

**Life History Aspects and Phylogeography of *Exyra semicrocea* (Lepidoptera:
Noctuidae) and their Implications for Pitcher Plant Bog Conservation**

by

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Abstract

Informed conservation decisions involve synthesizing information from a variety of disciplines. These include, but are not limited to, topics on life history, current anthropogenic threats, community interactions, and ecological processes. Here, I provide not only a robust life history of *Exyra semicrocea*, an endemic arthropod associate within pitcher plants, but also elucidate genetic connectivity of this species in order to develop a holistic approach to conservation of pitcher plant bogs throughout the southeastern United States Coastal Plain. Findings indicate that *E. semicrocea* populations have a genetic divergence across the Mississippi alluvial plain and a transition zone found near the Apalachicola-Chattahoochee-Flint River Basin. In addition, there is strong genetic structure among the three populations in the West Gulf Coastal Plain. These results with the natural history information that suggests weak dispersal ability as well as possible extirpation from multiple populations highlight the need to conserve not only the plants of this community but to take into account other endemic and associated community members as well when conserving and restoring pitcher plant bogs.

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Chapter Two: Genetic Differentiation and Structure among Populations of *Exyra semicrocea* (Lepidoptera:Noctuidae) and their Implications for Pitcher Plant Bog Conservation

Figure 1 Shaded region represents historical range of *Exyra semicrocea* across the southeastern United States (Folkerts and Folkerts 1996). Hash marks indicate historical range that no longer contains bog. Sample locations for this study are indicated by stars. The three localities west of the Mississippi River are Angelina National Forest (AN), Big Thicket Nature Preserve (BT), and Kisatchie National Forest (KN). Populations sampled across the eastern portion of the range are Abita Creek (AC), Grand Bay National Estuarine Research Reserve Site/National Wildlife Refuge (GB), Weeks Bay National Estuarine Research Reserve Site (WB), Conecuh National Forest (CF), Apalachicola National Estuarine Research Reserve Site/National Forest (AP), Okefenokee National Wildlife Preserve (OF), Francis Marion National Forest (FM), and Green Swamp Nature Preserve (GS). Bogs at Centre, Eller Seep, Prattville, and Reed Branch were visited, but no specimens were located 37

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Figure 4 Haplotype network depicting the nested levels used to infer historical processes in eastern populations of *Exyra semicrocea* across the southeastern Coastal Plain. Each circle represents a unique haplotype with size of the circle corresponding to haplotype frequency (see Supplemental Material for exact haplotype frequencies). The rectangle is the parsimonious ancestral haplotype or the haplotype with the highest outgroup probability according to TCS (Clement et al. 2000). Each branch represents one mutational difference between haplotypes with black circles representing missing or unsampled haplotypes. Colors correspond to location as designated in the legend 54

Chapter One: Life History Aspects of *Exyra semicrocea* (Pitcher Plant Moth)
(Lepidoptera: Noctuidae)

Introduction

Pitcher plant bogs (wet pine savannahs and other habitats dominated by carnivorous plants) of the southeastern United States Coastal Plain are composed of a diverse and complex community found nowhere else in the world. This region contains 29 or more carnivorous plants, with the more conspicuous genus *Sarracenia* (Pitcher Plants) containing eight or more of these species. Members of the genus are known to interact with a variety of organisms including bacteria (Koopman and Carstens in review, Kneitel and Miller 2002), protozoa (Hegner 1926), rotifers (Bateman 1987, Bledzki and Ellison 2003, Kneitel and Miller 2003), and most notably arthropods (Dahlem and Naczi 2006, Folkerts 1999, Goodnight 1940, Jones 1904, 1907, 1908). Pitcher plant interactions with arthropods as prey have garnered most attention (Heard 1998, Wolfe 1981), but it is important to note that these plants promote a diverse array of interactions with various arthropod species. These include herbivory, pollination, parasitism and predation of primary associates as well as capture interruption (Folkerts 1999). Some of these interactions are so specific that many of these arthropods are found only in pitcher plant bogs.

Pitcher Plant Moths (*Exyra* spp.) are the most noticeable of the 17 or more species of endemic arthropods within pitcher plants (Folkerts 1999, Rymal and Folkerts 1982) and are among of the more studied pitcher plant symbionts because of apparent damage caused by larval stages on pitchers (Atwater et al. 2006, Moon et al. 2008). These noctuid moths, first described by Gueneé (1852) and later revised by Grote (1879), consist of three described species: *Exyra fax* (Grote), *E. ridingsii* (Riley), and *E. semicrocea*

(Gueneé). *Exyra fax* is obligate in *Sarracenia purpurea* Linnaeus (Purple Pitcher Plant), *E. ridingsii* is obligate in *S. flava* Linnaeus (Yellow Pitcher Plant), and *E. semicrocea* is generally adapted to all species of *Sarracenia*. Initial behavioral observations on these moths were reported by Jones (1921) and followed by various reports of range and life history information (Benjamin 1922, Brower and Brower 1970, Folkerts and Folkerts 1996), but to date none have provided a complete life history of any of the three species.

In this study, I focused on *E. semicrocea* by presenting information from the unpublished portions of the thesis of Rymal¹ (1980) regarding (1) larva and adult morphology/development and (2) mortality factors across all life stages. Additional data is provided from recent observations made in 2010 on (3) adult behavior and (4) local abundance across the southeastern Coastal Plain. Together, with a thorough review of previously published information, I present a robust account of the biology of *E. semicrocea*.

Study Area

Life history and behavior observations were conducted at Packer Bog (1978-79), Splinter Hill Bog (2010), and Weeks Bay National Estuarine Research Reserve Site (2010) in Baldwin County, Alabama (Fig. 1). Historically, Baldwin County possessed a once large and continuous expanse of pitcher plant bog habitat including the now pastured Packer Bog, the recently preserved Splinter Hill Bog, and most likely the area now preserved in Weeks Bay National Estuarine Research Reserve Site (Folkerts 1982).

¹ D.R. Folkerts maiden name

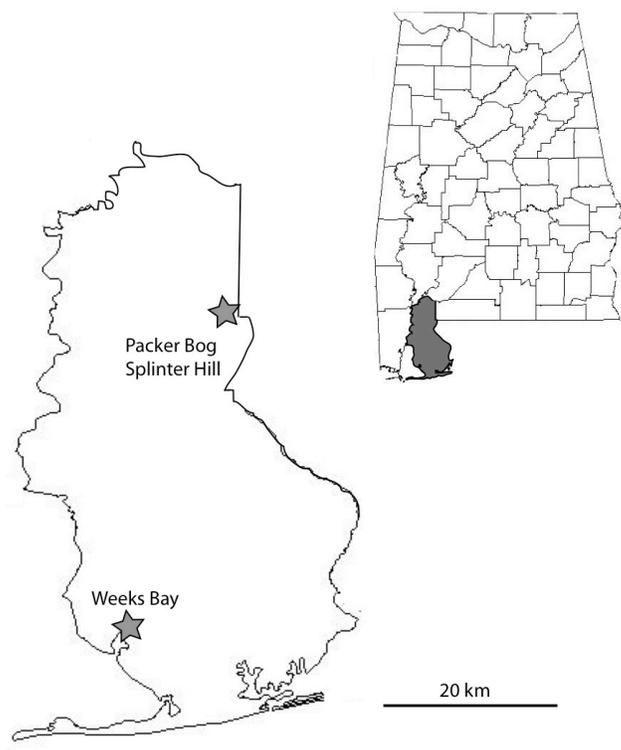


Figure 1. The three study sites located within Baldwin County, Alabama where behavioral observations of *Exyra semicrocea* were conducted.

Within the past 100+ years southern Alabama has undergone a reduction of habitat with remaining bog being managed by agencies such as Forever Wild, The Nature Conservancy, and the National Estuarine Research Reserve Sites. The bogs in this region are characterized by deep, poorly drained and strongly acidic soils found in seep areas at the bases of slopes and along drainages. The soil is fine sandy loam with 2 to 5 percent slopes and is very low in natural fertility, with low to medium content of organic matter. Runoff is slow and areas that are not ditched are usually saturated with water from seepage areas (McBride and Burgess 1964). The prominent pitcher plant species found at each of the three sites was *Sarracenia leucophylla* Raf. (White Topped Pitcher Plant) with *S. purpurea* Linnaeus (Purple Pitcher Plant), *S. rubra* Walt. (Red Pitcher Plant) and *S. psittacina* Michx. (Parrot Pitcher Plant) also occurring at these locations. Surveys of *E. semicrocea* distribution were made in 2010 across the entire southeastern Coastal Plain (Fig. 2).

Methods

Larval and adult morphology/development

Exyra semicrocea larvae were reared from fertilized eggs collected from Packer Bog in 1978 to 1) determine number of instars and 2) measure of head capsule width of each larval stadium. Larvae (n = 25) were fed in glass petri dishes with moist filter paper and cut portions of White Topped Pitcher Plant leaves at Auburn University. Daily observations were conducted to detect ecdysis and successive measures of head capsule width were made with an ocular micrometer. Head capsule width has been traditionally

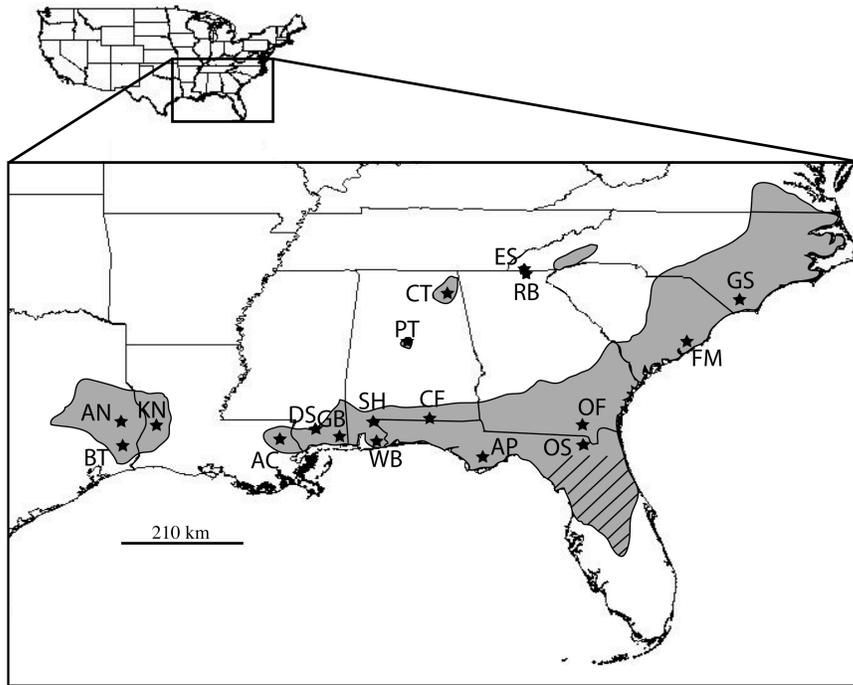


Figure 2. Shaded region represents historical range of *Exyra semicrocea* across the southeastern United States with hash marks indicating historical range that no longer contains bog. The eighteen sites visited in 2010 are represented by stars. The three localities west of the Mississippi River are Angelina National Forest (AN), Big Thicket Nature Preserve (BT), and Kisatchie National Forest (KN). Populations sampled across the eastern portion of the range are Abita Creek (AC), DeSoto National Forest (DS), Grand Bay National Estuarine Research Reserve Site/National Wildlife Refuge (GB), Weeks Bay National Estuarine Research Reserve Site (WB), Splinter Hill Nature Preserve (SH), Conecuh National Forest (CF), Apalachicola National Estuarine Research Reserve Site/National Forest (AP), Osceola National Forest (OS), Okefenokee National Wildlife Preserve (OF), Francis Marion National Forest (FM), Green Swamp Nature Preserve (GS), Prattville Bog (PT), Centre Bog (CT), Reed Branch Nature Preserve (RB), and Eller Seep Nature Preserve (ES).

used to determine instar stage because head capsule growth is discreet and occurs with each ecdysis (Harman 1970, Hoxie 1974, Watson 1974). The resulting measures were then used to estimate instar of larvae for subsequent field observations.

