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The Evolution And Reproductive Ecology Of Oenothera (Onagraceae)

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THE EVOLUTION AND REPRODUCTIVE ECOLOGY OF *OENOTHERA* (ONAGRACEAE)

By

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ABSTRACT OF THE DISSERTATION

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This dissertation describes the role of pollination in the floral diversification of *Oenothera* with an integration of both ecological and phylogenetic approaches. *Oenothera* (Onagraceae) is a model system for studying plant reproductive biology. It provides excellent examples of shifts in reproductive traits such as pollination and breeding system, features that have been important in angiosperm diversification. These systems are evolutionarily labile; they easily shift between different states. These different reproductive traits may shift in a concerted fashion; therefore, a more comprehensive approach to understanding the evolution of these plant systems simultaneously addresses shifts in pollination and breeding system. Using 54 species of *Oenothera*, I first collected detailed data describing the pollination systems, breeding systems, and floral traits associated with pollinator rewards; and second I determined the

phylogenetic structure, evolutionary history and relationships among these species. Finally, in that phylogenetic context, I examined the timing and position of transitions in reproductive traits and consider how these traits are associated with pollination and breeding systems.

My results offer new insights regarding the specialization of pollination systems and the predictive power of pollination syndromes. I find that specialization in pollination is not accurately characterized by visitation rates alone, and that considering functional groups of visitors to the flowers provides the most informative characterization of pollination systems. I also find that pollination syndromes do not sufficiently or accurately describe these pollination systems. My results also clarify phylogenetic relationships in the genus *Oenothera*, determine that there have been 13 independent transitions to self-compatibity, and provide the first phylogenetic tree for subsection *Kneiffia*. I find that pollination and breeding system do not correlate consistently with floral traits, and do not show an association with each other. Finally, I find that the transitions in the reproductive traits reveal a complex and diverse pattern in which shifts in floral traits occur prior and post a transition in pollination system. I also document an example of a rare transition from a generalized pollination system to a specialized pollination system. The placement of floral trait transitions with regards to pollinator shifts suggests selective pressures in floral traits that are predictable and follow transitions to novel dominant pollinator groups, rather than changes in pollination system.

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CHAPTER 1

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INTRODUCTION

Plant-pollinator interactions have long been used to examine broad themes of co-evolution and diversification. The field of pollination biology has moved from descriptive natural history to a hypothesis-driven field that helps explain the early radiation of angiosperms (Vamosi and Vamosi, 2010). Traditionally, there have been phylogenetic studies that examine patterns of pollinator- mediated angiosperm evolution, and ecological studies that focus on plant-pollinator interactions at a species or community level. Studies that combine the ecological and evolutionary approaches offer a better understanding of plant-pollinator interactions (Mitchell et al., 2009).

Two major evolutionary transitions in plant reproductive systems are in breeding system, the evolution of selfing from outcrossing, and the evolution of animal pollination (Barrett, 2010a). Pollination and breeding system, which are clearly correlated, have been important in angiosperm evolution, yet they are too often addressed separately (Fenster and Marten-Rodriguez, 2007). These systems are evolutionarily labile, easily shifting between different states. The different reproductive traits may shift in a concerted fashion; therefore a more comprehensive approach simultaneously addresses shifts in pollination and breeding system.

My objectives are to examine the role of shifts in reproductive biology in the evolution of *Oenothera* (Onagraceae). I use detailed plant-pollinator data to accurately define the degree of specialization of the pollination system. I develop a phylogenetic context and identify the placement and directionality of shifts in reproductive traits, and in doing so, assess the timing and pattern of floral trait evolution for this group. My results have broad implications for how plant-pollinator interactions are measured and

interpreted, especially with regards to the appropriate use of pollination syndromes and pollinator functional groups in studies of floral evolution.

Onagraceae, the evening primrose family, has long served as a model system for analyzing the role of reproductive biology in the evolutionary history of flowering plants. The genus *Oenothera* is widespread across western North America with some taxa extending to central Mexico and South America (Wagner et al., 2007). Recent phylogenetic analyses (Hoggard et al., 2004; Levin et al., 2004; Levin et al., 2003) resulted in a dramatic clarification of the relationships within *Oenothera*. Specifically, the formerly recognized genera *Gaura, Calylophus*, and *Stenisiphon* are now understood to be best viewed as elements within a more comprehensive but still monophyletic *Oenothera* (Wagner et al., 2007). The 45 taxa in subsections *Gauropsis, Hartmannia, Xanthocoryne, Leucocoryne, Kneiffia, Megapterium, Peniophyllum, Paradoxus* and *Gaura* encompass a broad array of floral form, including a transition from yellow, actinomorphic flowers to white, zygomorphic flowers. These *Oenothera* taxa have a diversity of pollination and breeding systems, and these systems have had repeated shifts in character state.

This dissertation has five chapters that present new data and analyses. Chapters 2 through 5 provide data that are ultimately united in the broader analyses of Chapter 6; however, each chapter addresses unique questions. Each chapter contains an introduction to the topic on which it focuses, as well as separate figures, tables, and literature-cited sections. Chapter 2 examines the question of generalization and specialization in pollination systems, and provides detailed descriptions of the pollination ecology for the *Oenothera* species considered. Chapter 3 addresses the use of pollination syndromes as a

predictive tool and how this concept relates to pollination ecology. Chapter 4 describes the phylogenetic relationships of these *Oenothera* taxa and the evolution of breeding system in this clade. Chapter 5 focuses on subsection *Kneiffia*, providing the first phylogeny based on molecular data for these taxa, and describing their reproductive biology. Together, Chapters 4 and 5 define the breeding system for these *Oenothera* taxa. Finally, Chapter 6 examines the transitions in *Oenothera* reproductive ecology in a phylogenetic context. I identify correlations and transitions in the breeding system, pollination system, and floral traits, and discuss key transitions in the evolution of the reproductive biology of these *Oenothera* taxa.

The use of *Oenothera* for a broad comparative study relies on detailed ecological data being placed in a phylogenetic context to examine floral evolution. This study would not have been possible if not for the decades of work already conducted on the reproductive biology of Onagraceae. The intensive studies of this family performed in the sixties by Peter Raven and David Gregory helped to establish Onagraceae as a model family for research in plant reproductive biology. It is upon that foundation that this dissertation builds.

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CHAPTER 2

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GENERALIST VS. SPECIALIST POLLINATION SYSTEMS IN *OENOTHERA* (ONAGRACEAE)

Introduction

The rapid rise of the angiosperms in the early Cretaceous is traditionally explained by the co-evolution of plants with their insect pollinators (Crane et al., 1995; De Bodt et al., 2005; Grimaldi, 1999; Solds et al., 2008; Soltis et al., 2008). Because plant-pollinator interactions have played such an important role in the evolution and ecology of plant species, defining these interactions has a long history dating back to Darwin (Darwin, 1862) and his study of orchids. Early studies focused on the tightly coupled relationships of a plant and its pollinator (Faegri and Pijl, 1966; Grant and Grant, 1965; Stebbins, 1970) and depicted these interactions as highly specialized, meaning that a given plant species relied on a small number of pollinator species. Beginning in the 1990's, pollination biology research expanded rapidly, challenging these traditional ideas and debating the specialization of pollination systems (Bascompte et al., 2003; Fenster et al., 2004; Johnson and Steiner, 2000; Mitchell et al., 2009; Ollerton, 1996; Sahli and Conner, 2006; Tripp and Manos, 2008; Waser et al., 1996).

