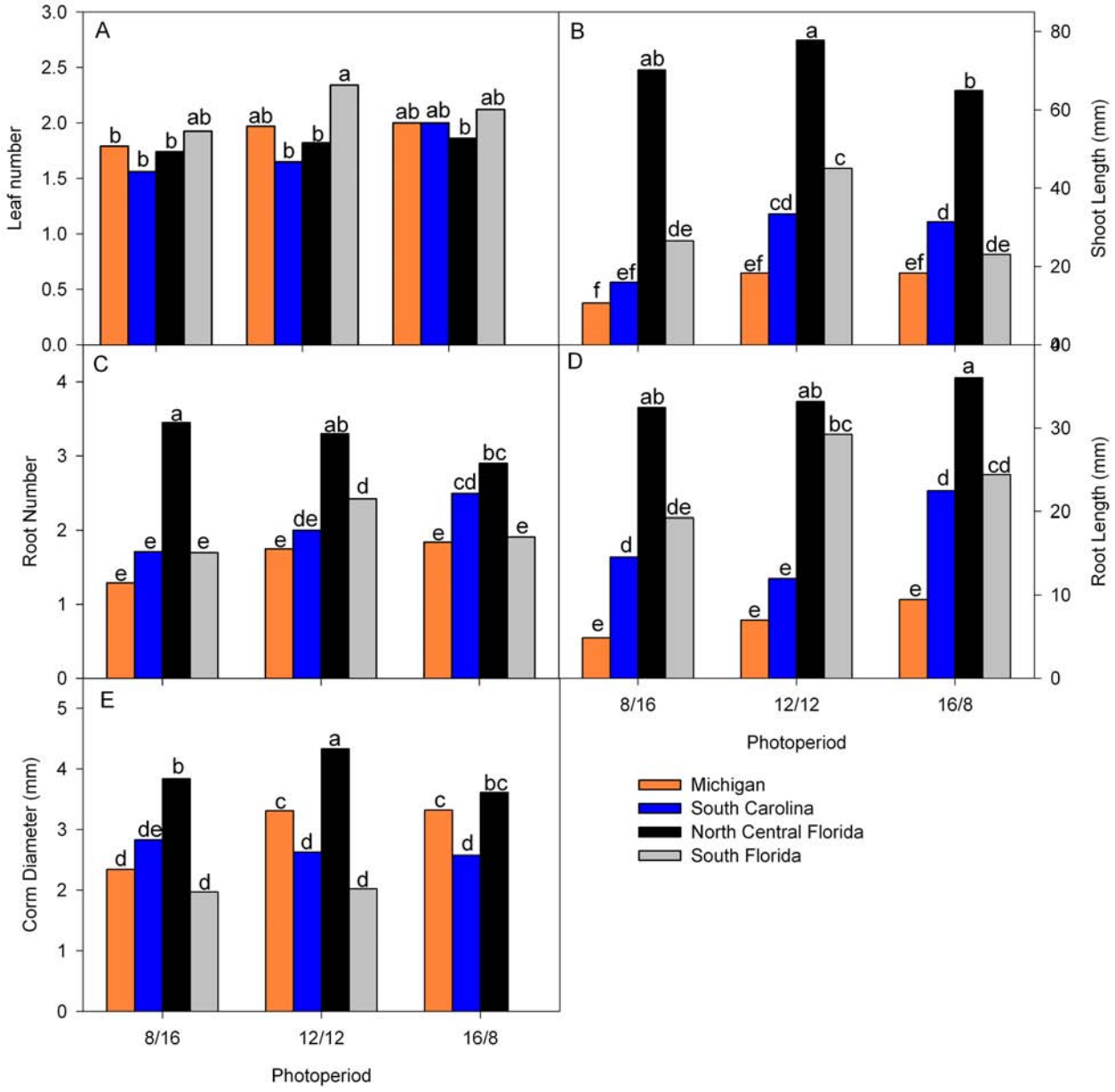

Figure 2-5. Comparative leaf number, shoot length, root number, root length, and corm diameter in *Calopogon tuberosus* seedlings from widespread populations. Data was collected after 16 weeks *in vitro* culture under short day (8/16 h light/dark), neutral day (12/12 h light/dark), and long day (16/8 h light/dark) photoperiods. Histobars represent the mean response of 50 seedlings. Histobars within with the same letter are not significantly different (α=0.05).

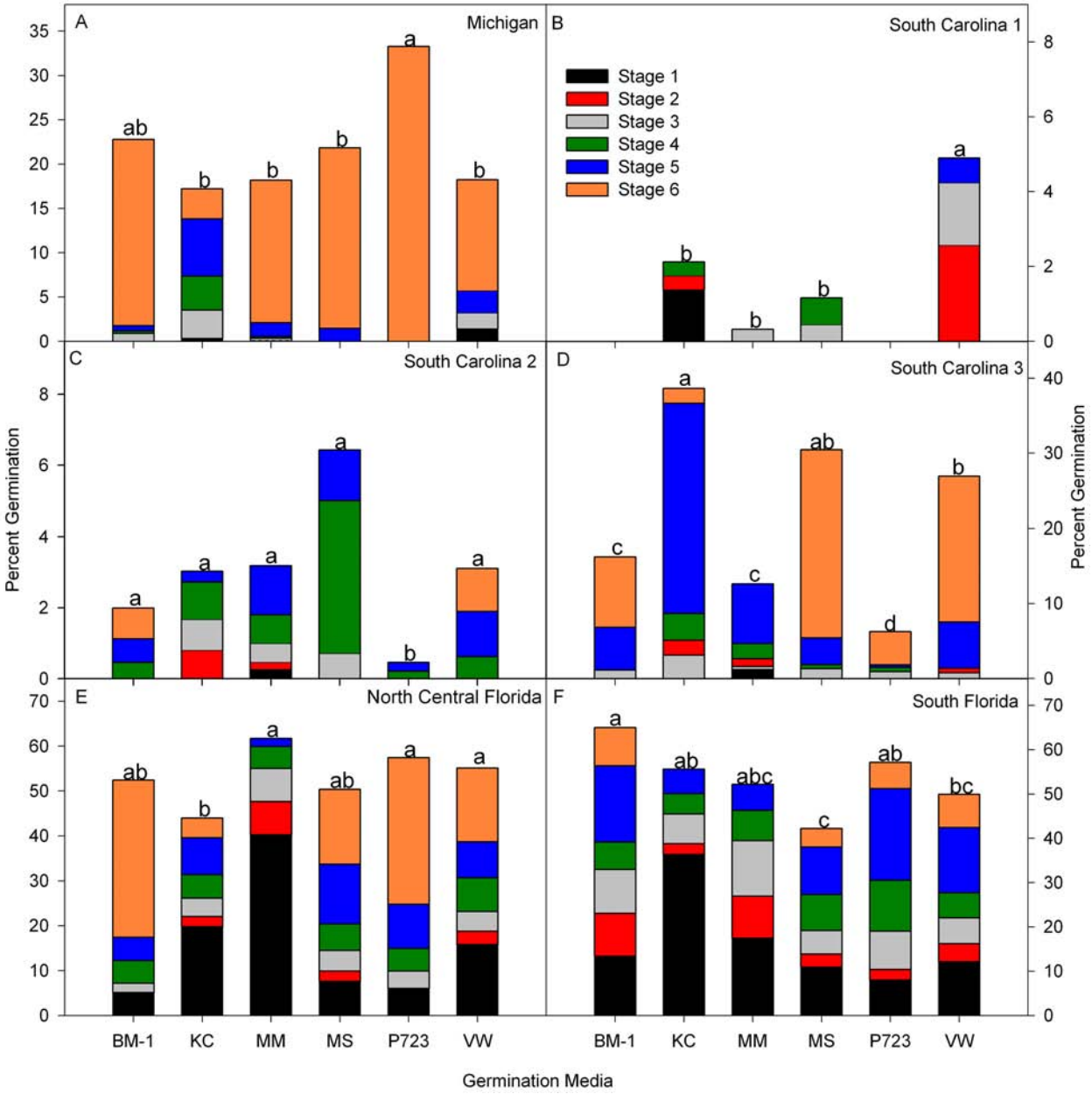

Figure 2-6. Effects of culture media on *in vitro* seed germination and subsequent development of *Calopogon tuberosus* from widespread populations. Data was collected after 8 weeks culture under a 12/12 h L/D photoperiod. A) Michigan population from Carney Fen. B) South Carolina population from Ashmore Heritage Preserve. C) South Carolina population from Site C. D) South Carolina population from Eva Chandler Heritage Preserve. E) North central Florida population from Goethe State Forest. F) South Florida population from the Florida Panther National Wildlife Refuge. Histobars (mean response of 5 replications with 3 subreplications each) within each seed source with the same letter are not significantly different (α=0.05). See Table 2-1 for stages of development. For media abbreviations and formulae see Table 2-2.

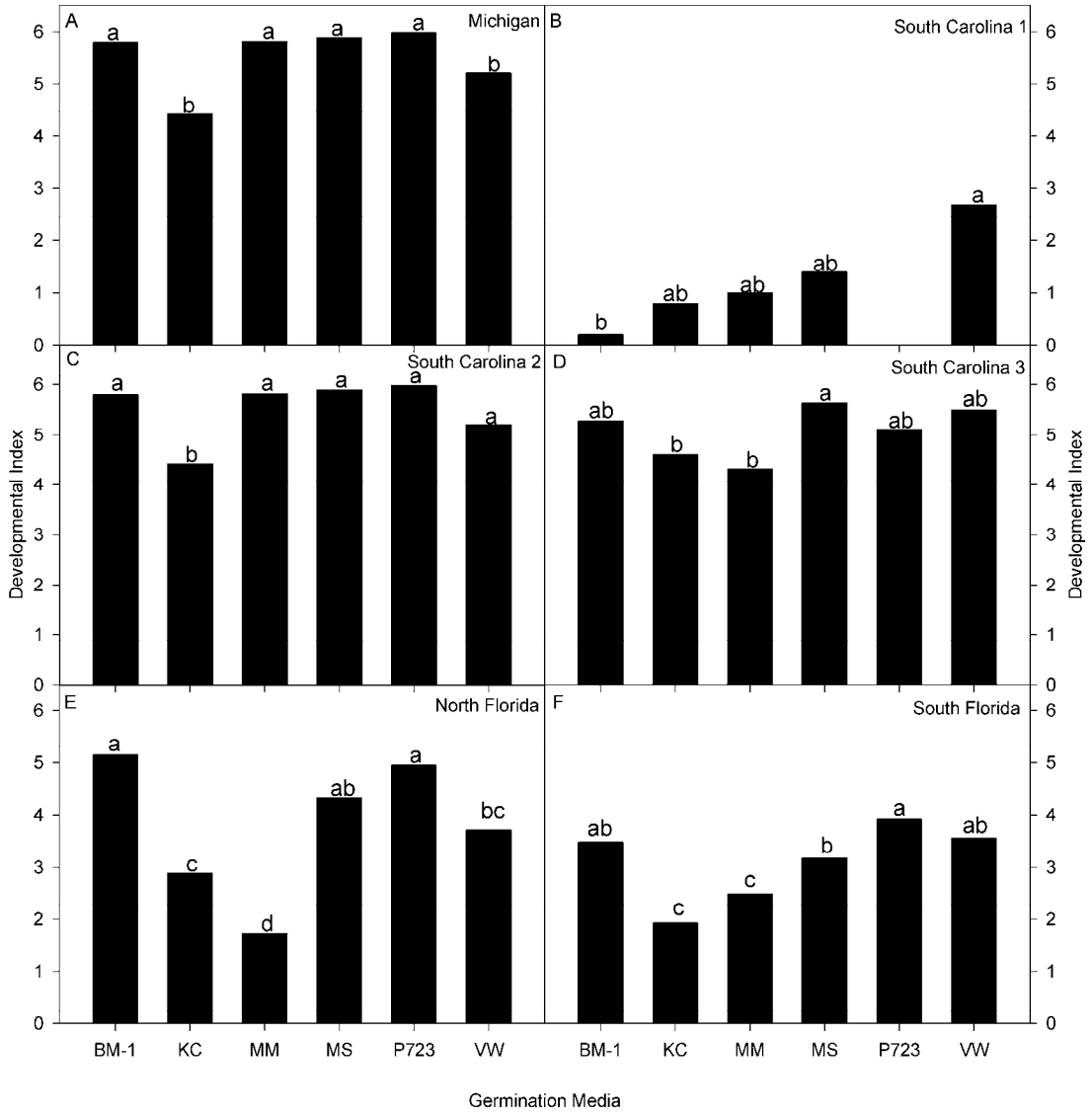

Figure 2-7. Effects of culture media on seedling developmental index of *Calopogon tuberosus* from different populations. Data was collected after 8 weeks culture under a 12/12 h L/D photoperiod. See equation 3-1 for developmental index. A) Michigan population from Carney Fen. B) South Carolina population from Ashmore Heritage Preserve. C) South Carolina population from Site C. D) South Carolina population from Eva Chandler Heritage Preserve. E) North central Florida population from Goethe State Forest. F) South Florida population from the Florida Panther National Wildlife Refuge. Histobars (mean response of 5 replications with 3 subreplications each) within each seed source with the same letter are not significantly different (α=0.05).

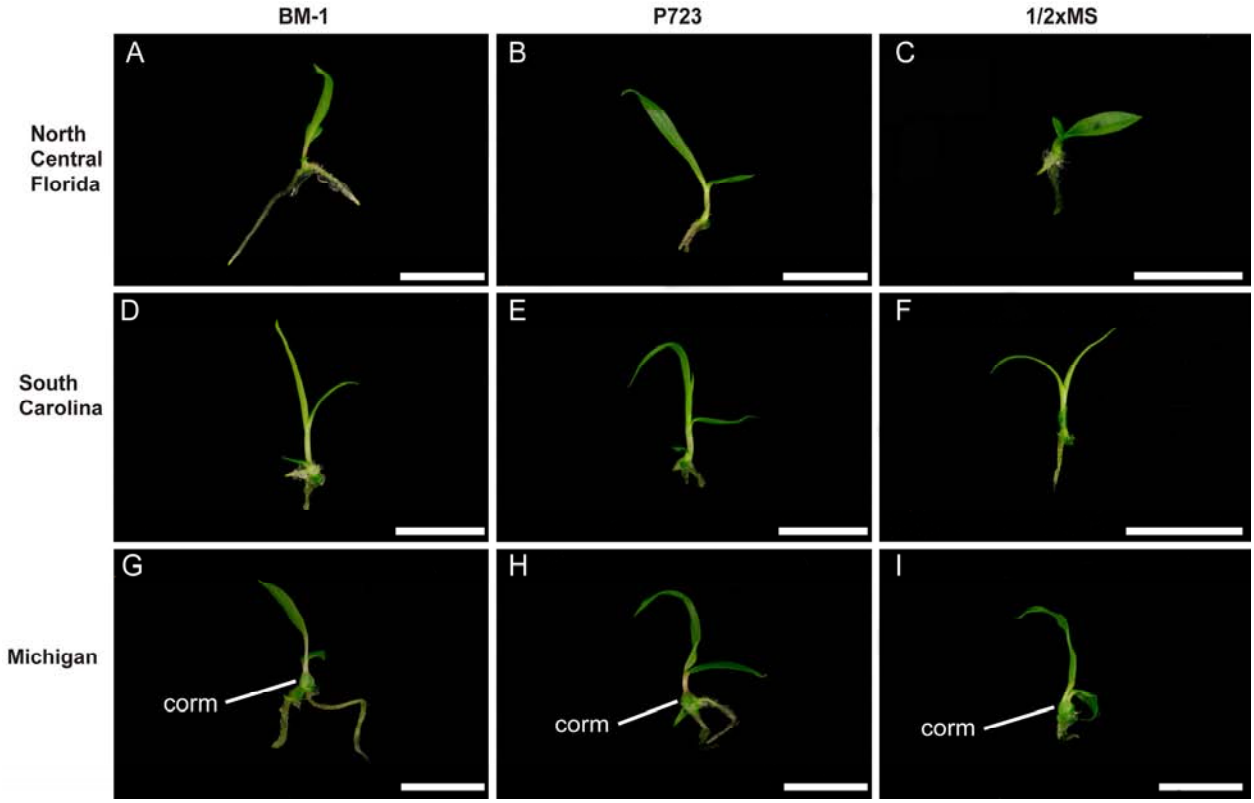

Figure 2-8. Culture media effects on early seedling development of *Calopogon tuberosus* from widespread populations. Seedlings representative average size after 8 weeks culture. A, B, C) North central Florida seedlings. D, E, F) South Carolina seedlings from Eva Chandler Heritage Preserve. G, H, I) Michigan seedlings. A, D, G) Seedlings cultured on BM-1 Terrestrial Orchid Medium. B, E, H) Seedlings cultured on P723 Orchid Seed Sowing Medium. C, F, I) Seedlings cultured on ½ MS. Scale bars = 1 cm.

CHAPTER 3
EFFECTS OF COLD STRATIFICATION AND DIURNAL TEMPERATURES ON *IN VITRO* GERMINATION OF *Calopogon tuberosus*

Introduction

Differences in photoperiod (Florida populations) and nutrient availability among germination media contributed to differences in seed germination and development among *Calopogon tuberosus* populations as found in Chapter 2. This may be caused by abiotic selection pressures such as soil nutrient availability and soil type as well as photoperiod influenced by latitude. Although different germination media increased germination of Michigan and South Carolina populations, germination was still relatively low compared to Florida populations. The low germination percentages may be influenced by a non-optimum temperature used in the previous experiments. Given that *C. tuberosus* populations are found in areas with different maximum and minimum temperatures, this may impose a selection pressure influencing ecotypic differentiation. Chilling length requirements may also be contributing to ecotypic differentiation due to the different winter conditions at each site.

Studying seed germination ecology has been reported as an effective method in detecting ecotypic differentiation in widespread plant populations (Seneca, 1972; Singh, 1973; Seneca, 1974; Probert *et al.,* 1985*b*). Understanding germination ecology is essential to further our knowledge about the timing of germination, seed maturation, seed dispersal, dormancy, and environmental cues that promote germination (Baskin and Baskin, 2001; Donohue, 2005). Studying the germination ecology of orchid seeds *in situ* is difficult and time consuming because orchid seeds are minute and germination is often low (Brundrett *et al.,* 2003; Zettler *et al.,* 2005*b*; Diez, 2007). Asymbiotic *in vitro* techniques were developed to successfully germination orchid seeds (Kauth *et al.,* 2008). *In vitro* conditions can also be manipulated to, at best, mimic *in situ* conditions such as photoperiod and temperature.

For many plant species temperature is a major factor responsible for the onset and breaking of seed dormancy (Baskin and Baskin, 2004). Unfortunately, the effects of temperature on orchid seed germination have largely been ignored compared to photoperiod and media nutrition. Furthermore, constant temperatures were used in orchid seed germination more often than fluctuating diurnal temperatures (Harvais, 1973; Rasmussen *et al.*, 1990*a*; Rasmussen and Rasmussen, 1991; Rasmussen, 1992; Mweetwa *et al.*, 2008). Using fluctuating temperatures was recommended over constant temperatures because seeds are not commonly exposed to constant temperatures in nature (Baskin *et al.*, 2006). However, constant temperatures improved *in vitro* germination of several orchids over alternating temperatures (van Waes and Debergh, 1986*a*). Regardless, selecting temperatures similar to those found *in situ* may lead to better insight into ecotypic differentiation by conducting *in vitro* common garden or transplant studies.

The role of cold-stratification in orchid seeds has also been examined, but its physiological role is not well-understood. In non-orchids cold-stratification was reported to decrease enzymatic reactions, slow metabolic processes that inhibit germination, or change enzyme production and concentration (Bewley and Black, 1994). However, reports of cold-stratification influence on orchid seed germination have been variable due to differences in the chilling length (Rasmussen, 1992; Tomita and Tomita, 1997; Miyoshi and Mii, 1998) and seed age (van Waes and Debergh, 1986*a*; De Pauw and Remphrey, 1993; Chu and Mudge, 1994). In addition, the effects of cold-stratification among distant populations of the same orchid species have not been reported. Examining the effects of chilling period on seed germination and embryo development may also provide further insight into ecotypic differentiation. Seeds from northern populations may require longer chill periods to increase germination since these seeds are exposed to longer winters.

The objectives were to: 1) Compare seed germination among latitudinally widespread *C. tuberosus* populations after chilling seeds; 2) Examine the role of diurnal temperatures on seed germination of latitudinally widespread *C. tuberosus* populations; 3) Examine differences in anatomy and morphology of *C. tuberosus* seeds from widespread populations; and 4) Validate the use of *in vitro* seed germination techniques to differentiate ecotypes.

Materials and Methods

Seed Source

Intact seed capsules (slightly yellow in color) were collected before dehiscence approximately 2 months after peak flowering. Capsules were collected from the Florida Panther National Wildlife Refuge (Collier County, Florida), Goethe State Forest (Levy County, Florida), Ashmore Heritage Preserve (Greenville County, South Carolina), Eva Chandler Heritage Preserve (Greenville County, South Carolina), Site C near Eva Chandler Heritage Preserve (Greenville County, South Carolina), and Carney Fen (Menominee County, Michigan). Non-dehisced capsules were collected to reduce the potential for surface contamination of individual seeds. Upon collecting and receiving capsules, seeds were pooled according to source and stored at 23°C over silica desiccant for 2 weeks. After 2 weeks, seeds were removed from the capsules and stored over silica desiccant at -11°C until use.

Media and Seed Preparation

BM-1 Terrestrial Orchid germination medium (chosen based on results from Chapter 2) supplemented with 0.1% activated charcoal was used as the germination medium for both the cold-stratification and temperature studies. The medium was prepared in 1000 mL batches, and the pH was adjusted to 5.7 with 0.1N KOH prior to autoclaving for 40 min at 117.7 kPa and 121°C. Forty mL sterile medium was dispensed into square 100x15 mm Petri plates with a 36-cell bottom (Integrid™ Petri Dish, Becton Dickinson and Company, Franklin Lakes, NJ, USA).

Mature seeds were surface sterilized in sterile scintillation vials for 3 min in a solution of 5 mL absolute ethanol, 5 mL 6% NaOCl, and 90 mL sterile dd water. Seeds were rinsed twice with sterile dd water after surface sterilization. Solutions were removed from the vials with sterile 5 mL glass Pasteur pipettes. Seeds were then placed on the surface of the germination medium with a 10µL sterile inoculating loop. The interior 16 cells of the Petri plates were used for subreplications to avoid areas of uneven medium drying at the edges. Petri plates were sealed with one layer of Nescofilm (Karlan Research Products, Santa Rosa, CA, USA). Seed germination and seedling development (Table 2-1) were monitored weekly for 8 weeks according to the six developmental stages described by Kauth (2005).

Cold-stratification Effects on Seed Germination

Upon seed inoculation, cultures were wrapped in aluminum foil and placed in continuous darkness at 9.9 ± 0.3°C for 2, 4, 6, or 8 weeks. A no chill control of 24.2 ± 0.2°C was also used. Five replications with three subreplications each were used for each seed source/chilling treatment combination. Approximately 57 seeds were sown onto each replicate plate (mean seeds per subreplication = 19) for an average of 285 seeds per treatment. After each chilling period, vessels were removed from cold-storage, unwrapped, and placed in a 12/12 h L/D photoperiod at 24.2 ± 0.2°C under cool-white fluorescent lights with a light level of 40 µmol m^{-2} s^{-1}. Culture vessels were initially scored upon removal from the dark-chill period. Germination and development were examined bi-weekly for 10 weeks after culture vessels were removed from cold storage.

Diurnal Temperature Effects on Seed Germination

Diurnal temperatures were chosen based on average seasonal day and night temperatures in Gainesville, FL. The following day/night temperature treatments were used: 33/24°C (summer); 29/19°C (spring); 27/15°C (fall); 22/11°C (winter). A constant temperature of 25°C was also

used. Five replications with three subreplications each were used for each seed source/chilling treatment combination. An average of 73.5 seeds were sown onto each plate (mean seeds per subreplication = 24.5) for an average of 367.5 seeds per treatment. BM-1 Terrestrial Orchid germination medium supplemented with 0.1% activated charcoal (B141; *Phyto*Technology Laboratories, Shawnee Mission, KS, USA) was used. Cultures were placed under a 12/12 L/D photoperiod and cool white fluorescent lights with a light level of 40 μmol m^{-2} s^{-1}. Cultures under fluctuating temperatures were incubated in Percival growth chambers (Model 130VL, Percival Scientific, Perry, IA, USA), while cultures in constant temperatures were placed in Percival growth chamber model 136LL. Cultures were incubated in light during higher temperature periods and incubated in darkness during the cooler temperature periods. Seed germination and seedling development were monitored bi-weekly for 8 weeks.

Scanning Electron Microscopy

Seeds from all six populations were observed by scanning electron microscopy (SEM). Seeds were not stored in fixative solutions, but prepared as dry seed. Seeds were stored at -11°C over desiccant until use as previously described. Seeds were gold sputter coated for 40 s at 45 mA before images were taken. Digital images were captured with a Hitachi S-4000 SEM (Hitachi Scientific Instruments, Danbury, CT) at 10.0 kV.

Histological Sectioning

Mature seeds stored at -11°C over desiccant were submitted for histological sectioning to the Interdisciplinary Center for Biotechnology Research Electron Microscopy and Bioimaging Laboratory at the University of Florida. Seeds were hydrated in distilled water for 24 h and fixed in formalin acetic acid alcohol (FAA) consisting of 50% ethanol (95%), 5% glacial acetic acid, 10% formalin (37% formaldehyde), and 35% distilled water. Seeds were then dehydrated in an ascending series of 95% and 100% ethanol followed by dehydration in 100% acetone. The ends

of the testae were cut to facilitate resin embedding. Dehydrated seeds were then embedded in Spurrs resin (Spurr, 1969) and cured at 60°C for 2 days. Polymerized blocks were trimmed and semi-thick sections (500 nm) were cur with a Leica Ultracut ultramicrotome R (Leica Microscopy and Scientific Instruments, Deerfield, IL). Sections were collected on glass slides and stained with toluidine blue. Sections were viewed under an Olympus BH2 brightfield microscope (Olympus America Inc., Center Valley, PA, USA). Images were captured with a Retiga-2000R Fast 1394 digital camera (QImaging, Surrey, British Columbia, Canada) and QCapture Pro 6 software (QImaging).

Statistical Analysis

Germination percentages were calculated by dividing the number of germinated seeds by the total number of seeds with an embryo in each subreplication. The percentage of embryos and seedlings in a developmental stage (Table 2-1) was calculated by dividing the number of seeds in a stage by the total number of seeds. A developmental index of germinated seeds was modified from Otero *et al.* (2004):

$$DI = \frac{(N_1 + N_2*2 + N_3*3 + N_4*4 + N_5*5 + N_6*6)}{(N_1 + N_2 + N_3 + N_4 + N_5 + N_6)}$$

where N_1 is the number of seeds in stage 1, etc. Stage 0 ungerminated seeds were excluded since only germinated seeds were of concern. Data were arcsine transformed to normalize variation. Germination, seedling development, and embryo viability data were analyzed using general linear model procedures and least square means using SAS v9.1 (SAS Institute, 2003).

Results

Effects of Cold-stratification on Seed Germination

Source ($F_4 = 44.2$, $p < 0.0001$), chilling period ($F_4 = 264.4$, $p < 0.0001$), and their interaction ($F_{16} = 22.4$, $p < 0.0001$) were highly significant for germination. Longer chilling

periods resulted in significantly higher germination percentages after 10 weeks culture with the exception of north central Florida (Figure 3-1). Lower germination was observed in unchilled seeds for all populations. In fact, no germination was observed in the control for South Carolina 2. In Michigan seeds, chill periods over 4 weeks promoted the highest seed germination. In both South Carolina populations chilling seeds for 6 or 8 weeks promoted the highest germination. No differences among chilling treatments were observed in north central Florida seeds, but germination of chilled seeds was significantly higher than unchilled seeds. Higher germination in south Florida seeds was observed following chilling periods of 4 weeks or longer. In south Florida (86.8%) and South Carolina 1 (86.4%) and 2 (88.1%), maximum germination was higher compared to 72.3% and 64.6% germination in Michigan and north central Florida seeds, respectively (Figure 3-1).

Effects of Diurnal Temperatures on Seed Germination and Early Development

Source ($F_5 = 410.4$, $p < 0.0001$), temperature ($F_4 = 10.6$, $p < 0.0001$), and their interaction ($F_{20} = 5.08$, $p < 0.0001$) were highly significant for germination. In all populations, cultures incubated at a constant 25°C reduced germination compared to at least one alternating temperature regime (Figure 3-2). Higher germination (>20%) in Michigan seeds occurred at temperatures below 33/24°C with the exception of 25°C (Figure 3-2A). Higher fluctuating temperatures promoted germination in all South Carolina populations. However, germination (<10%) was still low in South Carolina 1 and 2 populations regardless of temperature compared to all other seed sources (Figure 3-2B, C). Higher fluctuating temperatures (29/19°C and 33/24°C) promoted germination in north central Florida seeds (Figure 3-2E). Surprisingly, south Florida seed germination was highest at both 22/11°C and 33/24°C (Figure 3-2F). Among all populations, germination was highest in south Florida seeds, followed by north central Florida, South Carolina 3, Michigan, and South Carolina 1 and 2.

Source ($F_5 = 79.6$, $p < 0.0001$), temperature ($F_4 = 9.83$, $p < 0.0001$), and their interaction ($F_{20} = 4.38$, $p < 0.0001$) were highly significant for early development. Among seed sources, early development from weeks 1-8 was more advanced in Michigan (Figure 3-3A) and South Carolina 3 (Figure 3-3D) seedlings with the majority near or above stage 5. However, a large portion of embryos developed to stage 6 (Figure 3-3A, D). Above 22/11°C development was more advanced in Michigan, South Carolina 3, and north Central Florida (Figure 3-3E). Although South Carolina 1 and 2 embryos developed to stages 5 and 6 (Figure 3-3B, C), the average developmental index was less than 3 (Figure 3-3B, C). In fact, very few embryos among all populations developed to stages 5 and 6 (Figure 3-3). South Florida seedlings were more developed at temperatures below 33/24°C (Figure 3-3F), but the majority of embryos remained in stage 1 (Figure 3-3F).

Scanning Electron Microscopy

Overall Michigan and south Florida seeds were the smallest (Figure 3-4). The testae on all South Carolina seeds had cracks and holes, but this was not seen in seeds from Florida and only a few from Michigan. On average South Carolina seeds were the largest, followed by north central Florida, Michigan, and south Florida. South Florida seeds were consistently rounder than other seeds, while other seeds were more linear with tapered ends. Testae on Michigan and South Carolina seeds were more collapsed than Florida seeds, which may have been caused by the gold sputtering process or storing seed below freezing temperatures.

Histology

Seed cross sections revealed several interesting anatomical features. Seeds from Michigan and South Carolina were more oval shaped compared to the rounder seeds from Florida. The embryo was not evident in the Michigan seed possibly due to a poor sample (Figure 3-5A). The embryo contained few cells and was disorganized in the South Carolina 1 seed (Figure 3-5B).

Embryos in South Carolina 2 (Figure 3-5C), north central Florida (Figure 3-5D), and south Florida (Figure 3-5E) were well organized. Starch grains were evident in the north central Florida embryo (Figure 3-5D), while dehydration was noticeable in the south Florida and South Carolina 2 embryos. Testae surrounding the Florida seeds were generally one cell thick and thinner than other populations. The testa on the Michigan seed was approximately 15-30 µm thick, 5-20 µm on South Carolina 1 seed, 10-50 µm on South Carolina 2 seed, and 5-10 µm on both Florida populations.

Discussion

Beyond morphological variation reported (Goldman *et al.*, 2004*a*), differences in seed germination and seedling development in response to diurnal temperatures and cold-stratification among *C. tuberosus* populations provide further evidence for ecotypic differentiation. Morphological variation in *C. tuberosus* correlating to geographic location was likely caused by different selection pressures and abiotic factors (Goldman *et al.*, 2004*a*), but these selective pressures were not specifically explored with respect to ecotypic differentiation. Although photoperiod and nutrient availability were often concerns for successful orchid seed germination (for review see Kauth *et al.*, 2008), temperature should be included as well. The present results along with results from Chapter 2 indicate that photoperiod may not be as influential as temperature for *C. tuberosus* seed germination and development, especially for northern ecotypes.

Cold-stratification Effects on Seed Germination

Cold-stratification increased germination significantly compared to the control among all seed populations. This is not surprising since cold-stratification was reported to increase germination in numerous orchid species (De Pauw and Remphrey, 1993; Chu and Mudge, 1994; Tomita and Tomita, 1997; Miyoshi and Mii, 1998; Shimura and Koda, 2005; Øien *et al.*, 2008).

These reports were concerned with propagation and increasing germination, and not with the ecological aspect of chilling seeds with the exception of Øien *et al.* (2008). However, the present results first report the effects of chilling length on several populations of the same species. Although the exact mechanism of chilling in orchid seeds is not well-understood, cold-stratification likely breaks physiological dormancy under favorable environmental conditions (Baskin and Baskin, 2001). Breaking dormancy and germination requirements are factors in timing of *in situ* germination (Baskin and Baskin, 2001). Although a constant temperature was used after the chill period, previous research has indicated that cold-stratification may remove a requirement for fluctuating temperatures (van Assche *et al.*, 2003).

Calopogon tuberosus seeds exhibited some type of dormancy since chilling seeds increased germination significantly, and the degree of dormancy may differ among populations due to differences in testae thickness. Embryos may not be completely dormant, but rather the testa may be inducing physiological dormancy (Lauzer *et al.*, 2007). Once removed from the testa, embryos may germinate readily as reported for *Aplectrum hyemale* (Lauzer *et al.*, 2007). Delayed or non-existent germination may be caused by a high concentration of phenolic compounds in the testa, which reduces the permeability of the testa (Thompson *et al.*, 2001). Chilling seeds may release harmful phenolics by increasing the permeability of the testa (Thompson *et al.*, 2001).

South Carolina seeds required longer than 6 weeks chilling to obtain maximum germination, while Michigan and south Florida seeds required 4 weeks or longer, and north central Florida seeds required 2 weeks or longer. The longer chilling requirement in South Carolina seeds may be the influence of the micro-niche habitats that are mesic in spring and summer and xeric in fall and winter. Seeds likely require a longer chilling period to break

prolonged dormancy and germinate when the mesic environment and warmer temperatures return in spring.

The degree of dormancy is also likely different among populations since Michigan seeds did not germinate to the maximum percentages observed in other populations. Seeds from northern populations may require longer chilling periods that delay germination until late winter or spring and break dormancy (Meyer *et al.*, 1995; Allen and Meyer, 1998). Although non-chilled *C. tuberosus* seeds from Michigan did germinate, these seeds likely germinated quickly to form corms immediately in order to survive winter. South Florida seeds also required cold-stratification to promote maximum germination. Southern populations may require cold-stratification since fluctuating winter temperatures may lead to early seedling emergence and subsequent death from colder temperatures (Fowler and Dwight, 1694; Schütz and Milberg, 1997). The required chilling period may ensure that seeds germinate only upon experiencing temperatures (above 22/11°C) that do harm emerged seedlings. Nondormant seeds from south Florida germinate slowly with little embryo development indicating that autumn emergence is unlikely (Meyer *et al.*, 1995). South Florida seeds likely are not exposed to a long chilling period *in situ*, but long chilling periods lead to a breakdown of chilling cue resistance so seeds can germinate (Meyer, 1992).