Duration of developmental stages (i.e., egg, larval instars, pupa) for *E. semicrocea* was monitored within tagged pitchers during the 1978-79 field season (n = 72), and each larval stadia was determined by an estimation of head capsule width (see above). Further assessment of adult lifespan was conducted in 2010 with pupae collected from Splinter Hill Bog, AL (n = 16), and reared in the lab at Auburn University. Sex of *E. semicrocea* adults was determined by the presence of hair pencils (male) or ovipositor (female), a method used to sex other noctuid species (Birch and Poppy 1990). All adults that emerged in the lab received a nectar substitute and information regarding duration as adult and number of eggs oviposited was recorded. Eggs were kept in a growth chamber with adequate moisture to assess the possibility of parthenogenesis.

The original description of *E. semicrocea* was based on drawings and contained little information about larval morphology (Gueneé 1852). Therefore, I have included a detailed larval morphology description not reported elsewhere, along with an illustration of a fifth instar (Fig. 3). In addition, potential morphological adaptations in *E. semicrocea* adults that apparently facilitate walking on downward pointing cellular projections of the pitcher plant leaf conductive zone, were evaluated using scanning electron microscopy (SEM) in 1978 and again in 2010. The ability to walk on the interior surfaces of *Sarracenia* spp. pitchers is apparently unique to Pitcher Plant Moths (Folkerts 1999). Therefore, pretarsal characteristics were compared between *E. semicrocea* and specimens of a non-pitcher plant associating noctuid, *Helicoverpa zea* Boddie (Corn Earworm).

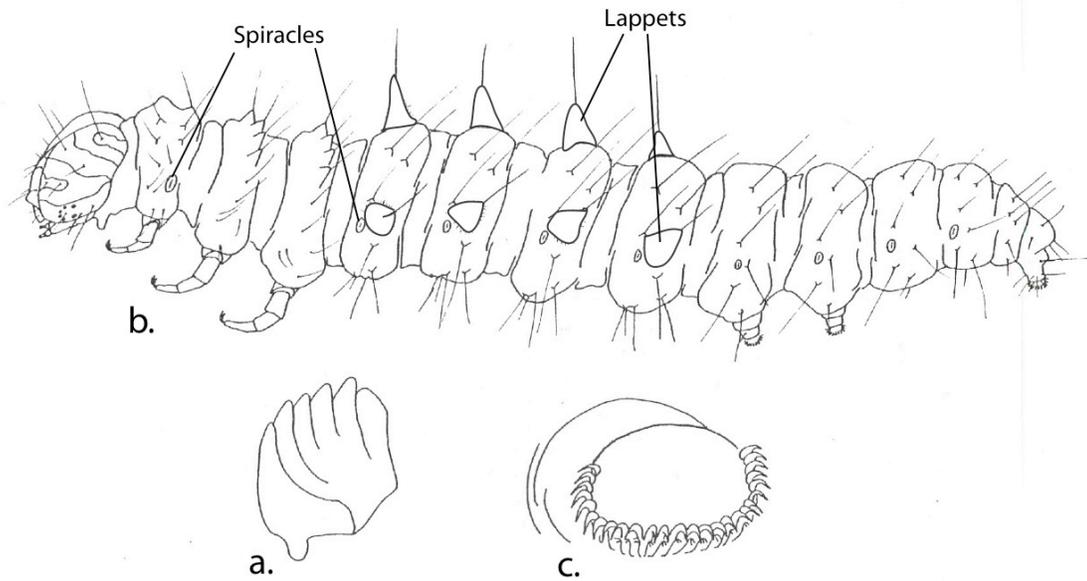


Figure 3. Depiction of *Exyra semicrocea* larva morphology. a. Mesal view of the right mandible, b. Lateral view of a fifth instar larva, c. Ventral view of the crochets of a proleg.

Adult behavioral observations

Observations on adult flight behavior were made twice a month from June through September 2010. Ten adults were marked during the day using a florescent dye powder (Magruder Color Company) and checked the following morning to assess flight emergence ($n = 60$). Additional adults were located and their respective pitchers flagged for nighttime observations on flight behavior. Each adult was sexed by making an incision into the wall of the pitcher and carefully using a hand lens to examine the lower abdomen. Leaves containing individuals were marked prior to dusk and watched for movement throughout the night. Sex of individual, time of emergence, duration of flight, number of pitchers visited, duration inside pitchers, flight distance from original pitcher to final pitcher, and number of eggs oviposited were recorded. T-tests were conducted in the R v2.6.2 statistical software environment (R Development Core Team 2008) to test whether there were differences between sexes regarding time of emergence and lifespan.

Mortality measures

Percentage mortality in egg, larval, and pupal stages were calculated from tagged samples in Packer Bog in 1978-79 ($n = 45, 287, 34$; respectively), and periodic collections of larvae displaying signs of parasitism were brought back to the lab to rear and identify parasitoids. Dead larvae were also collected in order to ascertain mortality factors. Additional incidents of predation, parasitism, and fungal pathogens were recorded from incidental field observations made from 1978 through 2010. Finally, a parasitoid was identified from amplification of the mitochondria (mtDNA) cytochrome *c*

oxidase subunit gene (COI) of a parasitized *E. semicrocea* larva from an ongoing study and compared to extant sequences in the NCBI database via BLAST (Altschul et al. 1990).

Population Density

Surveys of local abundance and possible extirpation across the southeastern Coastal Plain were prompted by reports of declines in *E. semicrocea* abundance (Folkerts and Folkerts 1996). Therefore during the summer of 2010, I sampled seventeen sites from eastern Texas through North Carolina in order to determine current status of *E. semicrocea* populations (Figure 2). Exhaustive searches of every pitcher were conducted in smaller bogs to determine total population number, while larger bog densities were estimated by extrapolating from counts in measured areas within the respective bogs. Information regarding life stage, host plant, female/male ratio, and presence/absence of congeners were recorded for each bog.

Results and Discussion

Development, morphology, and behavior

Egg

Descriptions of egg morphology have been presented previously (Folkerts and Folkerts 1996, Jones 1921), with both accounts detailing the spherical shape and yellow hue typical of Noctuidae. Eggs were an average diameter of 0.7 mm, with slightly undulating ridges radiating from a central point on the anterior surface to the opposite pole. These ridges contain tiny pores that are most likely part of the egg respiratory

mechanism (Wigglesworth 1972). Approximate time spent in the egg life stage was twelve days (Table 1).

An early report of *E. semicrocea* indicated that eggs are deposited singly due to the importance of having one larva per pitcher (Jones 1921). Observations by Folkerts and Folkerts (1996) indicated that number of eggs oviposited varied from one to several per pitcher. Results from 2010 support the latter, where females oviposit anywhere from 1-3 eggs per pitcher. While previous observations could not ascertain whether eggs were oviposited by multiple females, recent observations were conducted through close monitoring of female nocturnal behavior. Therefore I feel confident that eggs were all oviposited by a single female per pitcher in 2010.

Larva

Larval development of *E. semicrocea* spans five instars (determined from number of ecdyses in 1978-79) with head capsule size ranging between 0.32-1.78 mm (Table 2). Duration of stadia 1-3, stadia 3-4, and stadium 5 during summer months is roughly twelve, nine, and thirteen days, respectively (Table 1). These numbers are likely to increase during colder temperatures when quiescence occurs. Overall, the eruciform larva ranged from 4 mm in length during stadium one to 30 mm in length in stadium five with segments having a bright red coloration with white intersegmental areas (Folkerts and Folkerts 1996, Rymal and Folkerts 1982). The head capsule of *E. semicrocea* is ivory with four incomplete and irregular black bars, six pairs of ocelli, and mandibles each possessing four dentes (Fig. 3a). A ventral eversible gland is present on the ventromeson of the prothorax, between the legs and the head. Pairs of ventral prolegs occur on the

Table 1. *Exyra semicrocea* average length of time in life stages.

Developmental Stage	Ave. No. Days	Range	S.D.
Egg	12	6-20	3.9
Larval stadia 1-3	12	7-15	3.2
Larval stadia 3-4	9	7-15	2.5
Larval stadium 5	13	8-21	5.2
Pupa	14	8-20	5.5
Adult	10	7-16	2.8

Table 2. Average measurements of *Exyra semicrocea* head capsule widths of instars one through five.

Instar	Width of head capsule (mm)	SD
I	.32	.01
II	.65	.01
III	.89	.07
IV	1.32	.10
V	1.78	.21

fifth, sixth, and tenth abdominal segments (Fig. 3b), each with crochets in a biordinal mesoseries, the second series of claws being small and bifid (Fig. 3c). Dark brown elliptical spiracles are present on the prothorax and on abdominal segments one through eight. The entire body is covered with microsetae, which occur in especially dense patches on the dorsum of each segment and all primary setae are simple. The prespiracular group of setae on the prothorax is bisetose. Primary setae of the notum on the meso- and metathorax arise from chalazae. Paired lateral projections are found on abdominal segments one through four (Fig. 3b). These projections are termed “lappets” (Jones 1921) and are fleshy, elongate tubercles covered with microsetae. The rho seta is borne at the terminal end of the tubercle. Lappets are absent in first instar larvae, are relatively narrow and elongate in instars two and three, and become broader in instars four and five.

The ability of *E. semicrocea* larvae to avoid entrapment in pitcher plants may be due to the function of the lappets (Jones 1921, Folkerts 1999). Larvae, when disturbed from feeding or web spinning, will often curl their bodies and drop into the pitcher. The projecting lappets prevent larva from falling to the lowest, narrowest portion of the pitcher which contains long, stiff, downward pointing hairs. Other important entrapment-avoiding mechanisms of the larva include a dragline of silk that is often used inside the pitcher to pull the larva up from the pitcher bottom and feeding to destroy the trapping function of the leaf.

Damage to the leaves by *E. semicrocea* larvae has been well documented (Folkerts and Folkerts 1996, Folkerts 1999, Jones 1921, Rymal and Folkerts 1982, Moon et al. 2008). Typically, a first or second instar larva often cuts a narrow feeding channel

from within a young leaf, which encircles the pitcher and causes wilting above the channel, forming a hardened cap covering the mouth of the pitcher and thus creating a feeding chamber (Jones 1921, Folkerts 1999). In contrast, feeding on larger, mature pitcher plant leaves does not produce this wilting effect. Therefore, third, fourth, and fifth instar larvae create a fine, silken web often covering the opening of an inhabited pitcher (Folkerts 1999). A chamber of this kind usually has a web ceiling, a floor of frass which accumulates above the level of the prey mass, and walls formed by the membranous outer leaf surface which remain after the inner leaf layers have been removed by feeding (Folkerts 1999). The web ceiling was observed to be an effective shelter against water intrusion during a gentle rain and also excluded potential spider predators (Jones 1921). It has been suggested that dispersal of larvae only occurs once the entire pitcher has been consumed because of the inherent predation risk (Jones 1921), but Folkerts (1999) documented migration between several pitchers during development. For example, movement between pitchers occurs shortly after the first instar larvae hatches, between second or third instars, fifth instar, and finally before pupating, and in many cases the leaves are not completely consumed. Dispersal of first instar larvae partly explains why more than one larva is rarely found in a leaf, even though several eggs may have been present. More than one larva inhabiting a single pitcher has been found in a few instances, being separated from each other by webbing or by a layer of frass. This separation was also noticed in individuals reared in the lab in 2010.

Overwintering of *E. semicrocea* only occurs as second, third, fourth, or fifth instar larvae, with feeding ceasing in late November (Folkerts and Folkerts 1996). Various types of hibernaculae have been observed (Jones 1921). Small larvae are most often

found near the bottom of the pitcher, having burrowed beneath the accumulation of prey. Large larvae are sometimes buried in frass or cling to dry pitcher walls, well above the level of frass and prey. Occasionally, chambers have web ceilings with particles of frass incorporated into the silk.

Pupa

The medium brown pupa is of the oblong type, approximately 11 mm in length, with wing pattern discernable through the integument shortly before eclosion at approximately fourteen days (Table 1). Before pupation, fifth instar larvae disperse to pitchers that are usually undamaged and will often cut a small hole (approximately 2 mm in diameter) into the lower portion of the pitcher tube (Folkerts 1999). Pupal chambers are located above these holes, which are presumably for rainwater drainage. The cocoon varies from a few strands of supporting silk near the anterior end to a mesh of silken strands covering the entire pupa (Jones 1921). The simplest type of chamber contains a pupa, cephalic end up, suspended by silken strands attached to the interior wall of a pitcher with varying amounts of silk surrounding the pupa. A variant on this type of chamber is shielded above by a thin sheet of silk and bordered below by varying amounts of frass and silk (Jones 1921). The least common type of pupation chamber is found in pitchers with obvious feeding damage. In this case, the pupa is found beneath the level of frass in a silk lined chamber only slightly larger than the pupa itself.