Although generalists and specialists are often discussed as alternative states, the biological reality may be better viewed as a continuum of generalization to specialization (Johnson and Steiner, 2000). A major impediment to understanding the apparent paradox of specialized plants with generalized pollination systems is the lack of a standardized method for measuring pollination system specialization (Ne'eman et al.). Traditionally, one counted the number of pollinator taxa visiting a plant species (Waser et al., 1996). This method may be misleading in the case of a "generalist" plant species that is visited by multiple pollinator species if all of the pollinators belong to a functional group defined by a single morphology or foraging behavior. The use of pollinator functional groups,

which are defined as multiple taxa that share features (such as body size or tongue length) that determine their functionality as pollinators, provides a more accurate characterization of a plant's pollination biology (Fenster et al., 2004) and can drastically alter the perceived degree of specialization. For instance, Waser (1996) analyzed Robertson's (1928) pollinator survey and reported that 91% of 375 native plants in Illinois were visited by more than one insect species and therefore were generalist. Reanalysis of the same data indicated that when the insects were grouped into functional groups, 75% of the flowering plants only used one pollinator type and could therefore be considered specialized by that criterion (Fenster et al., 2004).

Calculating the degree of pollination specialization based solely on visitation, meaning the animals that land on the plant, can also be misleading because not all plant visitors are pollinators. A plant may be visited by dozens of potential pollinators, but critical pollen transfer may be accomplished by a single pollinator. In addition, a frequent visitor may carry a small pollen load, while a less frequent visitor may carry a large pollen load (Mayfield, Waser and Price 2001). In *Oenothera cinerea,* when both visitation and pollen load were examined, Clinebell et al. (2004) found a high degree of specialization to a few major pollinators: of 45 species of floral visitor, only 5 carried major pollen loads, and 32 carried little or no pollen. However, few studies evaluate pollination based on both visitation and pollen load, and failure to account for pollen load can lead to inaccurate assumptions regarding the number of pollinators with which a plant species actually interacts.

In addition, many angiosperm traits, including pollination system, are shared due to common ancestry, and results from comparative studies can be biased by phylogenetic

constraint and niche conservatism (Sanderson and Donoghue 1996, Sakai et al. 1997, Freckleton 2000, Vamosi et al. 2003, Machado and Lopes 2004). A well-resolved phylogeny can provide a framework for comparing pollination systems while controlling for shared evolutionary history (Nosil and Mooers, 2005).

Onagraceae, specifically the genus *Oenothera*, has long served as a model system for the evolution of flowering plant reproductive biology (Clinebell et al., 2004; Hoch et al., 1993; Raven, 1979; Raven, 1988). The diversity of pollination systems within *Oenothera* make it ideal for testing hypotheses of pollination system specialization. Recent molecular phylogenetic studies have clarified phylogenetic relationships within *Oenothera* (Hoggard et al., 2004; Levin et al., 2004; Levin et al., 2003; Wagner et al., 2007), notably, the once segregate genera *Gaura, Calylophus* and *Stenisiphon* now appear within a monophyletic *Oenothera* (Raven, 1988; Raven and Gregory, 1972). We focused on 26 species in sections *Kneiffia, Megapterium, Peniophyllum, Paradoxus* and *Gaura*, hereafter referred to as the "*Gaura* clade." The 26 species of the *Gaura* clade are widely-distributed in North America and Mexico (Raven, 1979; Raven and Gregory, 1972; Straley, 1977), and they exhibit a broad array of floral form, both diurnal and nocturnal flowering, and diverse pollinators, including noctuid moth, antlion, bee, fly, wasp, butterfly, and hawkmoth (Clinebell et al., 2004; Moody-Weis and Heywood, 2001; Nonnenmacher, 1999; Raven, 1979; Raven and Gregory, 1972; Straley, 1977).
This *Gaura*- clade provides a system in which we can make a more rigorous assessment of *Oenothera* pollination systems to clarify the degree of specialization while controlling for similarity due to shared ancestry. We examined the pollination systems of 26 species of taxa in the *Gaura*- clade of *Oenothera*. First, we describe the current measures of

specialization utilized in contemporary pollination studies. Second, we use the 26 focal species of *Oenothera* to test the hypothesis that visitation is sufficient to characterize pollination system specialization. Finally, we test the hypothesis that most flower species have generalized pollination systems by examining the distribution of the pollination system of these 26 *Oenothera*. We expect that defining pollination systems using pollinator functional groups will result in a distribution that shows that most pollination systems for these *Oenothera* are specialized, and that functional groups will be informative about which pollinator group a plant interacts with the most often. We predict that considering specialization in terms of morphological adaptations to pollinators, functional groups are a better metric than counting the number of pollinator species.

Materials/Methods

Study System

We studied 26 species of *Oenothera* in sites throughout the Northeast and Midwest of the United States. Fieldwork was conducted from April 2007 to August 2010. For each population, we conducted pollination observations and collected insects for later pollen load analyses. Vouchers of the *Oenothera* species were collected from each site and deposited with the Missouri Botanical Garden herbarium (MO).

The focal population for *O. macrocarpa* was located in Franklin Co., MO at Shaw Nature Reserve (38° 27’ 58.69” N, 90° 49’ 13.45” W). The three focal populations of *O. filiformis* were in Franklin Co., MO on private lands in Gray Summit, MO (38° 28.395N, 91° 6.035W, 38° 32’ 04”, 90° 20’ 25” W and 38° 39’ 43” N, 90° 18’ 59”W). The two focal populations of *O. linifolia* were located on the same private lands in Gray Summit,

MO (38° 32’ 04”, 90° 20’ 25” W) and in Clinton Co., IL (38° 29’ 18.39” N, 89° 33’ 52.6” W). *Oenothera triangulata* and *O. patriceae* were growing in a sympatric population in Tulsa Co., OK within the city limits of Tulsa, OK (36° 10’ 35.67” N, 90° 49’ 13.45”W). *Oenothera sinuosa* was located in Murray Co., OK along Interstate 35 (34° 22’ 56.26” N, 95° 48’ 44.64” W). The focal population for *O. suffulta ssp. suffulta* was in Murray Co., OK along Interstate 35 near Davis, OK (34° 25’ 34.32” N, 97° 8’ 41.6” W). The focal population for *O. demareii* was in McCurtain Co., on the outskirts of Broken Bow, OK (34° 1’ 44.8”N, 94° 43’ 6.28”). The focal population for *O. gaura* was located in Hampshire Co., MA within the city of Belchertown (42° 17’ 16” N, 72° 24’ 24” W). The focal populations for *O. suffulta ssp. neallyi, O. havardii* and *O. arida* were located in Brewster Co., TX. *Oenothera havardii* and *O. arida* were in a sympatric population at the outskirts of Alpine, TX (30° 22’ 27.46” N, 103° 39’ 39.69” W). *Oenothera suffulta ssp. neallyi* was found within the city limits of Alpine, TX at multiple locations (30° 21’ 28.21” N, 103° 39’ 14.4” W and 30° 22’ 0.54” N, 103° 39’ 39.85” W). The focal population for *O. coloradoensis. ssp. neomexicana* was located in Rio Arriba Co., NM on private lands 4 miles east of Cloudcroft, NM (32° 57’ 53” N, 105° 41’ 23” W). The focal population for *O. xenogaura* was located in Starr Co., TX along Hwy 1430 4 miles east of Rio Grande City, TX (26° 20’10.79” N, 98° 43’ 56.9” W). The focal populations for *O. simulans* were located in New Hanover Co., NC along the roadside near Island Creek (N 34° 22’ 02”, W 77° 48’ 54”) and in Pender Co., NC on Sloop Point Rd, Surf City, NC (34° 26’ 4.21” N, 77° 37’ 51.03”W). The focal populations of *O. pilosella* were located in SE Washington Co. IL, 3 miles south of Posen, IL (38° 15’ 33.08”N, 89° 18’ 12.85”W), and Jefferson Co., IL along Co. Hwy 9 (38 ° 15’ 53.82” N,