Germination of chilled seeds exceeded the tested embryo viability in all populations, but more so in the northern *C. tuberosus* populations. Seeds from these populations may require a longer treatment in $CaOCl_2$ than the 3 h period to further degrade the testa. The thicker testae observed on seeds from northern populations may be a result of harsher climates during winter months providing extra protection for the embryo. Longer chilling periods may aid in weakening

the thicker testae of northern populations leading to increased germination. The well-organized embryos of Florida seeds may also lead to higher germination percentages in unchilled seeds.

Diurnal Temperature Effects on Seed Germination

The highest germination percentages were achieved under fluctuating diurnal rather than the constant temperatures. Since constant temperatures are not normally found *in situ* (Baskin *et al.*, 2006), the decrease in germination observed at 25°C is not surprising. Previous research with non-orchids indicated that fluctuating temperatures promoted germination more than constant temperatures (Thompson *et al.*, 1977; Thompson and Grime, 1983; Probert *et al.*, 1986). Although seed germination of *C. tuberosus* still occurred at 25°C, the presence of light may have removed the requirement for fluctuating temperature (Thompson and Grime, 1983). Given that temperature fluctuations occur more often at the soil surface, increased germination under fluctuating temperatures may represent a mechanism to prevent germination of deeply buried seeds (Thompson and Grime, 1983). Constant temperatures increased germination in *Dactylorhiza majalis*, which was attributed to germination and mycorrhizal fungi infection at deeper soil depths (Rasmussen and Rasmussen, 1991). However, *D. majalis* seeds exposed to fluctuating temperatures where not exposed to light, which may have enhanced germination (Toole *et al.*, 1955). Seeds near the soil surface are exposed to fluctuating temperatures, light, and higher nitrate concentrations than seeds buried deeper potentially leading to higher germination (Roberts and Benjamin, 1979).

In Michigan, *C. tuberosus* flowers into July with seed dehiscence occurring into September. Higher *in vitro* germination at 22/11°C and 29/19°C corresponds to late or early summer and early fall germination *in situ*. Seedling development was more advanced at temperatures over 22/11°C, which corresponds to summer development. Germination of Michigan seeds was much faster than other populations and corm development occurred within

6-8 weeks from initiation of germination. The present results also reaffirm that Michigan seeds germinate and develop to advanced stages more quickly than other seed sources. The onset of lower temperatures, which happens rapidly during September in Michigan, may cue seed dormancy or seedling leaf senescence. Seeds that do not germinate and develop storage organs may require cold-treatment to germinate the following spring. Once seeds germinate in spring, seedling development may be enhanced at higher temperatures. Determining when seeds germinate in nature may be elucidated by conducting *in situ* germination studies.

All South Carolina populations germinated best at higher temperatures. However, germination was still low in South Carolina 1 and 2 populations, which may have been caused by physiological dormancy. Populations from South Carolina 1 and 2 are located in micro-island habitats with shallow soil overlaying granite in close proximity to seasonal flowing water (Porcher and Rayner, 2001). Seeds may likely only germinate at higher temperatures because of the micro-niche habitat in these areas. Since the soil is shallow, colder temperatures may impact soil conditions more than areas with deeper soils. Also, since granite is found directly below the soil, the cataract bogs may be exposed to larger temperature fluctuations.

Seeds in north central Florida likely germinate in late summer or spring as indicated by the maximum in vitro germination at 29/19°C and 33/24°C. Seedling development was more advanced at 27/15°C and 29/19°C corresponding to spring and fall development. Since seeds in north central Florida are subject to a longer growing season than Michigan and South Carolina, thus fall development is likely. Seeds from north central Florida that germinate *in vitro* developed relatively fast with corms forming after 12 weeks culture, ensuring that seedlings have adequate storage organs to survive winter. Viable seeds that do not germinate in fall likely germinate and develop the following spring.

Germination of south Florida seeds was highest at 22/11°C and 33/24°C, while development was lowest at 33/24°C. This indicated that most germination may take place in the cooler winter months or warmer summer months, and that seeds may have cold- or warm-stratification requirements. Although germination at 33/24°C was equal 22/11°C, development was suppressed at 33/24°C. Warmer temperatures may place physiological stress on smaller embryos and seedlings, leading to embryo and seedling development under cooler temperatures in late winter. In South Florida, *C. tuberosus* seedlings from the previous growing season re-emerge in mid-to-late February when temperatures are not cold enough to damage seedlings (see Appendix A). Higher germination at 25°C compared to other populations may be a consequence of burial. Seeds may not germinate at the soil surface under higher temperatures, but rather germinate slowly underneath the soil and emerge under cooler temperatures (Probert *et al.*, 1985*b*).

Conclusions

These results provide insight into the nature of orchid ecology and physiology, and specifically germination requirements within a widespread species. Along with our previous results, these results provide further evidence of ecotypic differentiation for temperature within the geographic range of *C. tuberosus*. The results of both experiments indicates that *C. tuberosus* seeds may germinate before winter months and spring, and those seeds that germinate in spring require some period of chilling. However, chilling period and optimum temperatures differ depending on seed source, and local environmental cues promote germination when conditions are optimal for seedling survival (Bischoff *et al.*, 2006). Given that cold-stratification increased germination significantly, chilling seeds is an absolute requirement to break seed dormancy in Michigan and South Carolina seeds. Once dormancy is broken, seeds may become more

responsive to wider environmental conditions such as photoperiod; thus, the results from Chapter 2 may be different with chilled seeds.

When interpreting *in vit*ro data, it must be scrutinized closely since *in vitro* common garden studies like other controlled common garden studies can oversimplify the interaction among environmental factors. Under controlled environments, environmental conditions are controlled for to simplify complex interactions that would otherwise confound the experiments. However, environmental factors do not act alone, but photoperiod, soil nutrients, and temperature all interact and form a complex network directing ecotypic differentiation. *In vitro* seed germination is only one technique that can be utilized to differentiate ecotypes, although it is a reliable method. Combining *in vitro* results with *in situ* field germination data may provide more understanding into ecotypic differentiation since conditions experienced in the field differ from those *in vitro*.

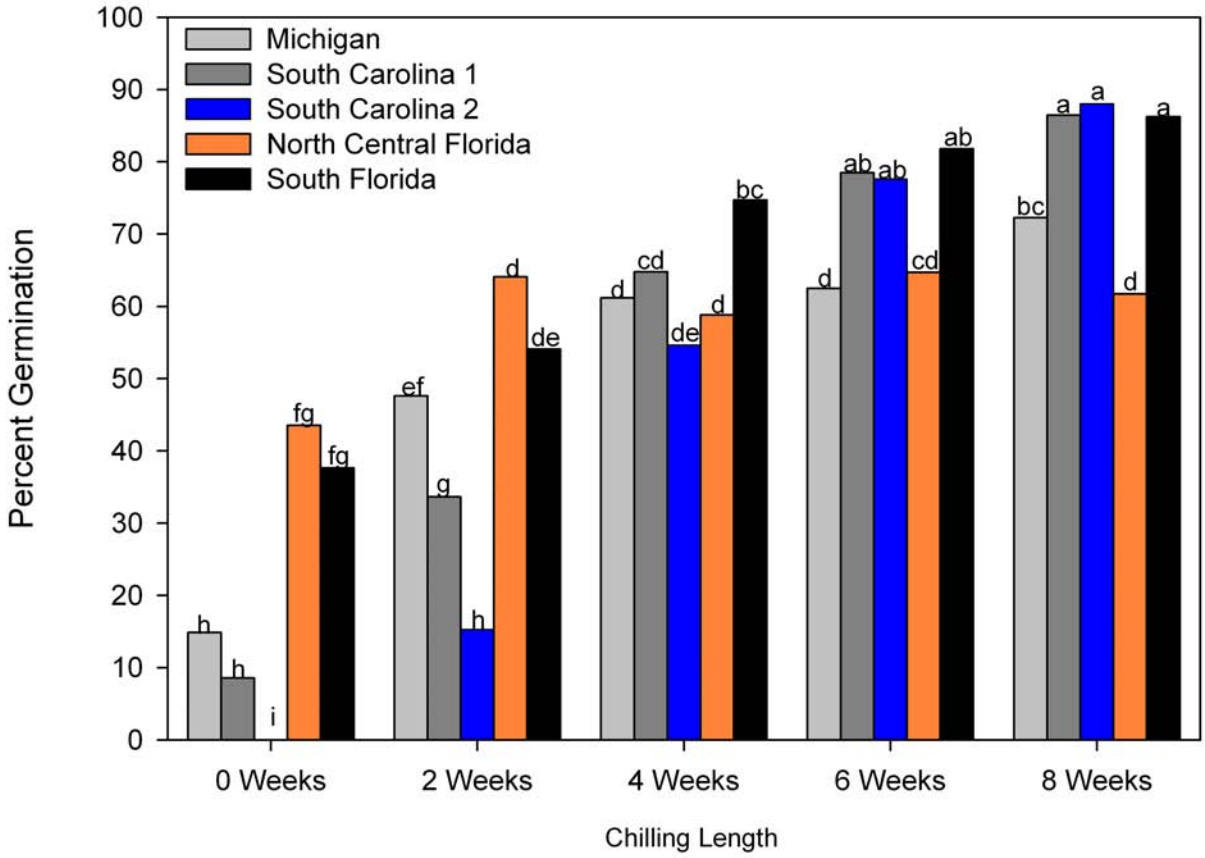

Figure 3-1. Effects of chilling seeds at 10°C in darkness on germination of *Calopogon tuberosus* seeds from distant populations. Data was collected after 10 weeks culture. Cultures were incubated under a 12 h photoperiod at 24°C following the chilling treatment. Histobars (mean response of 5 replications with 3 subreplications each) with the same letter are not significantly different (α=0.05).

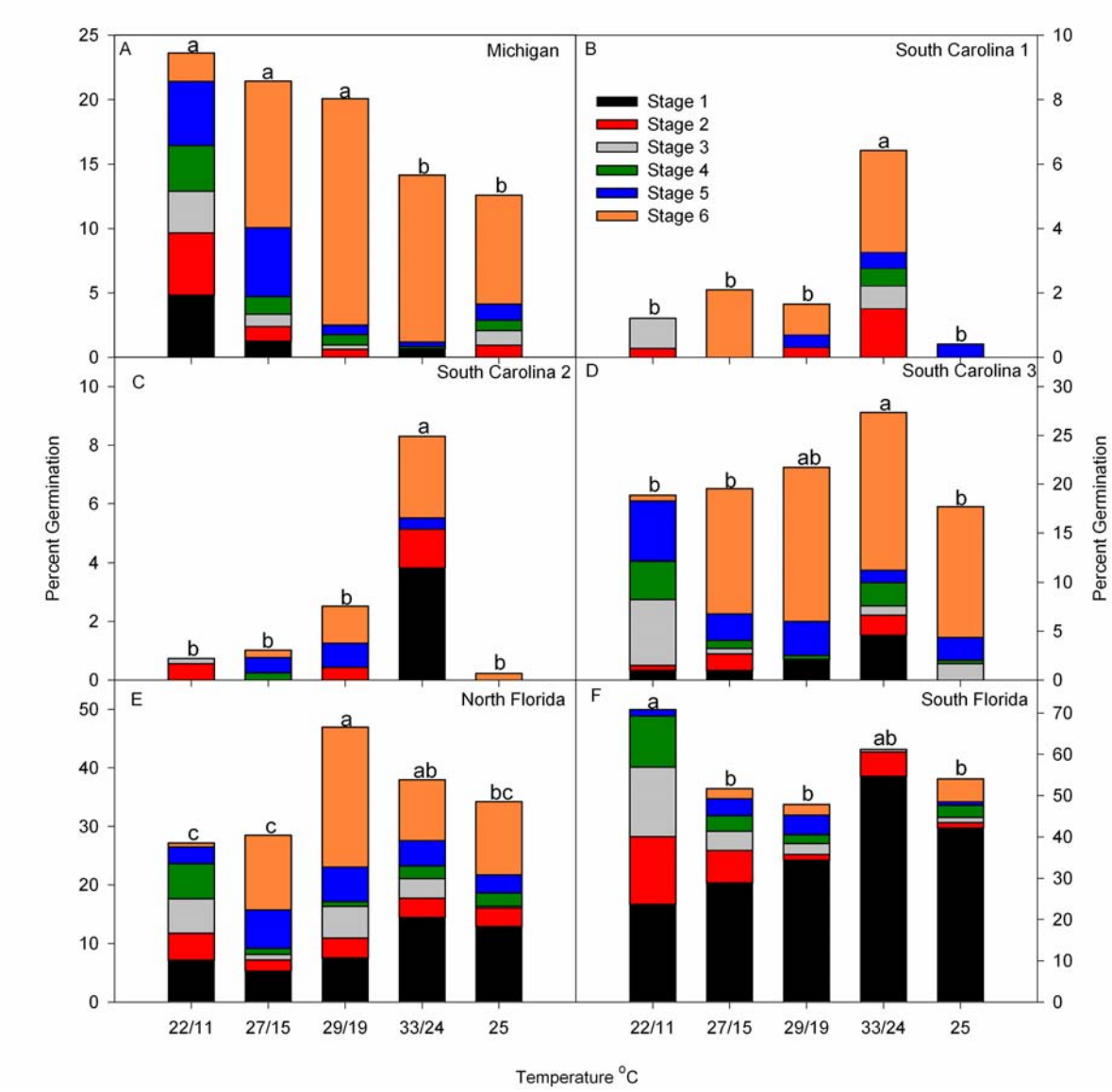

Figure 3-2. Diurnal temperature effects on germination and development of unchilled seed and *Calopogon tuberosus* seeds from different populations. Data was collected after 8 weeks culture under a 12 h photoperiod. A) Michigan population from Carney Fen. B) South Carolina population from Ashmore Heritage Preserve. C) South Carolina population from Site C. D) South Carolina population from Eva Chandler Heritage Preserve. E) North central Florida population from Goethe State Forest. F) South Florida population from the Florida Panther National Wildlife Refuge. Histobars (mean response of 5 replications with 3 subreplications each) within each seed source with the same letter are not significantly different (α=0.05). See Table 2-1 for stages of development.

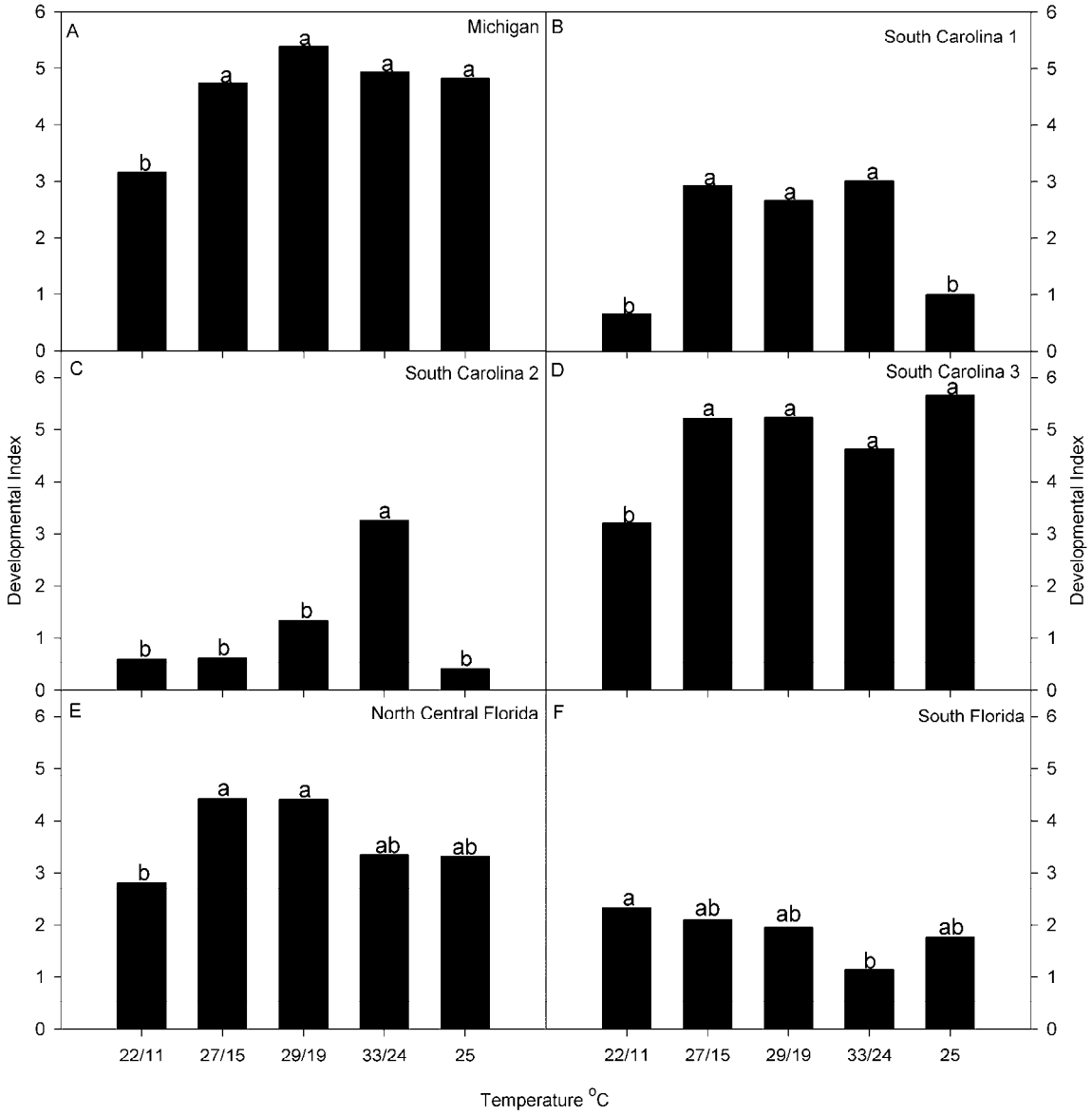

Figure 3-3. Developmental index of unchilled *Calopogon tuberosus* seeds from widespread populations. Seeds were incubated under diurnal temperatures for 8 weeks under a 12 h photoperiod. A) Michigan population from Carney Fen. B) South Carolina population from Ashmore Heritage Preserve. C) South Carolina population from Site C. D) South Carolina population from Eva Chandler Heritage Preserve. E) North central Florida population from Goethe State Forest. F) South Florida population from the Florida Panther National Wildlife Refuge. Histobars (mean response of 5 replications with 3 subreplications each) within each seed source with the same letter are not significantly different (α=0.05). Developmental index was calculated as follows: DI = (N_1 + N_2*2 + N_3*3 + N_4*4 +N_5*5 + N_6*6)/ (N_1 +N_2 + N_3 + N_4 + N_5 + N_6) where N_1 is the number of seeds in stage 1, etc.

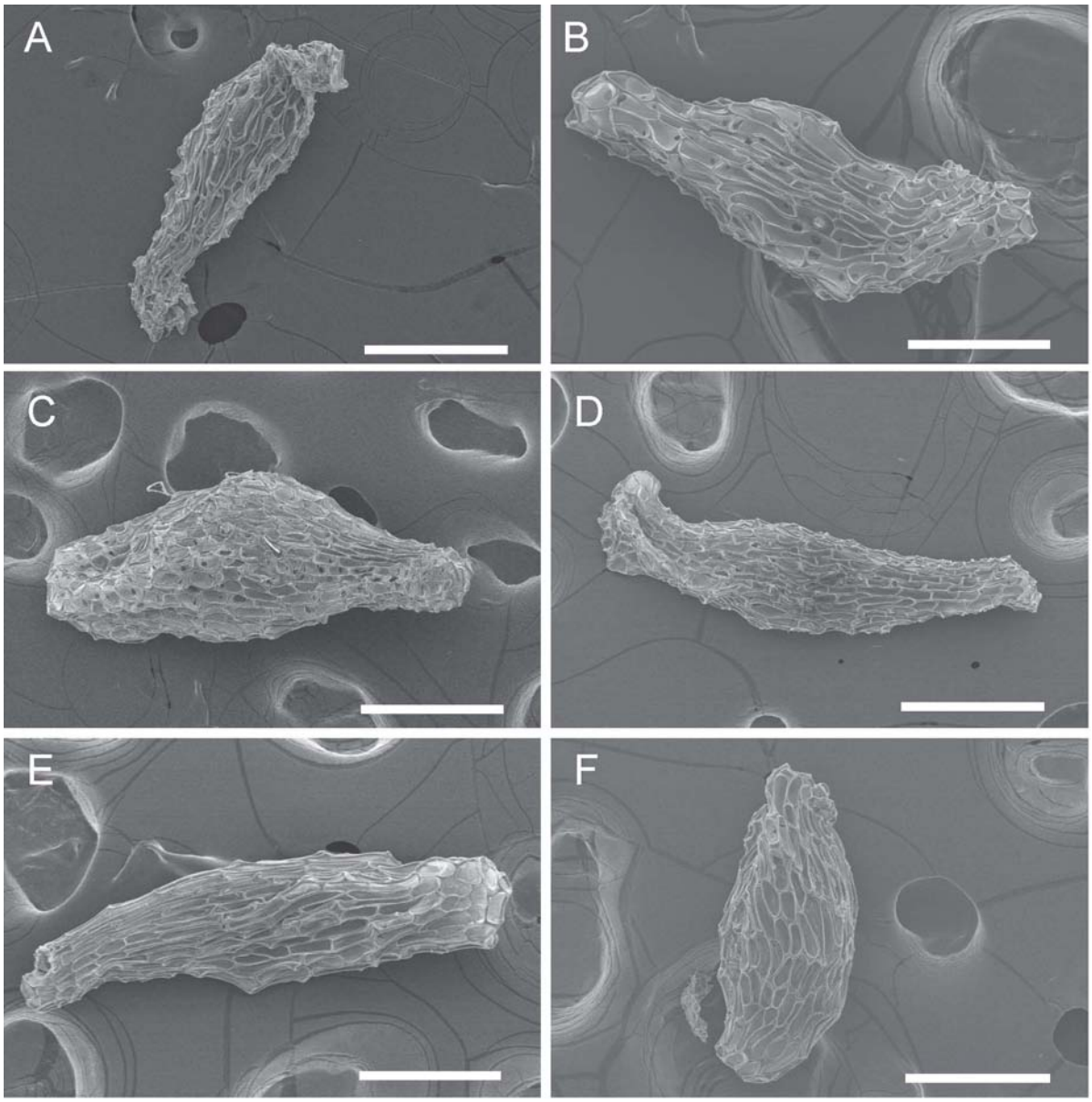

Figure 3-4. Comparative scanning electron microscopy of seeds from widespread populations of *Calopogon tuberosus*. A) Michigan population from Carney Fen. Scale bar = 150 µm at 200x. B) South Carolina population from Ashmore Heritage Preserve. Scale bar = 120 µm at 250x. C) South Carolina population from Site C. Scale bar = 200 µm at 150x. D) South Carolina population from Eva Chandler Heritage Preserve. Scale bar = 200 µm at 1500x. E) North central Florida population from Goethe State Forest. Scale bar = 167 µm at 180x. F) South Florida population from the Florida Panther National Wildlife Refuge. Scale bar = 167 µm at 180x.

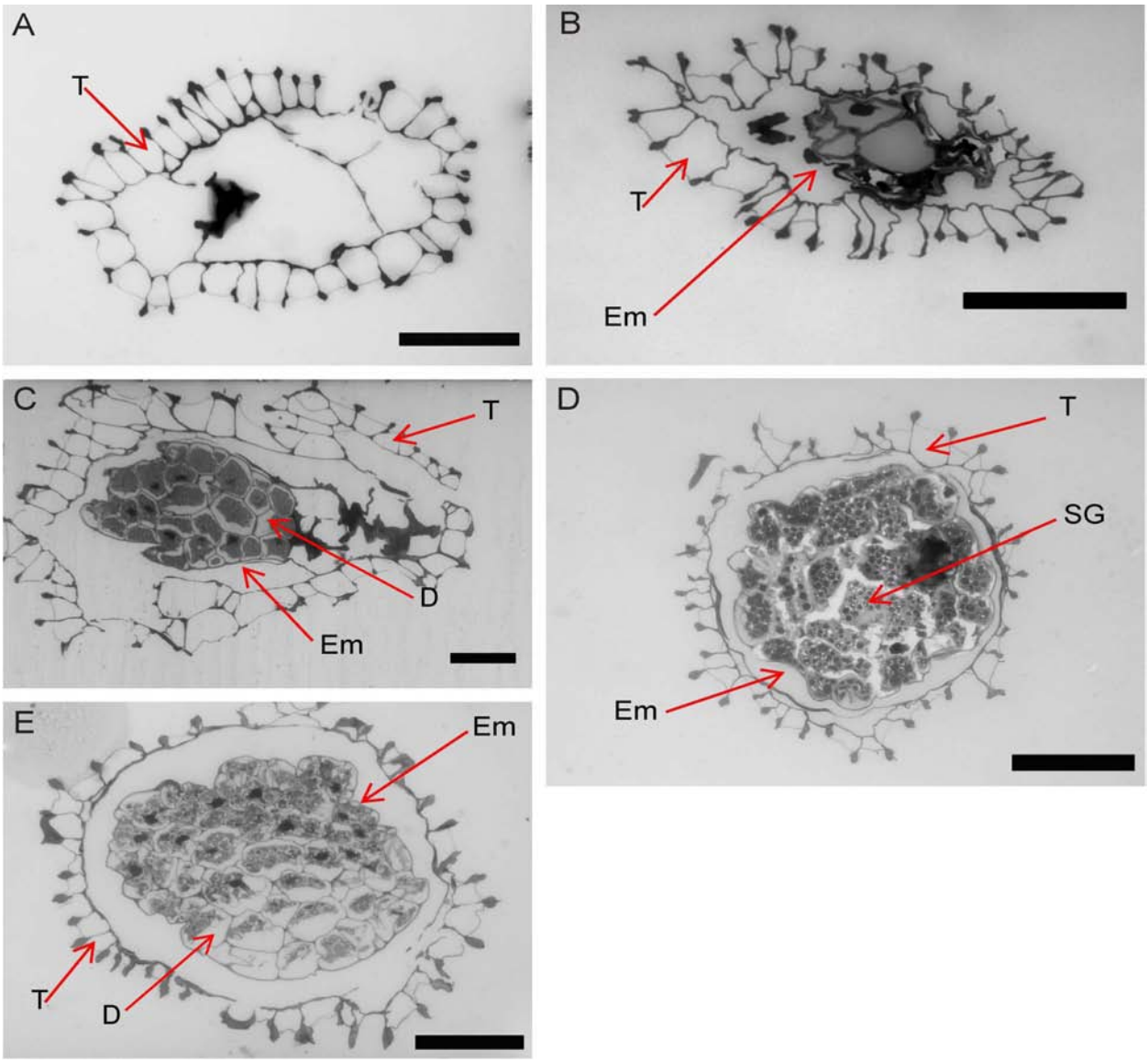

Figure 3-5. Light micrograph cross sections of mature *Calopogon tuberosus* seeds from widespread populations. A) Michigan seed at 40x. The lack of embryo is possibly due to a poor sample. B) South Carolina 1 seed at 40x. C) South Carolina 2 seed at 20x. D) North Central Florida at 40x. E) South Florida at 40x. T = testa; Em = embryo; SG = starch grains. Scale bars = 50 µm.

CHAPTER 4
COMPARATIVE SEEDLING BIOMASS ALLOCATION AND CORM FORMATION AMONG WIDESPREAD *Calopogon tuberosus* POPULATIONS

Introduction

A similar trend was observed throughout the experiments presented in Chapters 2 and 3. Regardless of seed germination treatment (photoperiod, germination media, temperature), Michigan seeds germinated quickly and seedlings developed corms before all other populations followed by South Carolina, north central Florida, and south Florida. The growing conditions at each population are markedly different with a short growing season and long winter in Michigan and a long growing season and short winter in south Florida. Thus timing of corm formation and differences in biomass allocation may be influenced by length of growing season, which in turn may be responsible for ecotypic differentiation.

Widely distributed plant species evolved the ability to survive wide environmental conditions leading to local adaptation to biotic and abiotic conditions (Linhart, 1995; Joshi *et al.*, 2001; Sanders and McGraw, 2005). Local adaptation in plants was first examined using common garden studies by Turesson (1922*a*), who first used the term *ecotype*, and by Clausen et al. (1941) using reciprocal transplant studies. These studies showed that local adaptation to environmental conditions, such as altitude and temperature, were influencing differences in growth and development of plant species. In recent years, the use of locally adapted plant material for restoration purposes was highlighted for the purpose of maintaining ecosystem function and stability because non-locally adapted ecotypes can reduce plant population fitness (Linhart and Grant, 1996; Hufford and Mazer, 2003; McKay *et al.*, 2005).

Local adaptation has been studied in numerous species through common garden and reciprocal transplant experiments (Nuismer and Gandon, 2008). Common garden studies test for adaptation and fitness of individuals from local or distant habitats in a common environment,

while transplant studies examine the role of non-local conditions on adaptation and fitness. Common garden studies may more efficiently test the genetic contribution to fitness while minimizing environmental impacts on fitness (Nuismer and Gandon, 2008). Transplant studies may better estimate environmental variation since individuals are transplanted to habitats with environmental conditions not experienced in the natural habitat (Nuismer and Gandon, 2008). Local adaptation can be studied by examining performance of ecotypes under different photoperiods (Howe *et al.*, 1995; Kurepin *et al.*, 2007), temperatures (Seneca, 1972; Probert *et al.*, 1985*b*), and soil regimes (Grześ, 2007; Sambatti and Rice, 2007).

Differences in biomass allocation were proposed as an important aspect of ecotypic differentiation. Northern ecotypes of *Spartina alterniflora* allocated more biomass to underground organs including roots and rhizomes (Gallagher, 1983; Gallagher and Howarth, 1987; Gross *et al.*, 1991). Greater biomass allocation to underground organs in northern ecotypes of several species was due to a shorter growing season (Potvin, 1986; Sawada *et al.*, 1994; Kane *et al.*, 2000; Liancourt and Tielbörger, 2009) and a higher allocation of carbohydrate reserves to overwintering structures (Mooney and Billings, 1960). Biomass allocation was also correlated with various reproductive strategies in ecotypes. Ecotypes found in fields or areas of younger succession allocated more biomass to reproductive organs than those in wooded habitats that allocated more biomass to vegetative structures (Abrahamson, 1975, 1979). Marsh plants that occupied areas of greater disturbance allocated more biomass and carbohydrate reserves to underground storage organs (Sun *et al.*, 2001; Peñas-Fronteras *et al.*, 2009)

Common garden and transplant studies can be performed in greenhouses, growth chambers, natural habitats, and outdoor plots (Gallagher *et al.*, 1988; Howe *et al.*, 1995; Majerowicz *et al.*, 2000; Suzuki, 2008), but obtaining permits to collect and transplant protected,

rare, threatened, or endangered species, as many orchids are, is difficult. Seeds can be used to produce mature plants for common garden and transplant studies. While this may be an effective method for quick-growing species, orchids often require four or more years to flower from initial seed germination (Stoutamire, 1964). Additionally, *in situ* orchid seed germination is difficult and time consuming since germination is often low (Brundrett *et al.*, 2003; Zettler *et al.*, 2005*b*; Diez, 2007). Alternatively, *in vitro* techniques can be used to study environmental requirements for orchid seed germination (Kauth *et al.*, 2008) as well as seedling growth and development (Dijk and Eck, 1995*b*).

Many *in vitro* culture techniques can be grouped under the discipline of *in vitro* ecology. *In vitro* ecology has been previously defined to include environmental and exogenous factors (i.e., temperature, light, gas phase, culture media) that affect *in vitro* growth and development (Hughes, 1981; Williams, 2007). Here, we further define *in vitro* ecology to include the evaluation and use of *in vitro* culture techniques to identify, propagate, evaluate, and select plant genotypes and ecotypes for ecological purposes. Specifically, *in vitro* ecology studies can be used to correlate environmental and genetic variables that affect plant growth and development *in vitro* with ecological factors affecting growth and development *in situ*. *In vitro* ecology could also be used to assess ecotypic differentiation for habitat restoration and plant reintroduction programs by conducting *in vitro* common garden studies under controlled environmental conditions. Since this use of *in vitro* ecology is a new area of research its validity must be verified.

Based on morphological variation, Goldman *et al.* (2004*a*) defined three distinct geographic areas of *Calopogon tuberosus*: northern plants in glaciated areas, southwest populations west of the Mississippi Embayment, and southeast populations east of the

Mississippi River and south of the glaciated zone. However, Goldman *et al.* (2004*a*) did not classify *C. tuberosus* ecotypes, but stated that variation in *C. tuberosus* could be caused by environmental conditions. Further ecotypic differentiation has not been previously explored in *C. tuberosus*. Although morphological and genetic variation exists in *C. tuberosus*, all plants throughout its range form corms. Differences in biomass allocation among *C. tuberosus* populations were reported in chapter 2. However, a detailed timecourse comparison for *C. tuberosus* seedling development has not been reported, and little information exists regarding this area of research for ecotypes in general.

Evaluation of *in vitro* seedling development with regards to biomass allocation and timing of corm formation from several *C. tuberosus* populations from diverse geographic sources might clarify the extent of ecotypic differentiation across its range. Seedling development in relation to the effects of photoperiod, germination media, and temperature was examined in Chapters 2 and 3. However, a detailed comparison of seedling biomass allocation and corm formation was not examined over several weeks. The objectives were to: 1) Validate the use of *in vitro* common garden studies to differentiate ecotypes; 2) Examine differences in the timing of corm formation among widespread *C. tuberosus* populations; and 3) Compare seedling biomass allocation among widespread *C. tuberosus* populations.

Materials and Methods

Seed Source

Seeds were collected from the following locations: Carney Fen (Menominee County, Michigan, USA), Eva Chandler Heritage Preserve (Greenville County, South Carolina, USA), Goethe State Forest (Levy County, Florida, USA), Florida Panther National Wildlife Refuge (Collier County, Florida, USA). Seed lots from Ohio, Oklahoma, and Central Florida were obtained from cultivated plants that were hand pollinated under greenhouse conditions (D.

Goldman). Seeds from greenhouse plants were used to determine if germination and development would still be different after years of *ex situ* cultivation. Seed capsules from all populations were collected before complete dehiscence and were stored at 23°C over silica gel for 2 weeks. Seeds were then removed from capsules, pooled by geographic source, and stored in complete darkness at -11°C until used.

Seed Germination and Medium Preparation

Seeds were surface disinfected in sterile scintillation vials for 3 minutes in a solution of 5 mL absolute ethanol, 5 mL 6% NaOCl, and 90 mL sterile distilled-deionized (dd) water. Seeds were rinsed with sterile dd water after surface sterilization, and solutions were removed with sterile Pasteur pipettes. Seeds were transferred with a sterile inoculating loop to BM-1 Terrestrial Orchid Medium (*Phyto*Technology Laboratories, Shawnee Mission, KS, USA) in 100 x 15 mm Petri plates (Fisher Scientific, Pittsburgh, PA, USA). The medium was supplemented with 1% activated charcoal. Medium pH was adjusted to 5.7 with 0.1N KOH prior to autoclaving for 40 minutes at 117.7 kPa and 121°C. Ten replicate Petri plates with 30 mL medium each were used for each seed source with approximately 100 seeds per plate. Cultures were placed in an environmental growth chamber (#I-35LL; Percival Scientific, Perry, IA, USA) under cool-white fluorescent lights in a 12/12 hr photoperiod at 24.2 ± 0.2°C and a light level of 40 µmol m^{-2} s^{-1}.