Adult

Adults of the species average 11 mm in length and have the most variable wing color patterns of the three *Exyra* spp. (Folkerts and Folkerts 1996). The common type has a black distal half of the forewing with an ivory basal half and hind wings completely black or gray in color. In addition, the head and prothorax are black with the remaining body, including the filiform antennae, ivory (Folkerts 1999, Jones 1921, Rymal and Folkerts 1982; Fig. 4). Two other forms have varying degrees of mottling on the black portions of the forewing with additional shading at the base of the hindwing and were first described as different subspecies (Benjamin 1922, Dyar 1904, McDunnough 1938), but the variation is continuous across the range, and therefore, these delineations are probably unwarranted (Folkerts 1999, Forbes 1954).

Regarding trap avoidance, *Exyra* adults have the apparently unique ability to walk along the interior of pitcher plants leaves (Folkerts 1999). While it is difficult to examine pretarsal position of a live adult within a pitcher, using scanning electron micrographs (SEM) and comparative measurements I can make assumptions about the potential function of pretarsi in relation to pitcher plant walls. SEM reveals a difference in the shape of the pretarsal claws of *E. semicrocea* when compared to *Helicoverpa zea* (Corn Earworm) of the same family (Fig. 5). Unlike in the Corn Earworm (Fig. 5a), the metathoracic pretarsal lobes of *Exyra* (Fig. 5b) have bifid claw tips of equal length, and claws project laterally causing the empodium and aroliar pad to extend beyond the claws. This lateral projection is also seen in the mesothoracic (not pictured) and prothoracic pretarsi (Fig. 6), which also have a thicker base with bifid tips. Although it is impossible



Figure 4. *Exyra semicrocea* in copulation. The lower and lighter moth is the male.

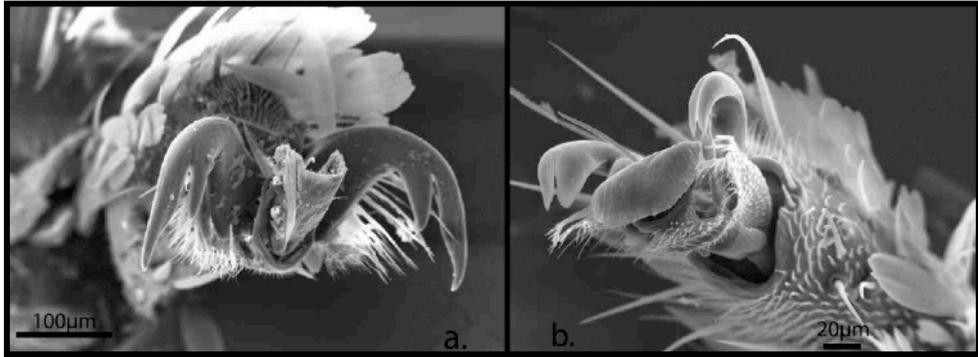


Figure 5. Scanning electron micrograph of the pretarsus of the adult metathoracic leg of a. *Helicoverpa zea* (magnification 500x) and b. *Exyra semicrocea* (1000x).

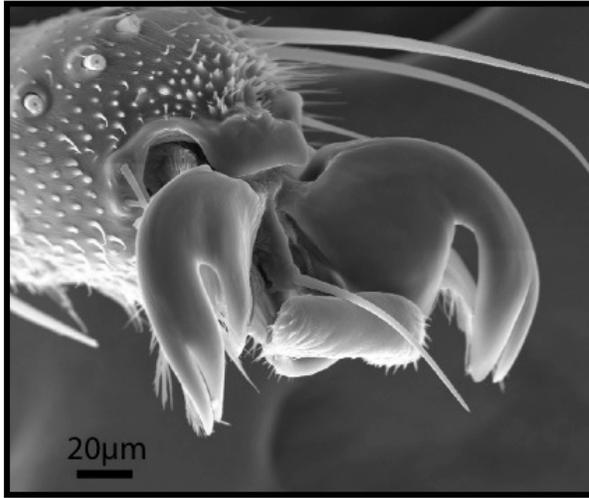


Figure 6. Scanning electron micrograph of the pretarsus of the adult prothoracic leg of *Exyra semicrocea* (1000x).

to conclusively determine the function of these structures in *Exyra*, comparative measurements of claws and leaf projections lead me to believe they are possibly used in placement of the pretarsus on the leaf surface. Cellular projections of the conductive zone in the White Topped Pitcher Plant are ~10 μm wide at the base, tapering to a point and ~20 μm apart. Moth pretarsi on all legs have two notches between claw tips ~10 μm wide with a width between tips of ~20 μm wide which may be used in grasping these projections (measurements were taken at multiple SEM angles not shown). Additional behavior modifications appear to be vital in trap avoidance, as *E. semicrocea* adults are always in an upright position within pitchers, even entering pitchers backward. Moreover, they copulate at a right angle (Fig. 4), which is in contrast to the end-to-end coupling typical in noctuids. This copulation position ensures neither moth is oriented with head downward in the pitcher. Together, it is likely that a combination of morphological and behavioral traits play a role in trap avoidance.

Additional behavioral observations of *E. semicrocea* suggest moths are mostly stationary and well below the orifice of the pitcher throughout the day, possibly to avoid predation. Nighttime observations in 2010 revealed that moths begin to move prior to dusk, feeding on the extrafloral nectaries near the orifice of the pitcher before quickly retreating back to their sedentary position, thus confirming descriptions made by Jones (1921). This feeding behavior typically occurs one to six times until flight emergence. Flight emergence occurred in all florescent dye-marked individuals ($n = 60$) as none were found in their respective pitchers the following morning. Descriptions of nightly flight emergence made in 1980 indicated that *E. semicrocea* females emerge from the pitchers

shortly after dusk, with males emerging soon after to find females within pitchers. Observations made in 2010 supported these inferences and reveal that female moths tended to leave ~24 minutes after sunset (range of 9-55 min). Females tended to fly between nearby pitcher plants, rarely venturing high above the bog, and flight time was from 2-30 minutes ($\bar{x} = 13$) with an average distance from starting pitcher of 16.84 m ($n = 12$). During this time, females exhibited similar behavior, often visiting multiple pitchers during flight ($\bar{x} = 4$). Duration within these pitchers typically lasted less than 1 minute; although three females stayed in a visited pitcher for over 30 minutes. Three out of seven females that were observed with males during the day oviposited 1-3 eggs within pitchers. Oviposition by females in the lab revealed female clutch size ranging from 0-93 ($\bar{x} = 41$, $n = 5$) eggs with no indication of parthenogenesis. This large number suggests that females have the ability to lay numerous eggs per pitcher over the average ten-day lifespan as adults (no difference in male and female lifespan $t = -0.9976$, $df = 14$, $P = 0.3354$; Table 1).

In contrast to females, males possessed a more erratic flight pattern and were therefore harder to observe. On average, males emerged approximately 3 hours and 32 minutes after sunset with the shortest time being 1 hour and 5 minutes. This emergence time is significantly different from females ($t = -8.4349$, $df = 15.477$, $P < 0.001$; Fig. 7). Measures of male flight time and distance were unattainable due to the quickness of males and their tendency to fly several meters above the bog. Males tend to be lighter in color than females, likely due to a loss of wing scales during flight (Fig. 4). This observation, when compared to sedentary captive males that retain their dark coloration in the lab, indicates that males potentially engage in more flight than females, as is

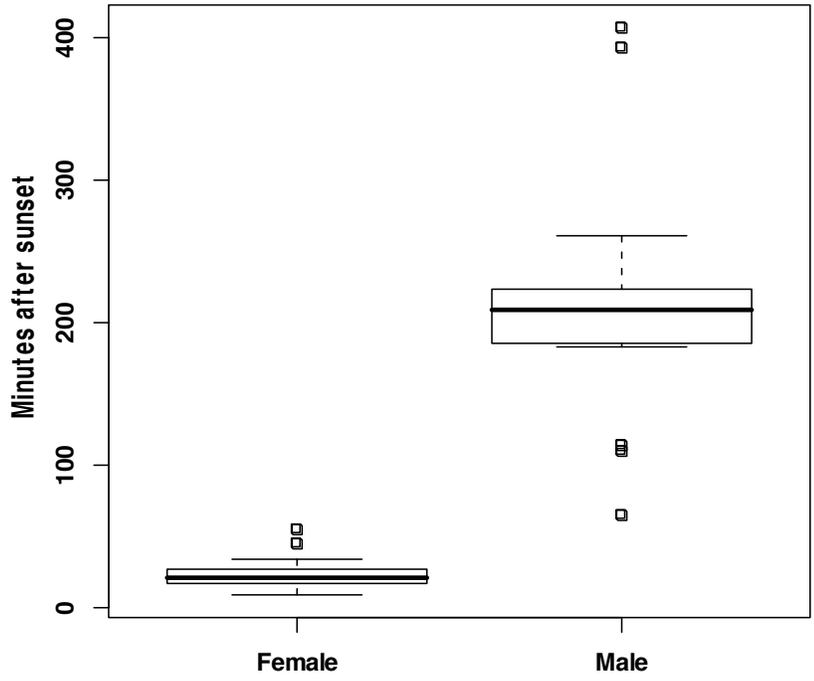


Figure 7. Emergence from pitchers as measured by minutes after sunset of male and female *Exyra semicrocea* in Baldwin County, Alabama.

necessary in the search for receptive females. This is further supported by a light trap study of *E. fax*, which consistently caught mainly males, indicating that in that species, males also fly more frequently (Brower and Brower 1970).

Mortality measures

Mortality in the egg stage was recorded at 56% in 1978-79 (Table 3). This high rate of mortality is assumed to be the combined results of infertility, predation, fungal pathogen and parasitism. Although egg predation was never witnessed, several small spiders capable of preying upon eggs in pitchers were observed. Eggs appearing to have been parasitized were distinguished by a silvery-black color and tiny exit hole in the eggshell. The egg parasitoid was never captured and identified but is believed to be a hymenopteran. This is supported by observations of *E. semicrocea* egg parasitism by a species of *Chalcis* (Chalicidae) in a bog in North Carolina (Jones 1921).

Larval mortality is difficult to ascertain, as many “missing” larvae potentially dispersed to surrounding pitchers. Therefore, disappearance rate was calculated at 60% with known mortality at 15% (Table 3). Predation of dispersing larvae was observed on multiple occasions between 1978 and 2010 by spider predators (i.e., *Peucetia viridans* (Green Lynx Spider), *Strotarchus piscatoria* (Bent-leaf Spider) and *Phidippus audax* (Common Jumping Spider)) and the Red Imported Fire Ant, *Solenopsis invicta* Buren (Formicidae). These predators have also been seen preying on *E. semicrocea* larvae within pitchers. Additional mortality of *E. semicrocea* larvae within pitchers was due to

Table 3. Mortality factors for *Exyra semicrocea* in *Sarracenia leucophylla*, Baldwin County, Alabama.

Lifestage	N	Mortality	Mortality factors	Noted predators & parasites
Egg	45	56%	Infertility, fungal pathogen, parasitism, predation	unknown
Larval	287	75%*	Dispersal factors, fungal pathogens, predation	<i>Strotarchus piscatoria</i> , <i>Peucetia viridans</i> , <i>Phidippus audax</i> , <i>Solenopsis invicta</i> , <i>Neumuraea rileyi</i> , tachinid (unidentified), braconid (unidentified)
Pupa	34	29%	Parasitism, predation	<i>Strotarchus piscatoria</i>
Adult	N/A	N/A	Dispersal factors, predation	Lycosids, Libellulids, <i>Peucetia viridans</i> , Chiroptera

* 60% missing, 15% known mortality

an unidentified tachinid parasite, braconid parasite, and the fungal pathogen, *Nomuraea rileyi* Farlow (Moniliaceae). The fungal pathogen attacks larvae of all instars and was most commonly seen in late fall. The tachinid parasite emerges as a larva from the fifth instar caterpillar and was most commonly seen during the overwintering period. The braconid parasite was collected as a pupa from a third instar larva in 1978. In addition to predation and parasitism, larvae have been witnessed to drown in pitchers after severe storms.