89° 2’ 23.6”W). The focal population of *O. perennis* was located in Middlesex Co, MA at the Great Meadows National Wildlife Refuge (42°23’32.6 N, 71° 22’ 55.1 W). Our focal populations of *O. sessilis* were located in Prairie Co., AR at Downs Praire Natural Area (34° 46’ 43” N, 91° 21’ 44” W) and Railroad Prairie Natural Area (34° 46’ 59” N, 91° 29’ 44” W). Our focal populations of *O. riparia* were located in New Hanover Co., NC on the banks of Island Creek (N 34° 22’ 02”, W 77° 48’ 54”), Pender Co., NC (34° 14’ 40” N, 78° 00’ 59” W), and New Hanover Co., NC along the banks of Upper Smith Creek (34° 15’44 N, 77° 53’ 15” W). The focal population for *O. curtiflora* was located in Woodward Co., OK at the Selman Living Laboratory (36° 42’ 46.227” N, 99° 15’ 28.1” W).

The pollination system data for *O. cinerea ssp. cinerea, O. hexandra ssp. hexandra, O. glaucifolia, O. suffrutescens, O. lindheimeri,* and *O. anomala* were conducted by the late R. Clinebell of the Missouri Botanical Garden. His data and collections were used in this study to determine the pollination rates and pollen load for these species. His collection methods were consistent with those studies conducted on the *Oenothera* listed above. In addition, some pollination data were included from collections of P. Raven and D. Gregory, which are stored at the Missouri Botanical Garden.

Oenothera cinerea ssp. cinerea was studied during the flowering seasons of July 1999, Sept 2000, Sept 2001, and July 2003. Focal populations were located in Morton Co., KS at Cimmaron National Grasslands (37° 7’ 16”N, 101° 53’ 40” W), and Union Co., NM at Kiowa National Grasslands near Carrizo Creek. *Oenothera cinerea ssp. cinerea* was also studied in June 1966 at a focal population in Crane Co., TX 13 miles west of Monahan on route 1053. *Oenothera lindheimeri* was studied during the flowering

seasons of June 1964 and 1965 at focal populations in Chambers Co., TX 6.5 miles N of High Island, Liberty Co., TX, and Fort Bend Co., TX. *O. anomala* was studied during the flowering seasons of July-September of 2000 and 2001, and June-September of 1966. The focal populations were located in Durango, Mexico (23° 47' 34" N, 104° 45' 40" W and 23° 48; 04" N, 104° 46' 00" W) and at a population 28 miles west of Durango on Mex 40. *Oenothera hexandra ssp. hexandra* was studied during the flowering season of July 2000 at a focal population in Durango, Mexico (23° 56' 13.7" N, 104° 52' 1.4" W) and a population near Llano Grande, Mexico (23° 52' 1.6" N, 105° 12' 52.7" W). *O. glaucifolia* was studied during the flowering seasons of July-Sept 2002, 2003, and 2004 at a focal population in McClain Co., OK at Kessler's Farm. *Oenothera suffrutescens* was studied during the flowering seasons of May-August 1964, 1966, and 1998 at focal populations in Clark Co., NV at Five State Park, Boaca Co., CO near Comance National Grasslands, 16 miles East of San Luis Potosi, Mexico, Oaxaca, Mexico, 5 miles north of Nochixtian, Reeves Co., TX, and Larimer Co., CO on the roadsides of Ft. Collins.

Characterization of pollination system in contemporary published studies

Using the Boolean search terms "pollination AND ecology", we searched in Web of Science (Thomas Reuters 2010) for all publications from the 2004 to 2009. We examined the main questions of 425 records and found that 144 of these records measured pollination systems as part of their research goals (Supplementary Table 2-2). For these 144 records, we read the methods and determined whether the number of animal visitors alone was used to characterize the pollination systems, or whether both visitation and pollen load were used to determine the pollination system.

Measuring Pollination

Pollination system was determined based on both visitation rates and pollen load analysis. For each population of *Oenothera* we conducted 20 min observations of multiple randomly chosen inflorescences and recorded the total number of visits, type of visitor, and behavior of visitors. We recorded observations of physical contact between an insect and the receptive stigma. These observations were conducted four times during each species flowering season, and took place at peak pollinator activity times of the day or night. The numbers of observations performed per species and the number of insect visitors collected are recorded in Table 2-1.

Average pollen load was determined from a collected sample of insect visitors that made stigma contact. The insect visitors to the flower were collected using a net and a killing jar charged with ethyl acetate. Insects were pinned and taken to the lab to quantify the amount and location of pollen carried. To assess the identity and number of pollen grains carried by each visitor to an *Oenothera* species we made a library of pollen grains from flowering plants at each study site. Dehiscent stamens were placed on glass slides. The pollen was teased out with probes, stained with 1-2 drops of Calbera's fluid to make a semi-permanent mount (Bernhardt et al., 2003; Goldblatt et al., 1998b) and labeled to species for future reference. Each euthanized insect collected on the *Oenothera* species was placed on a separate glass slide and washed in a few drops of 70% EtOH. The insect specimen was removed from the slide and the slide was allowed to air dry. Washed insect specimens were then dried, pinned, and saved for identification by regional entomologists. The pollen on the slide was stained with one or two drops of Calbera's fluid (Goldblatt et al., 1998b) and a cover slip was applied to the surface of the

drop. All pollen identified under light microscopy was compared to the pollen library. The type and amount of pollen on the legs, thorax, and proboscis was recorded.

Earlier collections of insect visitors collected off various *Oenothera* species are stored at the Missouri Botanical Garden. These include collections by R. Clinebell, P. Raven, and D. Gregory. I conducted the pollen load analysis on these insect collections. Visitation rates for these visitors are found in records kept at the Missouri Botanical Garden (unpublished data).

Quantifying specialization: "S-score" and "F-score"

The degree of pollinator specialization of a plant species, which I have termed the S-score, is defined as the number of taxa that account for 95% of the pollen flow. Pollen flow was calculated by combining visitation rate and pollen load to correct for the disparity between frequency and efficacy of pollinators. To calculate this S-score, we combine the visitation rate (visits/inflorescence/20min) with the pollen load (number of pollen grains carried by an animal visitor) summed across visitor species. Where:

$$\text{Pollen Flow} = \sum (\text{Visitation Rate}_{spx} * \text{PollenLoad}_{spx}).$$

We then determined the number of animal visitors that accounted for 95% of the total pollen flow, and designated that as the "S-score" for that specific *Oenothera* taxa. We also measured pollination by placing the visitors into functional groups based on taxa and size. For example, all noctuid moths of a similar size that visited during the same time period were considered as one functional group. These data are summarized in Table 2-1. We then determined the number of functional groups that accounted for 95% of the total pollen flow and designated that as the "F-Score".