Seedling Transfer and Data Collection

After 6 weeks culture seedlings were transferred from Petri plates to *Phyto*Tech Culture Boxes (*Phyto*Technology Laboratories) containing 100 mL of BM-1 medium. Medium was prepared as described previously. Uniform-sized seedlings with developing leaves were then transferred to individual culture boxes. Three *Phyto*Tech Culture Boxes with nine seedlings each were prepared per seed source per week. A total of 21 *Phyto*Tech Culture Boxes were prepared

per seed source. Cultures were completely randomized within the growth chamber under the same conditions previously described.

Data were collected bi-weekly on three replicate *Phyto*Tech Culture Boxes containing nine seedlings each per seed source. Data were taken on 27 seedlings per seed source each week. Data for week 10 South Carolina seedlings were collected on two replications due to contamination of one replicate. Also, data was collected on two replications of Oklahoma and Ohio from weeks 12-20 due to the limited number of seeds that germinated and developed into seedlings.The following data were collected: shoot length, root number, root length, corm diameter, and dry weight. Shoot, root, and corm dry weights were measured after tissues were dried for 24 hours at 60°C. Seedling percent biomass allocation was determined by dividing corm, root, and shoot weights by the total seedling weight. Shoot length, root number, root length, corm diameter, and biomass data were statistically analyzed using general linear procedures, ANOVA, and Tukey's HSD test at α=0.05 in SAS v9.1 (SAS Institute, 2003). Regression and Pearson's correlation analyses were performed on corm biomass allocation and growing season length reported in Table 1-1. Corm biomass allocation data were arcsine transformed prior to regression analysis.

Results

Corm Formation

Timing of corm formation differed significantly by source ($F_6 = 48.2$, $p < 0.0001$), week ($F_6 = 80.9$, $p < 0.0001$), as well as source by week ($F_{36} = 8.97$, $p < 0.0001$). Corm formation on Michigan seedlings was evident by week 8, week 10 on South Carolina, Ohio, and Oklahoma seedlings, week 14 on north central Florida seedlings, week 16 on central Florida seedlings, and week 18 on south Florida seedlings (Table 4-1). Initial mean corm diameter on Michigan and South Carolina seedlings was similar until week 16. Mean corm diameter was similar in Michigan and south Florida seedlings, but south Florida seedlings continued to grow after week

20 (personal observation). Mean corm diameter was largest on South Carolina, north central and central Florida seedlings at week 20. Ohio seedling corms were only smaller than north central Florida seedling corms, while corms on Oklahoma seedlings were only larger than Michigan and south Florida corms by week 20 (Table 4-1).

Shoot Length

Shoot lengths were significantly different among sources ($F_6 = 266.7$, $p < 0.0001$), week ($F_6 = 256.9$, $p < 0.0001$), and source by week ($F_{36} = 45.4$, $p < 0.0001$). Initial shoot lengths on Michigan and South Carolina seedlings were larger than both Florida populations (Table 4-2). After week 12, mean shoot length on Michigan seedlings were the shortest of all seedlings. Shoot growth on Michigan seedlings did not significantly increase from week 8 to 16, but did decrease significantly there after. Similarly, shoot growth did not increase significantly on South Carolina seedlings from week 12 to 20. Shoots on north central and central Florida seedlings were the largest by week 14, and were the largest by week 20 as well. Shoot growth on Ohio and Oklahoma seedlings was similar to South Carolina. Shoot growth on south Florida seedlings was initially small, and only north central Florida seedlings exceeded mean shoot length of south Florida seedlings at week 20 (Table 4-2). Although leaf width measurements were not collected, leaves on Oklahoma seedlings were very thin compared to all other seedlings (personal observation).

Shoot senescence, characterized by yellowing and browning of leaves, began on Michigan seedlings after 16 weeks culture, and by week 20 almost 100% of shoots senesced (Figure 4-1M). Shoot senescence was observed at week 20 on Ohio seedlings. Shoot senescence was delayed in southern populations, with no senescence occurring by week 20 on any other seedling source.

Root Length and Number

Source ($F_6 = 160.9$, $p < 0.0001$), week ($F_6 = 105.8$, $p < 0.0001$), and the interaction between source and week ($F_{36} = 19.4$, $p < 0.0001$) all significantly influenced root number. By week 18, north central and central Florida seedlings had the most roots and this was seen at week 20 as well (Table 4-3). Michigan seedlings had the lowest number of roots throughout the experiment, and root number declined after week 16 due to root die-back. Root development was similar on Ohio and Oklahoma seedlings throughout and similar to South Carolina and south Florida seedlings by week 18 and 14, respectively (Table 4-3).

Root length was significantly influenced by source ($F_6 = 161.1$, $p < 0.0001$), week ($F_6 = 209.7$, $p < 0.0001$), and source by week ($F_{36} = 16.8$, $p < 0.0001$). At week 20, the longest roots were observed on north central and central Florida seedlings, and the shortest roots on Michigan seedlings (Table 4-4). By week 14, mean root length was longest on north central and central Florida seedlings. Root elongation was similar on Ohio, Oklahoma, South Carolina, and south Florida seedlings after week 16 and continued until week 20.

Biomass Allocation

ANOVA results revealed that percent biomass allocation to shoots, corms, and roots differed significantly among seed sources (Table 4-5). Corm biomass allocation was inversely related to latitude with the highest allocation being observed on Michigan seedlings. Approximately 97% biomass was allocated to corms in Michigan seedlings by week 20, which was significantly higher than the 77% in South Carolina seedlings, 74% in Ohio and Oklahoma seedlings, 53% in north central Florida seedlings, 35% in central Florida seedlings, and 7% to corms in south Florida seedlings (Table 4-6). By week 8, greater corm biomass allocation was evident on Michigan seedlings compared to all other populations. Higher corm biomass allocation compared to shoot and root biomass allocation was evident on Michigan seedling by

week 10. Corm biomass allocation on South Carolina, Ohio, and Oklahoma seedlings was significantly greater than all Florida populations after 20 weeks culture (Table 4-6). Allocation to corms was lowest in south Florida seedlings followed by central and north central Florida seedlings. Ohio, Oklahoma, and central Florida donor plants were under cultivation for more than 10 years, but still maintained genetic identity.

Percent shoot biomass allocation declined as greater biomass was allocated to corms regardless of population (Table 4-6). However, shoot biomass allocation was significantly higher on south Florida seedlings than all other seedling sources. Shoot biomass allocation of Ohio and Oklahoma seedlings was generally greater than Michigan, similar to South Carolina, and less than all Florida populations. Michigan seedlings had the lowest shoot biomass allocation. South Florida seedlings allocated more biomass to shoots compared to roots and corms over the entire 20 week period. Root biomass allocation was significantly higher on central Florida seedlings compared to all other populations, while root biomass was lowest on Michigan seedlings. Root biomass allocation on Ohio, Oklahoma, and South Carolina seedlings was similar throughout the experiment and was higher than Michigan seedlings, but lower than all Florida seedlings (Table 4-6).

Correlation analysis revealed a strong negative correlation between corm biomass allocation and growing season length: higher corm biomass allocation was correlated with shorter growing season lengths. Growing season was considered the number of days between the first spring and last fall frost. Pearson's correlation coefficients (all p values < 0.0001) were as follows: -0.73 (all weeks), -0.67 (week 8); -0.81 (week 10); -0.87 (week 12); -0.93 (week 14); -0.96 (week 16); -0.91 (week 18); -0.95 (week 20). Regression analysis also revealed a negative trend for all weeks (Figure 4-2). With the exception of week 8 and 10, regression models

accounted for much of the data variance with strong r^2 values over 0.75 (Figure 4-2). Due to the lack of corm formation in week 8 and 10 data, r^2 values were not as strong. When weekly data were combined the r^2 was 0.54, but the model was significant.

Discussion

This represents the first application of *in vitro* ecology to assess the extent of ecotypic differentiation of a latitudinally widespread orchid species through studying biomass allocation. Although information relating timing of biomass allocation to ecotypic differentiation is scarce (Gallagher, 1983; Gallagher and Howarth, 1987; Gross *et al.*, 1991; Seliksar *et al.*, 2002; Yoshie, 2007), timing of corm formation is an important factor in the ecotypic development of *C. tuberosus*. Few reports exist that utilize *in vitro* techniques to correlate ecotypic life history traits with *in vitro* growth strategies of orchids (Dijk and Eck, 1995*a*). The present results also show the potential use of *in vitro* common garden studies to detect unique growth strategies. In particular biomass allocation in *C. tuberosus* ecotypes is most likely influenced by growing season length.

Biomass allocation dynamics and storage organ function have been previously described *in situ* for single orchid populations (Whigham, 1984; Snow and Whigham, 1989; Zimmerman and Whigham, 1992; Tissue *et al.*, 1995; Øien and Pederson, 2003, 2005). However, biomass allocation in orchids has not been explored with respect to ecotypic differentiation. In the present study, *C. tuberosus* biomass allocation to corms ranged from 7% to 97%, depending on seed source. Whigham (1984) reported nearly 80% of biomass in a single *Tipularia discolor* population was allocated to underground storage organs. Zimmerman and Whigham (1992) reported that 61% and 66% of the total non-structural carbohydrates were allocated to the youngest corms in vegetative and dormant plants, respectively. In a detailed analysis of biomass allocation in *T. discolor*, 66% of the total biomass was allocated to corms during fruit maturation

and 80% during leaf senescence (Tissue *et al.*, 1995). These data are comparable to *C. tuberosus* since more biomass was allocated to corms just prior to and during leaf senescence. Although carbohydrate analysis of *C. tuberosus* was not investigated, reallocation of carbohydrates from leaves to corms might explain increased corm biomass allocation in *C. tuberosus* as was similarly reported for *Dactylorhiza lapponica* tubers (Øien and Pederson, 2005).

Regardless of orchid species, storage organs such as corms represent ecological adaptations to ensure survival during unfavorable growing conditions. In *T. discolor* corms are vital to support growth and reproduction (Zimmerman and Whigham, 1992; Tissue *et al.*, 1995), and serve as sinks for nutrient reserves (Whigham, 1984). Corms may also aid in long term survival by protecting the shoot meristem during periods of stress (Whigham, 1984).

Greater and faster biomass allocation to underground organs in northern *C. tuberosus* ecotypes followed a similar trend to ecotypes of *Spartina alterniflora* (Gallagher, 1983; Gallagher and Howarth, 1987; Gross *et al.*, 1991) and *Sagittaria latifolia* (Kane *et al.*, 2000; Kane *et al.*, 2003). The faster biomass allocation to corms in *C. tuberosus* is likely a selection pressure favored by a shorter growing season in more northern latitudes as reported with ecotypes of *S. alterniflora* (Seliksar *et al.*, 2002), *Plantago asiatica* (Sawada *et al.*, 1994), grass species (Potvin, 1986; Liancourt and Tielbörger, 2009), and *Eriophorum vaginatum* (Fetcher and Shaver, 1990). Northern ecotypes of *C. tuberosus* may allocate larger carbohydrate reserves in storage organs to survive winter conditions, and subsequently reallocate those carbohydrates to rapid growth the following spring (Seliksar *et al.*, 2002). Greater corm biomass in northern *C. tuberosus* ecotypes could be influenced by faster reallocation of carbohydrates from shoots to corms leading to faster shoot senescence compared to southern ecotypes (Mooney and Billings, 1960). Further investigation may also determine whether northern ecotypes are more tolerant to

freezing temperatures due to higher corm carbohydrate reserves (Hofgaard *et al.*, 2003; Shahba *et al.*, 2003), and thus are better able to survive in colder climates.

A short life cycle from initial shoot production to shoot senescence as well as low temperature tolerance is an adaptation to northern environments where the growing season is short (Potvin, 1986). Even under the same environmental conditions *in vitro*, northern *C. tuberosus* ecotypes expressed a shorter growth cycle and faster corm biomass allocation. Since seeds were collected directly from wild populations, pre-conditioned environmental carry-over effects may have explained this adaptation. It is interesting to note that seedlings from Ohio, Oklahoma, and central Florida were morphologically different even after the seed donor plants had been in greenhouse culture for more than 10 years (D. Goldman, personal communication). A long-term genetic adaptation to shorter growing seasons may also explain the differences in development (Shaver *et al.*, 1986), and plants from northern latitudes may always express the characteristics of a shorter life cycle and greater corm biomass allocation regardless of environmental conditions. The adaptation may also be a consequence of primary productivity, and plants from northern latitudes may not be able to take advantage of increased temperatures or constant growing conditions (Fetcher and Shaver, 1990).

Greater biomass to corms may also represent a successful survival strategy under periods of environmental stress such as flooding. The populations used in the present study from Michigan and South Carolina have long periods of water availability in the form of ground water (Nelson, 1986; Cohen and Kost, 2008), while populations in Florida experience distinct dry seasons (Davis, 1943). Ecotypes in areas prone to flooding allocated more biomass and carbohydrates to corms and tubers indicating a vegetative growth strategy (Li *et al.*, 2001; Sun *et al.*, 2001; Peñas-Fronteras *et al.*, 2009). Higher biomass to underground storage organs may be a

response to prolonged flooding when plants would need a readily available source of carbohydrates (Peñas-Fronteras *et al.*, 2009). Growth differences may be related to reproductive strategy as well. Florida populations in the present study produce more flowers and seed capsules than the plants in Michigan and South Carolina, which may lead to higher seed production (Peñas-Fronteras *et al.*, 2009). Higher seed production may be necessary in order to colonize areas of earlier succession such as prairies and non-wooded areas in south Florida (Abrahamson, 1975, 1979).

Differences in root number, length, and biomass of *C. tuberosus* ecotypes may be related to soil nutrient and water availability. Biomass allocation to roots was greater in several annual plant species and *Populus davidiana* ecotypes under low nutrient and water stressed soils (McConnaughay and Coleman, 1999; Zhang *et al.*, 2005). Massachusetts ecotypes of *S. alterniflora* were found to have shorter roots due to the shallow, organic soils compared to the deeper sand-based soils in Georgia (Seliksar *et al.*, 2002). Longer or deeper roots on southern *C. tuberosus* ecotypes may be an adaptation to water-stressed environments where the upper soil layers are less hydrated (Kondo *et al.*, 2003).

Shoot biomass as well as shoot length on *C. tuberosus* was highest in Florida populations that experience higher growing temperatures. The larger shoots on Florida *C. tuberosus* seedlings may be a selection pressure to maximize photosynthesis to outcompete vegetation during a longer growing season (Gallagher and Howarth, 1987). A higher shoot biomass may be a requirement to reach reproductive size to set seed before adverse environmental conditions are experienced (Rice *et al.*, 1992). Faster shoot growth in Michigan seedlings may be due to earlier carbohydrate allocation.

Common garden studies are useful tools to detect local adaptation influenced by genetics, but often disregard the relative impact of environmental conditions *in situ* depending on source (Nuismer and Gandon, 2008). Transplant and reciprocal transplant studies better indicate environmental effects on local adaptation (Nuismer and Gandon, 2008). Cultural conditions *in vitro* can be controlled to represent *in situ* conditions by controlling environmental conditions experienced across a species' distribution such as photoperiod, temperature, and humidity. Using a captive generation of seeds may be necessary to further investigate the role of environment and genetics on local adaptation. Growing *C. tuberosus* seedlings *in vitro* under different temperatures or photoperiods may lead to different results. However, biomass allocation in the South Carolina and Florida populations were unaffected by different photoperiods after 16 weeks culture (Figure 2-4). Greater biomass allocation to shoots occurred in Michigan seedlings under short days, while neutral and long days promoted higher corm biomass allocation (Figure 2-4).

Conclusions

Screening for ecotypic differentiation is an exciting application for *in vitro* culture, and *in vitro* common garden studies can be effective at detecting different growth strategies. Biomass allocation and corm formation results along with data from Chapters 2 and 3 provide strong evidence that, based on the differences in biomass allocation and timing of corm formation, *C. tuberosus* ecotypes exist. The ecological adaptations presented are likely influenced by differences in growing season length influenced by both photoperiod and temperature. Due to shorter growing seasons prior to winter dormancy, seedlings from northern populations displayed an adaptation for accelerated corm initiation and development under *in vitro* conditions. Conversely, seedlings from southern populations demonstrate a delay in corm formation possibly an adaptation to a longer growing season.

Table 4-1. Comparative changes in mean corm diameter of *Calopogon tuberosus* seedlings of different geographic sources during 20 weeks *in vitro* culture. Means with the same letter among populations by week are not significantly different according to Tukey's HSD test at α=0.5. All units in mm.

	Michigan	Ohio	Oklahoma	South Carolina	North Central Florida	Central Florida	South Florida
Week 8	1.42 a	0 b	0 b	0 b	0 b	0 b	0 b
Week 10	1.73 a	2.09 a	1.85 a	1.78 a	0 b	0 b	0 b
Week 12	2.11 c	3.20 a	2.69 b	2.18 c	0 d	0 d	0 d
Week 14	2.37 ab	3.23 a	2.40 ab	2.75 ab	2.05 b	0 c	0 c
Week 16	2.60 b	3.37 ab	3.29 ab	3.90 a	3.59 a	2.63 b	0 c
Week 18	2.72 cd	4.04 a	3.17 bc	4.18 a	4.49 a	3.73 ab	2.34 d
Week 20	2.77 d	4.60 b	3.62 c	4.76 ab	5.48 a	4.88 ab	2.59 d

Table 4-2. Comparative change in mean shoot length of *Calopogon tuberosus* seedlings of different geographic source during 20 weeks *in vitro* culture. Means with the same letter among populations by week are not significantly different according to Tukey's HSD test at α=0.5. All units in mm.

	Michigan	Ohio	Oklahoma	South Carolina	North Central Florida	Central Florida	South Florida
Week 8	13.4 abc	14.8 a	11.9 bc	13.8 ab	10.6 cd	8.15 d	5.04 e
Week 10	14.4 de	31.9 b	44.9 a	29.0 b	22.4 c	18.9 cd	9.56 e
Week 12	14.7 d	37.4 c	63.3 a	50.2 b	40.0 b	35.8 c	22.8 d
Week 14	14.9 d	37.9 c	56.4 b	52.2 b	74.4 a	62.6 ab	29.3 c
Week 16	11.7 c	38.1 b	46.6 b	50.0 b	111.1 a	93.4 a	41.8 b
Week 18	6.00 c	41.7 b	46.7 b	49.9 b	137.2 a	128.4 a	52.4 b
Week 20	2.19 d	47.9 c	49.6 c	48.0 c	131.9 a	142.4 a	90.7 b

Table 4-3. Comparative changes in mean root number of *Calopogon tuberosus* seedlings of different geographic source during 20 weeks *in vitro* culture. Means with the same letter among populations by week are not significantly different according to Tukey's HSD test at α=0.5.

	Michigan	Ohio	Oklahoma	South Carolina	North Central Florida	Central Florida	South Florida
Week 8	1.22 ab	1.14 ab	1.35 a	1.33 ab	1.04 ab	1.00 b	0.33 c
Week 10	0.96 d	2.33 b	2.19 b	2.83 a	2.33 b	1.59 c	0.56 d
Week 12	1.33 b	2.56 a	2.72 a	2.52 a	2.56 a	1.78 b	1.37 b
Week 14	1.07 d	2.00 c	1.78 c	2.74 ab	3.00 a	2.78 a	2.15 bc
Week 16	1.04 e	2.18 cd	2.06 d	2.89 bc	3.85 a	3.63 ab	2.22 cd
Week 18	0.82 c	2.44 b	2.17 b	2.85 b	4.85 a	4.33 a	2.63 b
Week 20	0.33 c	2.22 b	2.00 b	2.67 b	4.11 a	4.67 a	2.59 b

Table 4-4. Comparative changes in mean root length of *Calopogon tuberosus* seedlings of different geographic source during 20 weeks *in vitro* culture. Means with the same letter among populations by week are not significantly different according to Tukey's HSD test at α=0.5.All units in mm.

	Michigan	Ohio	Oklahoma	South Carolina	North Central Florida	Central Florida	South Florida
Week 8	10.0 ab	7.29 bc	7.35 bc	10.7 a	10.0 ab	6.22 c	1.26 d
Week 10	9.56 c	27.5 a	23.4 ab	19.2 b	23.3 ab	17.5 b	5.37 c
Week 12	16.8 d	36.9 a	32.6 ab	27.1 bc	29.1 bc	31.1 ab	27.7 cd
Week 14	14.9 d	37.9 c	56.4 b	52.2 b	74.4 a	62.6 ab	29.3 c
Week 16	13.1 c	29.3 b	36.6 b	38.6 b	52.4 a	64.4 a	31.0 b
Week 18	17.3 d	40.1 c	29.9 cd	39.7 c	58.5 b	73.9 a	30.9 cd
Week 20	7.22 c	47.7 b	34.1 b	41.1 b	65.5 a	80.3 a	43.9 b

Table 4-5. ANOVA results for *Calopogon tuberosus* seedling biomass allocation after 20 weeks *in vitro* culture.

	Shoot			Root			Corm	
Variation	df	F	p	df	F	p	df	F	p
Source	6	272.5	< 0.0001	6	167.1	< 0.0001	6	412.3	< 0.0001
Week	6	266.9	< 0.0001	6	65.4	< 0.0001	6	260.1	< 0.0001
S*W	36	6.68	< 0.0001	36	14.3	< 0.0001	36	10.5	< 0.0001

Table 4-6. Comparative biomass allocation to shoots, roots, and corms of *Calopogon tuberosus* seedlings of different geographic source. Means with the same letter among populations by week (horizontal) are not significantly different according to Tukey's HSD test at α=0.5. All units in percent.

	Michigan	Ohio	Oklahoma	South Carolina	North Central Florida	Central Florida	South Florida
Shoot							
Week 8	37.9 e	57.9 cd	53.1 d	64.6 c	75.5 b	52.2 d	90.4 a
Week 10	27.2 d	37.2 cd	38.5 bc	47.8 bc	50.4 b	50.0 b	93.7 a
Week 12	17.9 e	22.9 de	32.5 cd	37.6 bc	55.8 a	47.8 ab	53.5 a
Week 14	10.1 d	26.1 c	26.2 c	30.7 c	49.0 b	40.1 b	60.6 a
Week 16	14.1 c	15.9 c	16.6 c	20.7 c	37.7 b	42.5 ab	50.2 a
Week 18	7.78 d	12.1 cd	16.8 c	14.9 cd	32.3 b	29.1 b	45.4 a
Week 20	4.24 d	10.1 c	12.7 c	11.3 c	25.3 b	23.8 b	54.2 a
Root							
Week 8	35.7 bc	42.1 ab	46.9 a	5.4 bc	24.5 d	47.8 a	28.8 cd
Week 10	17.8 d	44.5 ab	37.8 bc	44.9 ab	49.6 a	50.0 a	29.5 c
Week 12	14.9 e	34.9 d	33.6 cd	40.6 bcd	44.2 abc	52.2 a	50.2 ab
Week 14	10.5 e	41.8 b	25.7 d	32.2 cd	42.4 b	59.3 a	39.4 bc
Week 16	10.2 d	22.3 c	19.2 c	21.9 c	36.2 b	52.7 a	49.8 a
Week 18	10.5 d	20.1 cd	21.0 c	16.7 cd	29.2 b	45.5 a	35.8 b
Week 20	9.25 d	17.9 bc	13.5 bc	12.6 c	22.0 b	41.0 a	38.2 b
Corm							
Week 8	32.4 a	0 b	0 b	0 b	0b	0 b	0 b
Week 10	59.3 a	29.4 c	39.9 b	18.7 d	0 e	0 e	0 e
Week 12	67.2 a	46.1 b	33.9 c	21.9 d	0 e	0 e	0 e
Week 14	76.0 a	32.2 c	48.1 b	37.2 c	14.6 d	0 e	0 e
Week 16	80.5 a	66.7 b	61.2 bc	57.3 c	26.2 d	12.9 e	0 f
Week 18	89.4 a	67.8 b	62.2 b	68.4 b	38.5 c	35.4 c	33.7 c
Week 20	97.3 a	73.8 b	73.8 b	77.3 b	52.7 c	35.2 d	20.5 e

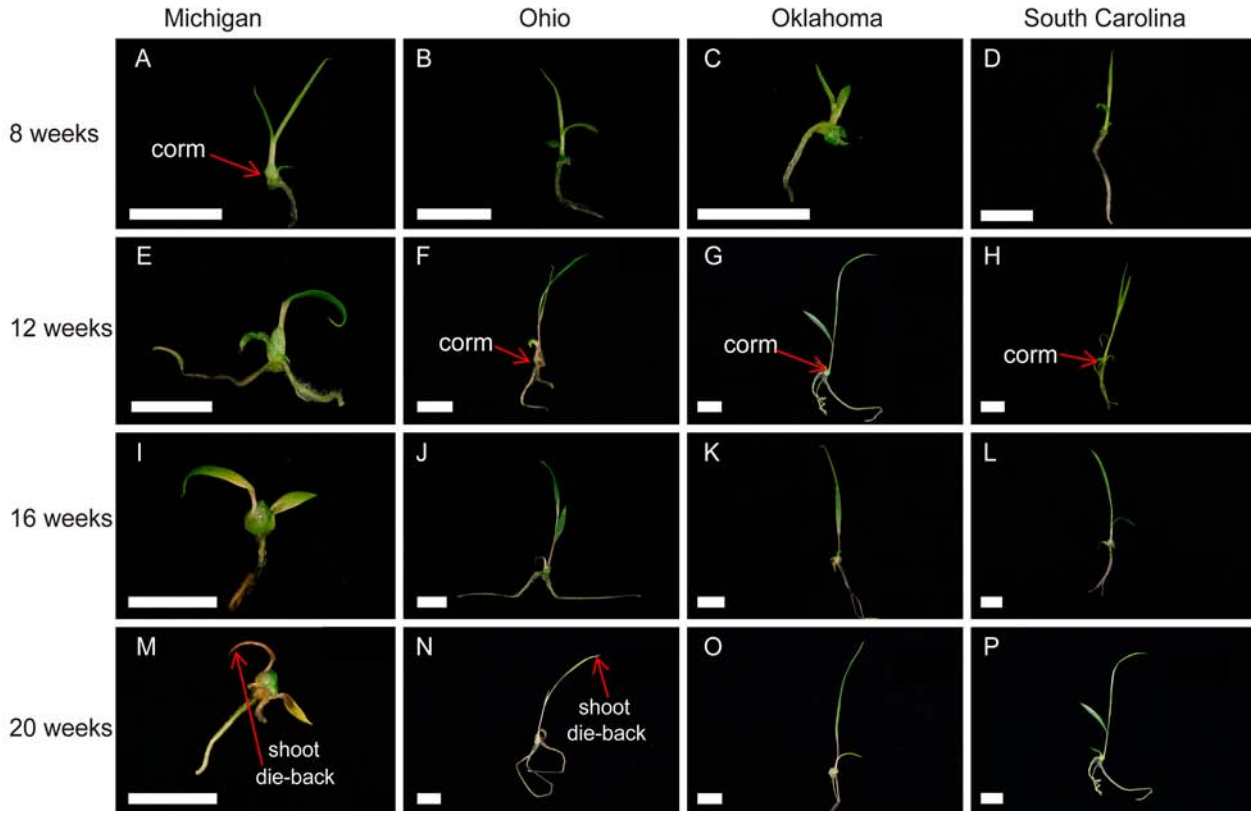

Figure 4-1. *In vitro* seedling development of *Calopogon tuberosus* from widespread populations. Note the progressively long delay in corm formation in southern populations. Shoot die-back was characterized by yellowing and browning of leaves. A-D, Q-S) Seedlings after 8 weeks culture. E-H, T-V) Seedlings after 12 weeks culture. I-L, W-X) Seedlings after 16 weeks culture. M-P, Z-BB) Seedlings after 20 weeks culture. A, E, I, M) Michigan seedlings. B, F, J, N) Ohio seedlings. C, G, K, O) Oklahoma seedlings. D, H, L, P) South Carolina seedlings. Q, T, W, Z) North Central Florida seedlings. R, U, X, AA) Central Florida seedlings. S, V, Y, BB) South Florida seedlings. Scale bars = 1 cm.

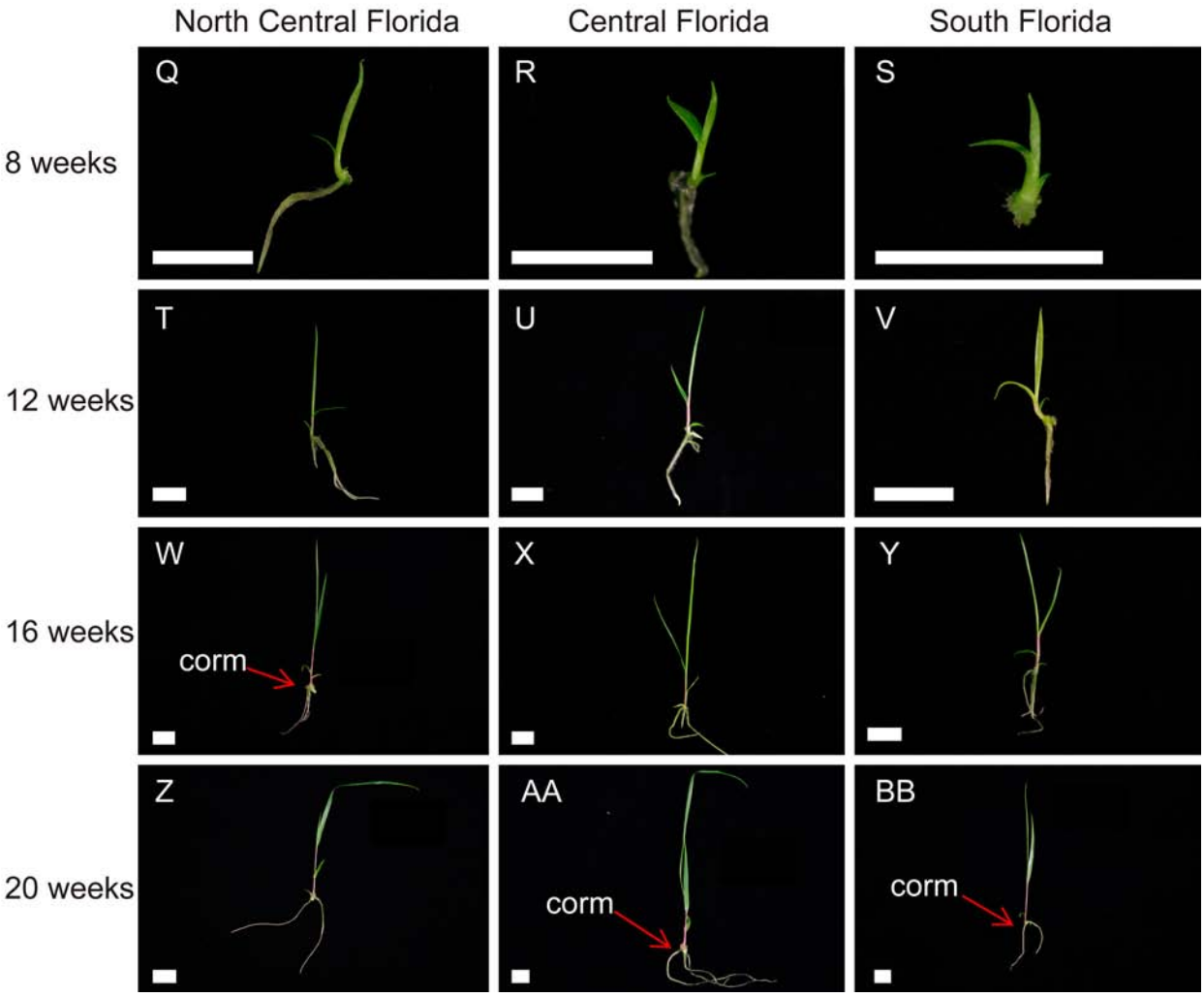

Figure 4-1. Continued.

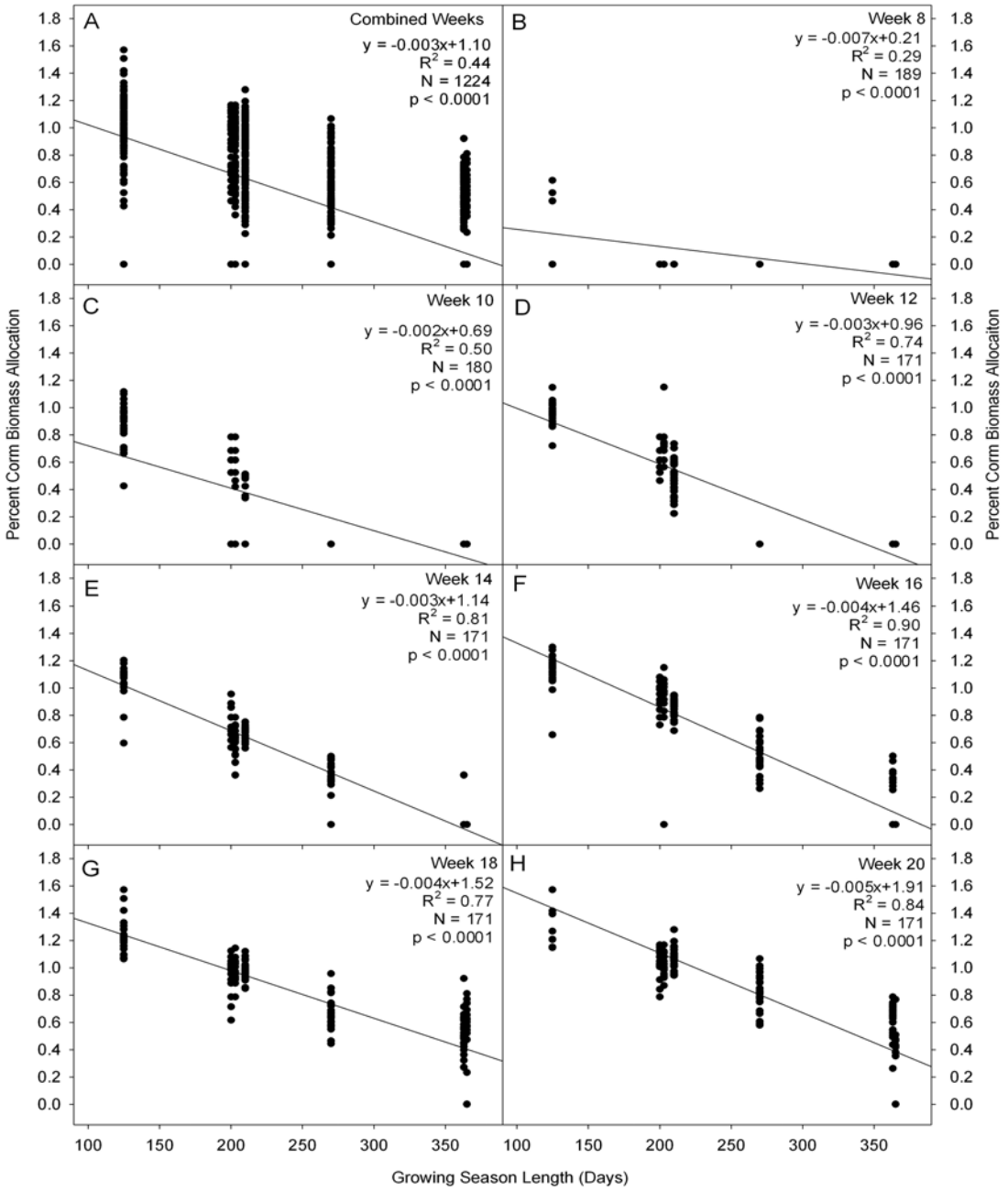

Figure 4-2. Correlation of growing season length and percent corm biomass allocation of *Calopogon tuberosus* seedlings from widespread populations. Percent biomass allocation is represented as mg of dry weight per total dry weight. A) Combined biomass allocation. B) Biomass allocation after 8 weeks *in vitro* culture. C) Biomass allocation after 10 weeks *in vitro* culture. D) Biomass allocation after 12 weeks *in vitro* culture. E) Biomass allocation after 14 weeks *in vitro* culture. F) Biomass allocation after 16 weeks *in vitro* culture. G) Biomass allocation after 18 weeks *in vitro* culture. H) Biomass allocation after 20 weeks *in vitro* culture. Each point represents the mean response of three replications with nine seedlings each. Corm biomass percentages were arcsine transformed prior to regression analysis.