The pupal stage incurred 29% mortality and was primarily the result of spider predation (Table 3). For example, Bent-leaf Spiders preyed upon 50% of the tagged pupae during a one-week period. In addition, a hymenopteran parasitoid mitochondrial cytochrome oxidase subunit I (COI) sequence (97% match with *Diadegma semiclausum* within the NCBI database) was discovered in one pupa sample from an ongoing study in 2010 (data not shown).

Percent mortality in the adult stage could not be estimated, as adults could not be tracked in the field. However, a variety of predators have been witnessed in incidental field observations. *Exyra semicrocea* does not fly during the daytime unless disturbed from the pitcher. When this occurs, various libellulid dragonflies have been observed to be diurnal predators. Nocturnal predators are generally bats and spiders. For example, predation of a female moth in flight by a wolf spider was observed during the 2010 observations. It has also been noted on several occasions that dead adult *Exyra semicrocea* were present and still in the upright position within the pitcher, with no apparent cause of death.

Population density

Numbers, life stages and sex ratios varied substantially across all sampled sites (Table 4), which is typical for a multivoltine insect. Individuals were not found at four localities (i.e., CT, PT, RB, ES), although *E. semicrocea* was recorded previously at those sites (personal communication D. Folkerts and M. Hodges). These sites are home to small, isolated pitcher plant bogs composed of two federally endangered pitcher plants (i.e., *S. oreophila* (Green Pitcher Plant), *S. alabamensis* (Alabama Pitcher Plant), USDA 2009). An exhaustive search of all pitchers at AC, AN, OS, OF, FM, and GS bogs revealed low abundance, potentially due to limited habitat. For example, bog habitat in OF, OS, and FM was mostly found along roadsides and AN has recently undergone bog restoration. On the other hand, other bog habitats, such as AC, were large but had not been burned recently and therefore pitcher plants were less dense. The low population numbers of *E. semicrocea* at GS and FM may be influenced by competition with a sister species *E. ridingsii*. *Exyra ridingsii* only inhabits the Yellow Pitcher Plant, which extends from Alabama into North Carolina. The abundance of *E. ridingsii* appears to shift with increase in latitude, from being rare to absent in the southernmost portion of Yellow Pitcher Plant range to replacing *E. semicrocea* in leaves of Yellow Pitcher Plant in the northern portion of the range (personal observation). While this observation may not be the case year round, this apparent competitive exclusion of *E. semicrocea* from Yellow Pitcher Plant was also noted in another population in South Carolina in the early 1900s (Jones 1921). The abundance of *E. ridingsii* throughout its range, as well as effects on *E. semicrocea*, has not been studied.

Table 4. The eighteen sites (see figure 2 for site localities) surveyed for *E. semicrocea* during May-June 2010.

Site	N	Area surveyed (ha.)	Density (#/per ha.)	# larvae	# Pupae	#adults	Fem:Male ratio
AN*	3	0.25	12	3	0	0	n/a
BT	30	0.15	300	13	3	14	3:4
KN*	32	0.25	128	0	0	32	1:1
AC*	30	3	9	21	0	9	2:7
DS	30	2	16	28	0	2	1:1
GB	30	2.75	11	0	0	30	1:2
WB	54	1.21	44	15	1	38	11:8
SH	27	1.11	24	9	1	17	7:10
CF	29	0.74	39	28	0	1	1:0
CT*	0	0.10	0	0	0	0	n/a
PT*	0	0.08	0	0	0	0	n/a
AP	30	0.52	60	0	0	30	1:2
OS*	5	0.04	125	5	0	0	n/a
OF*	22	0.36	61	19	3	0	n/a
RB*	0	0.18	0	0	0	0	n/a
FM*	18	0.16	113	11	5	2	1:1
GS*	17	2.75	6	17	0	0	n/a
ES*	0	0.28	0	0	0	0	n/a

* indicates sites that were exhaustively searched

Conservation implications

The lack of *E. semicrocea* at four sites surveyed, low abundance at other sites, and loss of bog habitat (e.g., Packer Bog and the peninsula of Florida) is of concern, especially with estimates predicting that perhaps ~3% of bog habitat remains due to fire restriction, urbanization, forestry, and agriculture (Folkerts 1982, Folkerts and Folkerts 1999). This decline in bog habitat has resulted in fragmentation of once continuous bog habitat (Folkerts 1982). Current management practices to preserve these fragments often involve prescribed burns conducted during the dormant season (i.e., winter burns). While these fires are beneficial to the plants, they may have negative effects on the arthropod community that are not adapted to winter burns. For example, *E. semicrocea* populations overwintering only as larvae, and therefore unable to disperse, are likely to be severely damaged by winter burns. When prescribed burns involve the entire bog area, as is often the case in small and isolated fragments, no insects survive to repopulate those areas after the burn. Therefore, more research to address winter fire effects on the pitcher plant community is warranted, especially given the great diversity of endemics and associates inhabiting pitcher plant bogs. Maintaining and restoring pitcher plant bogs around the southeast is vital to preserving these communities for future generations.

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Chapter Two: Genetic Differentiation and Structure among Populations of *Exyra semicrocea* (Lepidoptera: Noctuidae) and their Implications for Pitcher Plant Bog Conservation

Introduction

Currently, biodiversity losses are occurring at an alarming rate, exceeding anything in the geological past (Wilson 1988). These losses have further implications as they can exacerbate collapses of ecosystem functions. The most recognized and potentially well-documented examples of biodiversity and ecosystem loss have been in the tropics, home to half of the world's species (Wilson 1988). However, less well known is the fact that temperate zone ecosystems including marine (Worm et al. 2006), freshwater (Moyle and Williams 1990), and terrestrial (Norse 1990) environs are being adversely impacted at an even faster rate. Given that the majority of the world's affluent human populations have historically resided in the temperate region (Diamond 1997, Ehrlich and Ehrlich 1986; 1990), it is not surprising that many of its ecosystems have experienced anthropogenic driven environmental degradation, extensive species declines and habitat loss (Ehrlich and Ehrlich 1981, Soulé 1991, Wilson 1985). Such negative anthropogenic influences are well documented to have occurred in the United States, which has approximately 38 threatened, 58 endangered, and more than 30 critically endangered ecosystems (Noss et al. 1995).

Among the most critically endangered in the United States is the longleaf pine ecosystem, which historically occupied most of the southeastern Coastal Plain (Noss et al. 1995). Estimates suggest that there has been a >98% decline of pre-European settlement longleaf pine (*Pinus palustris*) forests in this area (Noss 1989, Ware et al.

1993). Encompassed within the longleaf pine ecosystem are a myriad of endangered habitats including carnivorous plant wetlands, commonly referred to as *pitcher plant bogs*. These bogs are typically found in wet pine flatwoods or seepage slopes and are characterized by wet, sandy and low nutrient soils (Folkerts and Folkerts 1993). In these habitats, many endemic plants have evolved a carnivorous lifestyle, obtaining nutrients through the capture and digestion of animal prey. Similar to the overall ecosystem, current estimates suggest that the pitcher plant habitat now occupies <3% of its former range largely due to restriction of natural fires, urbanization, forestry and agriculture (Folkerts and Folkerts 1993). Of the more than 29 carnivorous plant species representing five genera (Folkerts 1999, Schnell 2002), at least five species are federally endangered or threatened (USDA 2009) and all species in the genus *Sarracenia* (pitcher plants) are listed in The Convention on International Trade in Endangered Species (CITES, www.cites.org).

Many studies on carnivorous plants have focused on the investment in and evolution of carnivory (Brewer 2003, Brewer et al. 2011, Ellison and Gotelli 2009, Gibson and Waller 2009, Givnish et al. 1984, Zamora et al. 1998) that was pioneered in part by Charles Darwin's fascination with the subject (Darwin 1875). Other areas of interest include the genetics and population structure of these plant species since such information has implications for conservation of these unique organisms. For example, varying levels of genetic diversity have been reported across *Sarracenia* sp. and populations (Godt and Hamrick 1996, Godt and Hamrick 1998, Wang et al. 2004). Additionally, recent research examining *Sarracenia alata* (the winged pitcher plant), which ranges from eastern Texas to western Alabama, identified a phylogeographic break

and high genetic divergence centered on the Mississippi River alluvial plain, with additional population structure on either side of the break (Koopman and Carstens 2010). Such results highlight the potential uniqueness of individual pitcher plant populations that should be considered when developing management plans for these habitats.

While research and conservation efforts have primarily focused on the carnivorous plants, less attention has been given to other constituents of pitcher plant bogs. This is unfortunate because these bogs support a complex and intimately associated biotic community. For example, a number of arthropods have evolved characteristics for inhabiting *Sarracenia* sp. (Folkerts and Folkerts 1993). These include >17 species of endemic mites, flies and moths (Bradshaw and Creelman 1984, Dahlem and Naczi 2006, Folkerts 1999, Rymal and Folkerts 1982, Underwood 2009). Of these, the herbaceous noctuid moth *Exyra semicrocea* ranges across the United States southeastern Coastal Plain from eastern Texas to southern Virginia (Folkerts and Folkerts 1996, Lafontaine and Poole 1991) and is of conservation concern due to its obligate relationship with *Sarracenia* sp. like *S. alata* (Folkerts and Folkerts 1996, Jones 1921). Specifically, *E. semicrocea* is oligophagous, feeding exclusively on *Sarracenia* sp., and its life cycle occurs entirely inside pitcher plant leaves, from oviposition through larval development and mating. Because of this well documented relationship, I believe that the *E. semicrocea*/*Sarracenia* complex represents an ideal model examining a series of ecological and biogeographical questions. Here, I examine the genetic structure of *E. semicrocea* across the southeastern Coastal Plain. Given the tight relationship between this moth and its host plants, I hypothesize that the genetic structure of *E. semicrocea* is consistent with the major finding of Koopman and Carstens (2010) for *S. alata*, namely I

predict that a strong genetic break would be associated with the geographic region occupied by the Mississippi River alluvial plain.

Methods

Sample techniques, DNA extraction and polymerase chain reaction (PCR)

Specimens of *E. semicrocea* were collected across eight southeastern Coastal Plain states between May and August 2010 (Figure 1). *E. semicrocea* was not found at four of the visited sites (Centre, AL; Prattville, AL; Reed Branch, GA and Eller Seep, NC); thus, samples were acquired from 11 localities. At each site, attempts were made to collect 24 *E. semicrocea* individuals, however, the actual number acquired per population ranged from 3-24 ($\bar{x} = 21$) specimens. Individuals were preserved in 95% ethanol in the field and DNA extraction from each *E. semicrocea* following the methods described by Coffroth et al. (1992). The resulting DNA extractions served as templates for amplifying a ~680 bp fragment of the mitochondrial (mtDNA) cytochrome *c* oxidase subunit I (COI) gene. The primer pair for subsequent polymerase chain reaction (PCR) was LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3'). The COI region was chosen because it can reveal both historical and current genetic patterns (Avice et al. 1987) and has been shown to be informative in other Lepidoptera species (Meng et al. 2008, Roehrdanz et al. 1994, Vandewoestijne et al. 2004). Each PCR was conducted in 25 μ l volumes containing ~10-30 ng of template DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.001% gelatin, 2.0 mM MgCl₂, 200 mM dNTPs, 1 U *Taq* DNA polymerase and 0.4 mM of each primer. PCR was conducted with a PTC-100 Thermal Cycler (MJ Research) using the