Analyses

To test whether visitation alone was sufficient to characterize pollination systems, we compared the number of total visitors with the number of pollinators, defined as those visitors that carried the plant species pollen and made stigma contact. We log-transformed the data [ln (x +1)] for normalization, and then used a paired t-test to test for differences in these two ways of characterizing pollination systems. We also performed a Wilcoxon Sign Rank test to check for differences between visitation and pollination, and between S-scores and F-scores.

To determine how specialized *Oenothera* pollination systems are when defined by pollinators, not just visitors, we used regression on the log-transformed data [ln (x +1)] for S-scores.

Results

Characterization of pollination systems in contemporary published studies

Of the 144 records examined, 62.5% used only observed visitation rates of insects or birds to plants as a method to characterize pollination (Suppl. Table 2-1).

Pollination System

Of the 26 *Oenothera* species examined, *O. curtiflora*, *O. sessilis*, and *O. simulans* were completely autogamous. *Oenothera simulans* and *O. sessilis* had visitors, but none that carried any pollen and contacted a stigma. *Oenothera macrocarpa*, *O. suffulta ssp. nealleyi*, *O. filiformis*, *O. coloradoensis ssp. neomexicana*, and *O. gaura* all used both night and day pollinators. *Oenothera linifolia, O. pilosella, O. perennis, O. riparia, O. glaucifolia, O. demareei* and *O. lindheimeri* were all day pollinated. *Oenothera patriciae, O. triangulata, O. xenogaura, O. suffulta ssp. suffulta, O. sinuosa, O. cinerea ssp.*

cinerea, O. hexandra ssp. hexandra, O. havardii, O. arida, O. anomala, and *O. suffrutescens* were all exclusively night pollinated.

The main pollinator groups and F and S scores for all 26 species are listed in Table 2-1. The full list of taxa pollinating these *Oenothera* species is listed in Supplemental data Table 2-3.

Visitation vs. Pollination

For 3 of the *Oenothera* species, the number of visitors equaled the number of pollinators. In all other species, visitation alone was not sufficient to accurately describe the pollination system. There was a statistical difference between visitation and S-score (P = 0.000002) The Wilcoxon Sign-Rank test showed a statistical difference between visitation and S-score (Prob |z| < .00001). In addition, visitation was not proportional to S-score, in other words a high number of visitors did not equal a high number of pollinators. (Fig. 2-1)

Taxa vs. Functional Groups

The regression analysis using the S-score shows a pattern of more *Oenothera* species having specialized rather than generalized pollination systems (R^2= 0.640, P= 0.000198) (Fig 2-2). Regression analysis using the F-score also show that *Oenothera* pollination systems are more specialized (R^2= 0.682, P= 0.000081). There is a significant difference between how specialized the pollination systems are when calculated using taxa (S-score) and when using functional groups (F-score) (P= 0.000089). The Wilcoxon Sign-Rank test also shows a significant difference between the S-score and F-score (Prob |z| = 0.0001) (Fig. 2-3).

Discussion

The present study, integrating new datawith previous results, can help us understand the pollination system of *Oenothera*, and it provides insight into how specialization of pollination systems is measured. The pollination systems of *Oenothera* have been studied for several decades and serve as a model system for studying plant reproduction (Raven, 1979; Raven, 1988; Raven and Gregory, 1972; Wagner et al., 2007). *Oenothera* species have a broadly diverse number of pollination systems. Bee-pollination is most likely the ancestral state, with hawkmoth pollination as a derived state that has arisen multiple times (Raven and Gregory 1972; Raven 1979). In this study, we looked at the pollination systems of 26 taxa of *Oenothera* and focused on how to most accurately define the degree of specialization of these pollination systems. We found that these species attract a wide range of main pollinator groups including fly, bee, moth, hawkmoth, wasp, and antlion. These species cover a broad range of pollination system types, both in temporal and spatial variation, and are a good representation of North American pollination.

Understanding the degree of specialization of pollination systems is important when making inferences about a plant's evolutionary history. Pollinators are not the only factor in the adaptation of floral forms. For example, life history, breeding system, successional status, and abundance all play a role, but pollinators are described as a dominant influence in the evolution of floral specialization (Crane et al., 1995; De Bodt et al., 2005; Endress, 1994; Soltis et al., 2008). How specialized a pollination system is also plays a critical role when making conservation decisions for plant species (Ashworth et al., 2009; Bascompte, 2009; Biesmeijer et al., 2006; Johnson and Steiner, 2000; Winfree, 2008). Concluding that a plant has a generalized pollination system, when it

may be highly specialized, could lead to poor management decisions and result in a loss of plant diversity.

For several of the species, we have multiple years of pollination data, but with such a broad study, this was not available with all the taxa. This is a potential limitation of the study. To gauge the degree of pollination visitation variability from year to year, we compared pollination data between two years for *O. filiformis* and *O. macrocarpa.* We found that although the taxa or functional group of pollinator differed, the total number of pollinator species or functional groups active in a single year did not change. This is in agreement with recent pollination network studies that show while the type of species interacting may change from year to year, the overall number of interactions tends to remain constant (Memmott et al., 2004; Petanidou et al., 2008). Therefore, we determined that a 'snapshot' approach, involving a single season of detailed pollination data, is sufficient for the broad scale comparison of this project. When looking at functional groups, a snapshot approach can be sufficient to characterize a pollination system in terms of how specialized it may be (Alarcon et al., 2008).

Measuring how specialized a pollination system is has important implications when looking at the evolution of a lineage. If pollinators are a selective pressure that has led to such a diversity of floral form, then plant- pollinator interactions are expected to be highly specialized, and a specialized pollination systems would be seen for a majority of flowering species (Ollerton, 1996). One reason that pollination systems are often seen as generalized is because they are defined only using visitation rate of a potential pollinator to a plant. In this study, we found that the majority of pollination studies in the last five years only used visitation rate to characterize a pollination system. However, we found

that visitation rate highly over-estimates the number of taxa pollinating a plant species. Insects visit flowers for a variety of reasons (Buchmann and Nabhan, 1996). The flower may be a mating site or a source of food or shelter and all of the insects' interactions with the flower may not involve stigma contact. In this study, we only collected potential pollinators which were observed making regular stigma contact. Of the 26 *Oenothera* species we studied, for only 3 of those species did the number of visitors equal the number of pollinators. When pollen load and stigma contact are measured as well, the number of actual pollinators is significantly lower than the number of visitors for the other 20 *Oenothera* species (3 species were completely autogamous). In contrast to Waser (1996), we find that *Oenothera* pollination systems, as representative of North American pollination systems, are more specialized than generalized.

It has been suggested that visitation is still an accurate way to measure specialization of pollination systems because the number of visitors is proportional to the number of actual pollinators (Cayenne Engel and Irwin, 2003), and so one could make relative comparisons between plant species based on just visitation. However, in this study, we find that there is not a correlation between the number of visitors and the number of pollinators (Fig. 2-1). For example, *O. cinerea ssp. cinerea* has the highest number of visitors, 73, but an S-score of 9; while *Oenothera macrocarpa* uses the highest number of pollinators, with an S-score of 13, but only has 18 species of visitors. We conclude that not only does visitation highly over-estimate the number of pollinators, but it is also not a sufficiently accurate measurement of specialization of pollination systems in a proportional or comparative way either.