CHAPTER 5
EFFECTS OF CHILLING AND CUTTING CORMS ON CORM DORMANCY AMONG WIDESPREAD POPULATIONS OF *Calopogon tuberosus*

Introduction

The results of Chapter 4 indicated that differences in biomass allocation and timing of corm formation were influenced by growing season length, but the actual dormancy of the corms produced was not investigated. Because Michigan plants are located in a climate with a longer and colder winter, they may exhibit longer corm dormancy that may only be broken by long chilling periods compared to more southern plants. The next sequential step is an examination of corm dormancy in widespread populations of *Calopogon tuberosus*.

The issue of dormancy in plants has been debated because the term often has conflicting meanings based on the plant, plant part, whole plant or cellular level, and seasonality (Rohde and Bhalerao, 2007). Amen (1968) defined dormancy as "endogenously controlled, but environmentally imposed temporary suspension of growth." However, complete suspension of growth is difficult to assess (Rees, 1981). Three types of dormancy have been previously identified including ecodormancy, paradormancy, and endodormancy (Lang, 1987). Ecodormancy is influenced by unfavorable environmental conditions, paradormancy occurs due to an inhibition from another part of the plant, and endodormancy is found in the dormant structure (Lang *et al.*, 1987). Seed dormancy is defined as the inability of a viable seed to germinate under favorable conditions (Bewley, 1997). When considering the whole plant level dormancy may be defined as the "inability to initiate growth from a meristem under favorable conditions" (Rohde and Bhalerao, 2007).

Many temperate plant species form overwintering structures such as buds, tubers, rhizomes, and corms (Garbisch *et al.*, 1995; Rohde and Bhalerao, 2007). These structures are formed during the growing season before unfavorable growth conditions are encountered

(Garbisch *et al.*, 1995). The dormant structures continue to remain dormant until favorable growth conditions are encountered the following growing season (Garbisch *et al.*, 1995). In order to break dormancy, chilling is required (Rohde and Bhalerao, 2007). Longer chilling periods are often required to break dormancy in tubers and corms of temperate species, but extended periods often inhibit growth and development (Clark, 1995; Yañez *et al.*, 2005; Fukai *et al.*, 2006). However, chilling period requirement may be different according to plant provenance in that southern species may require shorter chill periods (Perry and Wang, 1960; Garbisch *et al.*, 1995).

Chilling has been explored in ecotypic differentiation of tree species (Perry and Wang, 1960; Kriebel and Wang, 1962), aquatic species (Garbisch *et al.*, 1996), and forage grasses (Silsbury, 1961; Cooper, 1964; Eagles, 1967*a*, *b*; MacColl and Cooper, 1967). *Acer rubrum* ecotypes from Florida required no chilling to break dormancy, but longer chill periods were required to break dormancy in northern ecotypes (Perry and Wang, 1960). *Acer saccharum* ecotypes Georgia and Tennessee required shorter chill periods to break dormancy than ecotypes in Michigan and Ohio (Kriebel and Wang, 1962). In several species of forage grasses, relative growth rate of Mediterranean populations was higher at cooler temperatures compared to north European populations that had a higher growth rate at warmer temperatures (Cooper, 1964). Prolonged chilling decreased both survival and shoot growth of aquatic plant ecotypes from Florida (Garbisch *et al.*, 1996).

Several hormones have been shown to be involved in storage organ dormancy. However, with the majority of research has been conducted with potato tubers. Low levels of indole-3-acetic acid (IAA) were found in dormant tubers while higher levels were present once shoot regrowth occurred (Hemberg, 1949). Abscisic acid (ABA) levels were relatively high in dormant tubers (Coleman and King, 1984), inhibited tuber sprouting (Suttle and Hultstrand, 1994), and

subsequently declined during storage (Suttle, 1995). Gibberellic acid (GA_3) broke tuber dormancy by increasing shoot regrowth, and cytokinins increased the activity of mersitematic areas (Suttle, 2004). GA has been shown to stimulate cell division, increase glucose levels near buds, and increase synthesis of DNA and RNA (Burton, 1989; Taiz and Ziegler, 1998; Alexopoulos *et al.*, 2007). Ethylene has been shown to increase as tuber dormancy decreased, and subsequently promoted shoot regrowth compared to control treatments (Rylski *et al.*, 1974). However, tuber response to ethylene may be cultivar dependent (Alam *et al.*, 1994). In addition to hormonal control, cutting tubers increased bud sprouting due to a possible wound response or removal of endogenous inhibitory hormones (Alexopoulos *et al.*, 2008).

Dormancy in corm-forming species, such as many terrestrial orchids, has not been fully examined. *Calopogon tuberosus* is a model orchid species to study the extent of corm dormancy since it is a widespread species. Studying corm dormancy, the chilling period length necessary to break dormancy, and shoot emergence may provide insight into *C. tuberosus* ecotypic differentiation. Southern ecotypes may require a shorter chilling period to break dormancy and initiate shoot regrowth compared to northern ecotypes that are exposed to longer winters. The effects of cutting corms on dormancy of widespread *C. tuberosus* populations were also examined. This research also further validates the use of *in vitro* ecology to screen for ecotypic differentiation.

The objectives were to: 1) Determine the degree of corm dormancy in *C. tuberosus* ecotypes; 2) Verify the length of chilling that is appropriate to break corm dormancy in *C. tuberosus* ecotypes; and 3) Determine whether cutting *C. tuberosus* corms can effectively relieve dormancy. Given that southern ecotypes do not experience long winters, shorter chilling periods may be sufficient to break corm dormancy.

Materials and Methods

Chilling Effects on Corm Dormancy and Shoot Regrowth

Seeds were used from the following locations: Carney Fen (Menominee County, Michigan, USA), Eva Chandler Heritage Preserve (Greenville County, South Carolina, USA), Goethe State Forest (Levy County, Florida, USA), Florida Panther National Wildlife Refuge (Collier County, Florida, USA). Seed capsules from all populations were collected before complete dehiscence and were stored at 23°C over silica gel for 2 weeks. Seeds were then removed from capsules, pooled by geographic source, and stored dry in the dark at -11°C until used.

Seeds were surface disinfected in sterile scintillation vials for 3 minutes in a solution of 5 mL absolute ethanol, 5 mL 6% NaOCl, and 90 mL sterile dd water. Seeds were rinsed with sterile dd water after surface sterilization. Solutions were removed with sterile Pasteur pipettes. Seeds were transferred with a 10µL sterile inoculating loop onto BM-1 Terrestrial Orchid Medium contained in 100 x 15 mm Petri plates. The medium was supplemented with 1% activated charcoal. Medium pH was adjusted to 5.7 with 0.1N KOH prior to autoclaving for 40 minutes at 117.7 kPa and 121°C. Ten replicate Petri plates with 30 mL medium each were used for each seed source with approximately 100 seeds per plate (Figure 5-1A). Cultures were placed in an environmental growth incubator (#I-35LL; Percival Scientific, Perry, IA, USA) under cool-white fluorescent lights in a 12 h photoperiod at 24.2 ± 0.2°C and 40 µmol m^{-2} s^{-1}.

After 8 weeks culture, seedlings (Figure 5-1B) were transferred to larger culture vessels for further growth and development. Nine seedlings were transferred to individual *Phyto*Tech Culture Boxes containing 100 mL of BM-1 Terrestrial Orchid Medium. Five replicate vessels were prepared for each treatment and seed source combination for a total of 45 seedlings per treatment. A total of 25 vessels with a total of 225 seedlings were prepared for each seed source.

A total of 900 seedlings were transferred. Seedlings grew *in vitro* for another 12 weeks, for a total of 20 weeks culture. Environmental conditions were the same as described previously.

After the 20 weeks (Figure 5-1C), shoots and roots on seedlings were removed so that only corms remained (Figure 5-1D). The nine corms in each *Phyto*Tech box were transferred to Sigma Phytatrays I (#P1552, Sigma-Aldrich, St. Louis, MO) containing 100 mL of moist, sterilized vermiculite (Figure 5-1E). Five Phytatrays I (114 mm x 86 mm x 63.5 mm) were prepared for each treatment. Cultures containing the corms were subsequently stored at 10 ± 0.3°C for 2, 4, 6, and 8 weeks in complete darkness; a control of no cold storage was also used. Five culture vessels per seed source were allocated to each chilling period treatment.

After the each chilling period, the five culture boxes for each time source*time treatment were removed. Corms were subsequently planted in a 9-cell pack containing Fafard 2 (Conrad Fafard, Inc., Agawam, MA, USA). Corms were planted in a randomized complete block design with block designated as the chill treatment so that block 1 was the control, etc. Each seed source was allocated to each block, and blocks were replicated five times. Corms were buried approximately 1 cm below the soil line. Trays were placed in a walk-in growth chamber (Figure 5-1F) under a 16/8 h L/D photoperiod at 27 ± 2.2°C and an average relative humidity of 85%. Four 400-watt metal halide bulbs (Sylvania, Danvers, MA, USA) provided a light level of 167 µmole m^{-2} s^{-1}. Corms were watered as needed and as frequently as daily.

Data Collection and Statistical Analysis

Shoot emergence date was recorded by the presence of the new shoot breaking the soil surface. Every 2 weeks, starting upon emergence and continuing until week 16, shoot length was measured from the soil surface to the shoot apex. At the final data collection, leaf number, leaf width, shoot height, root number, root length, corm diameter, and dropper formation were recorded. Droppers (Figure 5-2) are axillary shoots growing from storage organs to form new

storage organs (Dixon and Pate, 1978; Hollick *et al.*, 2001). Percent shoot emergence and survival, noted by the presence of a corm beneath the soil surface, were recorded. Logistic regression was used to assess the affect of chilling treatment and source on percent shoot emergence, percent survival, and percent dropper formation using the generalized linear mixed model procedure (proc glimmix macro) in SAS v9.1. Least-square means (lsmeans) were used to assess mean separation. Endpoint measurement data were analyzed using the general linear procedure (proc glm), ANOVA, and least-square means in SAS v9.1.

Effects of Cutting Corms on Corm Dormancy and Shoot Regrowth

Seeds were used from the following locations: Carney Fen (Menominee County, Michigan, USA), Eva Chandler Heritage Preserve (Greenville County, South Carolina, USA), Goethe State Forest (Levy County, Florida, USA), Florida Panther National Wildlife Refuge (Collier County, Florida, USA). In addition, seeds from Oklahoma, Ohio, and Central Florida were obtained from cultivated plants that were hand pollinated under greenhouse conditions (D. Goldman). Seed capsules from all populations were collected before complete dehiscence and were stored at 23°C over silica gel for 2 weeks. Seeds were then removed from capsules, pooled by geographic source, and stored in the dark at -11°C until used.

Seeds were surface disinfected in sterile scintillation vials for 3 minutes in a solution of 5 mL absolute ethanol, 5 mL 6% NaOCl, and 90 mL sterile dd water. Seeds were rinsed with sterile dd water after surface sterilization. Solutions were removed with sterile Pasteur pipettes. Seeds were transferred with a 10µL sterile inoculating loop onto BM-1 Terrestrial Orchid Medium contained in 100 x 15 mm Petri plates. The medium was supplemented with 1% activated charcoal. Medium pH was adjusted to 5.7 with 0.1N KOH prior to autoclaving for 40 minutes at 117.7 kPa and 121°C. Ten replicate Petri plates with 30 mL medium each were used for each seed source with approximately 100 seeds per plate. Cultures were placed in an

environmental growth incubator (#I-35LL; Percival Scientific, Perry, IA, USA) under cool-white fluorescent lights in a 12 h photoperiod at 24.2 ± 0.2°C and light levels of 40 µmol m^{-2} s^{-1}.

After 8 weeks culture, seedlings were transferred to Sigma Phytatrays II (#P5929, Sigma-Aldrich, St. Louis, MO) with 100 mL BM-1 Terrestrial Orchid Medium. Ten Phytatrays (114mm x 86 mm x 102 mm) with nine seedlings were prepared per seed source. After 20 weeks culture shoots and roots were removed from seedlings, and corms were transferred to fresh BM-1 Terrestrial Orchid Medium in Phytatrays II. Corms were either left whole or cut in half longitudinally. Previous experiments using the north central Florida population indicated that no difference was found between shoot regrowth on the bottom or top corm half (unpublished data). Both the top and bottom halves were randomly distributed throughout the culture vessels. For each seed source five replicate vessels with nine propagules each were prepared for each corm treatment. Cut corms were transferred cut-side down. Environmental conditions were the same as previously mentioned.

Data Collection and Statistical Analysis

Shoot regrowth was monitored bi-weekly and percent shoot regrowth was recorded. After 8 weeks final data was collected including percent shoot regrowth, number of shoots per explant, shoot length, root length and number, and corm formation on new shoots. Final endpoint measurement data as well as the days to shoot emergence were analyzed in SAS v9.1 using ANOVA, general linear model procedure (proc glm), and least-square means. Logistic regression was used to assess the affect of cutting corms on percent shoot regrowth using generalized linear mixed model procedure (proc glimmix macro) in SAS v9.1. Least-square means (lsmeans) were used to assess mean separation.

Results

Chilling Effects on Corm Dormancy and Shoot Regrowth

Chilling treatment ($F_4 = 61.2$, $p < 0.0001$), source ($F_3 = 11.1$, $p < 0.0001$), and the interaction between source and chilling treatment ($F_{12} = 4.29$, $p < 0.0001$) all significantly influenced the number of days to shoot emergence. The average number of days to shoot emergence was less under the longer chill periods of 6 and 8 weeks, regardless of source (Figure 5-3A). Corms subjected to no chilling and the 2 week chilling period exhibited the slowest shoot emergence. South Carolina and south Florida corms chilled longer than 6 weeks exhibited the quickest shoot emergence. Michigan corms required 4 weeks or longer for quickest shoot emergence, while shoots emerged faster when north central Florida corms were chilled for 8 weeks (Figure 5-3A).

Percent shoot emergence was highly influenced by chilling treatment ($F_4 = 56.0$, $p < 0.0001$), but source ($F_3 = 0.65$, $p = 0.58$) and the interaction between chilling treatment and source ($F_{12} = 1.66$, $p = 0.07$) were not significant. Lower percent shoot emergence was observed when corms were chilled for shorter periods (Figure 5-3B). Less than 20% shoot emergence was observed in unchilled corms and following the 2 week chilling period among all populations. In fact, only one shoot from South Carolina emerged in the control and only one shoot from north Central Florida emerged in the 2 week chilling treatment. Chilling periods longer than 6 weeks provided the highest percent shoot emergence in Michigan corms (Figure 5-3B), while 8 weeks chilling provided the highest shoot emergence for all other populations. Approximately 90% shoot emergence was observed for South Carolina, north central Florida, and south Florida corms, compared to 78% for Michigan corms (Figure 5-3B).

After 16 weeks, corm survival was high regardless of source or chilling length. Survival was measured by the presence of a viable corm below the soil surface, and not the presence the

emerged shoot. The original corm often remained viable, but did not form a shoot by the end of the experiment. Major differences in survival were not clearly evident according to source (F_3 = 0.00, p = 1.00), chilling treatment (F_4 = 0.03, p = 0.99), or their interaction (F_{12} = 0.37, p = 0.97). Differences in survival were only observed within Michigan and south Florida populations. Survival of south Florida propagules was highest with 4-8 weeks chilling, while Michigan corm survival was highest after 2 weeks chilling (Figure 5-3C). Near 100% survival was observed in South Carolina and North Central Florida corms regardless of treatment (Figure 5-3C).

Only source (F_3= 3.89, p = 0.009) significantly influenced dropper formation, while chilling treatment (F_4 = 0.12, p = 0.97) and their interaction (F_{12} = 0.93, p = 0.94) were not significant. Droppers were considered formed by the presence of a shoot connecting the original corm to the new shoot (Figure 2A, B). Dropper formation was prevalent in Michigan and South Carolina populations after 16 weeks (Figure 5-3D). Dropper formation was higher on corms that were chilled for 6 or 8 weeks compared to shorter chilling periods in Michigan and South Carolina seedlings (Figure 5-3D). No droppers formed on north central Florida and south Florida corms in the control, 2 weeks, and 4 weeks chilling periods (Figure 5-3D). Dropper formation in both Florida populations was highest when corms were chilled for 6 weeks, while 8 weeks chilling suppressed formation.

The interaction between source and chill treatment was significant (F = 2.45, p = 0.006) as well as source (F = 12.1, p < 0.0001) and chilling treatment (F = 5.26, p = 0.004) for shoot length. No differences in shoot length were observed among chilling periods in the Michigan and South Florida populations (Figure 5-4A). Few differences were observed in South Carolina and north central Florida populations. Michigan and south Florida shoots were generally the shortest of all populations and north central Florida the highest (Figure 5-4A).

The interaction between source and chilling treatment (F_{11} =1.19, p = 0.29) and chilling treatment (F_4 = 1.52, p = 0.19) did not significantly influence leaf number. However, source significantly influenced leaf development (F_3 = 6.87, p = 0.002). Michigan had the highest leaf number after 16 weeks followed by South Carolina and both Florida populations (Figure 5-4B). On average, less than two leaves were present on all seedlings regardless of source.

The interaction between source and chill treatment was significant for leaf width (F_{11} = 2.74, p = 0.002) as well as source (F_3 = 33.7, p < 0.0001), but not chilling treatment (F_4 = 0.40, p = 0.81). Average leaf width was lowest on south Florida plantlets (Figure 5-4C) while the widest leaves were observed on north central Florida and South Carolina populations (Figure 5-4C). No differences among treatments were observed on South Carolina and south Florida. Wider leaves were observed in chilling periods longer than 4 weeks on Michigan plantlets. Widest leaves were observed on north central Florida plantlets from the control, 2 weeks, and 8 weeks chill period.

The interaction between chilling treatment and source (F_{11} = 2.08, p = 0.02), source (F_3 = 12.5, p <0.0001) and chilling treatment (F_4 = 4.46, p = 0.002) significantly influenced root development. No differences were observed in root number for both South Carolina and Michigan populations, but more roots were observed in shorter chilling periods for both Florida populations (Figure 5-4D). The highest root number was observed in the control treatment in north central Florida plantlets, but South Carolina seedlings had the greatest root number on average.

Chilling treatment (F_4 = 4.27, p = 0.002), source (F_3 = 8.72, p < 0.0001), and their interaction (F_{11} = 2.72, p = 0.002) significantly influenced root length. Longer chilling periods promoted the longest roots on Michigan plantlets (Figure 5-4E). No difference was observed on South Carolina plantlets. Chilling periods less than 8 weeks promoted the longest roots on north

central Florida plantlets. South Florida plantlets from the no chill treatment did not form roots, while few differences were observed among chilling treatments (Figure 5-4E).

Source ($F_4 = 3.18$, $p = 0.03$) significantly influenced new corm development while chilling treatment ($F_4 = 1.73$, $p = 0.15$) and their interaction ($F_4 = 1.63$, $p = 0.09$) were not significant. Few differences were observed among chilling treatments within each source with the exception of South Carolina and north central Florida where the largest corms were observed in the control and control/2 weeks chilling treatment, respectively (Figure 5-4F). Regardless of treatment, smallest corms were observed on south Florida seedlings, and new corms did not form on seedlings in the control treatment. The largest corms were observed on South Carolina and north central Florida seedlings in the control and 2 weeks chilling treatment, while Michigan and South Carolina seedlings had the largest corms in the 4, 6, 8 week chilling treatments.

Effects of Cutting Corms on Corm Dormancy and Shoot Regrowth

Cutting treatment ($F_1 = 109.3$, $p < 0.0001$) had a highly significant effect on percent shoot growth, while source ($F_6 = 0.44$, $p = 0.86$) and the interaction between source and treatment ($F_6 = 1.18$, $p = 0.32$) did not have a significant effect. Cutting corms had a highly significant effect on shoot regrowth compared to whole corms for all sources (Figure 5-5). Less than 11% of uncut corms formed shoots among all populations. Percent shoot regrowth on cut corms was well over 50% for all populations except north central and south Florida, but this was only significantly different than corms from Michigan. Michigan had the highest shoot regrowth with 66.7% of propagules forming shoots, but this was not different than Ohio (58.3%), Oklahoma (62.2%), and South Carolina (53.3%). Northern populations generally expressed a higher percent shoot regrowth sooner than southern populations (Figure 5-6). At week 2, Florida populations had less than 20% shoot regrowth while other all other populations had over 20% shoot regrowth.

However, after week 4 shoot regrowth generally did not differ significantly within each population (Figure 5-6).

Cutting treatment ($F_1 = 23.1$, $p < 0.0001$) and the interaction between treatment and source ($F_6 = 3.28$, $p = 0.005$) significantly influenced shoot length, but source ($F_6 = 0.82$, $p = 0.56$) did not have a significant effect. Shoots produced on cut corms from Oklahoma exhibited the greatest mean shoot length (140 mm), as well as the smallest shoot length (10.5 mm) in the whole corm treatment (Figure 5-7A). However, shoot length on uncut Oklahoma corms was only significantly different than shoot length on uncut central Florida corms.

Within several weeks of shoot initiation, corms began to form at the base of the newly formed shoots. The interaction between source and cutting treatment ($F_6 = 4.69$, $p = 0.001$) significantly influenced new corm diameter, but source ($F_6 = 1.98$, $p = 0.07$) and cutting treatment ($F_1 = 2.04$, $p = 0.16$) did not. Few differences were observed among treatments and sources, but the smallest corms were observed on South Florida (cut treatment), Ohio (uncut treatment), and Michigan (uncut treatment) shoots. No corms formed in the uncut treatment on south Florida and Oklahoma shoots (Figure 5-7B).

Mean root number was significantly influenced by source ($F_6 = 4.52$, $p = 0.0003$) and cutting treatment ($F_6 = 12.2$, $p = 0.0006$), but not their interaction ($F_6 = 1.46$, $p = 0.19$). Root number on Ohio, Oklahoma, and north central Florida plantlets differed between treatments (Figure 5-7C). The highest number of roots was observed in the cut treatment, regardless of source, as well as South Carolina and central Florida whole corm treatments. Root length was also significantly influenced by source ($F_6 = 4.50$, $p = 0.0003$) and treatment ($F_6 = 15.5$, $p = 0.0001$), but not their interaction ($F_6 = 1.24$, $p = 0.29$). Few differences were observed among

treatments and sources with the exception that the longest mean roots were observed on central Florida in the cut corm treatment (Figure 5-7D).

Discussion

This is the first study examining the role of chilling in orchid ecotypes, and one of a few that investigated the role of chilling on storage organs among plant ecotypes. Previous research on chilling of storage organs has focused on effects of temperature and chilling length on corm and tuber sprouting and flowering for horticultural purposes (Clark, 1995; Kim *et al.*, 1996; González *et al.*, 1998; Yañez *et al.*, 2005; Fukai *et al.*, 2006), but little information exists focusing on local adaptation to chilling (Cooper, 1964; Eagles, 1967*a*, *b*; MacColl and Cooper, 1967). While few differences in chilling were found among populations, local adaptation to different habitats and environmental conditions may, in part, explain the chilling requirement.

The Role of Chilling on Dormancy

Comparative influence of chilling storage organs such as tubers and corms resulting from ecotypic differentiation has received little attention. Bud chilling of tree ecotypes has been investigated extensively (Perry and Wang, 1960; Kriebel and Wang, 1962; Myking and Heide, 1995; Li *et al.*, 2003; Li *et al.*, 2005), but correlating chilling response of buds with underground storage organs may be difficult due to location of plant parts. Regardless, chilling requirements should be considered in a restoration context if plants are moved from their home-site since southern ecotypes may not be cold-hardy (Garbisch *et al.*, 1996). Clearly, further research is required on the ecological implications of chilling requirements on ecotypes.

Chilling of *C. tuberosus* corms followed a similar pattern to bud chilling in birch species (*Betula pendula* and *B. pubescens*). Longer chilling treatments reduced days to bud break regardless of source latitude, and the number of days to bud break was more pronounced for southern ecotypes (Myking and Heide, 1995). Longer chilling periods increased shoot

emergence and reduced the number of days to shoot emergence regardless of chilling length in ecotypes of *C. tuberosus.* Longer chilling treatments had a more pronounced influence on emergence days of southern *C. tuberosus* ecotypes. In addition, northern ecotypes of both *C. tuberosus* and *Betula* sp. generally broke dormancy earlier than southern ecotypes.

The requirement for a chill period longer than 6 weeks for the *C. tuberosus* Michigan population is not surprising. The long winters and relatively constant temperatures below freezing require plants to maintain dormancy until environmental conditions are appropriate (Garbisch *et al.*, 1996). No difference in shoot emergence was observed between the 6 and 8 week chilling period for Michigan plants, and shoot emergence was lower than all other populations in the 8 week chilling treatment. Being subjected to longer winters, Michigan plants may require a chill period longer than 8 weeks for maximum shoot regrowth.

The required chilling period for southern *C. tuberosus* ecotypes may be explained by differences in temperatures. Winter temperatures in the south often exceed 17°C, but temperatures may drop suddenly in subsequent days. Even in south Florida temperatures can drop below 0°C, albeit for shorter periods than northern climates. Longer chilling periods would ensure that plants in Florida do not initiate regrowth until the threat of freezing temperatures is surpassed (Garbisch *et al.*, 1996).

An interesting morphological feature observed in Michigan and South Carolina populations was the formation of droppers. Droppers, which are not exclusive to orchids, are axillary shoots that grow downward to form replacement storage organs for the next season's growth (Dixon and Pate, 1978; Hollick *et al.*, 2001). Most species that form droppers are tuber forming plants rather than corm forming. The axillary shoot that forms on *C. tuberosus* corms is not an actually dropper since the shoot grows upward toward the surface. Also, this may be an *ex*

situ phenomenon because axillary shoots have not been seen on wild plants from any location (personal observation). Michigan and South Carolina seedlings were more prone to form droppers, and more droppers were formed with longer chill periods regardless of seedlings source. Longer chilling treatments may have influenced northern *C. tuberosus* ecotypes to form axillary shoots in order to position the new season's growth to the soil surface where warmer temperatures exist. Further investigation into the dynamics of axillary shoot formation is warranted.

Clear differences in growth and development of plantlets after corm chilling were not evident. However, north central Florida and South Carolina plantlets generally were the largest after 16 weeks. This was also observed throughout numerous experiments (personal observation). Specifically, north central Florida plantlets from the control had the longest shoots and roots, largest number of roots, and largest corms. Several factors may have influenced the inconsistent seedling growth and development results. Plantlet numbers were rather low in the control and shorter chill treatments, thus error was much larger creating fewer significant results. The few plantlets that did emerge and develop in the shorter chilling treatments had a longer time to develop compared to those in the longer chilling treatments.

The Effect of Cutting Corms on Dormancy

Exogenous application of ethylene and GA has been shown to break potato tuber dormancy (Rylski *et al.*, 1974; Alam *et al.*, 1994; Alexopoulos *et al.*, 2007, 2008). Free abscisic acid (ABA) decreased during dormancy release in freesia corms, and exogenous application of the cytokinin benzlyadenine (BA) broke dormancy (Uyemura and Imanishi, 1987). In addition, ethylene production increased simultaneously as ABA decreased prior to freesia corm dormancy release, and the increase in ethylene was attributed to dormancy release (Uyemura and Imanishi, 1983).

Cutting corms may trigger a wound response leading to an increase in respiration and metabolic activity that could influence faster shoot regrowth (Passam *et al.*, 1977; Burton, 1989). Alexopoulos *et al.* (2008) found that cutting potato tubers prior to treatment with GA increased shoot production compared to whole tubers and cutting tubers prior to incubation in water. Also, an increase uptake of GA may have influenced shoot regrowth. Cutting *C. tuberosus* corms may lead to increased concentrations of ethylene or GA causing enhanced shoot regrowth, or decrease the amount of inhibitory hormones (ABA) blocking regrowth. Due to sugars and nutrients present in the medium, cutting *C. tuberosus* corms may lead to enhanced uptake of nutrients leading to increased shoot regrowth (Alexopoulos *et al.*, 2008). Initial differences in shoot regrowth among *C. tuberous* ecotypes could be explained by differential rates of ethylene evolution or GA accumulation upon cutting corms, and further investigation is warranted.

Conclusions

The results presented here are not entirely conclusive concerning *C. tuberosus* ecotypic differentiation with respect to chilling and corm dormancy, but the experiments provide an excellent foundation for further investigation. Areas of research to examine include the effects of temperature on corm chilling, longer chill periods, photoperiodic effects on regrowth of chilled corms, and exogenous hormones, such as GA, ABA, and cytokinins, on shoot regrowth. Given that the chilling temperature used (10°C) is moderately cool and this temperature is experienced throughout the distribution of *C. tuberosus*, chilling corms at colder temperatures may provide better insight into corm dormancy. Also, investigating low temperature tolerance of propagules may reveal whether southern ecotypes can survive long-term under colder conditions.

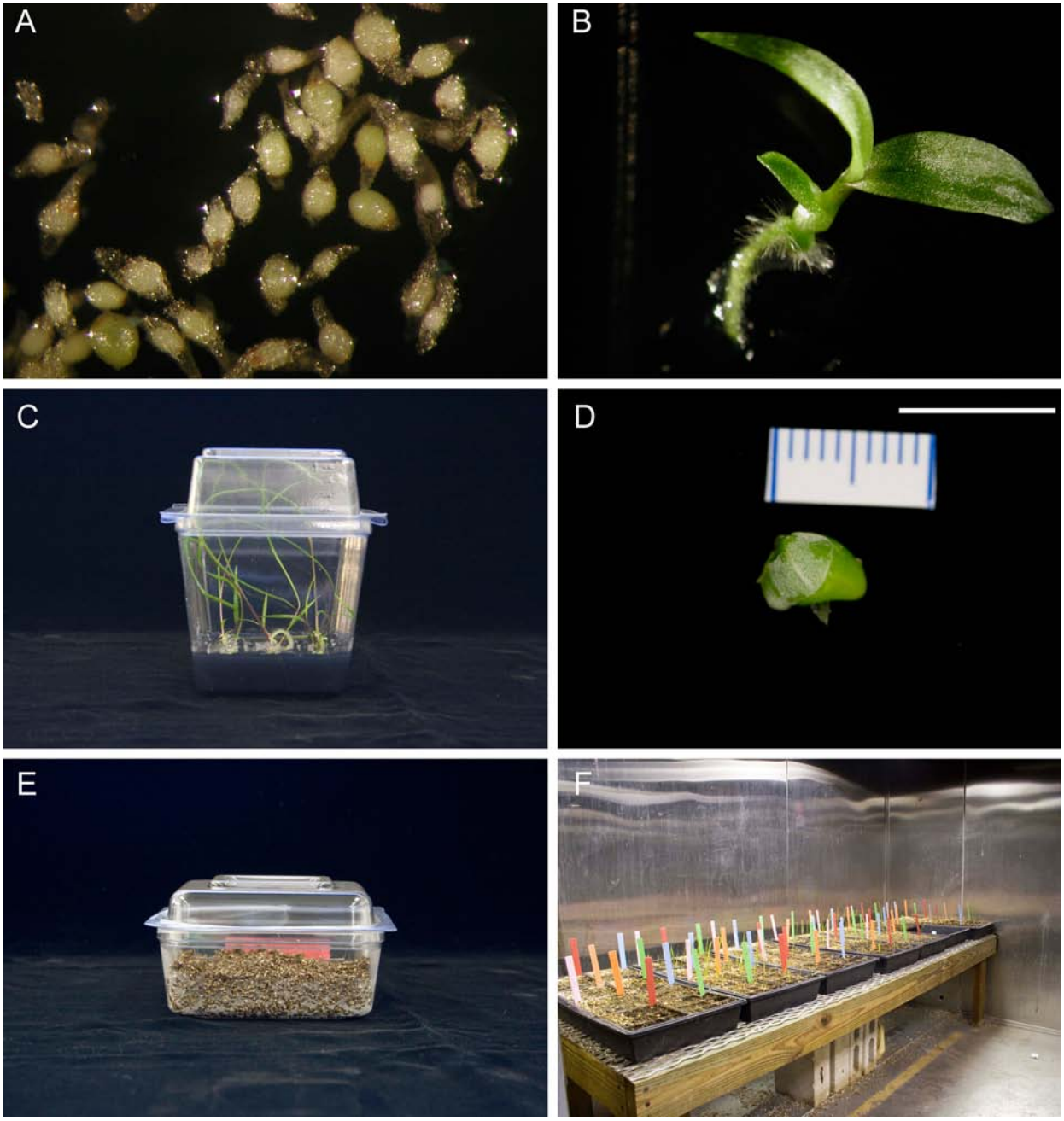

Figure 5-1. Outline of *in vitro* to *ex vitro* growth of *Calopogon tuberosus*. A) Germinating embryos in a Petri dish. Scale bar = 0.5 mm. B) *In vitro* seedling after 8 weeks culture and subsequently transferred to larger culture vessels. Scale bar = 0.5 cm. C) Seedlings after 20 weeks *in vitro* culture. D) Corm isolated from *in vitro* seedlings and placed in chilling conditions. Scale bar = 0.5 cm. E) Culture vessel containing sterilized vermiculite used to chill corms. F) Plantlets under *ex vitro* conditions in a walk-in growth chamber.