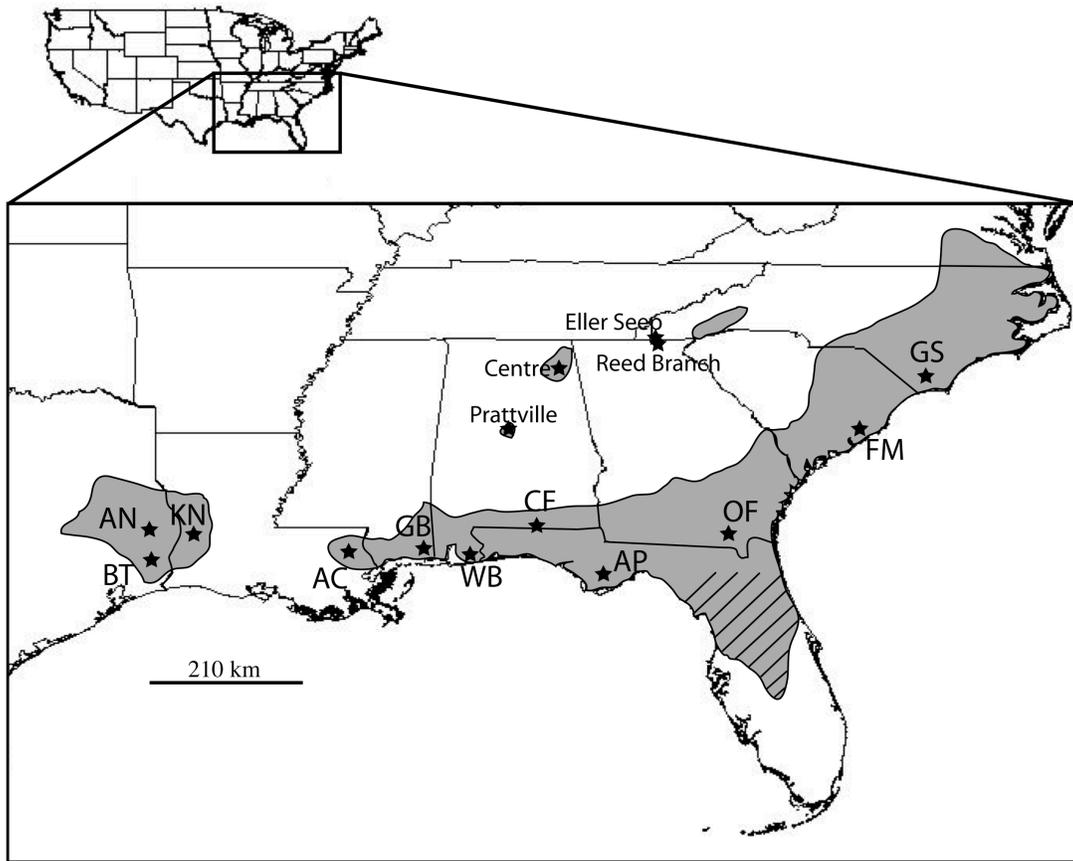


Figure 1. Shaded region represents historical range of *Exyra semicrocea* across the southeastern United States (Folkerts and Folkerts 1996). Hash marks indicate historical range that no longer contains bog. Sample locations for this study are indicated by stars. The three localities west of the Mississippi River are Angelina National Forest (AN), Big Thicket Nature Preserve (BT), and Kisatchie National Forest (KN). Populations sampled across the eastern portion of the range are Abita Creek (AC), Grand Bay National Estuarine Research Reserve Site/National Wildlife Refuge (GB), Weeks Bay National Estuarine Research Reserve Site (WB), Conecuh National Forest (CF), Apalachicola National Estuarine Research Reserve Site/National Forest (AP), Okefenokee National Wildlife Preserve (OF), Francis Marion National Forest (FM), and Green Swamp Nature

Preserve (GS). Bogs at Centre, Eller Seep, Prattville, and Reed Branch were visited, but no specimens were located.

following cycling profile: initial denaturing step of 94 °C for 5 min, 15 cycles of 94 °C for 45 s, 40 °C for 45 s, 72 °C for 60 s; 25 cycles of 94 °C for 45 s, 55 °C for 45 s, 72 °C for 60 s, and a final extension of 72 °C for 5 min. Five µl aliquots of PCR product from each reaction were electrophoresed in 1% agarose gels, stained using ethidium bromide, and viewed using shortwave (265 nm) UV to confirm PCR success. Prior to sequencing, amplification products were purified with MontageTM PCR Filter Units (Millipore) according to the manufacturer's specifications. Sequencing was conducted in both directions using Big-Dye Terminators v.3.1 and analyzed on a PRISM 3100 (Applied Biosystems, USA) at the Auburn University Genomics and Sequencing Laboratory or the University of Washington Genomics Unit. Forward and reverse sequence chromatograms were assembled and any ambiguities corrected using Sequencher v4.8 (Gene Codes Corporation, USA). All finished sequences were aligned manually using SE-AL version v2.0a11 (available at <http://tree.bio.ed.ac.uk/software/seal/>).

Data analysis

Genetic diversity, population structure and migration

Estimates of population genetic diversity were obtained using haplotype (h) and nucleotide (π) diversities calculated by Nei's (1987) method in the program DnaSP v5.10.01 (Rozas et al. 2003). Population structure was estimated using two approaches. First, the S_{nn} or 'nearest-neighbour' statistic (Hudson et al. 1992, Hudson 2000) was calculated as pairwise comparisons among sites, with 1,000 permutations to assess statistical significance, in DnaSP. Next, pairwise Φ_{ST} statistics based on haplotype

frequency and molecular divergence were generated using Arlequin v3.1 (Excoffier et al. 2005, Excoffier and Heckel 2006). To quantify the spatial distribution of genetic variation in *E. semicrocea*, analyses of molecular variance (AMOVAs, Excoffier et al. 1992) were conducted with Arlequin. Estimates of the relative contribution of molecular variance were assessed at three hierarchical levels using Φ -statistics: (i) between groups (Φ_{CT} , designated by the resulting population structure from pairwise Φ_{ST} and S_{nn} statistics); (ii) among populations within groups (Φ_{SC}); and (iii) within populations (Φ_{ST}). The significance of both the pairwise Φ_{ST} statistics and AMOVAs were assessed with 10,000 permutations. Lastly, Mantel (Mantel 1967) and 3-way (partial) Mantel (Smouse et al. 1986) tests were conducted with the Vegan package (Oksanen et al. 2010) in the R v2.6.2 statistical software environment (R Development Core Team 2008) with 10,000 permutations. A standard Mantel test was utilized to assess whether genetic and geographical distances are correlated. To determine if a potential transition zone among eastern populations influences the relationship between genetic and geographical distances, partial Mantel tests were conducted with an additional binary matrix defining populations as belonging to one of two groups (see Results). All of the above tests, when applicable, were conducted under Tamura and Nei's (1993) model of nucleotide evolution with rate variation among sites [TN + Γ] as selected by the Akaike Information Criterion (AIC) in ModelTest v3.6 (Posada and Crandall 1998).

To discriminate between the relative effects of ongoing migration ($M = 2N_e m$) versus recent divergence between pairs of *E. semicrocea* populations, a Markov Chain Monte Carlo (MCMC) based analytical method was utilized as implemented in MDIV (Nielsen and Wakeley 2001; available at <http://cbsuapps.tc.cornell.edu/>). Analyses were

conducted under the finite-site mutation model, which incorporates the possibility of multiple mutations per site, differences in nucleotide frequencies and the presence of transition/transversion bias. Three independent runs were conducted with the following conditions: $M_{max} = 50$, $T_{max} = 10$, length of Markov chain = 2×10^6 cycles, burn-in time = 5×10^5 cycles. Different random seeds were used in each run to check for consistency in the estimates.

Haplotype network, nested clade analysis and demographic analyses

Networks were generated to depict relationships among *E. semicrocea* haplotypes, based on the cladogram estimation algorithm of Templeton et al. (1992), with TCS v1.21 (Clement et al. 2000). The networks were constructed under the default setting (i.e., 95% parsimonious plausible branch connections). Reticulations in the networks, implying ambiguous connections between haplotypes, were resolved according to the suggestions of Crandall et al. (1994). Nested clade analysis (NCA; Templeton et al. 1995) was then conducted to separate population history from population structure (reviewed in Templeton 1998) in the program GeoDis v2.5 (Posada et al. 2000).

Haplotype nesting followed the methods of Templeton et al. (1987; 1995) and 5,000 permutations were employed in order to detect significant associations between particular clades within the nesting structure and their geographic locations. In situations when there were no interior clades (i.e., total cladogram), separate analyses were conducted switching each clade between the tip and interior positions. Measures of significant associations were assessed via contingency tables and these values were used to deduce

potential historical processes giving rise to current genetic patterns using the April 2009 NCA inference key (Posada and Templeton 2008; available at <http://darwin.uvigo.es/>).

Two approaches employing Fu's F_s (Fu 1997) and Tajima's D (Tajima 1989) were used to determine if patterns of genetic variation observed in the COI sequences were consistent with predictions under a neutrality model. While these tests are typically employed to detect selection (Fu 1997, Tajima 1989), they can also prove informative regarding the demographic history of a population (Akey et al. 2004). Both neutrality tests were conducted in Arlequin and significance ($P < 0.05$) assessed by 10,000 permutations.

Results

Genetic diversity of *Exyra semicrocea*

A total of 221 *Exyra semicrocea* were sampled from 11 localities across a range of ~1700 km. Overall, 51 (8.1%) nucleotide positions across the 630 bp COI fragment were variable and no stop codons were encountered. Nucleotide differences at the majority of these sites (50) represented 'silent' substitutions (i.e., would not change the encoded amino acid), with the remaining one being a non-synonymous substitution to an amino acid with similar biochemical properties (data not shown). These patterns suggest that all sequences were derived from mitochondrial copies of COI rather than nuclear copies of mitochondrial derived genes (numts; Buhay 2009, Lopez et al. 1994). From the 221 total individuals sampled, 54 unique haplotypes were identified with 34 occurring as singletons and the remaining 23 being identified more than once (See Appendix I). Sequences were deposited in GenBank under accession numbers HQ646110–HQ646163.

Table 1. Genetic diversity and tests of neutrality for *Exyra semicrocea* throughout the Southeast Coastal Plain.

Geographic region	Population	Genetic diversity				Neutrality tests	
		<i>n</i>	<i>nh</i>	π	<i>h</i>	Tajima's <i>D</i>	Fu's <i>F_s</i>
West Mississippi R.	AN	3	2	0.00317	0.66667	0.000	1.609
	BT	24	5	0.00189	0.67754	0.309	-0.432
	KN	24	6	0.00149	0.70290	-0.867	-2.162*
Total	3	51	13	0.00520	0.86510	0.158	-1.921
East Mississippi R.	AC	24	14	0.00411	0.89855	-1.548*	-7.981*
	GB	23	14	0.00305	0.91700	-2.002*	-10.786*
	WB	24	7	0.00167	0.55797	-1.957*	-3.265*
	CF	24	8	0.00434	0.89130	0.437	-7.981*
	AP	24	15	0.00400	0.91304	-1.465	-9.957*
	OF	21	5	0.00230	0.64286	0.125	-0.102
	FM	15	4	0.00160	0.71429	0.280	-0.064
	GS	15	3	0.00097	0.51429	-0.024	-0.064
Total	8	170	44	0.00457	0.89795	-1.632*	-26.374*

n, number of sampled individuals; *nh*, number of recovered haplotypes; π , nucleotide diversity; *h*, haplotype diversity

**P* < 0.05

Haplotype frequencies within populations varied from 2-15, with haplotype (h) and nucleotide (π) diversities ranging between 0.51-0.94 and 0.00097-0.00434, respectively (Table 1). Genetic distances between any two haplotypes ranged from 0 (0%) to 19 (3.0%) variable sites, with sequence divergence across the Mississippi River alluvial plain being between 1.9-3.0% ($\bar{x} = 2.5\%$). Arthropod COI mutation rates have been estimated at 2.3% sequence divergence per million years (Brower 1994), and more specifically, between 0.78-1.02% per million years for the family Papilionidae (Lepidoptera; Zakharov et al. 2004). Use of these rates would yield an estimate that the populations separated by the Mississippi River alluvial plain diverged within a range of 1.0-3.2 mya. Consistent with this estimate was the finding that no haplotypes were shared across the alluvial plain; based on this result all subsequent population genetic analyses treated the western and eastern populations of *E. semicrocea* separately.

Genetic differentiation, structure, and migration rates

Pairwise Φ_{ST} or S_{nn} statistics revealed significant genetic differentiation in 46 of 62 pairwise population comparisons of *E. semicrocea* (Tables 2 and 3). For example, all three populations west of the Mississippi River alluvial plain (KN, BT and AN: Fig 1) were significantly differentiated from one another for both estimates, with comparisons between BT and KN (~150 km) and BT and AN (~75 km) having S_{nn} values of 1.0 (Table 2). This implies a complete lack of haplotype exchange between these populations. Estimates of migration (M) via MDIV further support this, suggesting no female (since mtDNA is maternally inherited) migrants ($M = 0$) had been exchanged

Table 2. Measures of genetic differentiation for populations of *Exyra semicrocea* in Texas and western Louisiana as measured with S_{nn} (upper right triangle) and pairwise Φ_{ST} (lower triangle).