Pollination system is most often measured as the number of taxa involved in the plant-pollinator interaction. However, the use of functional groups, in which the visiting taxa are grouped by some morphological characteristic that afffects how pollen load is delivered to a plant species, is perhaps a more informative way to examine pollination systems. A pollination system is considered specialized when a single functional group is responsible for greater than 75% of the pollination visits (Fenster et al., 2004). In this study, when we grouped the insect visitors by major taxon groups and size, we found that the *Oenothera* pollination systems are more specialist than generalist. By the definition of Fenster et al. (2004), 17 of the *Oenothera* species have specialist pollination systems. Of the remaining species, 8 use only 2 functional groups of pollinators to reach 75% of the pollination visits, and only one species, *O. gaura*, uses 3 functional groups. We decided to measure pollination specialization in a way that would show the continuous nature of pollination systems. We calculated for each *Oenothera* species an "F-score," which placed them along a continuum. The majority of the *Oenothera* species were toward the specialist end of the continuum (Fig. 2-2). The highest F-score was a 5, which means it took 5 functional groups to account for 95% of the pollen flow, and only two species, *O. macrocarpa* and *O. glaucifolia*, had this score.

Placing pollinators into functional groups is not just creating a subset of pollinators measured by the number of visiting taxa. The number of functional groups is not always just a proportionally smaller set of the pollinators (Fig. 2-3). Of the 26 *Oenothera* pollination systems we studied, 8 species had the same number of pollinating taxa as they did functional groups. Functional groups can also give more information as to which species are actually the important pollinators. Some taxa do not contribute

sufficiently to the pollen flow to be included in the S-score; however, when the taxa are grouped by functional groups, they can become the dominant contributor to the pollen flow. For example, *O. cinerea ssp. cinerea* is pollinated by several species of small halictid bees, bumble bees and noctuid moths. If the data are examined looking only at S-score values, two halictid bees appear to be the dominant pollinators. But when the taxa are grouped into functional groups, the 14 taxa of noctuid moths collectively become the second most important group contributing to pollen flow. When only assessed by taxa, the noctuid moths are not seen as important pollinators because each species only carries a small pollen load, and there are many species involved. Overall, we find that the use of functional groups gives the most accurate representation of how specialized these *Oenothera* pollination systems are, with respect to morphological specialization to a specific type of pollinator.

One difficulty in applying these results to other floral systems is that *Oenothera* pollen are large compared to other flowering species. The size of the pollen would be a trait that would limit the number of pollinators that could manipulate and carry pollen. This could possibly filter out smaller visitors that would be pollinators if the pollen were smaller. It may be that in a different floral system with smaller pollen, there would be more species with more generalized pollination systems. In addition, the viscine threads that hold together *Oenothera* pollen could possibly affect the size of pollen load carried by a pollinator. Future studies should look at a comparison of specialization of pollination systems between different floral systems.

In conclusion, we find that for *Oenothera*, the number of visitors alone highly overestimates the number of pollinators and is inadequate for determining pollination

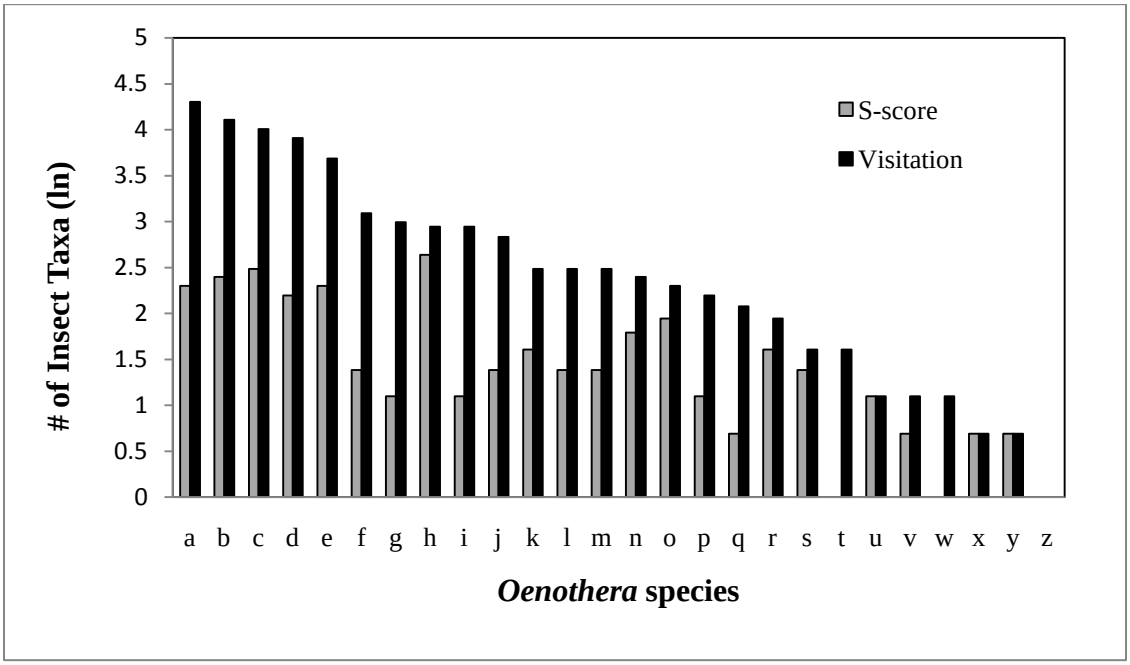
system specialization. When both pollen load and visitation rate were used to calculate specialization, we found that the pollination systems were distributed across a continuum from highly specialized to generalized, with the majority of the pollination systems being specialized. In addition, we find that functional groups provide the most informative characterization of pollination systems, especially when determining which pollinator group a plant interacts with the most often. These results are important when making broad conclusions regarding the evolution of this group, as well as for making conservation and management decisions. Finally, this study serves as an example of how to determine pollinators for future studies that consider specialization of pollination systems.

Table 2-1. The total number of insect taxa visiting each species of *Oenothera*. F and S scores for the 26 *Gaura*-clade members of *Oenothera*, as based on the visitor observations and pollen load data. The main pollinator functional groups are listed.