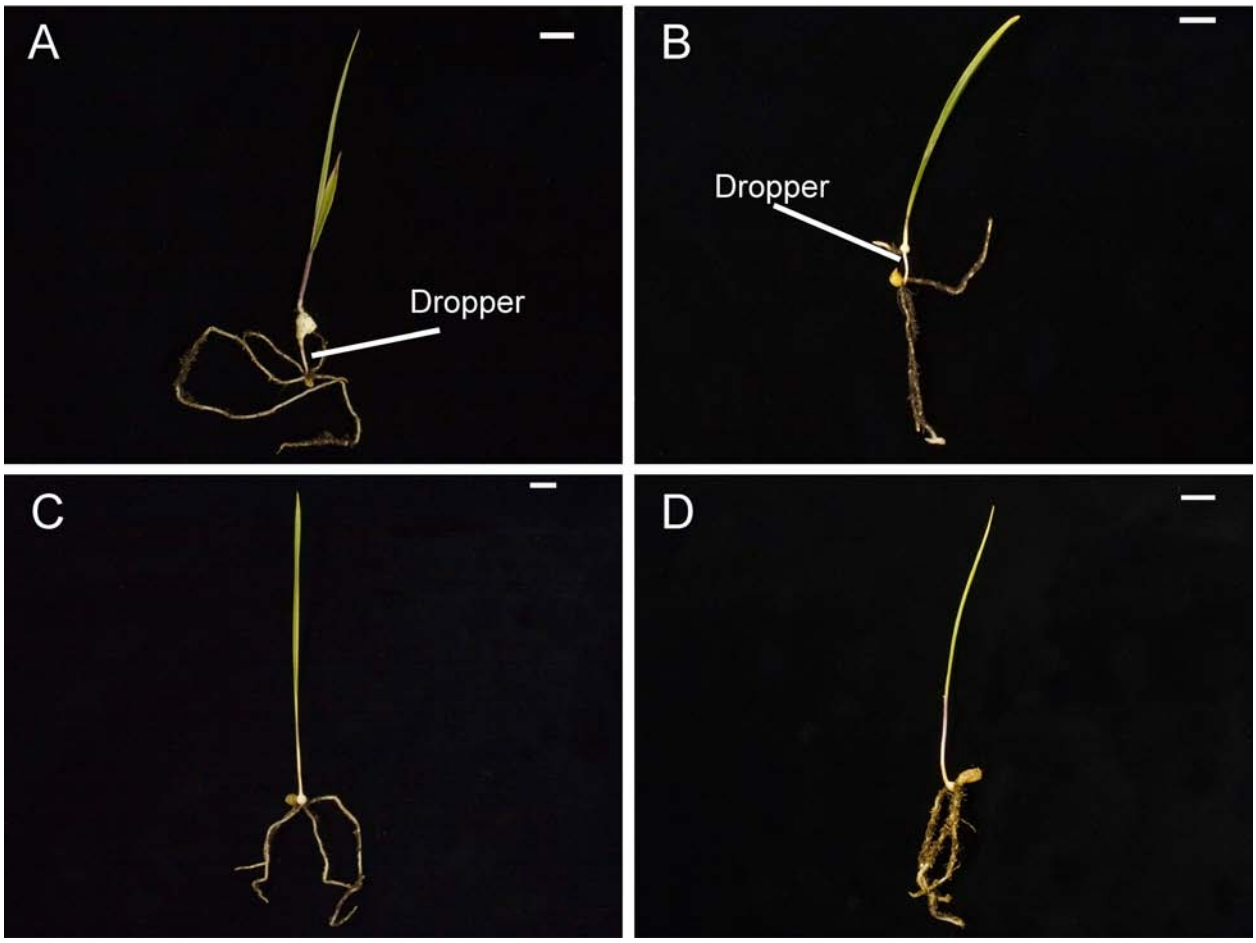

Figure 5-2. *Ex vitro* growth comparison of representative *Calopogon tuberosus* plantlets. Plantlets represent average size after 16 weeks growth in a walk-in growth chamber. Plantlets were generated after chilled corms were planted under *ex vitro* conditions. A) Michigan plantlet with dropper formed between the original and new corm. B) South Carolina plantlet with dropper formed between the original and new corm. C) North Central Florida plantlet. D) South Florida plantlet. Scale bars = 1 cm.

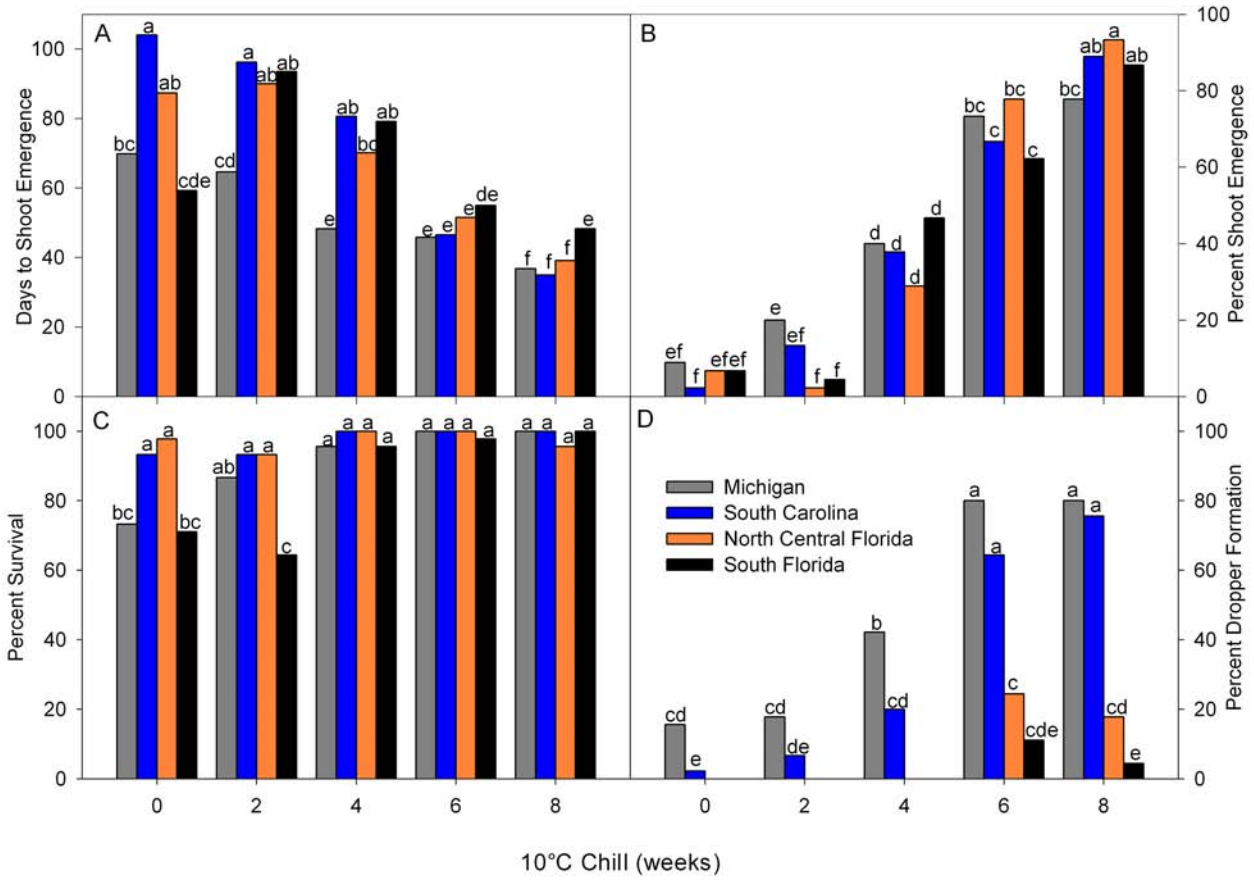

Figure 5-3. Effects of chilling corms at 10°C on shoot emergence after 16 weeks of *ex vitro* growth of *Calopogon tuberosus* plantlets. A) Number of days to shoot emergence. B) Percent shoot emergence recorded by the presence of a shoot emerged from the soil. C) Percent survival measured by the presence of a corm beneath the soil. D) Percent dropper formation. Each histobar represents the mean response of five replications with nine plantlets for a total of 45 plantlets per treatment*source. Means with the same letter are not significantly different at $\alpha = 0.05$.

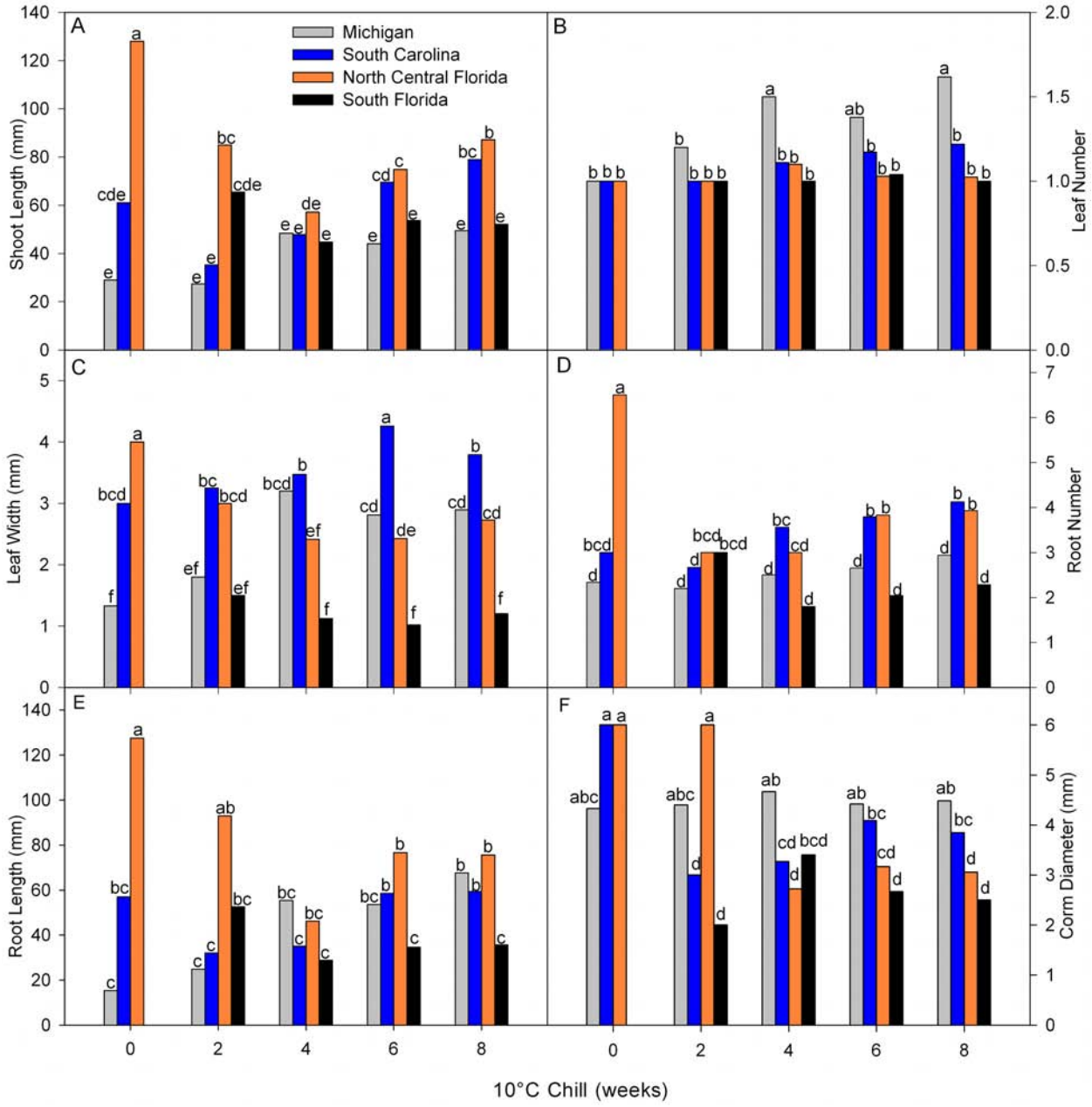

Figure 5-4. Effects of chilling corms at 10°C on growth and development of *Calopogon tuberosus* plantlets. Data was collected after 16 weeks *ex vitro* growth. A) Shoot length measured from the soil surface to the tip of the longest leaf. B) Leaf number. C) Leaf width measured at the widest point of the widest leaf. D) Root number. E) Root length of the longest root. F) Corm diameter of the new corm measured horizontally at the widest point. Each histobar represents the mean response of five replications with nine plantlets for a total of 45 plantlets per treatment*source. Means with the same letter are not significantly different at $\alpha = 0.05$.

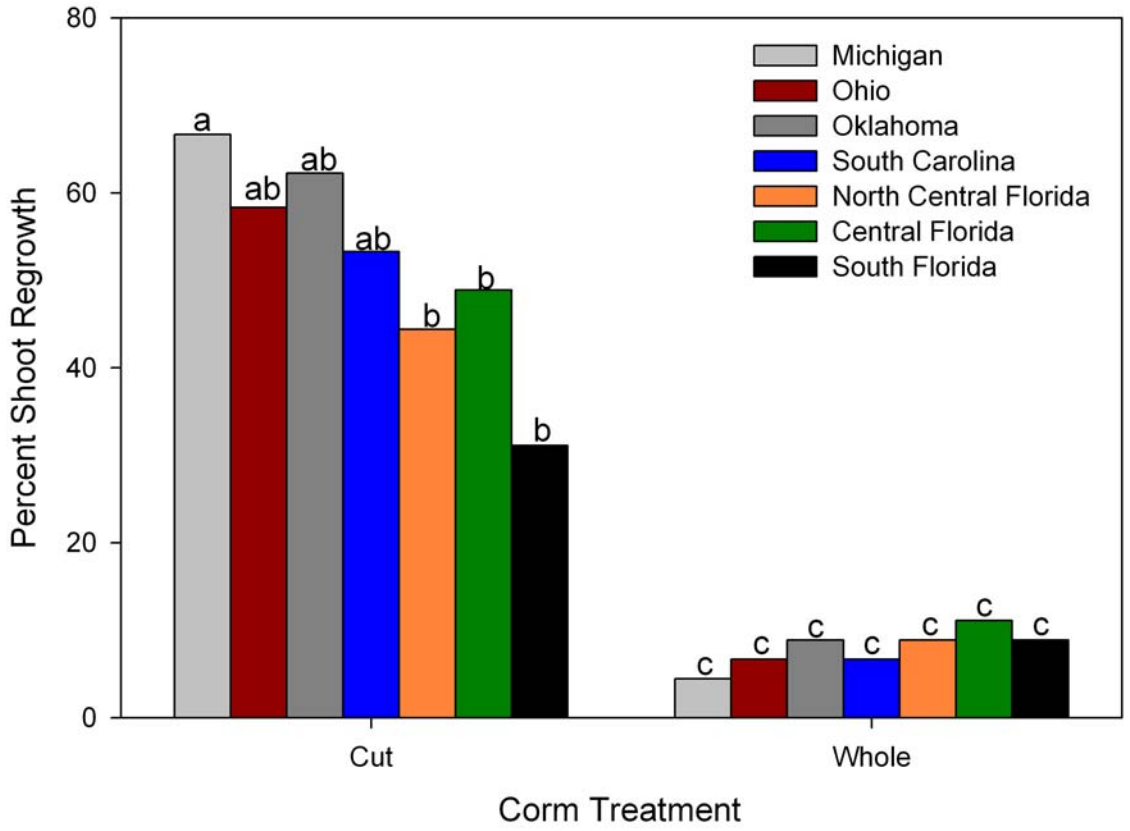

Figure 5-5. Effects of cut and uncut unchilled corms on regrowth of *Calopogon tuberosus* shoots. Data was collected after 8 weeks *in vitro* culture. Histobars represent the mean response of five replications with nine propagules for 45 total measurements per treatment*source. Histobars with the same letter are not significantly different $\alpha = 0.05$.

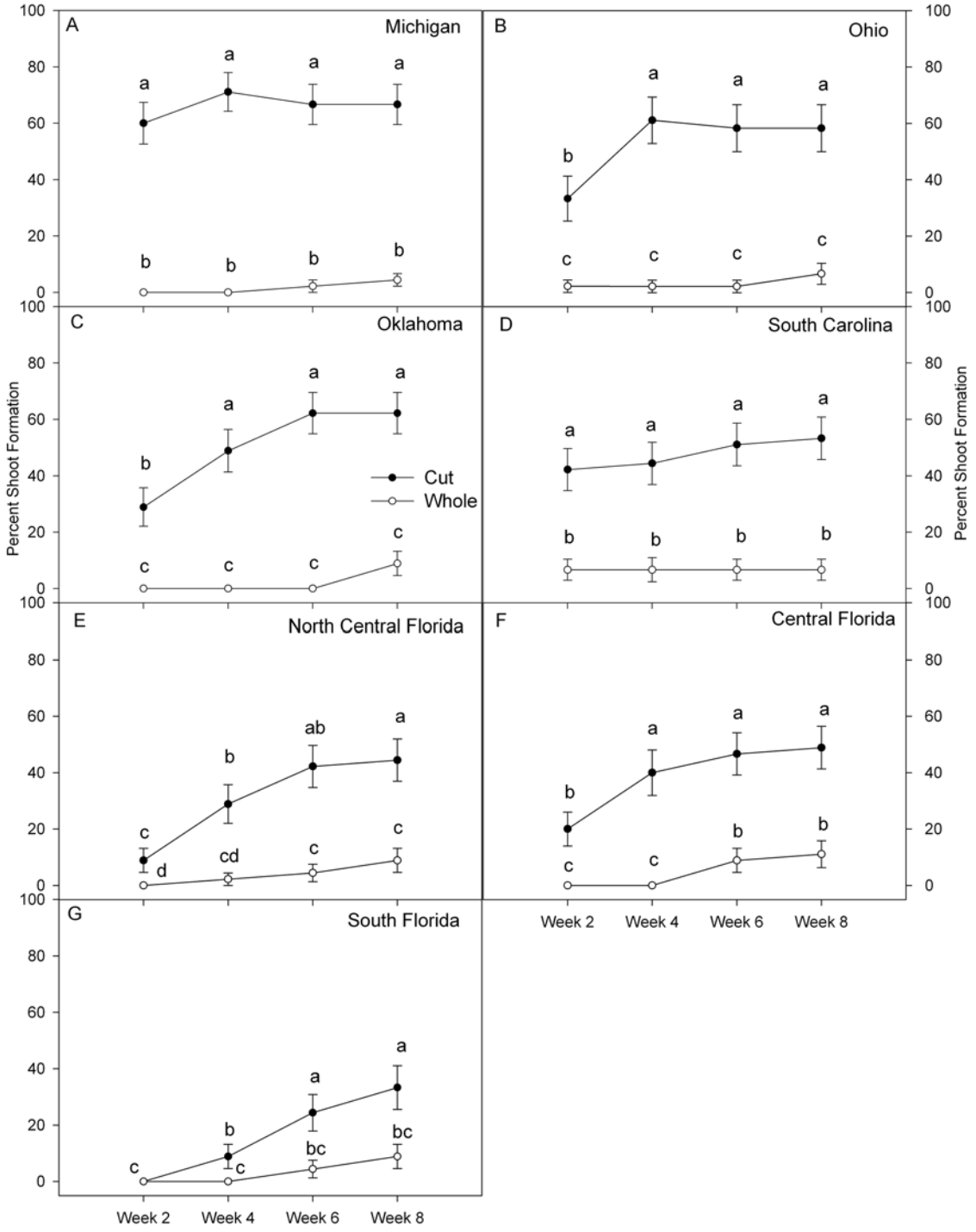

Figure 5-6. Percent shoot regrowth from cut and uncut corms of *Calopogon tuberosus* over 8 weeks *in vitro* culture. A) Michigan. B) Ohio. C) Oklahoma. D) South Carolina. E) North Central Florida. F) Central Florida G) South Florida. Each data point represents the mean response ± S.E. of five replications with nine propagules. Points with the same letter are not significantly different at α=0.05.

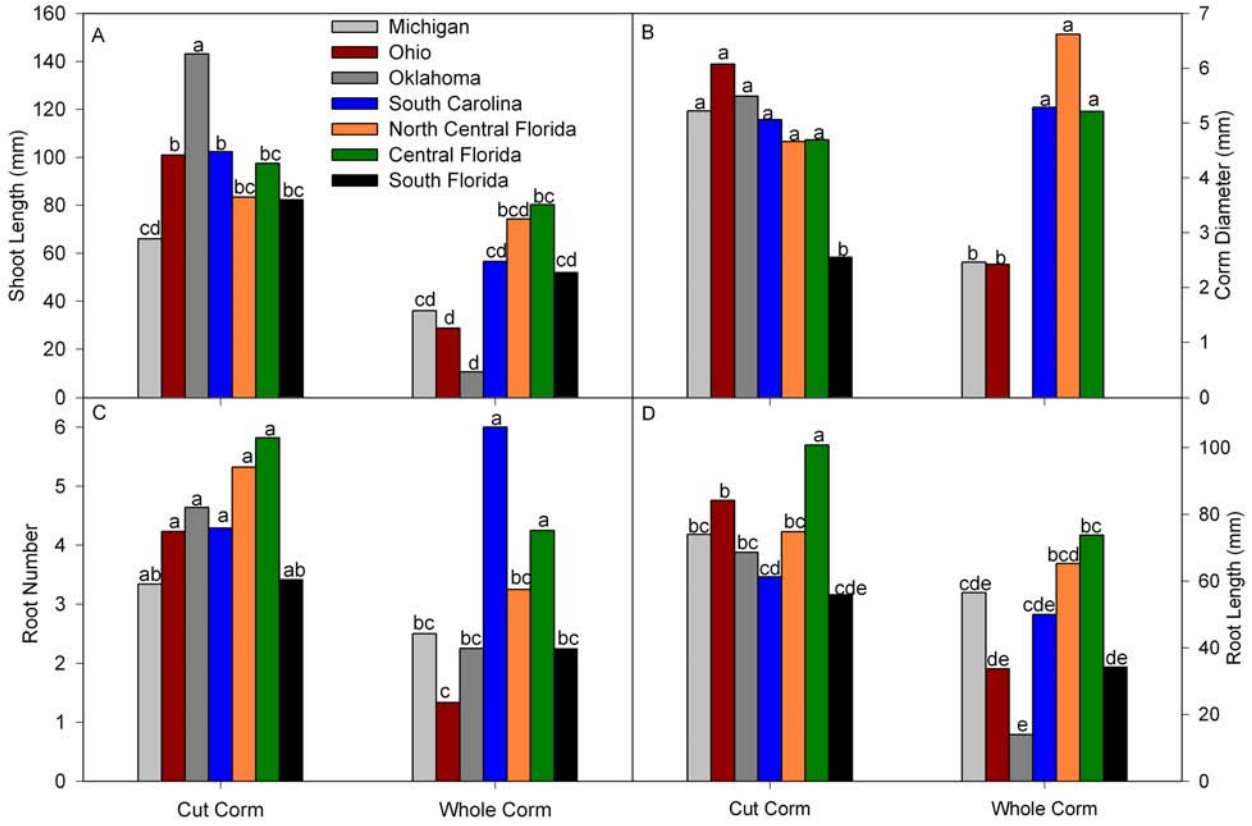

Figure 5-7. Comparative regrowth from cut and whole corms of *Calopogon tuberosus*. Data was collected after 8 weeks *in vitro* culture. A) Shoot height measured from the above the corm to the tip of the longest leaf. B) New corm diameter measured horizontally from the widest point. C) Root number. D) Root length of the longest root. Histobars represent the mean response of five replications with nine propagules for 45 total measurements per treatment*source. Histobars with the same letter are not significantly different $\alpha = 0.05$.

CHAPTER 6
SUMMARY AND CONCLUSIONS

The Orchidaceae is a high profile plant family that is imperiled worldwide due to habitat loss and illegal collecting. Because orchids have captured the attention of many individuals and organizations, their conservation is at the forefront of many concerns. While many organizations are involved in conserving and purchasing land to save orchids, others focused their attention on the restoration of rare and threatened species. In order to restore population numbers, the propagation of orchids has received much attention. *In vitro* methods were developed beginning in the early 1900s to germinate orchid seed. *In vitro* germination methods are popular tools for germinating orchid seed to produce plants for population restoration. However, the source of plant material for orchid population restoration is often not a concern.

For this reason, differences in local adaptation of *Calopogon tuberosus* to local environmental conditions were studied using *in vitro* methods. By controlling conditions such as photoperiod and temperature, *in vitro* methods were shown to be efficient and effective at differentiating ecotypes of *Calopogon tuberosus* based on seed germination, seedling development, biomass allocation, and corm formation. This area of research known as *in vitro* ecology utilizes methods to study the unique growth and development of plant species based on geographic source.

The effects of photoperiod and germination media on orchid seed germination were researched extensively over the past 100 years. However, my results indicate that photoperiod is not crucial for the germination of *C. tuberosus* seeds. Although seed germination percentages from Florida were higher under a short day photoperiod, differences in photoperiodic response of northern populations were not significant. *In vitro* responses to germination media contributed to differences in germination and development. These differences reflected the varying habitats,

soil types, and soil nutrient availability at each individual site. Different temperature treatments were a strong influence on seed germination compared to photoperiod. In addition, cold-stratification significantly increased germination by removing physiological dormancy. Germination results indicate that *C. tuberosus* ecotypes are influenced more by soil type and nutrients as well as temperature compared to photoperiod. However, photoperiod can not be ruled out as a major selection pressure influencing the growth and development of adult plants. Once dormancy is removed from seeds, they may be able to germinate under a wider range of conditions leading to different results reported in Chapters 2 and 3. A different approach would be to stratify seeds on several germination media followed by a photoperiod and temperature screen.

Throughout the germination experiments, Michigan seeds germinated and developed corms quicker than all other populations, while south Florida seeds germinated slowly. A detailed timecourse biomass allocation study showed that Michigan seedlings allocated more biomass to corms faster than all other populations, while corm formation was delayed in southern plants. The differential corm biomass allocation was positively correlated with a shorter growing season. Thus, the more rapid seed germination, corm formation, and corm biomass allocation in northern plants reflects adaptive responses to more severe winter conditions. Although all *C. tuberosus* populations required a chilling period to break corm dormancy, the ecological strategy for a chilling requirement is different. Southern plants require a chilling period to protect from sudden temperature fluctuations during winter months, while northern plants require a chilling period to remain dormant during a long winter.

The results from this research indicate that *Calopogon tuberosus* ecotypes do exist, but further investigation is still necessary to determine whether these ecotypes demonstrate

phenotypic plasticity and the ability to adapt to non-local environmental conditions. Data indicate that even under *in vitro* conditions, *C. tuberosus* ecotypes maintain their unique characteristics. For this reason, using local seed for population restoration projects is recommended. Local adaptation of *C. tuberosus* is, at least, based on temperature, growing season length, habitat type, and soil type and nutrient availability. These selection pressures do not interact as single entities, but rather form a complex web of selection pressures that influence the development of ecotypes. Biotic selection pressures, such as pollinators, likely influence differences in morphology, seed viability, and capsule set that could influence ecotypic differentiation. *In vitro* ecology methods are effective to differentiation ecotypes, and should be applied worldwide as a component of sound conservation management plans to recover at-risk species.

APPENDIX A
FIELD TRANSPLANT OF *Calopogon tuberosus* IN SOUTH FLORIDA

Introduction

Reintroducing and augmenting plant populations is a necessary step to maintain and reinstate ecosystem diversity (Maunder, 1992). Reintroducing and establishing new populations of charismatic species, such as terrestrial orchids, serve as conservation symbols and to divert attention from a vulnerable population (Maunder, 1992). The worldwide loss of orchid taxa has led to an abundance of research focused on their conservation, ecology, and field transplantation (Ramsay and Dixon, 2003). Unfortunately, few reports exist that detail management methods for both orchid populations and their habitat (Stewart, 2007). Successful establishment of plants into their habitats to augment or replace extent population is often the culmination and goal of conservation research (Batty *et al.*, 2006*a*). Field establishment of orchids is challenging since complex ecological requirements of individual taxa are not well-understood (Scade *et al.*, 2006).

Field establishment terrestrial orchids has been previously attempted, but only for a few species (McKendrick, 1995; Ramsay and Stewart, 1998; Stewart *et al.*, 2003; Batty *et al.*, 2006*b*; Scade *et al.*, 2006; Yamato and Iwase, 2008), and few involve successful field establishment of North American species (Stewart, 2007). Long-term survival of field translocated orchids is often very low because efficient methods for establishing orchids are lacking (Batty *et al.*, 2006*a*). The influence of abiotic and biotic factors on successful field establishment of orchids has not been studied in detail (Scade *et al.*, 2006). However, field establishment of orchids could be an important tool for not only conserving orchids, but also to further our knowledge of orchid ecology (McKendrick, 1995).

A major obstacle to field establishment is initial survival of propagules. Only a few articles discuss techniques for increasing survival of orchid seedlings under *in situ* conditions (Batty *et*

al., 2006*b*; Scade *et al.*, 2006; Smith *et al.*, 2009). Batty *et al.* (2006*b*) reported higher survival of several Australian orchid species when dormant tubers were used rather than seedlings. However, observations that *Thelymitra manginiorum* seedlings established more readily than tubers indicates that field performance of different propagule type is species specific. Smith *et al.* (2009) found that actively growing plants established readily compared to dormant tubers. Competition may also be an important factor to consider for successful establishment (McKendrick, 1995). Dense coverage by native species and less weed coverage increased survival of field transplanted orchid species (McKendrick, 1995; Scade *et al.*, 2006; Yamato and Iwase, 2008), but areas of highest vegetation coverage impeded total survival (McKendrick, 1995).

Calopogon tuberosus var. *tuberosus* (referred to as *Calopogon tuberosus*) is a corm forming species found throughout eastern North America including southwest Florida. In south Florida the flowering season begins in April and continues through the end of May. Typical south Florida habitat includes mesic-alkaline prairies surrounded by pine flatwoods growing in full sun. The ease of asymbiotic seed culture and rapid corm formation *in vitro* makes this orchid species an excellent candidate for development of a model system to test field establishment methods.

Two separate experiments were conducted to: 1) Establish *C. tuberosus* seedlings at the Florida Panther National Wildlife Refuge (FPNWR); 2) Compare survival of seedlings and corms of *C. tuberosus*; 3) Compare survival and growth of seedlings in burned and unburned areas; and 4) Recommend management practices for establishing terrestrial orchids at the FPNWR. In addition, the methods used can be transferred to other terrestrial orchids worldwide.

Materials and Methods

Study Site

The Florida Panther National Wildlife Refuge is located in southwest Florida in Collier County (Figure A-1). The field plots were established in the marl prairie in Unit 23. This area contains the largest population of *C. tuberosus* on the FPNWR.

Seed Source and Propagation

Seed was collected from the FPNWR as previously described in chapter 3. Only propagules derived from the FPNWR were used to avoid any potential cross-pollination effects by introducing no-local ecotypes. For the 2008 experiment, seeds were germinated beginning March 2007 on P723 medium (*Phyto*Technology Laboratories, Shawnee Mission, KS) in square Petri dishes as part of experiments in Chapter 2. After 8 weeks culture (May 2007), seedlings were transferred to *Phyto*Tech Culture Boxes (*Phyto*Technology Laboratories, Shawnee Mission, KS) containing 100 mL P723 medium. After an additional 20 weeks culture, corms were chilled from October 2007 to January 2008. This was accomplished by removing the shoots and roots from the seedlings, and transferring corms to fresh P723 medium contained in *Phyto*Tech Culture Boxes. After the chilling period at 10°C, corms were transferred to fresh P723 medium contained in *Phyto*Tech Culture Boxes for an additional 12 weeks. Seedlings were subsequently moved to greenhouse conditions April 2008. Seedlings were planted in 9-cell pack trays (Model #IKN0809, Hummert International, Earth City, MO) containing Fafard 2 soilless potting mix (Conrad Fafard, Inc., Agawam, MA). Seedlings were covered with clear vinyl humidity domes to prevent desiccation and placed under 50% shade cloth and a natural photoperiod. Average light levels were 300 µmol m^{-2} s^{-1} measured at 12 noon, and average temperatures ranged from 21.6 ± 2°C to 29.3 ± 3°C. After 1 week humidity domes were removed, and seedlings watered as needed.

Seedlings from the 2009 experiment were initially started January 2008 as part of experiments from Chapter 3. Seeds were sown on BM-1 Terrestrial Orchid Medium (*Phyto*Technology Laboratories, Shawnee Mission, KS) in square Petri dishes. After 8 weeks culture, seedlings were transferred March 2008 to *Phyto*Tech Culture Boxes containing 100 mL BM-1 medium. After 30 weeks culture, corms were transferred to new *Phyto*Tech Culture Boxes containing 100 mL BM-1 medium and chilled at 10°C from October 2008 to December 2008. Seedlings were moved to greenhouse conditions December 2008 until ready for field establishment February 2009. Greenhouse transfer procedures were similar to those previously described. Average light levels were 253 µmol m^{-2} s^{-1} measured at 12 noon, and average temperatures ranged from 20.8 ± 2.3°C to 28.8 ± 2.8°C.

Field Establishment

Planting occurred in successive years in April 2008 and February 2009. For the 2008 planting, differences in survival of field transplanted seedlings and corms were examined. For the 2009 planting, the response of planting seedlings in a burned and unburned area was studied. In all experiments square quadrats 30 cm x 30 cm were constructed from PVC piping (1.5 cm diameter). Each quadrat was divided into 16 sections approximately 7.5 cm x 7.5 cm by using 14 gauge coated electrical copper wire. A HOBO H8 Pro weather station (www.microdaq.com, Ltd., Contoocook, NH) was placed at the site to record daily temperatures and relative humidity (Figure A-2).

Comparison of propagule type on field survival

Three 10 m transects were establish in unit 23 on April 23, 2008. Each transect contained four quadrats that were 2.5 m apart. A randomized block design was used to plant propagules. Corms and seedlings were assigned randomly to a quadrat and quadrat section. Sixteen propagules were used in each quadrat (8 seedlings and 8 corms per quadrat). A total of 192

propagules were planted. Propagules were irrigated with distilled water upon initial planting. Data was collected on May 20th 2008, July 9th 2008, February 27th 2009, and April 23rd 2009.

Seedling survival in a burned and unburned field plot

In January 2009, unit 23 was burned except the area where *C. tuberosus* was previously established in 2008. This presented a unique opportunity to compare the effects of planting seedlings in the burned and unburned areas in unit 23. Two 10 m transects were established in the burned area and unburned area. Three quadrats were allocated to each transect. Sixteen seedlings were planted in each quadrat for a total of 48 seedlings per transect and 192 seedlings for the experiment.

Data Recording and Statistical Analysis

Survival of all seedlings was recorded. Two different categories were classified in determining propagule survival. Percentage of actively growing shoots was recorded for those seedlings with an actively growing green shoot. Percentage of emergent shoots was recorded when shoots were present, but not necessarily actively growing shoots. Seedling leaf measurements were recorded before the February 2009 experiment, and again in April 2009. Shoot emergence data were analyzed using proc glimmix, logistic regression, and least-square means in SAS v9.1.

Results

Comparison of Propagule Type on Field Survival

Propagule type ($F = 0.50$, $p = 0.48$) did not influence survival, but date of planting was significant ($F = 20.4$, $p < 0.0001$). At the initial data collection in May 2008, a higher proportion of seedlings (43.8%) had actively growing shoots compared to corms (32.3%) (Figure A-3). After 1 month of field establishment, less than 50% of all propagules had actively growing shoots regardless of treatment. In July 2008, all shoots had either senesced or were present but

not actively growing. A higher proportion of emergent shoots were observed on seedlings (22.9%) compared to corms (12.5%). Data collected during February 2009 occurred during the early growing season in south Florida. Actively growing shoots were higher on corms (12.5%) compared to shoots (10.4%), but was not significantly different. In April 2009 no significant difference was observed between the survival of corms (6.25%) and seedlings (8.33%), and the presence of shoots further declined. At this time, one seedling in the early flowering stage established from a corm propagule was observed No shoots were observed at the data recording in June 2009. Combined survivorship percentages were as follows: 38.0% (May 2008), 18.9% (July 2008), 11.4% (February 2009), and 7.3% (April 2009).

Seedling Survival in a Burned and Unburned Field Plot

Burning significantly influenced percent of emerged shoots ($F = 48.7$, $p < 0.0001$), while not burning influenced the percentage of actively growing shoots ($F = 4.32$, $p = 0.04$). Two months after field establishment, the number of actively growing shoots declined in both plots. Approximately 3% and 11% of actively growing shoots was observed in the burned and unburned areas, respectively (Figure A-4A). However, senesced shoots were visible on seedlings in the burned plot, but none in the unburned plot (Figure A-4B). Total survivorship was 7.3% when combining all data. Of the actively growing shoots, shoot lengths were recorded in April 2009 (Table A-1). Shoot lengths on all seedlings with actively growing shoots in the burned plot increased, while three of the eleven recorded leaf measurements in the unburned plot decreased (Table A-1).