Populations	KN	BT	AN
KN	–	1.000***	0.920*
BT	0.800*	–	1.000***
AN	0.548*	0.694*	–

* $P < 0.05$; ** $P < 0.001$

Table 3. Measures of genetic differentiation for eastern populations of *Exyra semicrocea* as measured with S_{nn} (upper right triangle) and pairwise Φ_{ST} (lower triangle).

Populations	AC	GB	WB	CF	AP	OF	FM	GS
AC	–	0.565	0.555	0.663**	0.795***	0.795***	0.897***	0.900***
GB	0.012	–	0.527	0.643**	0.789***	0.830***	0.974***	0.974***
WB	0.012	0.008	–	0.796***	0.797***	0.830***	0.949***	0.944***
CF	0.052	0.100*	0.137*	–	0.661**	0.784***	0.858***	0.817***
AP	0.380*	0.470*	0.524*	0.242*	–	0.548	0.617*	0.543
OF	0.470*	0.575*	0.647*	0.344*	0.009	–	0.572	0.591*
FM	0.558*	0.661*	0.751*	0.434*	0.045	0.047	–	0.562*
GS	0.564*	0.674*	0.773*	0.430*	0.017	0.065	0.005	–

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

between the BT and KN populations in the recent past (data not shown). Analysis of molecular variance (AMOVA) found a significant proportion of genetic variation occurring between populations (i.e., BT vs. AN/KN; ~55%; $\Phi_{CT} = 0.552$; $P < 0.05$). Additionally, estimates for among populations within groups and within populations were significant ($P < 0.01$) and accounted for ~24% and ~21% of total genetic variation, respectively (Table 4).

Comparisons among the eastern populations found a lack of genetic structure among AC, GB, and WB for both the pairwise Φ_{ST} and S_{mn} statistics (Table 3). For AP, OF, FM, and GS, weak but significant structure was detected depending on the statistical estimate (Table 3). In contrast, both variance estimators placed CF distinct from all sites with the exception of AC (Table 3). Therefore, subsequent genetic analyses were conducted on the eastern population groups of AC/GB/WB, AP/OF/FM/GS and CF. In this context, a significant proportion of variation was partitioned within populations (~52%; $\Phi_{ST} = 0.523$; $P < 0.001$) and among groups (~47%; $\Phi_{CT} = 0.469$; $P < 0.05$), with a small (i.e., 0.75%; $\Phi_{SC} = 0.014$) but significant ($P < 0.001$) component among populations within groups (Table 4). Furthermore, a Mantel test identified an overall positive ($r = 0.542$) and significant ($P = 0.013$) correlation between genetic and geographical distances. Because of the strong biogeographic separation observed between the eastern and western populations I attempted, using a 3-way Mantel algorithm, to evaluate the relative contributions of isolation-by-distance versus historical isolation to the overall pattern. For this analysis, a third binary (1 = both populations in same group, 0 = different group) was created. The 3-way Mantel test detected a significant ($r = 0.899$, P

Table 4. Analysis of molecular variance (AMOVA) of *Exyra semicrocea* populations from across the southeastern United States.

Geographic region	Source of variation	df	Sum of squares	Variance component	% var.	Φ statistic
West Mississippi R.	Between groups	1	51.275	1.442	55.21	$\Phi_{CT} = 0.552^*$
	Among pops within groups	1	3.835	0.615	23.55	$\Phi_{SC} = 0.526^{**}$
	Within populations	48	26.619	0.555	21.24	$\Phi_{ST} = 0.788^{***}$
		50	81.728	2.611		
East Mississippi R.	Among groups	2	88.282	0.826	46.90	$\Phi_{CT} = 0.469^{**}$
	Among pops within groups	3	5.927	0.013	0.75	$\Phi_{SC} = 0.014^{***}$
	Within populations	162	149.409	0.922	52.35	$\Phi_{ST} = 0.477^{***}$
		169	243.663	1.762		

* = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$

= 0.024) correlation between genetic distance and the binary (the matrix defining the two groups with 3-4 populations, CF was excluded) but no significance correlation ($r = 0.122$, $P = 0.218$) between the geographic and genetic distance matrices. This result suggested that historical isolation of groups was more likely to be the primary agent responsible for the overall haplotype pattern observed than was pure geographic distance. These results provide further support for the existence of an abrupt transition between the two groups of eastern *E. semicrocea* approximately located in the western Florida panhandle/southern Alabama between WB and AP (Fig 1).

Due to the occurrence of CF within the proposed transition zone, pairwise estimates of migration (M) between all eastern populations were conducted relative to both AP and WB (i.e., the closest populations to CF). Population comparisons on either side of the potential transition zone (i.e., within groups) revealed plateaus in the distribution of posterior probabilities of M (Fig 2), suggestive of high levels of intraregional migration and consistent with the lack of genetic structure implied by the Φ_{ST} and S_{nn} statistics for these populations (see above). Conversely, clear peaks in the distribution of posterior probabilities for M were identified in population comparisons across the proposed transition zone (i.e., between groups; Fig 2). For example, population comparisons of WB relative to GS, FM, OF, and AP (i.e., across groups) produced estimates for M of 0.2, 0.2, 0.3, 0.5, respectively, with plateaus for OF, FM and GS (i.e., within groups; Fig. 2A). These results were inverted for population comparisons of AP (Fig. 2B), which was located on the opposite side of the proposed transition zone (Fig. 1). In both cases, CF remained an outlier with an estimated M approximately double that of

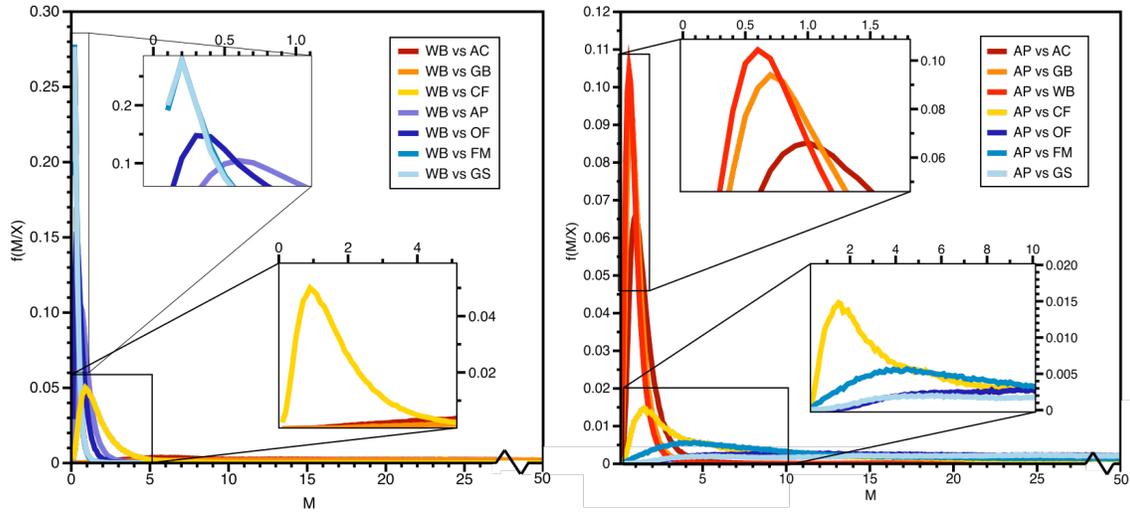


Figure 2. Posterior probability distributions of ongoing migration rate (M) for *Exyra semicrocea* populations. The distributions are an average of three independent runs utilizing identical starting conditions but different random seeds from the program MDIV (Nielsen and Wakeley 2001).

Table 5. Summary of nested clade analysis (NCA) using the inference key (Posada and Templeton 2008; available at <http://darwin.uvigo.es/>) for populations of *Exyra semicrocea* across the southeastern United States. Only those clades with significance ($P < 0.05$) based on 5,000 permutations are shown.

Geographic region	Clade	Inference chain	Inferred pattern
Western populations	2-1	1, 2, 11, 17, 4, No	RGF/IBD
	Total	1, 2, 3, 4, No	RGF/IBD
Eastern populations	1-6	1, 2, 3, 5, 6, 7, Yes	RGF/D w/LDD
	1-8	1, 2, 3, 5, 6, 7, 8, Yes	RGF/D w/LDD or PGF w/E
	2-2	1, 2, 3, 4, No	RGF/IBD
	Total	1, 2, 3, 5, 6, 15, 21, No	PREF or PLRE

PGF w/E, past gene flow followed by extinction of intermediate population; PLRE, past larger range followed by extinction in intermediate areas; PREF, past gradual range expansion followed by fragmentation; RGF/IBD, restricted gene flow with isolation by distance; RGF/D w/ LDD, restricted gene flow/dispersal but with some long distance dispersal over intermediate areas not occupied by the species.

population comparisons across the proposed transition zone (i.e., between groups; Fig. 2). Taken together, these estimates of M again imply a distinct shift in the level of migration in relation to the proposed transition zone, a patterns consistent with the 3-way Mantel results.

Haplotype networks and NCA

The Nested Clade Analysis (NCA) of the three western populations of *E. semicrocea* contained five 1-step clades, two 2-step clades and the total cladogram (Fig. 3). Only clade 2-1 and the total cladogram (i.e., 3-1) were significant (Table 5 and Appendix II), with both analyses of the total cladogram (i.e., alternating of clade 2-1 and clade 2-2 between tip and interior) yielding similar results. For these clades (i.e., 2-1 and 3-1), restricted gene flow with isolation by distance (Table 5) were inferred as probable drivers of the observed patterns, which are further supported by the clustering of haplotypes by locality (Fig. 3), significant genetic differentiation in both pairwise Φ_{ST} and S_{nn} statistics, and an absence of migration among these populations (see above).

The NCA for eastern populations contained eight 1-step clades, two 2-step clades and the total cladogram (Fig. 4) with significance for three lower level clades (i.e., clade 1-6, 1-8 and 2-2) and the total cladogram (Table 5 and Appendix III). For the three lower level clades, restricted gene flow/dispersal with isolation by distance and long distance dispersal were outcomes of the inference (Table 5) and the presence of several haplotypes (i.e., XXIX, XXXV, XXXIII, XXXVIII, XXXI) at low frequencies in populations west of the proposed transition zone (Fig. 4) were parsimonious with this conclusion. Both

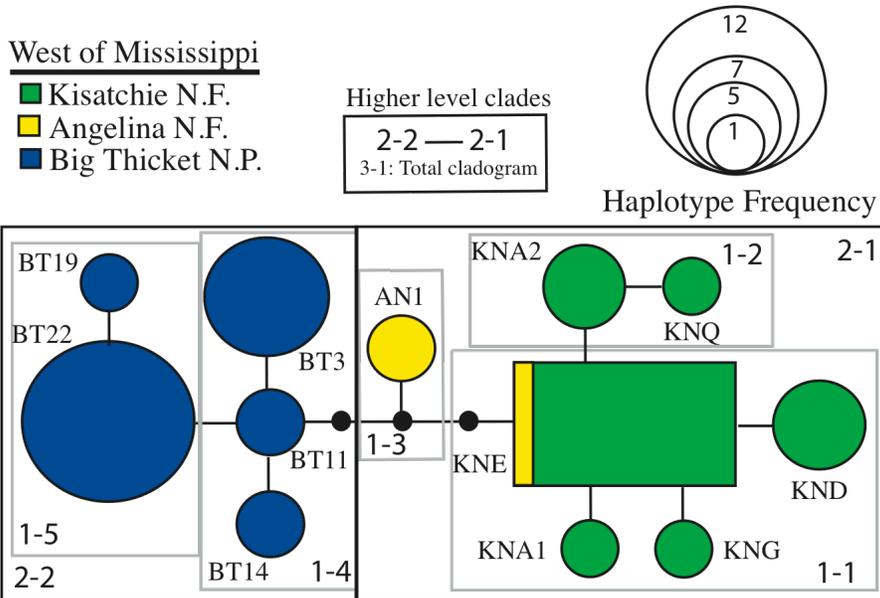


Figure 3. Haplotype network depicting the nested levels used to infer historical processes in western populations *Exyra semicrocea* across the southeastern Coastal Plain. Each circle represents a unique haplotype with size of the circle corresponding to haplotype frequency (see Appendix I for exact haplotype frequencies). The rectangle is the parsimonious ancestral haplotype or the haplotype with the highest outgroup probability according to TCS. Each branch represents one mutational difference between haplotypes with black circles representing missing or unsampled haplotypes. Colors correspond to location as designated in the legend.