Species	Visitor observations (n)	Insect Pollen Loads (n)	S-score	F-score	Pollinator Functional Groups
O. anomala	176	71	4	2	Hawkmoth/antion
O. arida	63	25	1	1	Noctuid moth
O. cinerea s. cinerea	904	330	9	3	Night-moth/Day-bee, bumble bee
O. coloradoensis s. neomexicana	308	66	2	2	Day-small bee/Night-moth
O. curtiflora	400	0	0	0	Autogamous
O. demareei	311	132	3	2	bee/bumble bee
O. filiformis	1110	212	10	2	Night-moth/Day-bee
O. gaura	241	126	6	4	moth/fly/wasp/bee
O. glaucifolia	228	182	11	4	bee/wasp/fly/small bees
O. harvardii	125	30	1	1	Hawkmoth
O. hexandra s. hexandra	236	108	8	3	moth/bee/fly
O. lindheimeri	180	72	5	2	bee/wasp
O. linifolia	152	52	2	2	Fly/Small halictid bee
O. macrocarpa s. macrocarpa	331	155	13	5	Night-moth, hawkmoth/Day-bee, wasp, small bee
O. patriciae	228	29	1	1	Noctuid moth
O. perennis	236	56	4	3	small bee/bee/bumble bee
O. pilosella s. pilosella	185	69	3	2	bee/small bee
O. riparia	285	41	3	3	bee/small bee/bumble bee
O. sessilis	137	0	0	0	Autogamous
O. simulans	290	19	0	0	Autogamous
O. sinuosa	330	50	1	1	Noctuid moth

O. suffrutescens	373	100	9	4	moth/bee/small bee/bumble bee
O. suffulta s. nealleyi	296	46	2	2	Night-moth/Day-bee
O. suffulta s. suffulta	133	109	3	2	Noctuid moth
O. triangulata	155	19	2	1	Noctuid moth
O. xenogaura	349	41	3	1	Noctuid moth

Figure 2-1 A comparison of number of insect visitors, which is often used as a substitute for number of pollinators, and the S-score, which is based on visitation rate and pollen load carried by insect, shows a statistical difference (P = 0.000002). Visitation is not proportional to pollination rate. Each pair of bars is for one of the 26 *Oenothera* taxa. The letter below each data pair corresponds to the *Oenothera* taxa indicated in the chart below.



Label	Species List
a	*O. cinerea s. cinerea*
b	*O. filiformis*
c	*O. glaucifolia*
d	*O. hexandra s. hexandra*
e	*O. suffrutescens*
f	*O. demareei*
g	*O. coloradoensis s. neomexicana*
h	*O. macrocarpa s. macrocarpa*
i	*O. suffulta s. nealleyi*

j	*O. pilosella s. pilosella*
k	*O. anomala*
l	*O. riparia*
m	*O. xenogaura*
n	*O. lindheimeri*
o	*O. gaura*
p	*O. triangulata*
q	*O. patriciae*
r	*O. perennis*
s	*O. suffulta s. suffulta*
t	*O. simulans*
u	*O. linifolia*
v	*O. harvardii*
w	*O. sessilis*
x	*O. arida*
y	*O. sinuosa*
z	*O. curtiflora*

Figure 2-2 Regression analysis on the S-score (log transformed data) show a pattern of more *Oenothera* having specialized rather than generalized pollination systems (R^2= 0.640, P = 0.000198).

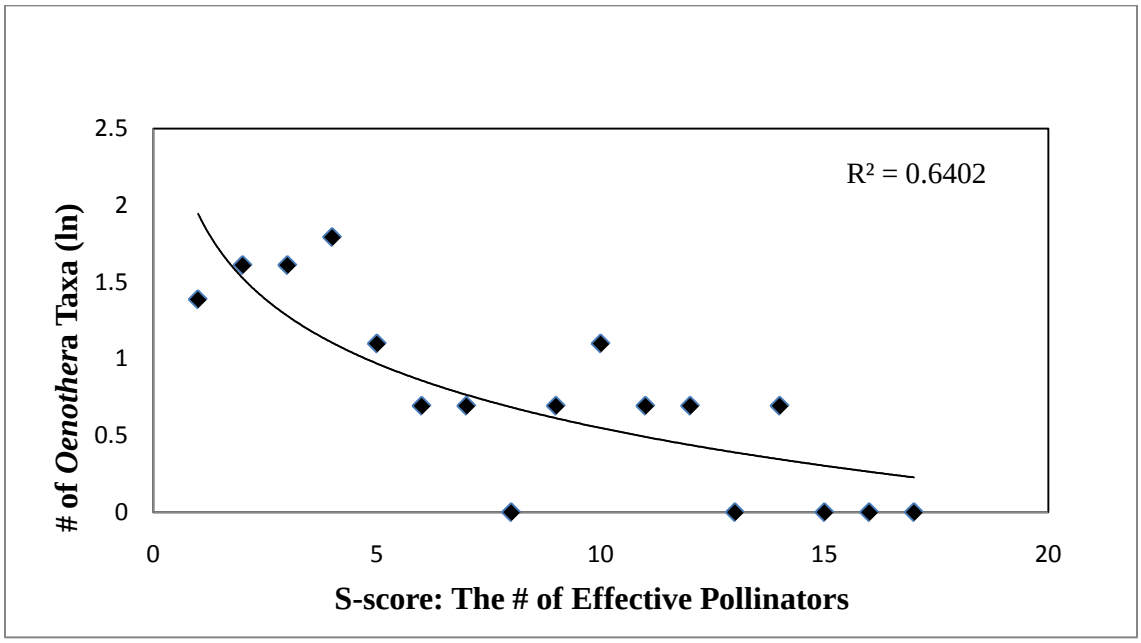
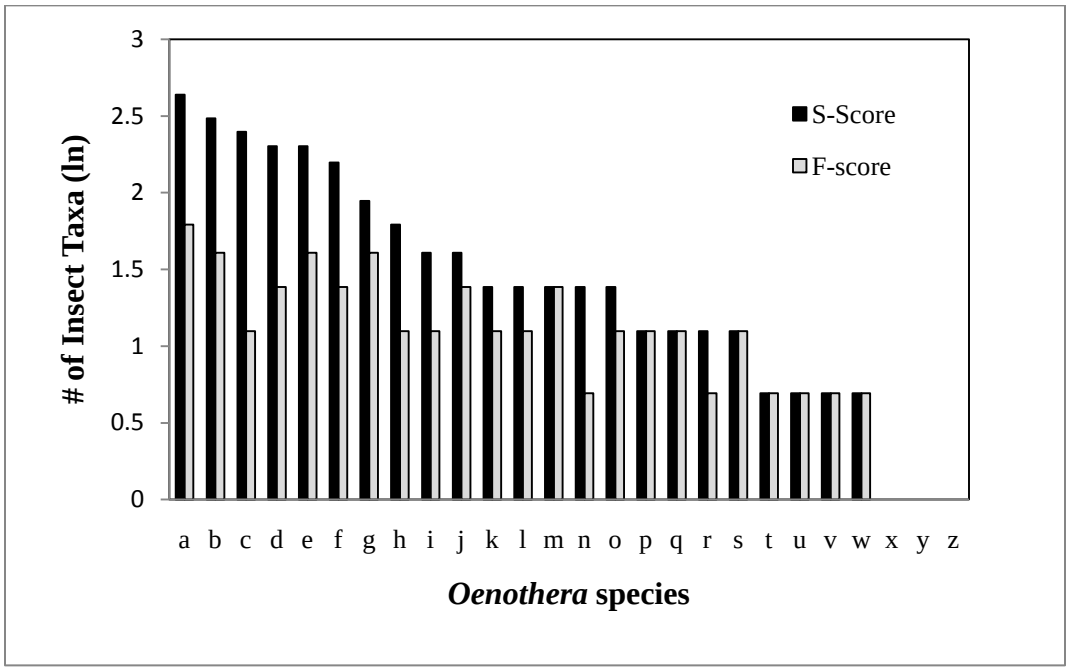


Figure 2-3 A comparison of the S-score and F-score for all 26 *Oenothera.* There is a significant difference between how specialized the pollination systems are when calculated using taxa (S-score) than when using functional groups (F-score) (*P*= 0.000089).