Discussion

This is the first documentation of a field establishment study involving *Calopogon tuberosus*, and one of the only scientifically documented orchid field establishment studies in North America (Stewart *et al.*, 2003; Zettler *et al.*, 2007). However, conclusive results were not

obtained due to the short-term nature of the study, but they are likely after several years of monitoring. Absence of an actively growing shoot did not indicate propagule death since corms may have been present beneath the soil surface, but their presence beneath the soil was not confirmed in order to minimize soil disturbance. In addition, shoots on field established seedlings may have senesced naturally because senescence naturally occurs in late May through early June.

Field establishment of orchids depends may depend on propagule type such as seedlings or storage organs. Dormant storage organs are often considered more likely to survive initial field establishment (Debeljak *et al.*, 2002; Batty *et al.*, 2006*b*). Dormant storage organs may be able to survive drought conditions better than seedlings (Batty *et al.*, 2006*b*); however, results are species specific. *Caladenia arenicola* and *Diuris magnifica* established more readily in the field when dormant tubers were planted rather than seedlings, but *Thelymitra manginiorum* established more readily from seedlings (Batty *et al.*, 2006*a*). However, no *C. arenicola* propagules survived into the third growing season and only 10% of *D. magnifica* tubers. Approximately 70% and 35% of *T. manginiorum* seedlings and tubers, respectively, survived into the third growing season (Batty *et al.*, 2006*a*). Likewise, Smith *et al.* (2009) found that 2-3 year old plants (35%) established more readily in the field compared to tubers (11%) after 4 years.

In the present study, no differences in shoot emergence were observed between corms (6.25%) and seedlings (8.33%) after the first year. The low rates of shoot emergence may have been caused by propagule death or dormancy of corms. Terrestrial orchids can remain dormant for several years (Kery and Gregg, 2004) so long-term monitoring is necessary to observe propagule survival. Throughout 2008-2009, south Florida experienced drought conditions which

may have promoted propagule death. The juvenile state of the propagules (1 year old) may have resulted in poor field establishment as well. Tuber size influenced the survival of several Australian orchids with larger tubers increasing survival compared to smaller tubers (Batty *et al.*, 2006*a*; Smith *et al.*, 2009). Likewise, larger *C. tuberosus* corms or more mature plants may have likely increased survival in the present study by increasing storage reserves that tubers can utilize to sustain drought conditions and initiate growth (Batty *et al.*, 2006*a*).

The influence of competition, shading, and weed coverage influences the establishment of orchids in the field (McKendrick, 1995; Scade *et al.*, 2006). The effects of establishing orchids in burned plots have not been reported previously, but the influence of competition has been examined. In the present study, more actively growing shoots on *C. tuberosus* were observed on seedlings in the unburned area during April 2009. Shoots on the seedlings in the burned area were brown and senesced with the exception of three plants. The surrounding native grasses in the unburned area likely shaded the seedlings providing increased survivorship. Seedlings in the unburned area did not receive any level of shading and likely caused seedling desiccation. Shading led to increased survival of several terrestrial orchids (McKendrick, 1995, 1996; Scade *et al.*, 2006; Yamato and Iwase, 2008), but areas of the densest shade and competition led to a decrease in orchid seedling survivorship (McKendrick, 1995; Yamato and Iwase, 2008).

Fire is a necessary natural disturbance in many ecosystems (Duncan *et al.*, 2008) including those found in south Florida. Competition with weeds and invasive species during field establishment often reduces the successful field establishment of seedlings (Moyes *et al.*, 2005). Native perennials were established readily in a burned grassland and dolomite glade areas, reduced weedy species, and prevented forest succession (Moyes *et al.*, 2005; Duncan *et al.*, 2008).

While the results of the study are mostly inconclusive due to the short-term monitoring of the plots, the techniques employed can be applied to other orchid species worldwide. More definitive results may be observed after another growing season when seedlings in the burned area may re-emerge. Due to the drought conditions the past 2 years in south Florida, additional irrigation may have improved propagule survival. In addition, using symbiotically grown seedlings or inoculating soil with mycorrhizal fungi may have improved seedling survival as well (Batty *et al.*, 2006*a*; Scade *et al.*, 2006; Smith *et al.*, 2009). Although *C. tuberosus* is not a threatened or endangered orchid, the species is still threatened by habitat loss, fragmentation, and illegal collecting. Augmenting current or creating new populations may be necessary for the overall success of the species.

Management Recommendations

In order to successfully establish *C. tuberosus* seedlings in the field the following management techniques are recommended: 1) Propagules approximately 2-3 years old should be used instead of using young seedlings or corms that do not have sufficient reserves to survive initial field establishment. 2) When planting dormant corms, larger corms should be planted. 3) Propagules should be planted at the beginning of the growing season, preferably mid to late February. 4) Due to frequent drought conditions in south Florida, supplemental irrigation should be applied when needed during propagule establishment. 5) Plots should be monitored for at least an additional year to observe successful field establishment. 6) If further field establishment is necessary, examine several different planting intervals after a burn (i.e. 6 months, 1 year).

Table A-1. Shoot lengths recorded for actively growing *Calopogon tuberosus* seedlings in February and April 2009. All measurements are in mm. Seedlings were measured in February under greenhouse conditions prior to transplant, and the April data collection was on seedlings after field transplant on the Florida Panther National Wildlife Refuge.

Treatment	Transect	Quadrat #	Seedling #	Height (Feb 2009)	Height (April 2009)
Unburned	1	1	14	85	28
	1	2	3	25	66
	1	3	3	100	75
	1	3	6	60	90
	1	3	13	90	108
	2	1	14	70	92
	2	2	4	62	220
	2	2	9	76	35
	2	2	10	63	72
	2	3	5	85	125
	2	3	12	26	102
Burned	1	1	13	52	91
	1	2	14	10	165
	2	1	3	95	140

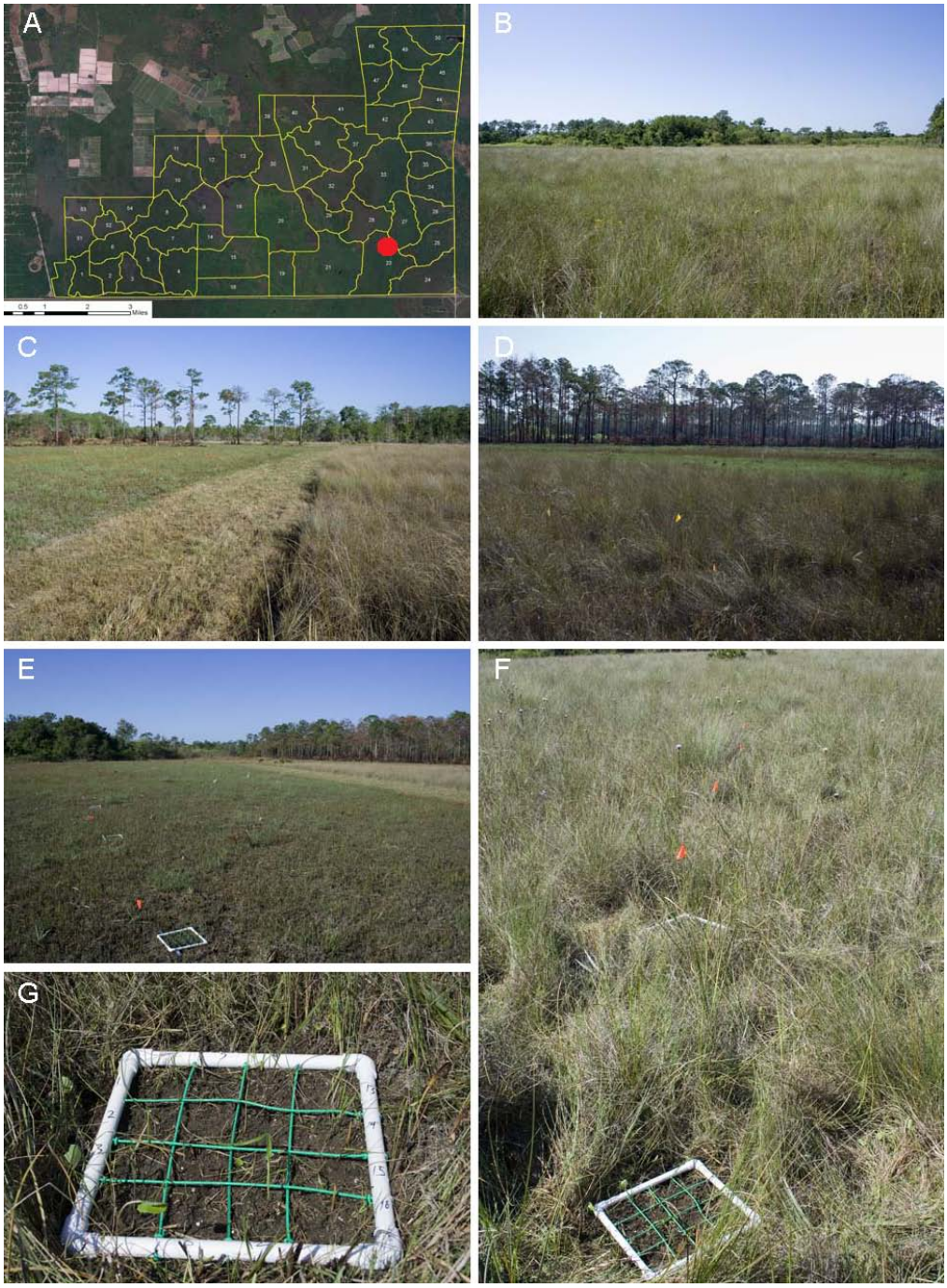

Figure A-1. Field translocation study at the Florida Panther National Wildlife Refuge. A) Map of the FPNWR. The red dot indicates the location of the prairie habitat in Unit 23. B) Prairie habitat where translocation study was conducted. C) Burned (left) and unburned (right) areas in February 2009. D) Burned (background) and unburned (foreground) areas. Yellow flags mark one of the transects. E) Transects and quadrats in the burned area. F) Transect and quadrats in the unburned area in April 2008. G) Close-up of a quadrat.

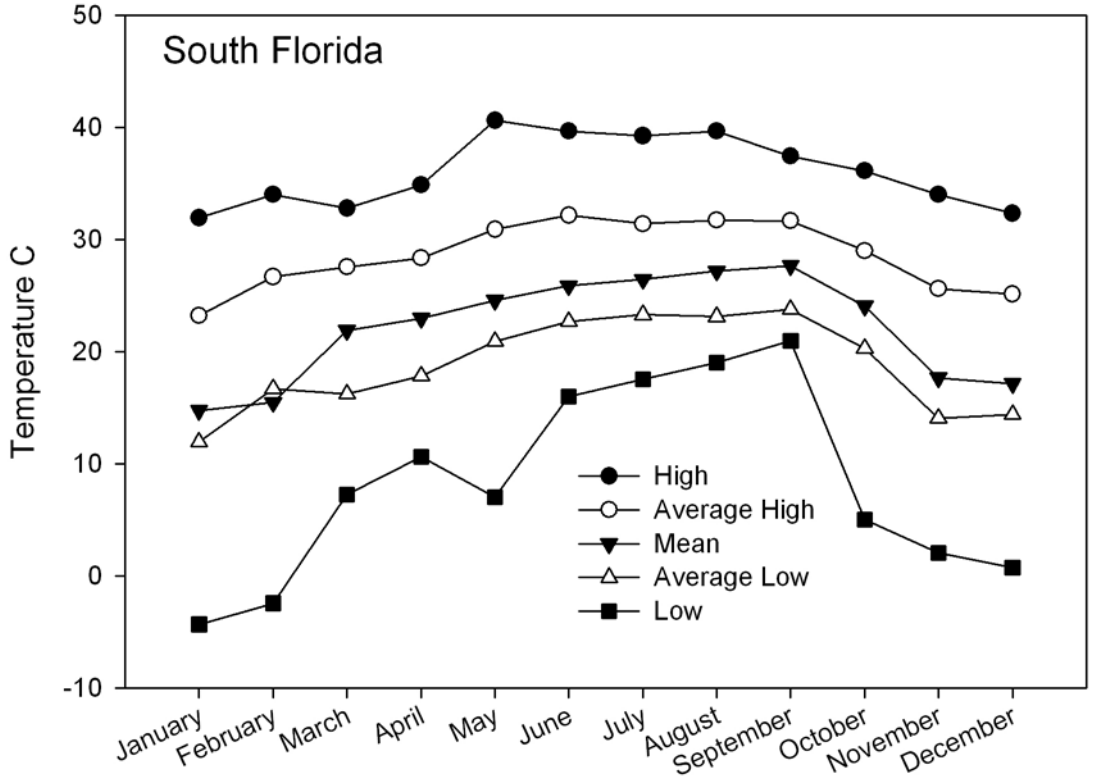

Figure A-2. Monthly temperatures recorded at Unit 23 in the Florida Panther National Wildlife Refuge. Average temperatures represent the mean daily high or low over the entire month. Data was collected with a HOBO H8 Pro series weather station.

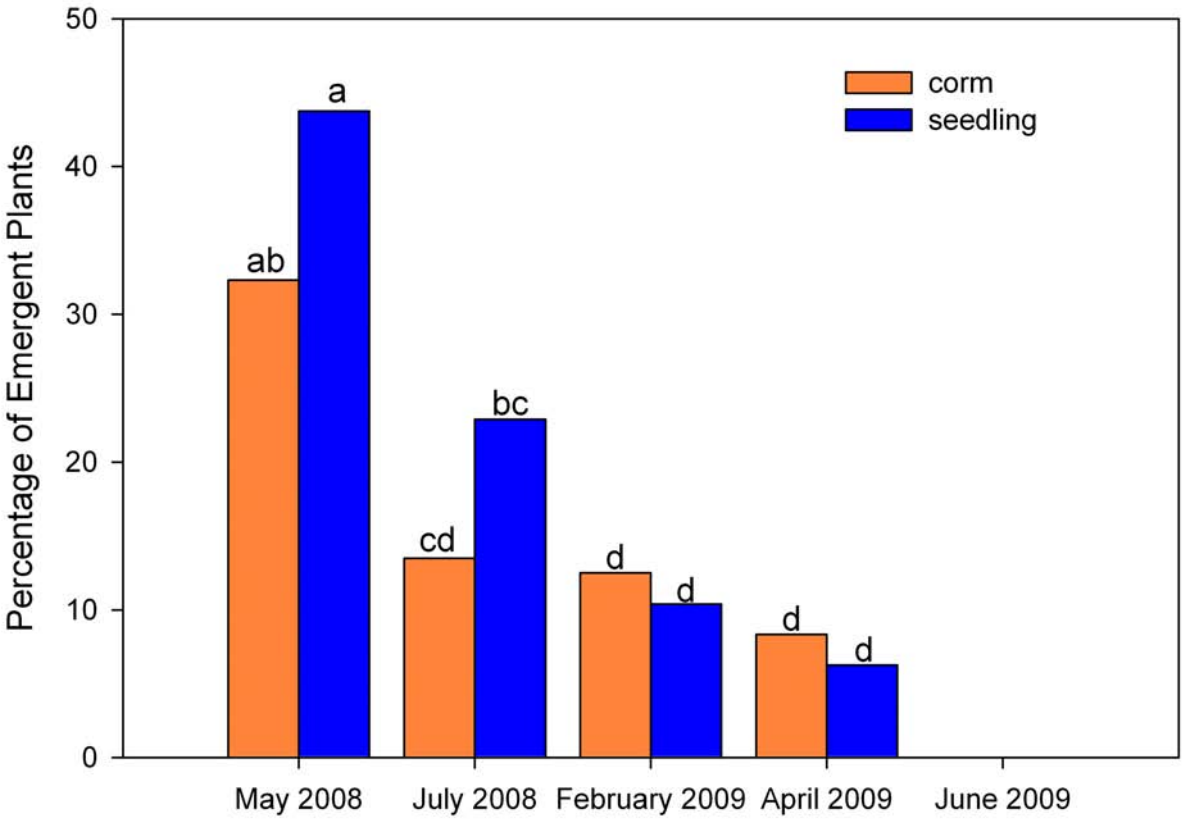

Figure A-3. Survival of *Calopogon tuberosus* propagules at the Florida Panther National Wildlife Refuge. Histobars are the average of three separate transects with four quadrats containing 16 propagules. A total of 96 propagules were planted per treatment for a total of 192 propagules.

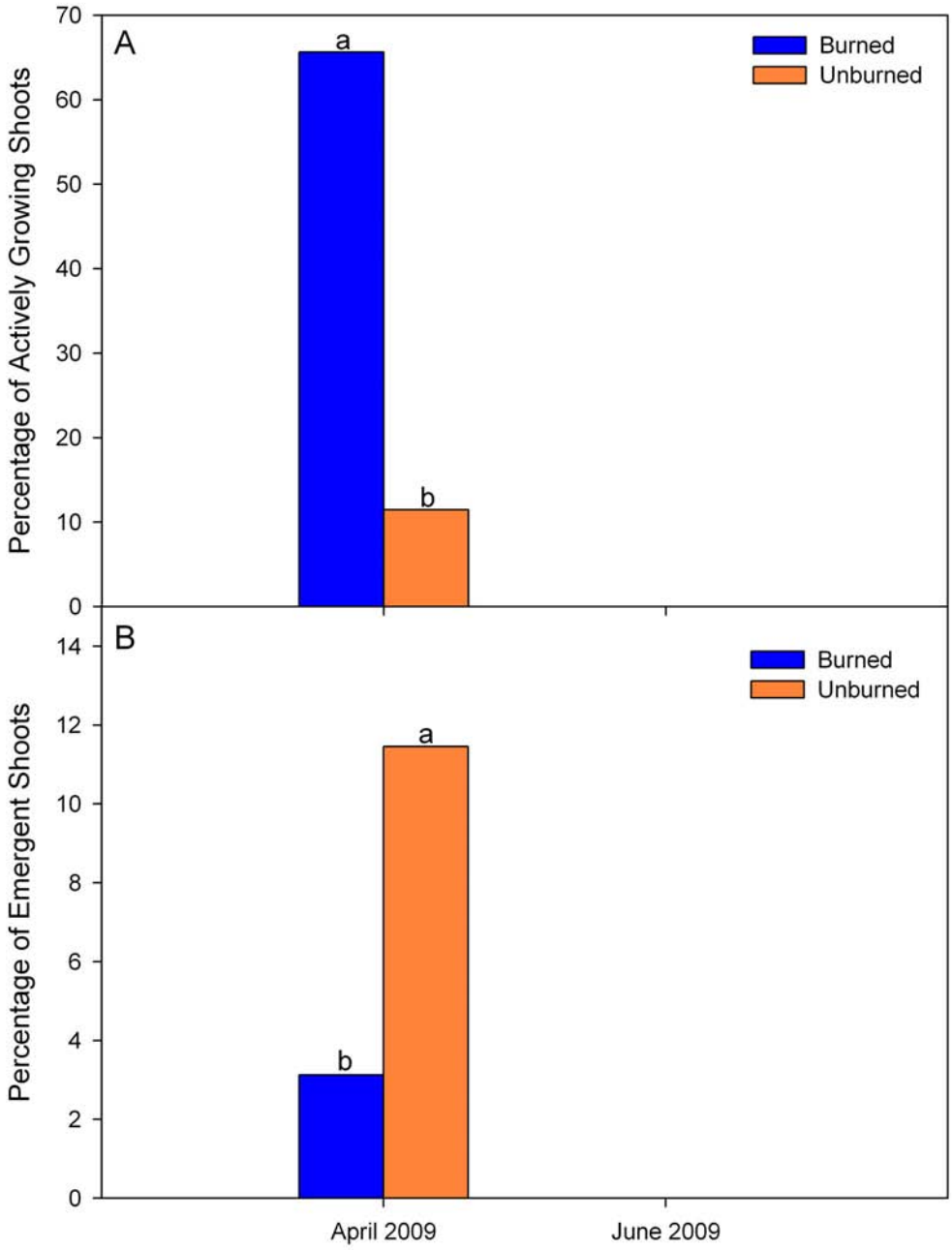

Figure A-4. Survival of *Calopogon tuberosus* seedlings in a burned and unburned plot at the Florida Panther National Wildlife Refuge. A) Percentage of plants with actively growing shoots marked by the presence of a growing green shoot. B) Percentage of plants with either have actively growing shoots or previously emerged shoots that senesced. Histobars represent the mean of two transects with three quadrats containing 16 seedlings. Ninety-six seedlings were planted in each treatment for a total of 192 total seedlings.

APPENDIX B
MORPHOMETRIC ANALYSIS OF C*alopogon tuberosus* POPULATIONS

Calopogon tuberosus is a morphologically diverse terrestrial orchid of eastern North America. This diversity can be explained in part due to population isolations caused by glaciation, limited suitable transitional habitats between the coastal plains and glaciated areas, and possible differences in bee pollinators (Proctor, 1998; Goldman *et al.*, 2004*a*). In a detailed morphometric treatment of *C. tuberosus*, Goldman *et al.* (2004*a*) distinguished plants from the northern, southeastern, and southwestern range. While many characteristics were not different, northern plants tended to be smaller in stature with shorter leaves, smaller flowers, and narrower labella. In addition, the shape of the labellum lobe (Figure B-1) differed. Northern plants had a more rounded and strongly mucronate (with an apex) labellum lobe, while southern plants had wider, broader, and flatter labellum lobe that was weakly mucronate.

A morphometric treatment based on Goldman *et al.* (2004*a*) was conducted on the populations from Michigan, South Carolina, and Florida. Ten plants from each population except Michigan were measured. Only two flowering plants were used for the Michigan population due to issues with encountering flowering plants. All data was collected on wild-growing plants during peak flowering season. The following data was collected: leaf number, leaf width, leaf height, inflorescence height, flower number, number of open flowers, flower width, flower height, lateral sepal length and width, dorsal sepal length and width, lateral petal length and width, labellum length and width, labellum lobe width, column length and width, and column lobe width (see Figures B-1, 2). Data was analyzed using Tukey's HSD test at $\alpha=0.05$.

Leaf number was consistent throughout the range, although plants in south Florida generally had more leaves. However, this was not significantly different from other sources (Figure B-3A). Leaf width was significantly different among sources with Michigan and south

Florida populations having the narrowest leaves (Figure B-3B). Leaf and inflorescence height were both shortest on Michigan plants and largest in Florida populations (Figure B-3C, D).

Flower number was highest on plants from north central Florida and lowest on Michigan plants, but no significant differences were observed between Michigan and South Carolina populations (Figure B-4A). While plants from north central Florida had approximately 10 flowers, only about two were open simultaneously (Figure B-4B). South Carolina plants generally had the highest number of open flowers while Michigan plants had the lowest number at one open flower. However, no significant differences were detected between Michigan and Florida populations.

No differences were observed in lateral sepal length (Figure B-5A) and dorsal width (Figure B-5C), and few differences existed in lateral sepal width (Figure B-5B) and dorsal sepal length (Figure B-5C). However, plants from north central Florida had longer dorsal sepals than Michigan and South Carolina populations and wider lateral sepals than the Michigan population. North central Florida plants also had the longest and widest lateral petals compared to all other populations (Figure B-5E, F).

Most of the characteristics measured were similar to those reported by Goldman *et al.* (2004*a*). Wider labella were observed in Florida populations compared to Michigan and South Carolina populations. Northern plants were also smaller in stature compared to southern plants. However, overall flower size was not significant different perhaps due to sample size differences. Differences in individual flower parts such may be due to different pollinators throughout the range of *C. tuberosus* (Thien and Marcks, 1972; Dressler, 1981; Firmage and Cole, 1988).

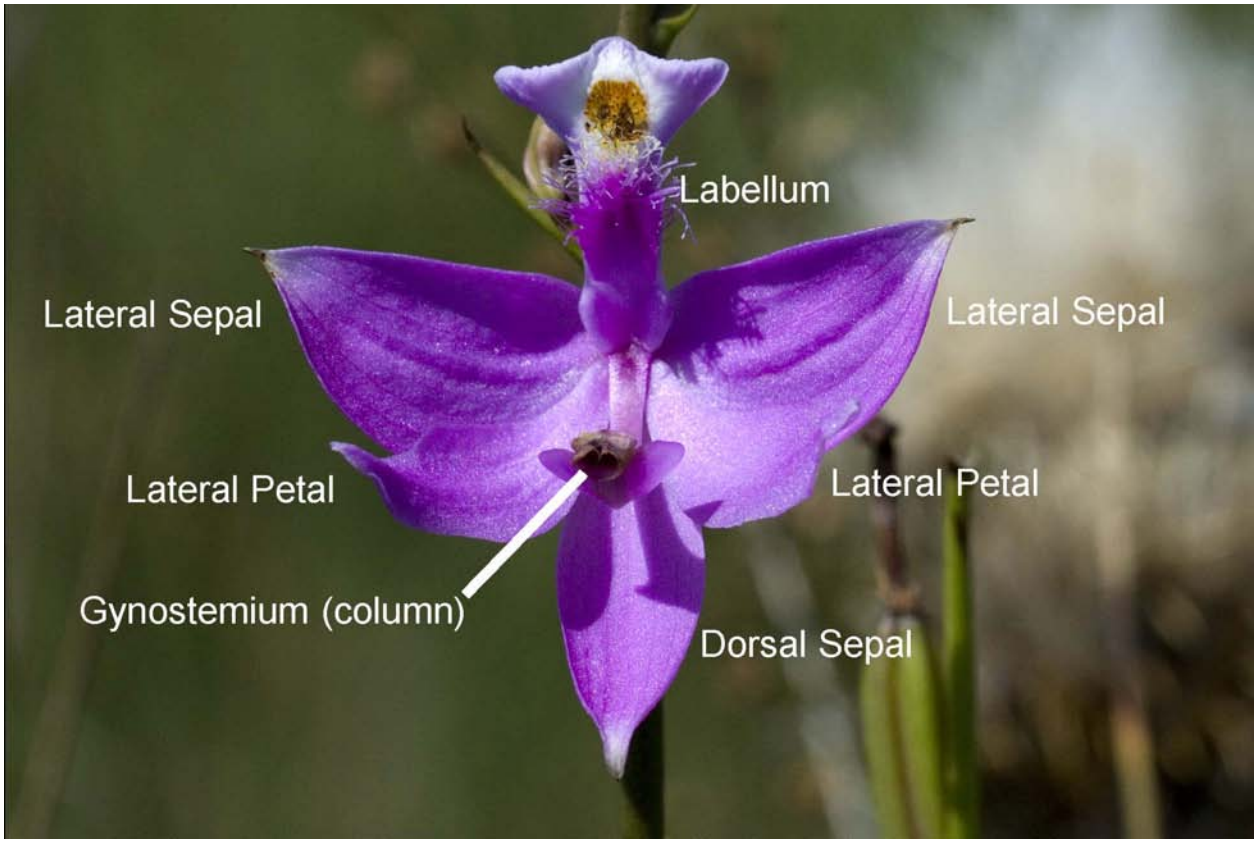

Figure B-1. Labeled parts of a *Calopogon tuberosus* flower measured.

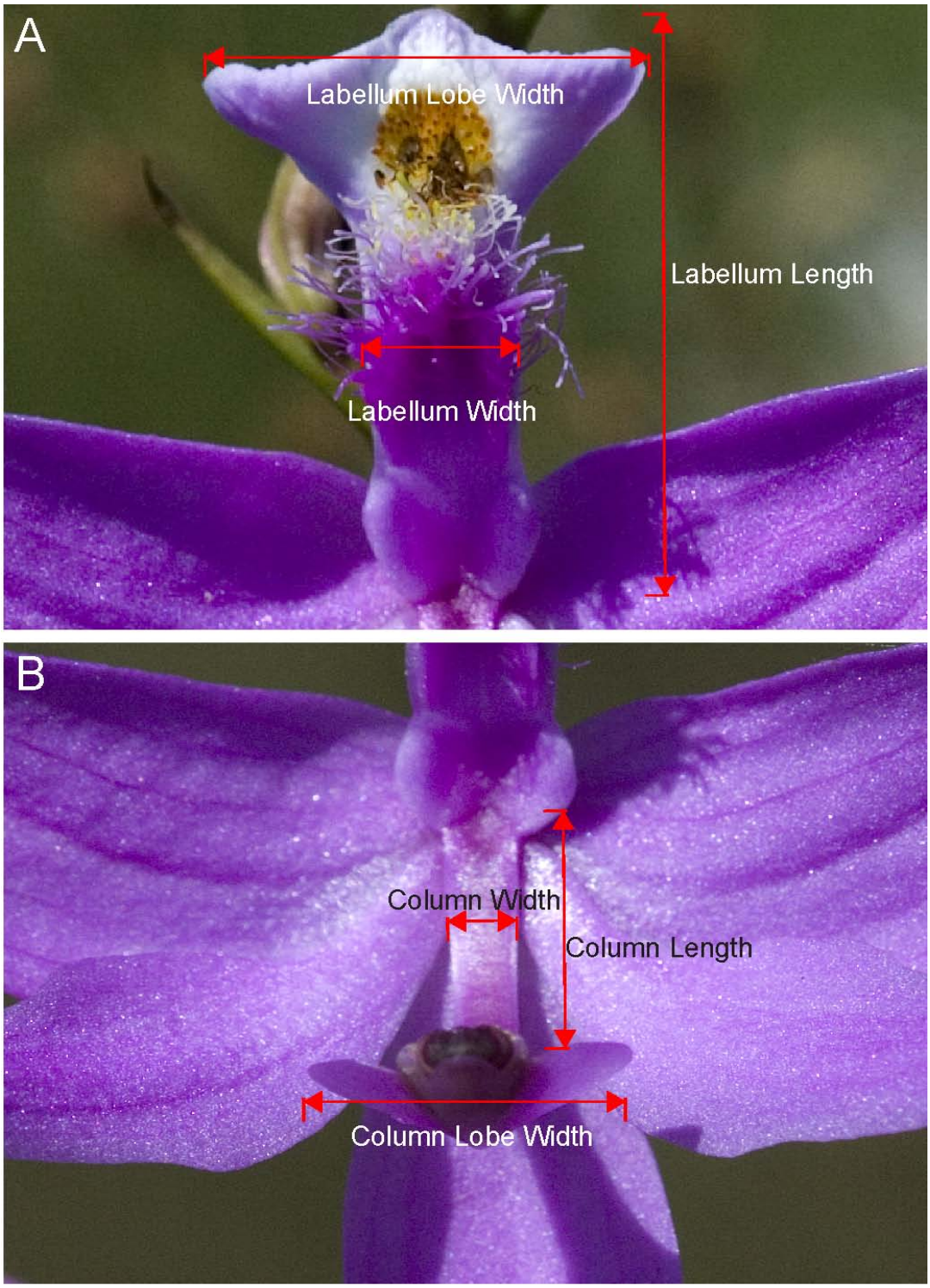

Figure B-2. Labeled close up of a *Calopogon tuberosus* flower. A) Labellum dimensions measured. B) Column dimensions taken.

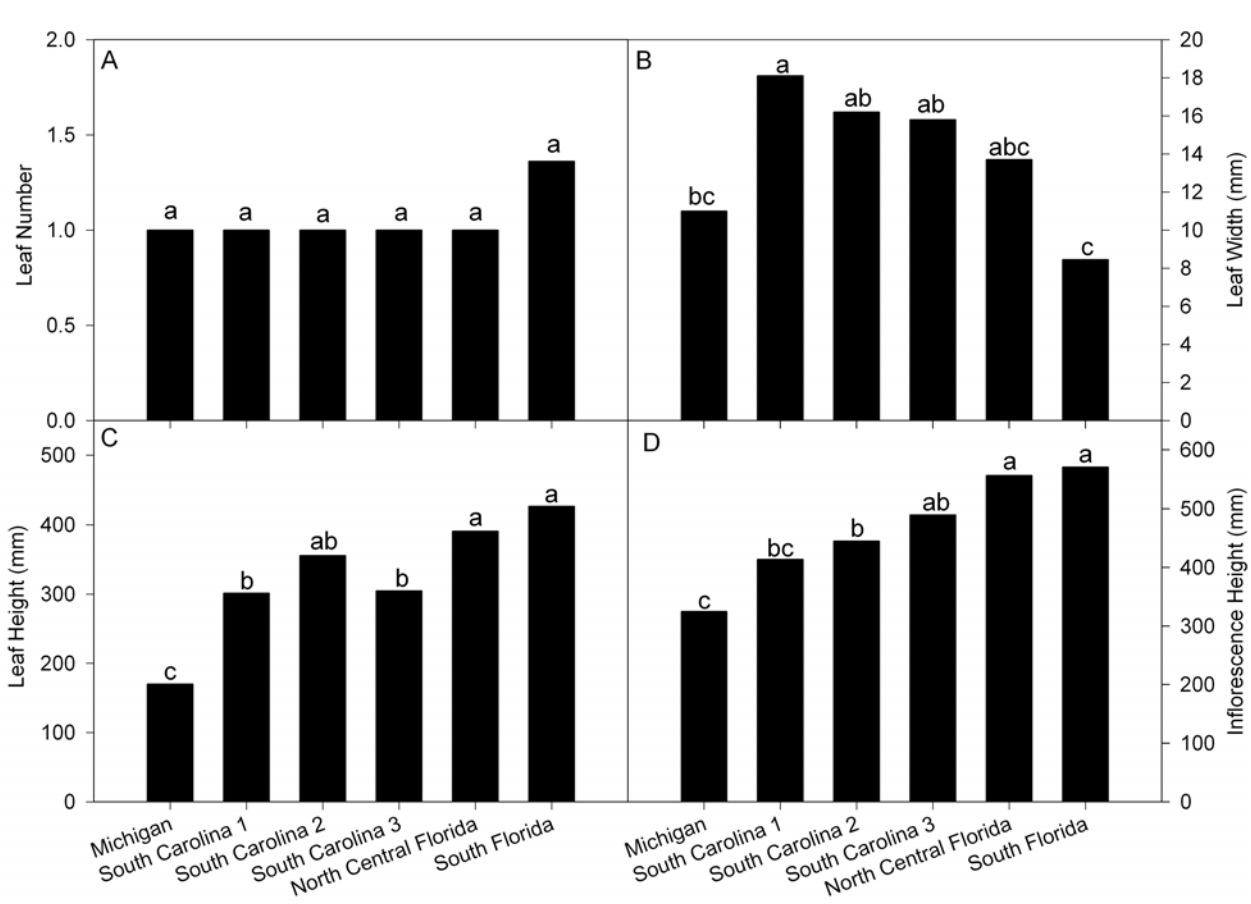

Figure B-3. Whole plant morphometrics analysis of *Calopogon tuberosus*. A) Leaf number. B) Leaf width measured at the widest point. C) Leaf height measured from soil to leaf apex. D) Inflorescence height measured from soil to inflorescence apex. Histobars with the same letter are not significantly different at α=0.05.