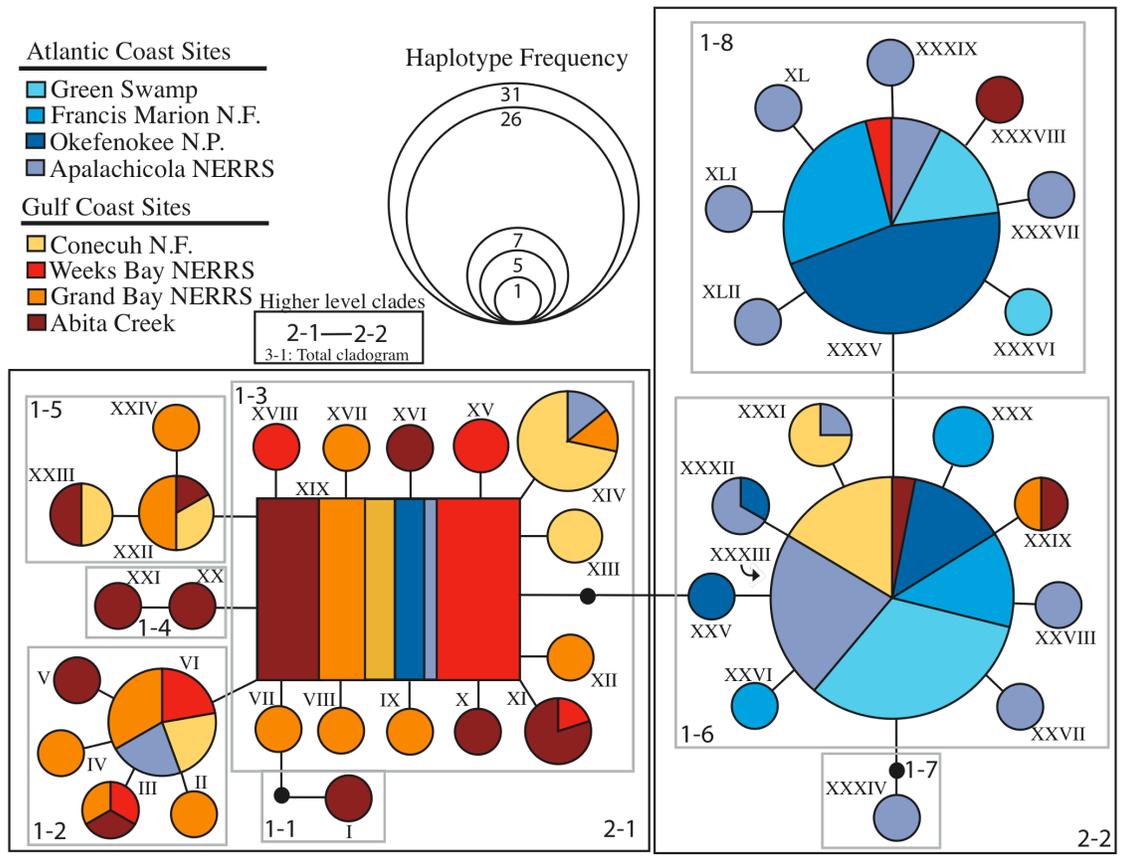


Figure 4. Haplotype network depicting the nested levels used to infer historical processes in eastern populations of *Exyra semicrocea* across the southeastern Coastal Plain. Each circle represents a unique haplotype with size of the circle corresponding to haplotype frequency (see Appendix for exact haplotype frequencies). The rectangle is the parsimonious ancestral haplotype or the haplotype with the highest outgroup probability according to TCS (Clement et al. 2000). Each branch represents one mutational difference between haplotypes with black circles representing missing or unsampled haplotypes. Colors correspond to location as designated in the legend.

analyses of the total cladogram (i.e., alternating of clade 2-1 and clade 2-2 between tip and interior) inferred either past gradual range expansion followed by fragmentation or a past larger range subsequently reduced by extinction in intermediate areas as the most likely explanations for current patterns. I hypothesize that the second possibility, namely a larger past range reduced by extinction in intermediate areas is the more plausible scenario. This conclusion based on recent observations of limited flight abilities (unpublished data) and western population structure (see above) of *E. semicrocea* which collectively suggest that long distance movements indicative of gradual range expansion followed by fragmentation are probably rare for this species.

Demography of *Exyra semicrocea*

Populations west of the Mississippi River alluvial plain had both positive and negative values for Tajima's D and Fu's F_S , with KN being the only population with a significant negative F_S value (Table 1). In contrast, five of eight populations east of the Mississippi River possessed significant negative F_S values, with three (i.e., AC, GB, WB) also significant for Tajima's D (Table 1). Significant negative values indicate an excess of rare polymorphisms within each of these populations and implied either positive selection or recent population expansion (Tajima 1989; Akey et al. 2004). Due to the near total absence of non-synonymous substitutions in the analyzed COI fragment (see above), it is surmised that recent population expansion is the most likely explanation for these values. Taken together, populations of *E. semicrocea* in the eastern portion of its range as a relative whole are undergoing expansion compared to those west of the Mississippi River (Table 1).

Discussion

West Gulf Coastal Plain and Mississippi River alluvial plain

Populations of *Exyra semicrocea* in the West Gulf Coastal Plain (WGCP, USGS designation) possess appreciable divergence from, and a clear break between, eastern populations across the Mississippi River alluvial plain. The identification of the Mississippi River alluvial plain as a significant barrier has been documented for a number of species (Al-Rabab'ah and Williams 2002, Brant and Ortí 2003, Burbrink et al. 2000, Leache and Reeder 2002), including other members of the longleaf ecosystem, such as *Pinus palustris* (longleaf pine), *Dichromona colorata* (white topped sedge), *Drosera capillaris* (pink sundew), *Rhexia alifanus* (a meadow beauty), *Utricularia* sp. (bladderworts) (Thomas and Allen 1993). Notably, an identical break is present in the host plant of *E. semicrocea* in this region (*Sarracenia alata*: Koopman and Carstens 2010) and results from an ~200 km wide swath of unsuitable soils and alluvial swamps that replace bog habitat (Sheridan 1991). Given that *S. alata* is monotypic throughout most of its range (MacRoberts and MacRoberts 1991) and therefore is the only host plant for populations of *E. semicrocea* in Texas and Louisiana, it is not surprising that this barrier has influenced a similar pattern in both species. Interestingly, the genetic structure of microbial communities found within the pitchers of *S. alata* also mirrors that of the plant (Koopman and Carstens in review) and, by extension, that of *E. semicrocea* (this study). Taken together, these patterns highlight the biological uniqueness and intimate associations among constituents of pitcher plant bog habitats in the WGCP.

In the west, the significant genetic structure among geographically close populations of *E. semicrocea* contrasts with an absence of genetic structure among similarly (or more widely) distanced populations in the east (Fig. 1). One explanation for this may be a lack of historical connectivity between patches of bogs in the west. While little is known concerning the history of pitcher plant bogs in this region, it is estimated that bogs of the WGCP may have occupied ~4,000-8,000 ha prior to European settlement (MacRoberts and MacRoberts 2001) and been distributed among sites in as many as 20 counties (e.g., 4 parishes in Louisiana, 16 counties in Texas; Sheridan 1991). However, areas that currently harbor locally abundant pitcher plant bogs are just two parishes in Louisiana (which encompasses KN) and four counties in Texas (which encompasses AN and BT). Unfortunately while historical records of small and/or rare populations can provide useful information for establishing past distributions, they may also artificially extend “known” ranges due in part to the reporting of erroneous localities, producing a false picture of historical abundance in the process (Miller et al. 2007). Such a situation has been reported for pitcher plant records in Texas (Sheridan 1991) and if this is the case, pitcher plant bogs in the WGCP might have been as patchy and historically isolated as they are today. Along with this, it is well known that floristic composition differs between eastern and western portions of the Coastal Plain (Bridges and Orzell 1989, Folkerts 1982, Folkerts 1991, Peet and Allard 1993) with these differences typically attributed to variations in soil (Folkerts 1982) and levels of rainfall (Bridges and Orzell 1989). Additionally, many western bogs are supported by spring water (Bridges and Orzell 1989) rather than rainfall, the common source for their eastern counterparts. Such environmental differences may have contributed to habitat patchiness and the paucity of

large, expansive bogs in the west relative to the east and are potential contributors to the local and significant population structure of *E. semicrocea* that likely extends to other obligate pitcher plant bog organisms.

Another explanation for the significant structure among western *E. semicrocea* populations is a possible difference in dispersal behavior resulting from genetic differentiation. Specifically, the COI sequence divergence between *E. semicrocea* from the WGCP and eastern Coastal Plain ranges from 1.9-3.0% and while sequence divergence values for Lepidoptera congeneric species pairs are generally greater than 3.0% (Hebert et al. 2003), values for Lepidoptera species known to be morphologically different can be lower than 3.0% (Brower and Egan 1997, Flanagan et al. 2004, Hebert et al. 2003, Hebert et al. 2004, Whinnett et al. 2005). The amount of difference seen in the COI gene indicates a long period of isolation between western and eastern *E. semicrocea* populations during which it is conceivable that behavioral differences may have evolved. If so, further investigations into potential morphological and behavioral differences between the eastern and western lineages of *E. semicrocea* are warranted since such information has important implications in future conservation efforts (see below).

Eastern population structure and Apalachicola-Chattahoochee-Flint River (ACF) break

Populations of *E. semicrocea* east of the Mississippi River alluvial plain are genetically structured into three distinct groups. These groups are situated within the East Gulf Coastal Plain (EGCP, USGS designation, encompassing AC/GB/WB), the South Atlantic Coastal Plain (SACP, USGS designation, encompassing AP/OF/FM/GS), and Conecuh National Forest (CF) in the proposed transition zone between the groups

(although it should be noted that CF is officially within the East Gulf Coastal Plain USGS designation, Fig 1). Located within the proposed transition zone is the Apalachicola-Chattahoochee-Flint (ACF) River basin, a well-documented area for genetic discontinuity for a number of vertebrates (Avisé et al. 1979, Blaney 1971; Means 1977, Conant and Collins 1998, Swenson and Howard 2005, Swift et al. 1986), invertebrates (Engle and Summers 2000), and plant (Soltis et al. 2006) species. Various hypotheses on why the ACF is an area of genetic discontinuity have been proposed. One hypothesis argues that the Apalachicola River and its resulting floodplain is one of the few systems completely bisecting the eastern Coastal Plain therefore it is likely to have served as a potential historical and contemporary physical barrier (Avisé 2000, Pauly et al. 2007). In addition, this system is responsible for draining the Appalachian Mountain massif, creating distinct soil differences on the resulting sides of the ACF (Folkerts 1982). Along with this, areas of the Coastal Plain were consistently inundated by the Gulf of Mexico during the late Pliocene and Pleistocene interglacial periods and it is thought that while these sea level increases did not completely cover the entire Coastal Plain, range fragmentations and occurred due to coastal advances (James 1961) and the inability of endemic taxa to shift their ranges further north (Pauly et al. 2007). Under this scenario refugia were likely to have been created both west and east of the ACF for these taxa during interglacial periods in the Pleistocene (Sorrie and Weakley 2001).

While an overall pattern of discontinuity at the ACF basin is well established, it is generally not congruent across taxa. In this study, *E. semicrocea* is structured across the ACF, but with CF as an outlier possessing haplotypes commonly found both west and east of the ACF basin (Fig. 4). In addition, migration estimates for CF undergo a distinct

transition in this area, suggesting the influence of a potential barrier. However, the exact mechanism(s) contributing to these trends in *E. semicrocea* are difficult to identify since the region between the Mississippi and Apalachicola Rivers has a history of variable phylogeographic patterns across many species (Soltis et al. 2005). For example, numerous studies of southeastern Coastal Plain fishes (Avice 1996, Bermingham and Avice 1986, Swift et al. 1986) and other vertebrate taxa (Avice 1996, Soltis et al. 2005), highlight variable genetic structure and patterns among taxa due to situations like potential, but numerous interglacial refugia leading to divergence in isolation. Thus, future phylogeographic studies of additional taxa from this region provide insight and further resolve the unusual nature of this area.