Label	Species List
a	*O. cinerea s. cinerea*
b	*O. filiformis*
c	*O. glaucifolia*
d	*O. hexandra s. hexandra*
e	*O. suffrutescens*
f	*O. demareei*
g	*O. coloradoensis s. neomexicana*
h	*O. macrocarpa s. macrocarpa*
i	*O. suffulta s. nealleyi*
j	*O. pilosella s. pilosella*
k	*O. anomala*
l	*O. riparia*
m	*O. xenogaura*

n	*O. lindheimeri*
o	*O. gaura*
p	*O. triangulata*
q	*O. patriciae*
r	*O. perennis*
s	*O. suffulta s. suffulta*
t	*O. simulans*
u	*O. linifolia*
v	*O. harvardii*
w	*O. sessilis*
x	*O. arida*
y	*O. sinuosa*
z	*O. curtiflora*

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CHAPTER 3

PAGES 36-66

TESTING POLLINATION SYNDROMES IN *OENOTHERA* (ONAGRACEAE)

Introduction

A pollination system is the interaction between a plant and its pollinator(s). Pollination biologists have always looked for a way to explain the floral diversity in angiosperm evolution and to predict the pollination system for a specific plant, and this led to the idea of "pollination syndromes." Pollination syndromes are groups of floral traits that correspond to specific type of pollinator or pollinator group. Darwin discussed how pollinators were the major selective agent for floral trait evolution (Darwin, 1877). This concept was developed in the late 1800's by scholars such as Herman Muller, Federico Delphino, and Paul Knuth, who made long lists of plant features and corresponding pollinator traits (Ollerton et al., 2009). These groupings were used as a way to organize and understand floral diversity. In 1954, Stefan Vogel coined the term "Pollination Syndrome" (Ollerton et al., 2009; Waser and Ollerton, 2006), and later, the seminal work of Faegri and van der Pilj outlined 11 pollination syndromes that became the standard in pollination biology studies (Faegri and Pijl, 1966; Faegri and van der Pilj, 1979). These 11 pollination syndromes described specific floral characteristics, mainly associated with reproduction, which were associated with groups of pollinator types.

The concept of pollination syndromes has played a central role in plant-pollinator studies. First, it has been used as a way of understanding the evolution of groups of floral traits, and is a classic example of convergent evolution (Fenster et al., 2004; Stebbins, 1970). Across distantly related taxa, there is a correlation between the floral traits and ecology, which provides evidence that there has been selection by specific types of pollinators. Many comparative studies showed that suites of floral traits do correspond to different pollinators (Fenster et al., 2004; Ollerton et al., 2009; Wilson et al., 2004; Wolfe

and Sowell, 2006), and broadly agreed with Stebbins (1970) “Most Effective Pollinator” principle which state that plants will specialize to the pollinator that is most responsible for pollen transfer (Ne'eman et al. 2010; Stebbins, 1974).

Second, pollination syndromes have been used to predict the plant-pollinator relationship. However, the use of pollination syndromes as a predictor of pollination system has several problems. Pollination syndromes are potentially too limited an explanation of the complex relationships between a plant and a visitor. There are multiple reasons a visitor might interact with a plant other than pollination, and these interactions can affect the evolution of floral traits (Ashman and Majetic, 2006; Chittka et al., 1999; Yang and Guo, 2005). Second, inherent to the concept of pollination syndromes is the idea that most plant-pollination interactions are highly specialized (Fenster et al., 2004; Ollerton et al., 2009; Reynolds et al., 2009); however, most interactions appear more generalized (Mitchell et al., 2009; Waser et al., 1996; Waser and Ollerton, 2006). Despite this discrepancy between the predicted pollinator and the current pollinator, pollination syndromes as a predictive tool have rarely been tested directly (but see (Hingston and Mc Quillan, 2000; Muchhala, 2006; Ollerton et al., 2009). More studies that use detailed pollination data to evaluate the degree to which pollination syndromes are useful for predicting plant pollinators are needed (Fenster et al., 2004; Waser et al., 1996).

A final concern is that many studies that use pollination syndromes as a tool to infer a plant’s pollinator, often define that syndrome by a suite of floral characters that consist of morphological measurements (DeWitt Smith, 2010; Smith et al., 2008b; Tripp and Manos, 2008; Whittall and Hodges, 2007). These quantitative floral trait measurements do not always overlap with the discrete floral traits that traditionally define

a pollination syndrome as described by Faegri and van der Pilj (1979). Thus, the use of quantitative floral trait measurements to infer a pollination system could lead to incorrect conclusions.

Onagraceae, the evening primrose family, is one of the major plant radiations in western North America (Raven, 1979; Raven and Gregory, 1972; Straley, 1977). The genus *Oenothera* is a model system for studying plant reproductive biology and floral evolution (Raven, 1988). *Oenothera* encompass a wide range of pollination systems including bee, bird, butterfly, wasp, moth, antlion, fly, and hawkmoth (Clinebell et al., 2004; Moody-Weis and Heywood, 2001; Nonnenmacher, 1999; Raven, 1979; Raven and Gregory, 1972; Straley, 1977). Recent studies have provided detailed empirical data on the pollination systems of sister taxa of *Oenothera* that also show diverse floral forms (Chapter 2). This provides an opportunity to rigorously test hypotheses about pollination syndromes.

Here, I ask how accurate pollination syndromes are at predicting current pollination systems. I evaluate the correspondence between morphology and pollinators by creating a phenotypic space using the traditional floral traits that define pollination syndromes. I then compare the pollination syndromes predicted for the 54 *Oenothera* species to the current pollination systems as defined by visitation and pollen deposition data. I also assessed the predictive power of current methodologies that use pollination syndromes as a way of defining pollination systems. I then ask the following questions: 1. Do most *Oenothera* species fit into traditional pollination syndromes? 2. Do these pollination syndromes accurately predict the dominant pollinator for each species? 3.

When using quantitative floral trait measurements, do *Oenothera* species form groups that correspond to their main pollinators?

Methods/Materials

Pollinator Data and Floral Traits

I used the 54 species of *Oenothera* in Subclade B (Levin et al., 2004) for this study. This clade has a diversity of floral forms and uses multiple pollinator types. A previous study gives detailed pollination data, including visitation, pollen load, and stigma contact of visitors, for 26 of these species (Chapter 2). The main pollinator group for the remainder of these species comes from other published pollination studies and unpublished data at the Missouri Botanical Garden. All pollination systems were determined using both visitation and pollen load data, and main pollinators were considered those that contributed to 95% of the total pollen flow. These pollinators were then grouped into functional groups (Fenster et al., 2004) of similar species and sizes.

I conducted the quanititative floral measurements on 10-15 flowers of each species of *Oenothera*. I measured floral tube length, floral tube mouth width, corolla span, stamen length, and style length. For *O. deserticola, O. canescens, O. rosea, O. speciosa, O. texensis, O. epilobiifolia, O. multicaulis, O. seifrizii, O. dissecta, O. kunthiana, O. orizabae, O. tetraptera, O. brachycarpa, O. coryi, O. howardii, O. spachiana, O. anomala, O. boquillensis, O. cinerea ssp. parksii, O. filipes,* and *O. mckelveyae*, I used herbarium sheets from the Missouri Botanical Garden to make these measurements. All other measurements were taken from the plant populations used for

pollinator data collection (Chapter 2) or with greenhouse populations. The average measurement of each trait for 15 individuals was used to represent each species.