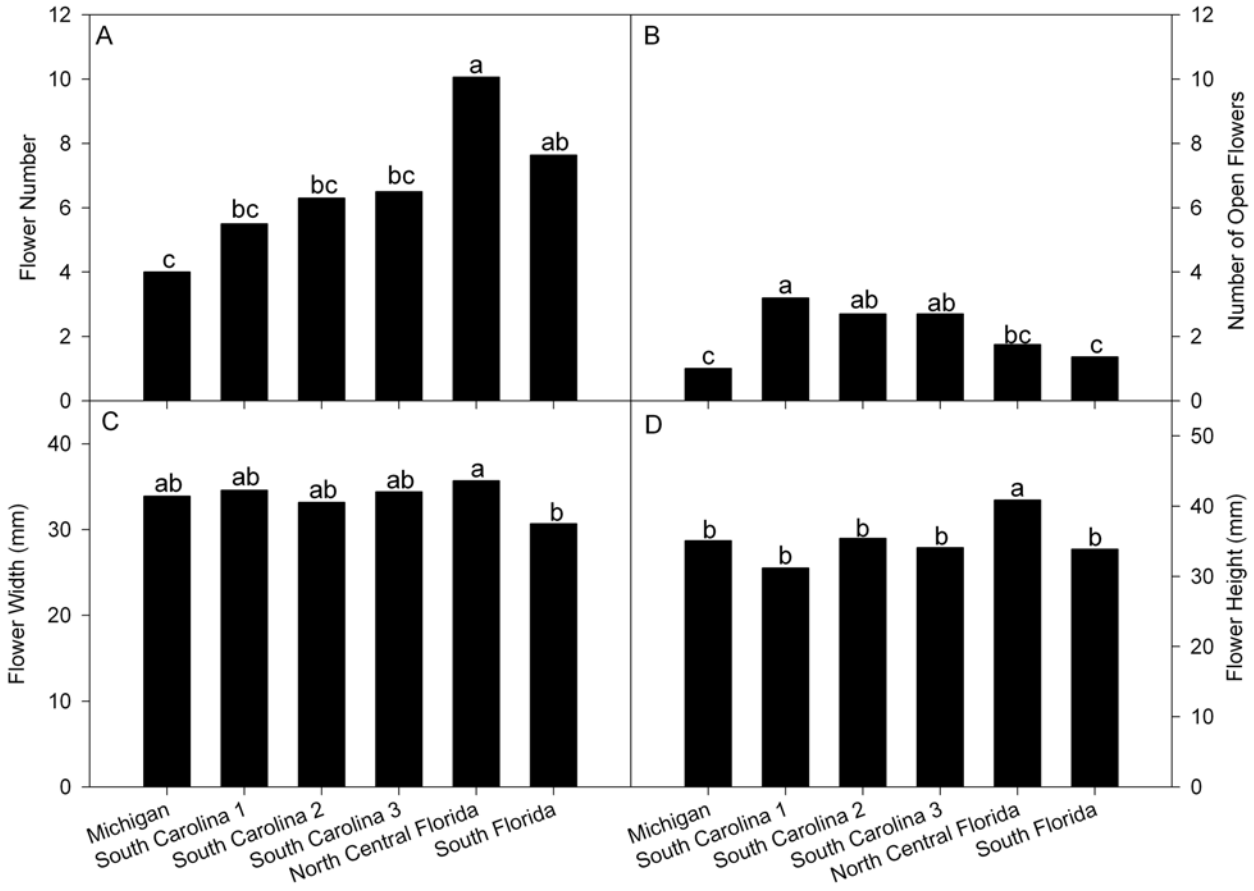

Figure B-4. Flower morphometrics of *Calopogon tuberosus*. A) Number of total flowers including open and closed. B) Number of flowers opened simultaneously. C) Flower width measured on a horizontal plane at the widest point. D) Flower height measured on a vertical plane from the widest point. Histobars with the same letter are not significantly different at $\alpha=0.05$.

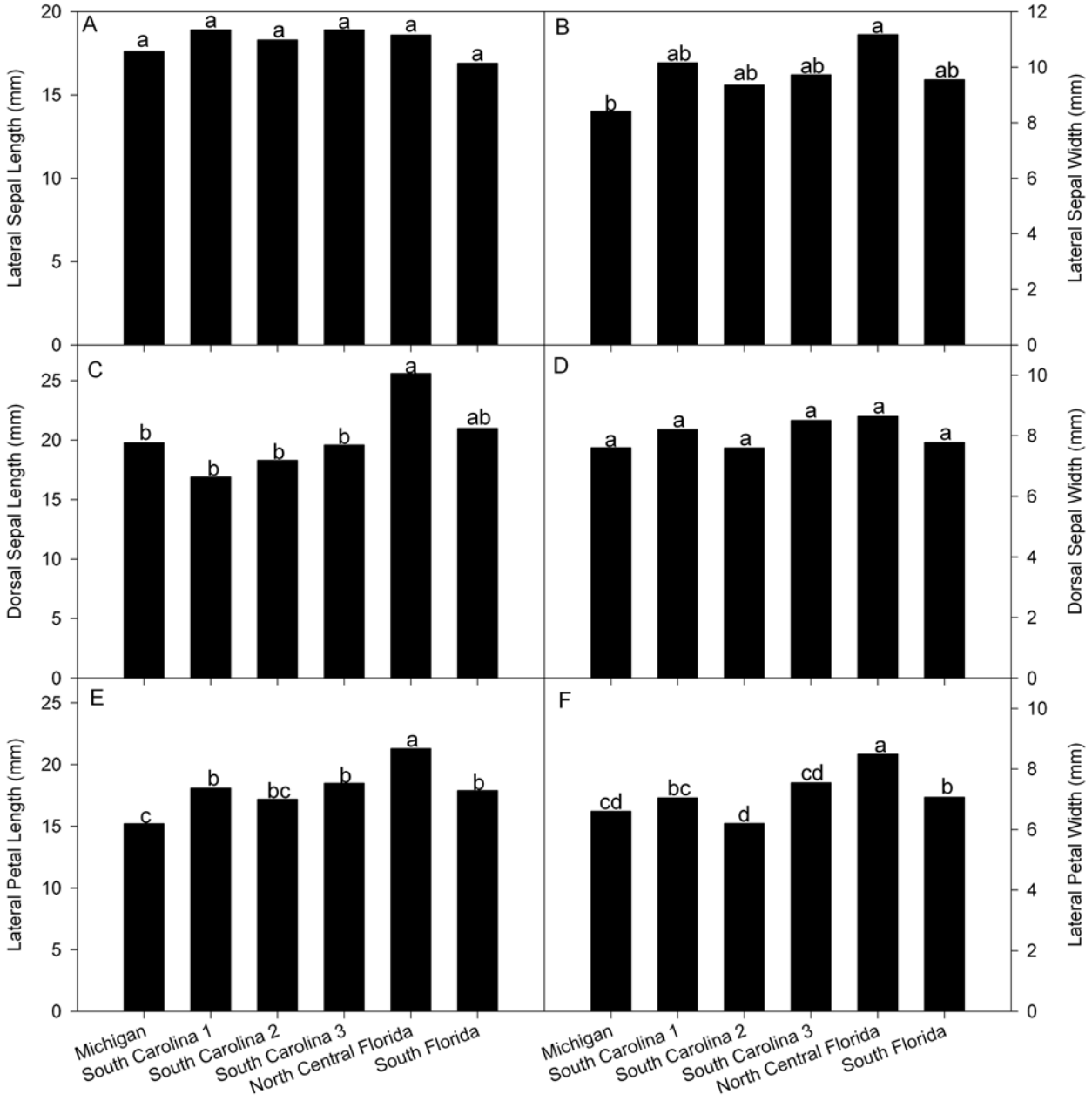

Figure B-5. Flower part morphometrics of *Calopogon tuberosus*. A) Lateral sepal length from point of attachment to apex. B) Lateral sepal width at the widest point. C) Dorsal sepal length from point of attachment to apex. D) Dorsal sepal width at the widest point. E) Lateral petal length from point of attachment to apex. F) Lateral petal width at the widest point. Histobars with the same letter are not significantly different at α=0.05. For floral part locations see Figure B-1.

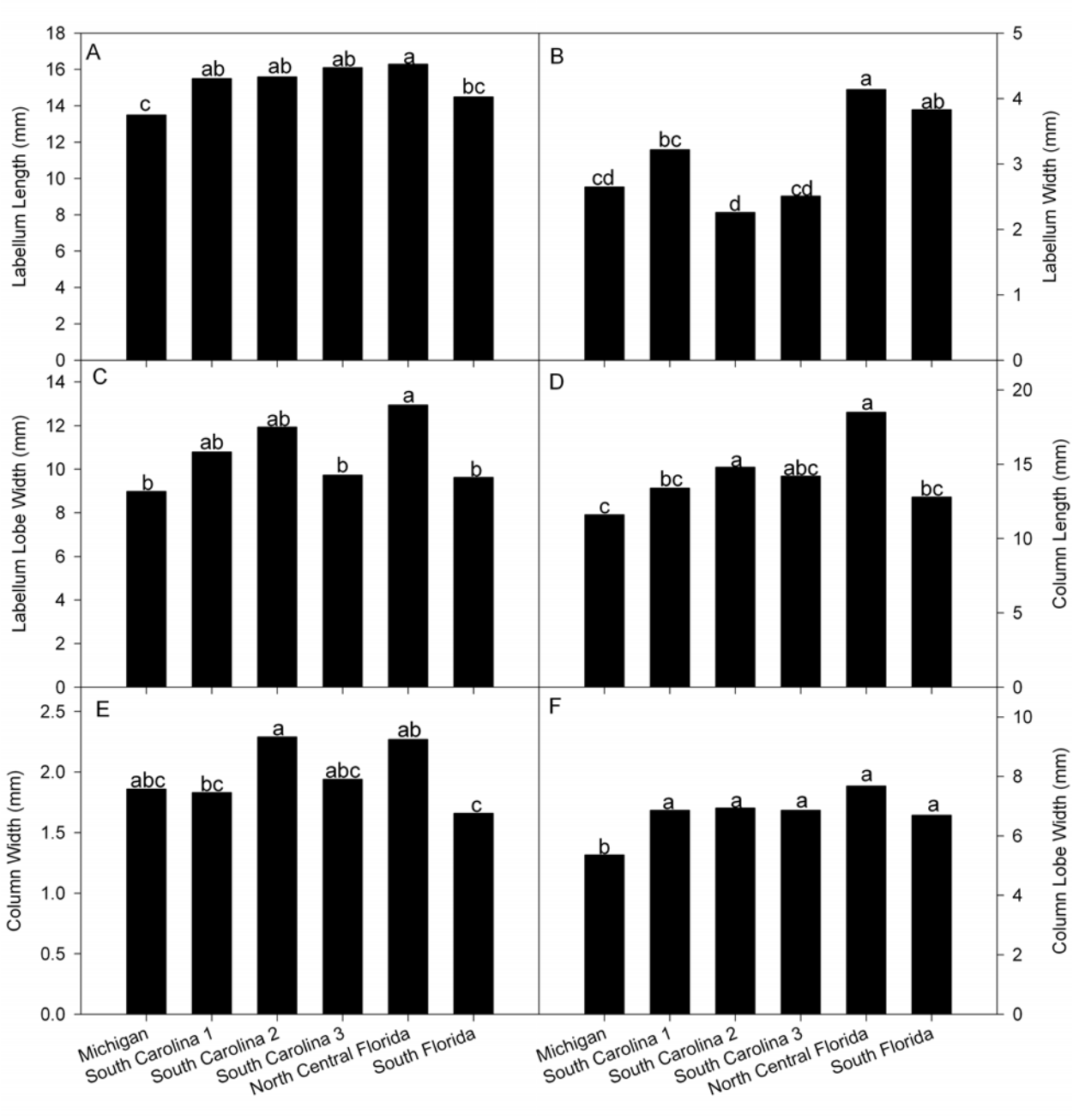

Figure B-6. Labellum and column morphometrics of *Calopogon tuberosus*. A) Labellum length from point of attachment to apex. B) Labellum width at the widest point. C) Labellum lobe width. D) Column length from point of attachment to apex. E) Column width at the widest point. F) Column lobe width at the widest point. Histobars with the same letter are not significantly different at α=0.05. For floral part measurement locations see Figure B-2.

LIST OF REFERENCES

Abrahamson WG. 1975. Reproduction of *Rubus hispidus* L. in different habitats. *The American Midland Naturalist* **95**: 471-478.

Abrahamson WG. 1979. Patterns of resource allocation in wildflower populations of fields and woods. *American Journal of Botany* **66**: 71-79.

Alam S, Murr D, Kristof L. 1994. The effect of ethylene and of inhibitors of protein and nucleic acid syntheses on dormancy break and subsequent sprout growth. *Potato Research* **37**: 25-33.

Alexandersson R, Agren J. 2000. Genetic structure in the nonrewarding, bumblebee-pollinated orchid *Calypso bulbosa*. *Heredity* **85**: 401-409.

Alexopoulos AA, Akoumianakis KA, Vemmos SN, Passam HC. 2007. The effect of postharvest application of gibberellic acid and benzyl adenine on the duration of dormancy of potatoes produced by plants grown from TPS. *Postharvest Biology and Technology* **46**: 54-62.

Alexopoulos AA, Aivalakis G, Akoumianakis KA, Passam HC. 2008. Effect of gibberellic acid on the duration of dormancy of potato tubers produced by plants derived from true potato seed. *Postharvest Biology and Technology* **49**: 424-430.

Allen PS, Meyer SE. 1998. Ecological aspects of seed dormancy loss. *Seed Science Research* **8**: 183-191.

Amen R. 1968. A model of seed dormancy. *The Botanical Review* **34**: 1-31.

Amon JP, Thompson CA, Carpenter QJ, Miner J. 2002. Temperate zone fens of the glaciated midwestern USA. *Wetlands* **22**: 301-317.

Anderson A. **1990**. Asymbiotic germination of seeds of some North American orchids. In: Sawyers C, ed. *North American native terrestrial orchids: propagation and production.* Chadds Ford, Pennsylvania: Brandywine Conservancy, 75-80.

Anderson A. **1996**. The reintroduction of *Platanthera ciliaris* in Canada. In: Allen C, ed. *North American native terrestrial orchids: propagation and production.* Germantown, Maryland: North American Native Terrestrial Orchid Conference, 63-71.

Arditti J. 1967. Factors affecting the germination of orchid seed. *The Botanical Review* **33**: 1-97.

Arditti J, Oliva AP, Michaud JD. 1985. Practical germination of North American and related orchids-3-*Calopogon tuberosus, Calypso bulbosa, Cypripedium* species and hybrids, *Piperia elegans* var. *elata, Piperia maritima, Platanthera hyperborea,* and *Platanthera saccata*. *American Orchid Society Bulletin* **54**: 859-872.

Arditti J, Ghani AKA. 2000. Tansley review No. 110 - Numerical and physical properties of orchid seeds and their biological implications. *New Phytologist* **145**: 367-421.

Ballard WW. **1990**. Further notes on *Cypripedium* germination. In: Sawyers CE, ed. *North American native terrestrial orchids: propagation and production*. Chadds Ford, Pennsylvania: Brandywine Conservancy, 87-89.

Baskin CC, Baskin JM. 2001. *Seeds: Ecology, biogeography, and evolution of dormancy and germination*. San Diego, California: Academic Press.

Baskin CC, Baskin JM. **2004**. Determining dormancy-breaking and germination requirements from the fewest seeds. In: Guerrant EO, Havens K, Maunder M, eds. *Ex situ plant conservation: Supporting species survival in the wild*. Washington DC: Island Press, 162-179.

Baskin CC, Thompson K, Baskin JM. 2006. Mistakes in germination ecology and how to avoid them. *Seed Science Research* **16**: 165-168.

Baskin JM, Baskin CC. 1973. Plant population differences in dormancy and germination characteristics of seeds: heredity or environment? *The American Midland Naturalist* **90**: 493-498.

Batty AL, Brundrett MC, Dixon KW, Sivasithamparam K. 2006*a*. New methods to improve symbiotic propagation of temperate terrestrial orchid seedlings from axenic culture to soil. *Australian Journal of Botany* **54**: 367-374.

Batty AL, Brundrett MC, Dixon KW, Sivasithamparam K. 2006*b*. *In situ* seed germination and propagation of terrestrial orchid seedlings for establishment at field sites. *Australian Journal of Botany* **54**: 375-381.

Bedford BL, Godwin KS. 2003. Fens of the United States: distribution, characteristics, and scientific connection versus legal isolation. *Wetlands* **23**: 608-629.

Berliocchi L. 2000. *The orchid in lore and legend*. Portland, Oregon: Timber Press.

Bernard N. 1899. Sur la germination du *Neottia nidus* avis. *Comptes Rendus de l'Académie des Sciences Paris* **128**: 1253-1255.

Bewley JD, Black M. 1994. *Seeds: Physiology of development and germination*. New York: Plenum Press.

Bewley JD. 1997. Seed germination and dormancy. *Plant Cell* **9**: 1055-1066.

Bischoff A, Vonlanthen B, Steinger T, Müller-Schärer H. 2006. Seed provenance matters -- effects on germination of four plant species used for ecological restoration. *Basic and Applied Ecology* **7**: 347-359.

Blakeman JP, Mokahel MA, Hadley G. 1976. Effect of mycorrhizal infection on respiration and activity of some oxidase enzymes of orchid protocorms. *New Phytologist* **77**: 697-704.

Boivin B. 1967. Enumeration des plantes du Canada, Monopsides. *Naturaliste Canadien* **94**: 131-157.

Bowles ML, Jacobs KA, Zettler LW, Delaney TW. 2002. Crossing effects on seed viability and experimental germination of the federal threatened *Platanthera leucophaea* (Orchidaceae). *Rhodora* **104**: 14-30.

Brown PM. 2002. *Wild orchids of Florida with references to the Atlantic and Gulf coastal plains*. Gainesville, Florida: University Press of Florida.

Bruland GL, Grunwald S, Osborne TZ, Ramesh Reddy K, Newman S. 2006. Spatial distribution of soil properties in water conservation area 3 of the Everglades. *Soil Science Society of America Journal* **70**: 1662-1676.

Brundrett MC, Scade A, Batty AL, Dixon KW, Sivasithamparam K. 2003. Development of *in situ* and *ex situ* seed baiting techniques to detect mycorrhizal fungi from terrestrial orchid habitats. . *Mycological Research* **107**: 1210-1220.

Burgeff H. 1909. Die Wurzelpilze der Orchideen, ihre Kultur und ihre Leben in der Pflanze. *G. Fischer Verlag*: 202.

Burton WG. 1989. *The Potato*. Essex, England: Longman Scientific and Technical.

Cameron D, Leake JR, Read DJ. 2006. Mutualistic mycorrhiza in orchids: evidence from plant-fungus carbon and nitrogen transfers in the green-leaved terrestrial orchid *Goodyera repens*. *New Phytologist* **171**: 405-416.

Cameron D, Johnson I, Leake JR, Read DJ. 2007. Mycorrhizal acquisition of inorganic phosphorus by the green-leaved terrestrial orchid *Goodyera pubescens*. *Annals of Botany* **99**: 831-834.

Cameron D, Johnson I, Read DJ, Leake JR. 2008. Giving and receiving: measuring the carbon cost of mycorrhizas in the green orchid, *Goodyera repens*. *New Phytologist* **180**: 176-184.

Campbell RK, Sorensen FC. 1973. Cold-acclimation in seedling douglas-fir related to phenology and provenance. *Ecology* **54**: 1148-1151.

Case FW. 1987. *Orchids of the western great lakes region. 2nd edn*. Bloomfield Hills, Michigan: Cranbook Institute of Science, Bulletin 48.

Catling PM, Lucas Z. 1987. The status of *Calopogon tuberosus* var. *latifolius* with comments on the application of varietal rank. *Rhodora* **89**: 401-412.

Chu CC, Mudge KW. 1994. Effects of prechilling and liquid suspension culture on seed germination of the yellow lady's slipper orchid (*Cypripedium calceolus* var. *pubescens*). *Lindleyana* **9**: 153-159.

Clark GE. 1995. Effects of storage temperature and duration on the dormancy of *Sandersonia aurantiaca* tubers. *New Zealand Journal of Crop and Horticultural Science* **23**: 455-460.

Clausen J, Keck DD, Hiesey WM. 1939. The concept of species based on experiment. *American Journal of Botany* **26**: 103-106.

Clausen J, Keck WM, Hiesey WM. 1941. Regional differentiation in plant species. *American Naturalist* **75**: 231-250.

Clements MA, Muir H, Cribb PJ. 1985. A preliminary report on the symbiotic germination of European terrestrial orchids. *Kew Bulletin* **41**: 437-445.

Cohen JG, Kost MA. 2008. Natural community abstract for northern fen. Michigan Natural Features Inventory, Lansing, MI. 18 p.

Coke JL. **1990**. Aseptic germination and growth of some terrestrial orchids. In: Sawyers CE, ed. *North American native terrestrial orchids: propagation and production.* Chadds Ford, Pennsylvania: Brandywine Conservancy, 90-91.

Coleman W, King R. 1984. Changes in endogenous abscisic acid, soluble sugars and proline levels during tuber dormancy in *Solanum tuberosum* L. *American Journal of Potato Research* **61**: 437-449.

Cooper JP. 1964. Climatic variation in forage grasses. I. leaf development in climatic races of *Lolium* and *Dactylis*. *Journal of Applied Ecology* **1**: 45-61.

Curtis JT. 1939. The relation of specificity of orchid mycorrhizal fungi to the problem of symbiosis. *American Journal of Botany* **26**: 390-399.

Curtis JT. 1947. Studies on the nitrogen nutrition of orchid embryos. I. Complex nitrogen sources. *American Orchid Society Bulletin* **16**: 654-660.

Daehler CC, Anttila CK, Ayres DR, Strong DR, Bailey JP. 1999. Evolution of a new ecotype of *Spartina alterniflora* (Poaceae) in San Francisco Bay, California, USA. *American Journal of Botany* **86**: 543-546.

Davis JH. 1943. *The natural features of southern Florida*. Tallahassee, FL: The Florida Geological Survey.

De Pauw MA, Remphrey WR. 1993. *In vitro* germination of three *Cypripedium* species in relation to time of seed collection, media, and cold treatment. *Canadian Journal of Botany* **71**: 879-885.

Debeljak N, Regvar M, Dixon KW, Sivasithamparam K. 2002. Induction of tuberisation *in vitro* with jasmonic acid and sucrose in an Australian terrestrial orchid, *Pterostylis sanguinea*. *Plant Growth Regulation* **36**: 253-260.

Diez JM. 2007. Hierarchical patterns of symbiotic orchid germination linked to adult proximity and environmental gradients. *Journal of Ecology* **95**: 159-170.

Dijk E, Eck N. 1995*a*. Axenic *in vitro* nitrogen and phosphorus responses of some Dutch marsh orchids. *New Phytologist* **131**: 353-359.

Dijk E, Eck N. 1995*b*. Ammonium toxicity and nitrate response of axenically grown *Dactylorhiza incarnata* seedlings. *New Phytologist* **131**: 361-367.

Dixon KW, Pate JS. 1978. Phenology, morphology, and reproductive biology of tuberous sundew, *Drosera erthryorhiza* Lindl. *Australian Journal of Botany* **26**: 441-454.

Dixon KW. **1987**. Raising terrestrial orchids from seed. In: Harris W, ed. *Modern orchid growing for pleasure and profit*. Adelaide, Australia: Orchid Club of South Australia, 47-100.

Donohue K. 2005. Seeds and seasons: interpreting germination timing in the field. *Seed Science Research* **15**: 175-187.

Downie DG. 1940. On the germination and growth of *Goodyera repens*. *Transactions and Proceedings of the Botanical Society of Edinburgh* **33**: 36-51.

Downs RJ, Bevington JM. 1981. Effect of temperature and photoperiod on growth and dormancy of *Betula papyrifera*. *American Journal of Botany* **68**: 795-800.

Dressler RL. 1981. *The orchids: natural history and classification*. Cambridge, Massachusetts: Harvard University Press.

Dressler RL. 1993. *Phylogeny and classification of the orchid family*. Portland, Oregon: Dioscorides Press.

Duever MJ, Carlson JE, Meeder JF, *et al.* 1986. The big cypress national preserve. National Audobon Society, New York.

Duncan RS, Anderson CB, Sellers HN, Robbins EE. 2008. The effect of fire reintroduction on endemic and rare plants of a southeastern glade ecosystem. *Restoration Ecology* **16**: 39-49.

Dutra D, Johnson TR, Kauth PJ, Stewart SL, Kane ME, Richardson L. 2008. Asymbiotic seed germination, in vitro seedling development, and greenhouse acclimatization of the threatened terrestrial orchid Bletia purpurea. *Plant Cell Tissue and Organ Culture* **94**: 11-21.

Eagles CF. 1967*a*. The effect of temperature on vegetative growth in climatic races of *Dactylis glomerata* in controlled environments. *Annals of Botany* **31**: 31-39.

Eagles CF. 1967*b*. Variation in the soluble carbohydrate content of climatic races of *Dactylis glomerata* (Cocksfoot) at different temperatures. *Annals of Botany* **31**: 645-651.

Ernst R. 1967. Effect of carbohydrate selection on the growth rate of freshly germinated *Phalaenopsis* and *Dendrobium* seed. *Bulletin of the American Orchid Society* **36**: 1068-1073.

Ernst R, Arditti J, Healey PL. 1971. Carbohydrate physiology of orchid seedlings. II. hydrolysis and effects of oligosaccharides. *American Journal of Botany* **58**: 827-835.

Ernst R, Arditti J. 1990. Carbohydrate physiology of orchid seedlings. III. Hydrolysis of maltooligosaccharides by *Phalaenopsis* seedlings. *American Journal of Botany* **77**: 188-195.

Fang W, Taub DR, Fox GA, Landis RM, Natali S, Gurevitch J. 2006. Sources of variation in growth, form, and survival in dwarf and normal-stature pitch pines (*Pinus rigida*, Pinaceae) in long-term transplant experiments. *American Journal of Botany* **93**: 1125-1133.

Fernald ML. 1946. Contributions from the Gray Herbarium of Harvard University: Identifications and reidentifications of North American plants. *Rhodora* **48**: 185-200.

Fetcher N, Shaver GR. 1990. Environmental sensitivity of ecotypes as a potential influence on primary productivity. *American Naturalist* **136**: 126-131.

Firmage DH, Cole FR. 1988. Reproductive success and inflorescence size of *Calopogon tuberosus* (Orchidaceae). *American Journal of Botany* **75**: 1371-1377.

Florida Division of Forestry. 2005. *Goethe State Forest*. http://www.fl-dof.com/state_forests/goethe.html. (9 July 2009).

Florida Natural Areas Inventory. 1990. *Guide to the natural communities of Florida*. http://www.fnai.org/PDF/Natural_Communities_Guide.pdf. (9 July 2009).

Fowler DP, Dwight TW. 1694. Provenance differences in the stratification requirements of white pine. *Canadian Journal of Botany* **42**: 669-675.

Friar EA, Ladoux T, Roalson EH, Robichaux RH. 2000. Microsatellite analysis of a population crash and bottleneck in the Mauna Kea silversword, *Argyroxiphium sandwicense* ssp *sandwicense* (Asteraceae), and its implications for reintroduction. *Molecular Ecology* **9**: 2027-2034.

Fukai S, Fujiwara K, Okamata O, Hasegawa A, Goi M. 1997. Effects of red and blue light on germination and protocorm growth of *Calanthe* Satsuma. . *Lindleyana* **12**: 169-171.

Fukai S, Kanechika R, Hasegawa A. 2006. Effect of low temperature on breaking dormancy and flowering of *Arisaema sikokianum* (Araceae). *Scientia Horticulturae* **111**: 97-100.

Gallagher JL. 1983. Seasonal patterns in recoverable underground reserves in *Spartina alterniflora* Loisel. *American Journal of Botany* **70**: 212-215.

Gallagher JL, Howarth RW. 1987. Seasonal differences in *Spartina recoverable* underground reserves in the Great Sippewissett marsh in Massachusetts. *Estuarine, Coastal and Shelf Science* **25**: 313-319.

Gallagher JL, Somers GF, Grant DM, Seliksar DM. 1988. Persistent differences in two forms of *Spartina alterniflora*: a common garden experiment. *Ecology* **69**: 1005-1008.

Garbisch EW, McIninch SM, Swartz HJ, Salvaggio GJ. 1995. Chilling responses for some herbaceous wetland plants. *Wetland Journal* **7**: 16-20.

Garbisch EW, McIninch SM, Swartz HJ, Salvaggio GJ. 1996. The effects of controlled chilling on five wetland herbaceous plant species. *Wetland Journal* **8**: 20-29.

Goldman DH, van den Berg C, Griffith MP. 2004*a*. Morphometric circumscription of species and infraspecific taxa in *Calopogon* R. Br. (Orchidaceae). *Plant Systematics and Evolution* **247**: 37-60.

Goldman DH, Jansen RK, van den Berg C, Leitch IJ, Fay MF, Chase MW. 2004*b*. Molecular and cytological examination of *Calopogon* (Orchidaceae, Epidendroideae): circumscription, phylogeny, polyploidy, and possible hybrid speciation. *American Journal of Botany* **91**: 707-723.

González A, Bañón S, Fernández JA, Franco JA, Casas JL, Ochoa J. 1998. Flowering responses of *Gladiolus tristis* (L.) after exposing corms to cold treatment. *Scientia Horticulturae* **74**: 279-284.

Gregor JW, Davey VM, Lang JMS. 1936. Experimental taxonomy. I. experimental garden technique in relation to the recognition of the small taxonomic units. *New Phytologist* **35**: 323-350.

Gregor JW. 1939. Experimental taxomony. IV. population differentiation in North American and European sea plantains allied to *Plantago martima* L. *New Phytologist* **38**: 19-322.

Gross M, Hardisky M, Wolf P, Klemas V. 1991. Relationship between aboveground and belowground biomass of *Spartina alterniflora* (Smooth Cordgrass). *Estuaries and Coasts* **14**: 180-191.

Grześ IM. 2007. Does rare *Gentianella germanica* (Wild.) Borner originating from calamine spoils differ in selected morphological traits from reference populations? *Plant Species Biology* **22**: 49-52.

Hadley G, Purves S. 1974. Movement of C-14 from host to fungus in orchid mycorrhiza. *New Phytologist* **73**: 475-482.

Hadley G, Ong SH. 1978. Nutritional requirements of orchid endophytes. *New Phytologist* **81**: 561-569.

Hadley G. **1982**. Orchid mycorrhiza. In: Arditti J, ed. *Orchid biology: reviews and perspectives*. Ithaca, New York: Cornell University Press.

Harvais G. 1973. Growth requirements and development of *Cypripedium reginae* in axenic culture. *Canadian Journal of Botany* **51**: 327-332.

Hemberg T. 1949. Significance of growth-inhibiting substances and auxins for the rest-period of the potato tuber. *Physiologia Plantarum* **2**: 24-36.

Henrich JE, Stimart DP, Ascher PD. 1981. Terrestrial orchid seed germination in vitro on a defined medium. *Journal of the American Society for Horticultural Science* **106**: 193-196.

Hijner JA, Arditti J. 1973. Orchid mycorrhiza - vitamin production and requirements by symbionts. *American Journal of Botany* **60**: 829-835.

Hofgaard IS, Vollsnes AV, Marum P, Larsen A, Tronsmo AM. 2003. Variation in resistance to different winter stress factors within a full-sib family of perennial ryegrass. *Euphytica* **134**: 61-75.

Hollick P, Senaratna T, McComb J, Bunn E, Dixon KW. 2001. Response to paclobutrazol of symbiotic mycorrhizal fungi and dropper (tuber stalk) formation of host orchid seedlings. *Plant Growth Regulation* **00**: 1-9.

Howe GT, Hackett WP, Furnier GR, Klevorn RE. 1995. Photoperiodic responses of a northern and southern ecotype of black cottonwood. *Physiologia Plantarum* **93**: 695-708.

Hufford KM, Mazer SJ. 2003. Plant ecotypes: genetic differentiation in the age of ecological restoration. *TRENDS in Ecology and Evolution* **18**: 147-155.

Hughes KW. 1981. *In vitro* ecology - Exogenous factors affecting growth and morphogenesis in plant culture systems. *Environmental and Experimental Botany* **21**: 281-288.

Jersáková J, Johnson SD, Kindlmann P. 2006. Mechanisms and evolution of deceptive pollination in orchids. *Biological Reviews* **81**: 219-235.

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

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

Joshi J, Schmid B, Caldeira MC, *et al.* 2001. Local adaptation enhances performance of common plant species. *Ecology Letters* **4**: 536-544.

Kamiya A, Ikegami I, Hase E. 1981. Effects of light on chlorophyll formation in cultured tobacco cells I. chlorophyll accumulation and phototransformation of protochlorophyll(ide) in callus cells under blue and red light. *Plant Cell and Physiology* **22**: 1385-1396.

Kane ME. 1996. Wetland plant micropropagation: issues and opportunities. *Aquatics* **18**: 4-11.

Kane ME, Gillis MR, Philman N, Campbell S. 2000. Seasonal differences in *ex vitro* growth and corm formation between two micropropagated *Sagittaria latifolia* ecotypes. *Acta Horticulturae* **520**: 229-237.

Kane ME, Philman NL, Emshousen C. 2003. Wetland plant propagation: comparative growth and reproduction of micropropagated *Sagittaria latifolia* ecotypes. *Combined Proceedings of the 2002 International Plant Propagators' Society* **52**: 453-459.

Kauth PJ. 2005. *In vitro* seed germination and seedling development of *Calopogon tuberosus* and *Sacoila lanceolata* var. *lanceolata*: two Florida native terrestrial orchids. University of Florida, Gainesville, FL, USA.

Kauth PJ, Vendrame WA, Kane ME. 2006. *In vitro* seed culture and seedlings development of *Calopogon tuberosus*. *Plant Cell, Tissue and Organ Culture* **85**: 91-102.

Kauth PJ, Dutra D, Johnson TR, Stewart SL, Kane ME, Vendrame WA. **2008**. Techniques and applications of *in vitro* orchid seed germination. In: Teixeira da Silva JA, ed. *Floriculture, ornamental and plant biotechnology: advances and topical issues*. Isleworth, UK: Global Science Books, 375-391.

Keller M, Kollmann J. 1999. Effects of seed provenance on germination of herbs for agricultural compensation sites. *Agriculture, Ecosystems & Environment* **72**: 87-99.

Kery M, Gregg K. 2004. Demographic analysis of dormancy and survival in the terrestrial orchid *Cypripedium reginae*. *Journal of Ecology* **92**: 686-695.

Kim HH, Ohkawa K, Sakaguchi K. 1996. Effects of storage temperature and duration on flower bud development, emergence and flowering of *Zephyra elegans* D. Don. *Scientia Horticulturae* **67**: 55-63.

Kindell CE, Winn AA, Miller TE. 1996. The effects of surrounding vegetation and transplant age on the detection of local adaptation in the perennial grass *Aristida stricta*. *Journal of Ecology* **84**: 745-754.

Knudson L. 1922. Nonsymbiotic germination of orchid seed. *Botanical Gazette* **73**: 1-25.

Knudson L. 1946. A new nutrient solution for the germination of orchid seed. *American Orchid Society Bulletin* **15**: 214-217.

Kondo M, Pablico PP, Aragones DV, *et al.* 2003. Genotypic and environmental variations in root morphology in rice genotypes under upland field conditions. *Plant and Soil* **255**: 189-200.

Koopowitz H, Lavarack PS, Dixon KW. **2003**. The nature of threats to orchid conservation. In: Dixon KW, Kell SP, Barrett RL, Cribb PJ, eds. *Orchid Conservation*. Borneo: Natural History Publications, 25-42.

Kost MA, Albert DA, Cohen JG, *et al.* 2007. Northern fen. Natural communities of Michigan: classification and description. Michigan Natural Features Inventory, Report Number 2007-21, Lansing, MI. 314 pp.

Kriebel HB, Wang CW. 1962. The interaction between provenance and degree of chilling in bud-break of sugar maple. *Silvae Genetica* **11**: 125-130.

Kurepin LV, Walton LJ, Reid DM, Chinnappa CC, Pharis RP. 2007. Photoperiod, light quality, and irradiance effects on flowering in the alpine and prairie ecotypes of *Stellaria longipes*. *Canadian Journal of Botany-Revue Canadienne De Botanique* **85**: 538-544.

Kushlan JA. **1990**. Freshwater Marshes. In: Myers RL, Ewel JJ, eds. *Ecosystems of Florida*. Orlando, FL: University of Central Florida Press, 324-363.