With the exception of CF, the other EGCP populations (i.e., AC, GB, WB) of *E. semicrocea* exhibit demographic expansion but no genetic structure among them, even though the distances between populations are similar to those west of the Mississippi River (Fig. 1). I hypothesize that these differences are most likely due to possessing much more contiguous bog habitat both historically and contemporarily across the EGCP. Specifically, early writings imply a large expanse of bog possibly stretching from Pensacola, Florida, west to Pascagoula, Mississippi (a span of 130 km) before the late 1800s (Bartram 1791, Harper 1918). Support for this comes from habitat suitability assessments and soil surveys of the EGCP indicating pitcher plant bogs might have occupied 293,500 ha across the region in pre-Columbian times (Folkerts 1982). In contrast to the EGCP, populations of *E. semicrocea* in the SACP (i.e., AP, OF, FM, GS) in some cases exhibited weak, but significant, genetic structure (Table 3). One potential driver for this pattern might again be historic habitat patchiness. For example, sites such

as OF and FM have minimal pitcher plant bog habitat due to large swaths of old swampy areas. Furthermore, in the northern portions of the SACP (i.e., South and North Carolina), regionally specific “bog-like” habitats known as pocosins and Carolina Bays (Sharitz and Gresham 1998) either exclude (i.e., pocosin) or create elliptical, irregular pitcher plant habitats (i.e., Carolina Bays; Folkerts 1991, Porcher 1966). These alternative habitat types as well as the effects of glacial advances and retreats over time in this region (Christensen 2000) may contributed to the generation of pitcher plant bogs with patchy distributions and initiated the process of differentiation among *E. semicrocea* populations in the SACP.

Conservation Implications

As mentioned previously, pitcher plant bogs of the southeastern United States Coastal Plain possess the most diverse assemblages of carnivorous plants in the world (Folkerts 1999) while also belonging to one of the most critically endangered ecosystems in North America (Noss et al. 1995). Thus, it is imperative to further develop sound management practices for maintaining the health and integrity of those habitats that remain. Established techniques towards this end are prescribed burns implemented by state and US Federal agencies. In contrast to the historical and natural burning that occurred every one to ten years during the growing season (Chapman 1932, Christensen 1981, Mattoon 1922), these regular, controlled burns are conducted in the winter (i.e., non-growing season). While it is assumed that the endemic members of this community, such as *E. semicrocea*, are most likely fire adapted (Rymal 1980), it remains unclear how these unnatural winter burns may impact the overall composition of pitcher plant bog

communities. However, detection of high mortality among larvae with even a low intensity, patchy fire and further observations of *E. semicrocea* overwintering only as 4th or 5th instar larva (Rymal 1980) suggest winter burns are potentially detrimental to local populations. For example, *E. semicrocea* was not found at four sites (i.e., Centre, AL; Prattville, AL; Reed Branch, GA and Eller Seep, NC) visited for this study in spite of previously being reported as occurring at these locations (pers. comm. D. Folkerts and M. Hodges). I propose that previous winter burns conducted at these four sites in conjunction with isolation and fragmentation may have contributed to the potential extirpation of their *E. semicrocea* populations. This possibility highlights the need for additional research specifically addressing the effects of fire on other constituents of pitcher plant bog communities and in the interim and until more information is gathered, it may be prudent to maintain unburned habitat during prescribed fire events (preferably during the growing season) as refugia for community members susceptible to such management practices.

The identification of *E. semicrocea* as encompassing six distinct population groups within two divergent lineages (i.e., western and eastern) should also be considered when developing management and conservation strategies. For example, while the taxonomic status of the two *E. semicrocea* lineages requires further examination (see above), they could minimally be considered Evolutionary Significant Units (ESUs) of the species. The recognition of ESUs, defined as one or a set of populations with a distinct evolutionary heritage (Ryder 1986), can aid managers in prioritizing sites and activities related to conservation (Moritz 1999, Moritz 2002). In this context, the three populations of *E. semicrocea* west of the Mississippi River (KN, AN, BT) are also distinctive due to their strong genetic structure and this is further supported by the comparative

phylogeographies of *E. semicrocea*, *S. alata*, and the microbial community within the pitchers representing unique and coevolving units in the WGCP (see above). Thus, steps should be taken to maintain the distinct genetic diversity at each of these sites. Similarly, the three population groups (i.e., EGCP, CF, SACP) of *E. semicrocea* in the eastern portion of its range deserve comparable attention. This region of the southeastern Coastal Plain has the highest biodiversity in the United States (Keddy 2009) and therefore future protection and/or restoration of this area has further implications for not only pitcher plant bogs and *E. semicrocea*, but also other taxa (Jones and Dorr 2004, Walters et al. 1988) and unique habitats (Holt et al. in press, Estill and Cruzan 2001, Platt and Schwartz 1990) as well.

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Appendix I

Distribution of *Exyra semicrocea* haplotypes at sampling localities across the southeastern Coastal Plain of the United States.

Haplotype Name	GenBank Accession	Localities											Individuals/haplotypes
		BT	AN	KN	AC	GB	WB	CF	AP	OF	FM	GS	
AC10/XXI	HQ646110				1								1
AC14/XVI	HQ646111				1								1
AC16/XX	HQ646112				1								1
AC18/XI	HQ646113				4		1						5
AC21/XXIX	HQ646114				1	1							2
AC24/I	HQ646115				2			2					4
AC26/XXIII	HQ646116				1								1
AC28/V	HQ646117				1								1
AC3/X	HQ646118				1								1
AC30/XXXVIII	HQ646119				1								1
AC7/XIX	HQ646120				7	6	16	3	1	3			36
AC8/XXII	HQ646121				1	3		2					6
AN1	HQ646122		2										2
AP3/XXXV	HQ646123						1		2	12	7	4	26
APA/XXVII	HQ646124								1				1
APC1/XXXIX	HQ646125								1				1
APF2/XL	HQ646126								1				1
API3/XLI	HQ646127								1				1
APN/XXVIII	HQ646128								1				1
APOXLII	HQ646129								1				1
APP1/XXXIV	HQ646130								1				1
APR1/XXXVII	HQ646131								1				1
BT11	HQ646132	2											2
BT14	HQ646133	2											2
BT19	HQ646134	1											1
BT22	HQ646135	12											12
BT3	HQ646136	7											7
CFF1/XIV	HQ646137					1		5	1				7
CFF6/XXXIII	HQ646138				1			5	7	4	4	10	31
CFL28/VI	HQ646139					3	2	2	2				9
CFL37/XXXI	HQ646140							3	1				4
CFL9/XIII	HQ646141							2					2
FM10/XXX	HQ646142										3		3
FM4/XXVI	HQ646143										1		1
GBA2/XXIV	HQ646144					1							1
GBC/IV	HQ646145					1							1
GBF/VIII	HQ646146					1							1
GBI/II	HQ646147					1							1
GBJ1/IX	HQ646148					1							1
GBN/VII	HQ646149					1							1
GBO1/XII	HQ646150					1							1
GBO2/XVII	HQ646151					1							1
GS2/XXXVI	HQ646152											1	1
KNA1	HQ646153			1									1
KNA2	HQ646154			4									4
KND	HQ646155			5									5
KNE	HQ646156		1	12									13
KNG	HQ646157			1									1

Haplotype Name	GenBank Accession	Localities											Individuals/ haplotypes
		BT	AN	KN	AC	GB	WB	CF	AP	OF	FM	GS	
KNQ	HQ646158			1									1
OF17/XXV	HQ646159									1			1
OF19/XXXII	HQ646160								2	1			3
WB3/XV	HQ646161						2						2
WB4/III	HQ646162				1	1	1						3
WB5/XVIII	HQ646163						1						1
Individuals/locations		24	3	24	24	23	24	24	24	21	15	15	

Appendix II

Nested Clade Analysis (NCA) flow chart of *Exyra semicrocea* COI haplotypes from the western populations of the southeastern Coastal Plain based on 5000 permutations in GeoDis.

0-step clades			1-step clades			2-step clades		
Name	D_C	D_N	Name	D_C	D_N	Name	D_C	D_N
KNE	61.73	59.06						
KNA1	0	38.11						
KNG	0	38.11						
KND	0	38.11	1-1	53.63	64.30			
<i>I-T</i>	61.73	20.95						
KNA2	0	0						
KNQ	0		1-2	0	64.31			
AN1	0	0	1-3	0	64.29S	2-1	64.30	67.85
			<i>I-T</i>	-42.90	-0.01S			
			1, 2, 11, 17, 4, No: RGF/IBD					
BT11	0	0						
BT3	0	0						
BT14	0	0	1-4	0	0			
BT22	0	0						
BT19	0	0	1-5	0	0	2-2	0S	65.52
						<i>I-T</i>	-64.30S	-2.33
						1, 2, 3, 4, No: RGF/IBD		
						*2-1	64.30	67.85
						2-2	0S	65.52
						<i>I-T</i>	64.30L	2.33
						1, 2, 3, 4, No: RGF/IBD		

Distances are given for both within (D_C) and nested clades (D_N). An 'L' indicates the distance is significantly large, while an 'S' indicates the distance is significantly small, at the 5% ($P < 0.05$) level. '*I-T*' indicates the average distance between tip and interior clades and shaded regions indicate interior groupings. *Analysis of total cladogram (i.e., 3-1) was conducted alternating between tip and interior (see methods).

Appendix III

Nested Clade Analysis (NCA) flow chart of *Exyra semicrocea* COI haplotypes from the eastern populations of the southeastern Coastal Plain based on 5000 permutation in GeoDis.

0-step clades			1-step clades			2-step clades		
Name	D_C	D_N	Name	D_C	D_N	Name	D_C	D_N
I	0	0	1-1	0	198.42			
II	0	58.31						
III	78.18	90.75						
IV	0	58.31						
V	0	207.60						
VI	122.51	112.41	1-2	106.93	106.32			
<i>I-T</i>	83.42	13.00						
VII	0	61.80						
VIII	0	61.80						
IX	0	61.80						
X	0	210.29						
XI	65.95	171.49						
XII	0	61.80						
XIII	0	133.56						
XIV	71.14	142.78						
XV	0	16.32						
XVI	0	210.29						
XVII	0	61.80						
XVIII	0	16.32						
XIX	131.58	126.40	1-3	124.03	122.34			
<i>I-T</i>	92.03	1.14						
XX	0	0						
XXI	0	0	1-4	0	198.42			
XXII	101.43	99.63						
XXIII	168.09	168.63						
XXIV	0	35.67	1-5	118.35	123.64	2-1	122.32S	335.83S
<i>I-T</i>	-33.04	-42.41	<i>I-T</i>	23.83	-0.07			
XXV	0	149.95						
XXVI	0	259.62						
XXVII	0	335.19						
XXIX	74.67	693.49L						
XXX	0	259.62						
XXXI	69.85	411.88						
XXXII	130.79	280.56						
XXXIII	350.22	362.07	1-6	357.10L	355.90L			
<i>I-T</i>	284.54L	-19.56						
1, 2, 3, 5, 6, 7, Yes: RGF/D w/ LDD								
XXXIV	0	0	1-7	0	364.22			
XXXV	238.99S	243.72S						
XXXVI	0	389.39						
XXXVII	0	387.35						

Appendix III *Continued*

0-step clades			1-step clades			2-step clades		
Name	D_C	D_N	Name	D_C	D_N	Name	D_C	D_N
XXXVIII	0	815.06						
XXXIX	0	387.35						
XL	0	387.35						
XLI	0	387.35						
XLII	0	387.35	1-8	280.05	284.38L	2-2	326.48	397.07L
<i>I-T</i>	238.99S	-205.02S	<i>I-T</i>	85.29L	69.18L	<i>I-T</i>	204.16L	61.24L
1, 2, 3, 5, 6, 7, 8, Yes: RGF/D w/LDD or PGF w/E			1, 2, 3, 4, No: RGF/IBD			1, 2, 3, 5, 6, 15, 21, No: PREF or PLRE		
						*2-1	122.32S	335.83S
						2-2	326.48	397.07L
						<i>I-T</i>	-204.16S	-61.24S
						1, 2, 3, 5, 6, 15, 21, No: PREF or PLRE		

Distances are given for both within (D_C) and nested clades (D_N). An 'L' indicates the distance is significantly large, while an 'S' indicates the distance is significantly small, at the 5% ($P < 0.05$) level. '*I-T*' indicates the average distance between tip and interior clades and shaded regions indicate interior groupings. *Analysis of total cladogram (i.e., 3-1) was conducted alternating between tip and interior (see methods).