Lakon G. 1949. The topographical tetrazolium method for determining the germinating capacity of seeds. *Plant Physiology* **24**: 389-394.

Lang GA. 1987. Dormancy: a new universal terminology. *Hortscience* **22**: 817-820.

Lang GA, Early JD, Martin GC, Darnell RL. 1987. Endo-, para-, and ecodormancy: physiological terminology and classification for dormancy research. *Hortscience* **22**: 371-377.

Lauzer D, Renaut S, St-Arnaud M, Barabe D. 2007. *In vitro* asymbiotic germination, protocorm development, and plantlet acclimatization of *Aplectrum hyemale* (Muhl. ex Willd.) Torr. (Orchidaceae). *Journal of the Torrey Botanical Society* **134**: 344-348.

Lee YI, Lee N, Yeung EC, Chung MC. 2005. Embryo development of *Cypripedium formosanum* in relation to seed germination *in vitro*. *Journal of the American Society for Horticultural Science* **130**: 747-753.

Lee YI, Yeung EC, Lee N, Chung MC. 2006. Embryo development in the lady's slipper orchid, *Paphiopedilum delenatii*, with emphasis on the ultrastructure of the suspensor. *Annals of Botany* **98**: 1311-1319.

Li B, Tomoko S, Yasuhiro Y, Toshihiko H, Masayuki Y. 2001. Interclonal differences, plasticity and trade-offs of life history traits of *Cyperus esculentus* in relation to water availability. *Plant Species Biology* **16**: 193-207.

Li C, Junttila O, Heino P, Palva ET. 2003. Different responses of northern and southern ecotypes of *Betula pendula* to exogenous ABA application. *Tree Physiology* **23**: 481-487.

Li C, Wu N, Liu S. 2005. Development of freezing tolerance in different altitudinal ecotypes of *Salix paraplesia*. *Biologia Plantarum* **49**: 65-71.

Li XG, Seliskar DM, Moga JA, Gallagher JL. 1995. Plant regeneration from callus cultures of salt marsh hay, *Spartina patens*, and its cellular based salt tolerance. *Aquatic Botany* **51**: 103-113.

Liancourt P, Tielbörger K. 2009. Competition and a short growing season lead to ecotypic differentiation at the two extremes of the ecological range. *Functional Ecology* **23**: 397-404.

Linhart YB. 1995. Restoration, revegetation, and the importance of genetic and evolutionary perspectives. US Department of Agriculture, Forest Service, Intermountain Research Station, General Technical Report Number INT-GTR-315, Odgen, Utah. pp. 271-287.

Linhart YB, Grant MC. 1996. Evolutionary significance of local genetic differentiation in plants. *Annual Review of Ecology and Systematics* **27**: 237-277.

Luer CA. 1972. *The native orchids of Florida*. New York: The New York Botanical Garden.

Luer CA. 1975. *The native orchids of the United States and Canada excluding Florida*. New York: The New York Botanical Garden.

MacColl D, Cooper JP. 1967. Climatic variation in forage grasses. III. seasonal changes in growth and assimilation in climatic races of *Lolium*, *Dactylis* and *Festuca*. *Journal of Applied Ecology* **4**: 113-127.

Macel M, Lawson CS, Mortimer SR, *et al.* 2007. Climate vs soil factors in local adaptation of two common plant species. *Ecology* **88**: 424-433.

Majerowicz N, Kerbauy GB, Nievola CC, Suzuki RM. 2000. Growth and nitrogen metabolism of *Catasetum fimbriatum* (Orchidaceae) grown with different nitrogen sources. *Environmental and Experimental Botany* **44**: 195-206.

Malmgren S. 1993. Large-scale asymbiotic propagation of *Cypripedium calceolus*-plant physiology from a surgeon's point of view. *Botanic Gardens Micropropagation* **1**: 59-63.

Malmgren S. **1996**. Orchid propagation: theory and practice. In: Allen C, ed. *North American native terrestrial orchids: propagation and production*. Germantown, Maryland: North American Native Terrestrial Orchid Conference, 63-71.

Manning JC, van Staden J. 1987. The development and mobilization of seed reserves in some African orchids. *Australian Journal of Botany* **35**: 343-353.

Maunder M. 1992. Plant reintroductions: an overview. *Biodiversity and Conservation* **1**: 51-61.

McConnaughay KDM, Coleman JS. 1999. Biomass allocation in plants: ontogeny or optimality? A test along three resource gradients. *Ecology* **80**: 2581-2593.

McCormick MK, Whigham DF, O'Neill J. 2004. Mycorrhizal diversity in photosynthetic terrestrial orchids. *New Phytologist* **163**: 425-438.

McKay JK, Christian CE, Harrison S, Rice KJ. 2005. "How local is local?"- a review of practical and conceptual issues in the genetics of restoration. *Restoration Ecology* **13**: 432-440.

McKendrick SL. 1995. The effects of herbivory and vegetation on laboratory-raised *Dactylorhiza praetermissa* (Orchidaceae) planted into grassland in Southern England. *Biological Conservation* **73**: 215-220.

McKendrick SL. 1996. The effects of shade on seedlings of *Orchis morio* and *Dactylorhiza fuchsii* in chalk and clay soil. *New Phytologist* **134**: 343-352.

McKinley T, Camper ND. 1997. Action spectra of *in vitro* asymbiotic germination of *Goodyera repens* var *ophioides*. *Lindleyana* **12**: 30-33.

Meyer SE. 1992. Habitat correlated variation in firecracker penstemon (*Penstemon eatonii* Gray: Scrophulariaceae) seed germination response. *Bulletin of the Torrey Botanical Club* **119**: 268-279.

Meyer SE, Kitchen SG, Carlson SL. 1995. Seed germination timing patterns in intermountain *Penstemon* (Scrophulariaceae). *American Journal of Botany* **82**: 377-389.

Miyoshi K, Mii M. 1998. Stimulatory effects of sodium and calcium hypochlorite, pre-chilling and cytokinins on the germination of *Cypripedium macranthos* seed *in vitro*. *Physiologia Plantarum* **102**: 481-486.

Molvray M, Kores PJ. 1995. Character analysis of seed coat in Spiranthoideae and Orchidoideae, with special reference to the Diurideae (Orchidaceae). *American Journal of Botany* **82**: 1443-1454.

Mondoni A, Rossi G, Hay F, Bonomi C. 2008. Habitat-correlated seed germination behaviour in populations of wood anemone (*Anemone nemorsa* L.) from northern Italy. *Seed Science Research* **18**: 213-222.

Mooney HA, Billings WD. 1960. The annual carbohydrate cycle of alpine plants as related to growth. *American Journal of Botany* **47**: 594-598.

Moyes AB, Witter MS, Gamon JA. 2005. Restoration of native perennials in a California annual grassland after prescribed spring burning and solarization. *Restoration Ecology* **13**: 659-666.

Mweetwa AM, Welbaum GE, Tay D. 2008. Effects of development, temperature, and calcium hypochlorite treatment on *in vitro* germinability of *Phalaenopsis* seeds. *Scientia Horticulturae* **117**: 257-262.

Myers PJ, Ascher PD. 1982. Culture of North American orchids from seed. *Hortscience* **17**: 550.

Myking T, Heide OM. 1995. Dormancy release and chilling requirement of buds of latitudinal ecotypes of *Betula pendula* and *B. pubescens*. *Tree Physiology* **15**: 697-704.

Mylavarapu RS, Kennelley ED. 2002. *UF/IFAS extension soil testing laboratory (ESTL) analytical procedures and training manual*. http://edis.ifas.ufl.edu/SS312. (21 Aug 2009).

Nagy ES, Rice KJ. 1997. Local adaptation in two subspecies of an annual plant: implications for migration and gene flow. *Evolution* **51**: 1079-1089.

National Climactic Data Center. 2009. *Climatological data annual summary 2007*. http://www.ncdc.noaa.gov/oa/climate/stationlocator.html. (18 Aug 2009).

Nelson JB. 1986. *The natural communities of South Carolina*. http://cricket.biol.sc.edu/acmoore/publications/JBNDoc.pdf. (10 July 2009).

Nelson JR, Harris GA, Goebel CJ. 1970. Genetic vs. environmentally induced variation in medusahead (*Taeniatherum asperum* [Simonkai] Nevski). *Ecology* **51**: 526-529.

Nieuwland JA. 1913. New plants from various places. II. *American Midland Naturalist* **3**: 129-133.

Nuismer SL, Gandon S. 2008. Moving beyond common garden and transplant designs: Insight into the causes of local adaptation in species interactions. *The American Naturalist* **171**: 658-668.

Øien DI, Pederson P. 2003. Seasonal pattern of dry matter allocation in *Dactylorhiza lapponica* (Orchidaceae) and the relation between tuber size and flowering. *Nordic Journal of Botany* **23**: 441-451.

Øien DI, Pederson P. 2005. Seasonal pattern of dry matter allocation in *Dactylorhiza lapponica* (Orchidaceae) and the relation between tuber size and flowering. *Nordic Journal of Botany* **23**: 441-451.

Øien DI, O'Neill JP, Whigham DF, McCormick MK. 2008. Germination ecology of the boreal-alpine terrestrial orchid *Dactylorhiza lapponica* (Orchidaceae). *Annales Botanici Fennici* **45**: 161-172.

Otero JT, Ackerman JD, Bayman P. 2004. Differences in mycorrhizal preferences between two tropical orchids. *Molecular Ecology* **13**: 2393-2404.

Otero JT, Bayman P, Ackerman JD. 2005. Variation in mycorrhizal performance in the epiphytic orchid Tolumnia variegata in vitro: the potential for natural selection. *Evolutionary Ecology* **19**: 29-43.

Passam HC, Hughes PA, Read SJ. 1977. Wound repair in yam tubers: the effect of inhibitors of respiration and protein synthesis on repair physiology. *New Phytologist* **79**.

Peñas-Fronteras JT, Villalobos MC, Baltazar AM, Merca FE, Ismail AM, Johnson D. 2009. Adaptation to flooding in upland and lowland ecotypes of *Cyperus rotundus*, a troublesome sedge weed of rice: tuber morphology and carbohydrate metabolism. *Annals of Botany* **103**: 295-302.

Perry TO, Wang CW. 1960. Genetic variation in the winter chilling requirement for date of dormacy break for *Acer rubrum*. *Ecology* **41**: 790-794.

Pillon Y, Qamaruz-Zaman F, Fay MF, Hendoux F, Piquot Y. 2007. Genetic diversity and ecological differentiation in the endangered fen orchid (*Liparis loeselii*). *Conservation Genetics* **8**: 177-184.

Porcher RD, Rayner DA. 2001. *A guide to the wildflowers of South Carolina*. Colombia, South Carolina: University of South Carolina Press.

Potvin C. 1986. Biomass allocation and phenological differences among southern and northern populations of the C4 grass *Echinochloa crus-galli Journal of Ecology* **74**: 915-923.

Pritchard HW. 1985. Determination of orchid seed viability using fluorescein diacetate. **8**: 727-730.

Probert RJ, Smith RD, Birch P. 1985*a*. Germination reponses to light and alternating temperatures in European populations of *Dactylis glomerata* L. II. the genetic and environmental components of germination. *New Phytologist* **99**: 317-322.

Probert RJ, Smith RD, Birch P. 1985*b*. Germination responses to light and alternating temperatures in European populations of *Dactylis glomerata* L. I. variability in relation to origin. *New Phytologist* **99**: 305-316.

Probert RJ, Smith RD, Birch P. 1986. Germination responses to light and alternating temperatures in European populations of *Dactylis glomerata* L .5. The principle components of the alternating temperature requirement. *New Phytologist* **102**: 133-142.

Proctor HC. 1998. Effect of pollen age on fruit set, fruit weight, and seed set in three orchid species. *Canadian Journal of Botany-Revue Canadienne De Botanique* **76**: 420-427.

Prutsch J, Schardt A, Schill R. 2000. Adaptations of an orchid seed to water uptake and -storage. *Plant Systematics and Evolution* **220**: 69-75.

Quinn JA, Colosi JC. 1977. Separating genotype from environment in germination ecology studies. *The American Midland Naturalist* **97**: 484-489.

Raghavan V. 1964. Effects of certain organic nitrogen compounds on growth *in vitro* of seedlings of *Cattleya*. *Botanical Gazette* **125**: 260-267.

Raghavan V, Torrey JG. 1964. Inorganic nitrogen nutrition of the seedlings of the orchid, *Cattleya*. *American Journal of Botany* **51**: 264-274.

Ramsay MM, Stewart J. 1998. Re-establishment of the lady's slipper orchid (*Cypripedium calceolus* L.) in Britain. *Botanical Journal of the Linnean Society*: 173-181.

Ramsay MM, Dixon KW. **2003**. Propagation science, recovery, and translocation or terrestrial orchids. In: Dixon KW, Kell SP, Barrett RL, Cribb PJ, eds. *Orchid Conservation*. Kota Kinabalu, Sabah, Malaysia: Natural History Publications (Borneo).

Rasmussen HN, Andersen TF, Johansen B. 1990*a*. Temperature sensitivity of *in vitro* germination and seedling development of *Dactylorhiza majalis* (Orchidaceae) with and without a mycorrhizal fungus. *Plant Cell and Environment* **13**: 171-177.

Rasmussen HN, Andersen TF, Johansen B. 1990*b*. Light stimulation and darkness requirement for the symbiotic germination of *Dactylorhiza majalis* (Orchidaceae) *in vitro*. *Physiologia Plantarum* **79**: 226-230.

Rasmussen HN, Rasmussen FN. 1991. Climactic and seasonal regulation of seed plant establishment in *Dactylorhiza majalis* inferred from symbiotic experiments in vitro. *Lindleyana* **5**: 221-227.

Rasmussen HN. 1992. Seed dormancy patterns in *Epipactis palustris* (Orchidaceae): Requirements for germination and establishment of mycorrhiza. *Physiologia Plantarum* **86**: 161-167.

Rasmussen HN. 1995. *Terrestrial orchids from seed to mycotropic plant*. Cambridge, Great Britain: Cambridge University Press.

Rees AR. 1981. Concepts of dormancy as illustrated by the tulip and other bulbs. *Annals of Applied Biology* **98**: 544-548.

Rice KJ, Black RA, Radamaker G, Evans RD. 1992. Photosynthesis, growth, and biomass allocation in habitat ecotypes of cheatgrass (*Bromus tectorum*). *Functional Ecology* **6**: 32-40.

Roberts EH, Benjamin SK. 1979. The interaction of light, nitrate and alternating temperature on the germination of *Chenopodium album*, *Capsella bursa-pastoris* and *Poa annua* before and after chilling. *Seed Science and Technology* **7**: 379-392.

Robertson C. 1887. Fertilization of *Calopogon parviflorus*. *Botanical Gazette* **12**: 288-291.

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

Robichaux RH, Friar EA, Mount DW. 1997. Molecular genetic consequences of a population bottleneck associated with reintroduction of the Mauna Kea silversword (*Argyroxiphium sandwicense* ssp. *sandwicense* [Asteraceae]). *Conservation Biology* **11**: 1140-1146.

Rogers SMD. 2003. Tissue culture and wetland establishment of the freshwater monocots Carex, Juncus, Scirpus, and Typha. *In Vitro Cellular and Developmental Biology-Plant* **39**: 1-5.

Rohde A, Bhalerao RP. 2007. Plant dormancy in the perennial context. *Trends in Plant Science* **12**: 217-223.

Rutchey K, Schall T, Doren RF, *et al.* 2006. Vegetation classification system for south Florida natural areas. United States Geological Survey, Open File Report 2006-1240, St. Petersburg, FL. 142 p.

Rylski I, Rappaport L, Pratt HK. 1974. Dual effects of ethylene on potato dormancy and sprout growth. *Plant Physiology* **53**: 658-662.

Sambatti JBM, Rice KJ. 2007. Functional ecology of ecotypic differentiation in the Californian serpentine sunflower (*Helianthus exilis*). *New Phytologist* **175**: 107-119.

Sanders S, McGraw JB. 2005. Population differentiation of a threatened plant: variation in response to local environment and implications for restoration. *Journal of the Torrey Botanical Society* **132**: 561-572.

SAS Institute. 2003. SAS for Windows, version 9.1. Cary, NC.

Sawada S, Nakajima Y, Tsukuda M, *et al.* 1994. Ecotypic differentiation of dry matter production processes in relation to survivorship and reproductive potential in *Plantago asiatica* populations along climatic gradients. *Functional Ecology* **8**: 400-409.

Scade A, Brundrett MC, Batty AL, Dixon KW, Sivasithamparam K. 2006. Survival of transplanted terrestrial orchid seedlings in urban bushland habitats with high or low weed cover. *Australian Journal of Botany* **54**: 383-389.

Schütz W, Milberg P. 1997. Seed dormancy in *Carex canescens*: Regional differences and ecological consequences. *Oikos* **78**: 420-428.

Seliksar DM, Gallagher JL, Burdick DM, Mutz LA. 2002. The regulation of ecosystem functions by ecotypic variation in the dominant plant: a *Spartina alterniflora* salt-marsh case study. *Journal of Ecology* **90**: 1-11.

Seliksar DM, Gallagher JL. 2000. Exploiting wild population diversity and somaclonal variation in the salt marsh grass *Distichlis spicata* (Poaceae) for marsh creation and restoration. *American Journal of Botany* **87**: 141-146.

Seneca ED. 1972. Germination and seedling response of Atlantic and Gulf coast populations of *Uniola paniculata*. *American Journal of Botany* **59**: 290-296.

Seneca ED. 1974. Germination and seedling response of Atlantic and Gulf coasts populations of *Spartina alterniflora*. *American Journal of Botany* **61**: 947-956.

Shahba MA, Qian YL, Hughes HG, Christensen D, Koski AJ. 2003. Cold hardiness of saltgrass accessions. *Crop Science* **43**: 2142-2147.

Sharma J, Zettler LW, van Sambeek JW, Ellersieck MR, Starbuck CJ. 2003. Symbiotic seed germination and mycorrhizae of federally threatened *Platanthera praeclara* (Orchidaceae). *American Midland Naturalist* **149**: 104-120.

Shaver GR, Fetcher N, Chapin FS. 1986. Growth and flowering in *Eriophorum vaginatum*: annual and latitudinal variation. *Ecology* **67**: 1524-1535.

Sheehan TJ, Black RJ. 2007. *Orchids to know and grow*. Gainesville, Florida: University of Florida press.

Shefferson RP, Wei M, Kull T, Taylor DL. 2005. High specificity generally characterizes mycorrhizal association in rare lady's slipper orchids, genus *Cypripedium*. **14**: 613-626.

Shefferson RP, Taylor DL, Wei M, *et al.* 2007. The evolutionary history of mycorrhizal specificity among lady's slipper orchids. *Evolution* **61**: 1380-1390.

Sheviak CJ. **1983**. United States terrestrial orchids: patterns and problems. In: Plaxton EH, ed. *North American Terrestrial Orchids*. Southfield, Michigan: Michigan Orchid Society, 49-60.

Shimura H, Koda Y. 2005. Enhanced symbiotic seed germination of *Cypripedium macranthos* var. *rebunense* following inoculation after cold treatment. *Physiologia Plantarum* **123**: 281-287.

Shimura H, Matsuura M, Takada N, Koda Y. 2007. An antifungal compound involved in symbiotic germination of *Cypripedium macranthos* var. *rebunense* (Orchidaceae). *Phytochemistry* **68**: 1442-1447.

Shoushtari BD, Heydari R, Johnson GL, Arditti J. 1994. Germination and viability staining of orchid seeds following prolonged storage. *Lindleyana* **9**: 77-84.

Silsbury JH. 1961. A study of dormancy, survival and other characteristics in *Lolium perenne* L. at Adelaide, S.A. *Australian Journal of Agricultural Research* **12**: 1-9.

Singh KP. 1973. Effect of temperature and light on seed germination of two ecotypes of *Portulaca oleracea* L. *New Phytologist* **72**: 289-295.

Smith SE. 1966. Physiology and ecology of orchid mycorrhizal fungi with reference to seedling nutrition. *New Phytologist* **65**: 488-&.

Smith SE. 1967. Carbohydrate translocation in orchid mycorrhizas. *New Phytologist* **66**: 371-&.

Smith SE. 1973. Asymbiotic germination of orchid seeds on carbohydrates of fungal origin. *New Phytologist* **72**: 497-499.

Smith ZF, James EA, McDonnell MJ, McLean CB. 2009. Planting conditions improve translocation success of the endangered terrestrial orchid *Diuris fragrantissima* (Orchidaceae). *Australian Journal of Botany* **57**: 200-209.

Snow AA, Whigham DF. 1989. Costs of flower and fruit production in *Tipularia discolor* (Orchidaceae). *Ecology* **70**: 1286-1293.

South Carolina Department of Natural Resources. *Eva Russell Chandler Heritage Preserve*. http://www.dnr.sc.gov/managed/heritage/chandler/description.html. (10 July 2009).

Spoerl E, Curtis JT. 1948. Studies on the nitrogen nutrition of orchid embryos. II. comparative utilization of nitrate and ammonium nitrogen. *American Orchid Society Bulletin* **17**: 111-114.

Spurr AR. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. *Journal of Ultrastructure Research* **26**: 31-43.

St-Arnaud M, Lauzer D, Barabe D. 1992. *In vitro* germination and early growth of seedlings of *Cypripedium acaule* (Orchidaceae). *Lindleyana* **7**: 22-27.

Stenberg M, Kane M. 1998. *In vitro* seed germination and greenhouse cultivation of *Encyclia boothiana* var. *erythronioides*, an endangered Florida orchid. *Lindleyana* **13**: 101-112.

Stewart SL, Zettler LW, Minso J, Brown PM. 2003. Symbiotic germination and reintroduction of *Spiranthes brevilabris* Lindley, an endangered orchid native to Florida. . *Selbyana* **24**: 64-70.

Stewart SL, Kane ME. 2006*a*. Asymbiotic seed germination and *in vitro* seedling development of *Habenaria macroceratitis* (Orchidaceae), a rare Florida terrestrial orchid. *Plant Cell, Tissue and Organ Culture* **86**: 147-158.

Stewart SL, Kane ME. 2006*b*. Symbiotic seed germination of *Habenaria macroceratitis* (Orchidaceae), a rare Florida terrestrial orchid. *Plant Cell, Tissue and Organ Culture* **86**: 159-167.

Stewart SL. 2007. Integrated conservation of Florida Orchidaceae in the genera *Habenaria* and *Spiranthes* : model orchid conservation systems for the Americas. University of Florida, Gainesville, FL, USA.

Stoutamire WP. 1964. Seeds and seedlings of native orchids. *Michigan Botanist* **3**: 107-119.

Stoutamire WP. **1974**. Terrestrial orchid seedlings. In: Withner CL, ed. *The orchids: scientific studies*. New York: John Wiley and Sons, 101-128.

Sun S-C, Cai Y-L, Liu H. 2001. Biomass allocation of *Scirpus mariqueter* along an elevational gradient in a salt marsh of the Yangtse River estuary. *Acta Botanica Sinica* **43**: 178-185.

Suttle JC, Hultstrand JF. 1994. Role of endogenous abscisic acid in potato microtuber dormancy. *Plant Physiology* **105**: 891-896.

Suttle JC. 1995. Postharvest changes in endogenous ABA levels and ABA metabolism in relation to dormancy in potato tubers. *Physiologia Plantarum* **95**: 233-240.

Suttle JC. 2004. Physiological regulation of potato tuber dormancy. *American Journal of Potato Research* **81**: 253-262.

Suzuki RO. 2008. Dwarf morphology of the annual plant *Persicaria longiseta* as a local adaptation to a grazed habitat, Nara Park, Japan. *Plant Species Biology* **23**: 174-182.

Swarts N. 2007. Integrated conservation of the rare and endangered terrestrial orchid *Caladenia huegelii* H.G. Reichb. University of Western Australia, Perth, Australia.

Taiz L, Ziegler E. 1998. *Plant Physiology*. Sunderland, MA, USA: Sinauer Associates, Inc.

Taylor DL, Bruns TD, Szaro TM, Hodges SA. 2003. Divergence in mycorrhizal specialization within *Hexalectris spicata* (Orchidaceae), a nonphotosynthetic desert orchid. *American Journal of Botany* **90**: 1168-1179.

Thien LB, Marcks BG. 1972. The floral biology of *Arethusa bulbosa*, *Calopogon tuberosus*, and *Pogonia ophioglossiodes* (Orchidaceae). *Canadian Journal of Botany* **50**: 2319-2325.

Thompson DI, Edwards TJ, van Staden J. 2001. *In vitro* germination of several South African summer rainfall *Disa* (Orchidaceae) species: Is seed testa structure a function of habitat and a determinant of germinability? *Systematics and Geography of Plants* **71**: 597-606.

Thompson K, Grime JP, Mason G. 1977. Seed germination in response to diurnal fluctuations of temperature. *Nature* **267**: 147-149.

Thompson K, Grime JP. 1983. A comparative study of germination responses to diurnally fluctuating temperatures. *Journal of Applied Ecology* **20**: 141-156.

Tissue DT, Skillman JB, McDonald EP, Strain BR. 1995. Photosynthesis and carbon allocation in *Tipularia discolor* (Orchidaceae), a wintergreen understory herb. *American Journal of Botany* **82**: 1249-1256.

Tomita M, Tomita M. 1997. Effects of culture media and cold treatment on germination in asymbiotic culture of *Cypripedium macranthos* and *Cypripedium japonica*. *Lindleyana* **12**: 208-213.

Toole EH, Toole VK, Borthwick HA, Hendricks SB. 1955. Interaction of temperature and light in germination of seeds. *Plant Physiology* **30**: 473-478.

Trapnell DW, Hamrick JL, Giannasi DE. 2004. Genetic variation and species boundaries in *Calopogon* (Orchidaceae). *Systematic Botany* **29**: 308-315.

Tremblay RL. 1992. Trends in pollination biology of the Orchidaceae: evolution and systematics. *Canadian Journal of Botany* **70**: 642-650.

Tremblay RL, Ackerman JD, Zimmerman JK, Calvo RN. 2005. Variation in sexual reproduction in orchids and its evolutionary consequences: a spasmodic journey to diversification. *Biological Journal of the Linnean Society* **84**: 1-54.

Turesson G. 1922*a*. The genotypical response of the plant species to the habitat. *Hereditas* **3**: 211-350.

Turesson G. 1922*b*. The species and the variety as ecological units. *Hereditas* **3**: 100-113.

U.S. Fish and Wildlife Service. 2009. *Florida panther national wildlife refuge fact sheet*. http://www.fws.gov/southeast/pubs/facts/flpcon.pdf. (8 July 2009).

Uyemura S, Imanishi H. 1983. Effects of gaseous compounds in smoke on dormancy release in freesia corms. *Scientia Horticulturae* **20**: 91-99.

Uyemura S, Imanishi H. 1987. Changes in abscisic acid levels during dormancy release in freesia corms. *Plant Growth Regulation* **5**: 97-103.

Valero-Aracama C, Kane ME, Wilson SB, Vu JC, Anderson J, Philman NL. 2006. Photosynthetic and carbohydrate status of easy- and difficult-to-acclimatize sea oats (*Uniola paniculata* L.) genotypes during *in vitro* culture and *ex vitro* acclimatization. *In Vitro Cellular & Developmental Biology-Plant* **42**: 572-583.

Valero-Aracama C, Wilson SB, Kane ME, Philman NL. 2007. Influence of *in vitro* growth conditions on in vitro and ex vitro photosynthetic rates of easy- and difficult-to-acclimatize sea oats (*Uniola paniculata* L.) genotypes. *In Vitro Cellular & Developmental Biology-Plant* **43**: 237-246.

van Assche JA, Debucquoy KLA, Rommens WAF. 2003. Seasonal cycles in the germination capacity of buried seeds of some Leguminosae (Fabaceae). *New Phytologist* **158**: 315-323.

van der Pijl L, Dodson CH. 1966. *Orchid flowers: their pollination and evolution*. Coral Gables, Florida: University of Miami press.

van Tienderen PH. 1992. Variation in a population of *Plantago lanceolata* along a topographical gradient. *Oikos* **64**: 560-572.

van Waes JM, Debergh PC. 1986*a*. *In vitro* germination of some Western European orchids. *Physiologia Plantarum* **67**: 253-261.

van Waes JM, Debergh PC. 1986*b*. Adaptation of the tetrazolium method for testing the seed viability, and scanning electron microscopy study of some Western European orchids. *Physiologia Plantarum* **66**: 435-442.

Vendrame WA, Carvalho VS, Dias JMM. 2007. *In vitro* germination and seedling development of cryopreserved *Dendrobium* hybrid mature seeds. *Scientia Horticulturae* **114**: 188-193.

Volis S, Mendlinger S, Ward D. 2002. Differentiation in populations of *Hordeum spontaneum* along a gradient of environmental productivity and predictability: life history and local adaptation. *Biological Journal of the Linnean Society* **77**: 479-490.

Wang J, Seliskar DM, Gallagher JL, League MT. 2006. Blocking *Phragmites australis* reinvasion of restored marshes using plants selected from wild populations and tissue culture. *Wetlands Ecology and Management* **14**: 539-547.

Wang J, Seliskar DM, Gallagher JL. 2007. Growth of tissue culture-regenerated salt marsh monocots, in a simulated marsh field plot: Implication for wetland creation and restoration. *Ecological Engineering* **29**: 8-16.

Whigham DF. 1984. Biomass and nutrient allocation of *Tipularia discolor* (Orchidaceae). *Oikos* **42**: 303-313.

Whitlow CE. **1996**. Mass production of *Calopogon tuberosus*. In: Allen C, ed. *North American native terrestrial orchids: propagation and production*. Germantown, Maryland: North American Native Terrestrial Orchid Conference, 5-10.

Williams RR. 2007. Environmental factors affecting plant tissue cultures. *Combined Proceedings International Plant Propagators' Society* **56**: 91-95.

Williams SL. 2001. Reduced genetic diversity in eelgrass transplantations affects both population growth and individual fitness. *Ecological Applications* **11**: 1472-1488.

Wotavová-Novotná K, Vejsadová H, Kindlmann P. 2007. Effects of sugars and growth regulators on *in vitro* growth of *Dactylorhiza* species. *Biologia Plantarum* **51**: 198-200.

Wynd FL. 1933. Sources of carbohydrate for germination and growth of orchid seedlings. *Annals of the Missouri Botanical Garden* **20**: 569-581.

Yamato M, Iwase K. 2008. Introduction of asymbiotically propagated seedlings of *Cephalanthera falcata* (Orchidaceae) into natural habitat and investigation of colonized mycorrhizal fungi. *Ecological Research* **23**: 329-337.

Yamazaki J, Miyoshi K. 2006. *In vitro* asymbiotic germination of immature seed and formation of protocorm by *Cephalanthera falcata* (Orchidaceae). *Annals of Botany* **98**: 1197-1206.

Yañez P, Ohno H, Ohkawa K. 2005. Temperature effects on corm dormancy and growth of *Zephyra elegans* D.Don. *Scientia Horticulturae* **105**: 127-138.

Yates RC, Curtis JT. 1949. The effect of sucrose and other factors on the shoot root ratio of orchid seedlings. *American Journal of Botany* **36**: 390-396.

Yeung EC, Zee SY, Ye XL. 1996. Embryology of *Cymbidium sinense*: embryo development. *Annals of Botany* **78**: 105-110.

Yoder JA, Zettler LW, Stewart SL. 2000. Water requirements of terrestrial and epiphytic orchid seeds and seedlings, and evidence for water uptake by means of mycotrophy. *Plant Science* **156**: 145-150.

Yoshie F. 2007. Length of the pre-reproductive period of *Plantago asiatica* L. from different latitudes. *Plant Species Biology* **22**: 135-139.

Zettler LW, McInnis TM. 1992. Propagation of *Platanthera integrilabia* (Correll) Luer, and endangered terrestrial orchid, through symbiotic seed germination. *Lindleyana* **7**: 154-161.

Zettler LW, McInnis TM. 1994. Light enhancement of symbiotic seed germination and development of an endangered terrestrial orchid (*Platanthera integrilabia*). *Plant Science* **102**: 133-138.

Zettler LW. 1997. Orchid-fungal symbiosis and its value in conservation. *McIlvainea* **13**: 40-45.

Zettler LW, Hofer CJ. 1997. Sensitivity of *Spiranthes odorata* seeds to light during in vitro symbiotic seed germination. *Lindleyana* **12**: 26-29.

Zettler LW, Hofer CJ. 1998. Propagation of the little club-spur orchid (*Platanthera clavellata*) by symbiotic seed germination and its ecological implications. *Environmental and Experimental Botany* **39**: 189-195.

Zettler LW, Stewart SL, Bowles ML, Jacobs KA. 2001. Mycorrhizal fungi and cold-assisted symbiotic germination of the federally threatened eastern prairie fringed orchid, *Platanthera leucophaea* (Nuttall) Lindley. *American Midland Naturalist* **145**: 168-175.

Zettler LW, Pearlman S, Dennis DJ, Hopkins SE, Poulter SB. 2005*a*. Symbiotic germination of a federally endangered Hawaiian endemic, *Platanthera holochila* (Orchidaceae), using a mycobiont from Florida: a conservation dilemma. *Selbyana* **26**: 269-276.

Zettler LW, Piskin KA, Stewart SL, Hartsock JJ, Bowles ML, Bell TJ. 2005*b*. Protocorm mycobionts of the federally threatened eastern prairie fringed orchid, *Platanthera leucophaea* (Nutt.) Lindley, and a technique to prompt leaf elongation in seedlings. *Studies in Mycology* **53**: 163-171.

Zettler LW, Poulter SB, McDonald KI, Stewart SL. 2007. Conservation-driven propagation of an epiphytic orchid (*Epidendrum nocturnum*) with a mycorrhizal fungus. *Hortscience* **42**: 135-139.

Zhang X, Wu N, Li C. 2005. Physiological and growth responses of *Populus davidiana* ecotypes to different soil water contents. *Journal of Arid Environments* **60**: 567-579.

Ziegler AR, Sheehan TJ, Poole RT. 1967. Influence of various media and photoperiod on growth and amino acid content of orchid seedlings. *American Orchid Society Bulletin* **36**: 195-202.

Zimmerman JK, Whigham DF. 1992. Ecological functions of carbohydrates stored in corms of *Tipularia discolor* (Orchidaceae). *Functional Ecology* **6**: 575-581.

BIOGRAPHICAL SKETCH

Philip was born and raised in Manitowoc, WI, and attended the University of Wisconsin-Stevens Point (UWSP). His research career began at UWSP where he worked on screening potato clones for disease resistance. He received several awards for his potato research allowing him to attend local, regional, and national meetings. Although he loved researching potato genetics, he soon developed a passion for Wisconsin's native orchids. Philip graduated from UWSP in 2003 with a major in biology and minor in chemistry. He joined the Plant Restoration, Conservation, and Propagation Biotechnology Program in the Environmental Horticulture Department at the University of Florida in August 2003, and earned a Master's degree in August 2005. His thesis was titled *In vitro* Seed Germination and Seedling Development of *Calopogon tuberosus* and *Sacoila lanceolata* var. *lanceolata*: Two Florida Native Terrestrial Orchids. Fortunately, *Calopogon* seeds were much easier to work with than *Sacoila* seeds. Philip then transitioned into a PhD program in August 2005, and continued to study *Calopogon tuberosus*. In his spare time Philip volunteers for a pet rescue organization, laughs at his dogs, enjoys photography, studies martial arts, and continues to write biographies in the third person, although this is probably the last one.