Analyses

To evaluate the predictive power of pollination syndromes, I first created a phenotypic space using discrete floral traits that characterize each of the 11 pollination syndromes described by Faegri and van der Pijl (1979). Because the traits for hawkmoth and moth have been found to be indistinguishable (Ollerton et al., 2009), I also combined them and used 10 syndromes, bat, bee, beetle, bird, butterfly, fly, moth, carrion fly, small non-flying mammal, and wasp. This matrix of idealized pollination syndrome traits is a modified version of Ollerton et al (2009), which gives multiple different versions of each idealized pollination syndrome (e.g. Bee 1, Bee 2, etc). This creates a broader, more realistic definition of the pollination syndromes and captures the variability of the floral traits associated with a syndrome. For example, a "bee" flower can be white or yellow. I used their multiple trait vector approach of 537 vectors across 10 syndromes, with each trait scored as present (score of 1) or absent (score of 0). However, I modified how the syndromes were characterized and used the following 9 floral traits: color at anthesis (yellow, white, red, pink, green, purple, brown, blue, orange), scent (sweet, fruity, fresh, musty, sour, decay, none), flower shape (dish, bell/funnel, trumpet, tube), symmetry (actinomorphic, zygomorphic), orientation (pendant, upright, horizontal), brightness (dull, vivid), anthesis time (day, night), nectar presence, and nectar location (hidden, accessible) (see Supplementary Data Table 3-1 for full matrix). I then used these 10 pollination syndrome traits to score the 54 *Oenothera* species such that each species was

described by a vector of 35 ones and zeroes (see Supplementary Data Table 3-2 for full matrix). All analyses were carried out in PC-ORD (McCune and Mefford, 2006).

To determine whether the different vectors for each of the 10 syndromes grouped into discrete groups, I used formal ordinations using non-metric multidimensional scaling (NMDS), which is appropriate for binary data (McCune and Grace, 2002; Ollerton et al., 2009). I used a Sorensen's index (Bray-Curtis) to express the distance relationships between the idealized pollination syndromes described by the binary data set. NMDS was used to find the best dimensional representation of the distance matrix. The NMDS analyses started with 250 runs of real data, which were then compared with a Monte Carlo test with 250 ordinations of randomized data. Mean stress did not decline after 3 dimensions, and so a 3-dimensional space was selected for the analyses (McCune and Grace, 2002). I then ran the final solution and assessed the stability of this solution by examining a Scree plot (final stress vs. the number of dimensions), and the final stability reported from the NMDS output. I assessed the final stress from the NMDS using Kruskal's stress formula and Clarke's rule of thumb (McCune and Grace, 2002).

This ordination of the idealized pollination syndromes created a three-dimensional space with each pollination syndrome represented by a cluster of the multiple traits combinations. Using these results and the matrix that scored the floral traits of the 54 *Oenothera* species, I used NMS Scores algorithm in PC-ORD 5.14 to calculate co-ordinates for the *Oenothera* species in that pollination syndrome space. Then, I calculated the Euclidean distance between each *Oenothera* species and the center of the nearest pollination syndrome cluster. I also calculated the second closest syndrome. An alternative method, discriminate function analysis (DFA) conducted in PC-ORD

(McCune and Mefford, 2006) did not yield different results. To determine how accurately the idealized pollination syndromes predicted the actual pollination systems, I compared the pollinator system predicted from these analyses to the pollination system as determined from the ecological pollination system data for each plant species.

It may be that the morphological measurements of a flower are better predictors of pollinator type than the traditional pollination syndrome floral traits. To determine whether *Oenothera* species form groups based on quantitative floral traits that correspond to their main pollinators, I used the methods most commonly employed by studies that infer pollination syndromes in this way (Tripp and Manos, 2008; Whittall and Hodges, 2007), which is principle component analyses (PCA). The PCA variables were the five floral measurements listed above. I log transformed the data and conducted a PCA using JMP, Version 8.0 (2009).

Results

Idealized syndromes and real flowers in phenotypic space

Ordination using NMDS of the traditional pollination syndromes produced a well-resolved 3-dimensional phenotypic space that accounted for nearly 75% of the variance of the among-syndrome variation (axis 1 $R^2 = 0.16$, axis 2 $R^2 = 0.28$, axis 3 $R^2 = 29$, cumulative $R^2 = 0.74$). After 279 iterations the instability was 0.00, and the final stress for the 3-dimensional solution was 15.31. Most ecological community data sets have solutions with stress between 10 and 20, and this data set falls within this range (Clarke, 1993), I deem this value acceptable. In agreement with the results for the idealized pollination syndromes used by Ollerton et al. (2009), I also find that the traditional

syndromes, which had multiple versions for each type, group into discrete areas of the multivariate space without overlap (Fig. 3-1). For example, all of the “bee” syndrome vectors group together, while all of the “moth” syndrome vectors group together and do not overlap with the “bee” syndrome. However, some syndrome groups are closer together, for example, non-flying mammal and bat.

If the *Oenothera* species conform to a specific pollination syndrome, and if the floral trait combination for a given species is similar to one of the defined traditional syndromes, I would expect the *Oenothera* species to fall within the cluster of a traditional syndrome. These results show that these 54 *Oenothera* species do not fall within any of the phenotypic spaces that represent traditional pollination syndromes (Fig. 3-2). There is no grouping in the phenotypic space between the subsections of *Oenothera* that reflects the phylogenetic relatedness. Subsections that are sister to one another are not near each other in the phenotypic space. However, the *Oenothera* do show some clustering within the subsections of the genus (Fig. 3-3). For instance, 24 of the 26 species in subsection *Gaura* cluster together and the 4 species in subsection *Megapterium* occupy the same phenotypic space exactly.

Predictions by traditional pollination syndromes compared to pollinator data

When I calculated the nearest traditional pollination syndrome for each *Oenothera* species, and compared that to current ecological pollinator data, I found that the pollinator syndromes accurately predicted the pollinator for only 48.2% of the species(Table 3-1). When I expanded this to look at the second closest pollination syndrome vectors, the pollination syndrome accurately predicted the pollinator for 72.2%

of the *Oenothera* species. Many of the species had equal Euclidean distance values from multiple pollination syndrome vectors. For instance, *O. gaura* was equally close to two "moth" traditional syndromes. For *O. glaucifolia, O. sinuosa,* and *O. cinerea ssp. cinerea* the nearest pollination syndrome vectors included both day and night pollinators, and did not provide sufficient predictive resolution. For *O. epilobiifolia ssp. epilobiifolia* and *O. multicaulis* multiple vectors were equally close; however, the accurate pollinator was not the dominant pollinator predicted (e. g. 2 "bird" and 9 "fly", but the actual pollinator is a bird). When I included the second closest pollination syndrome vectors, this could include up to six pollinator syndromes, which does not give enough resolution for a prediction. These results are summarized in Table 3-1.

The predictability of the pollination syndromes varied. Moth-pollinated plants (69.7%) and butterfly-pollinated plants (66.7%) were the most accurately predicted. Bird-pollinated plants were accurately predicted 33.3% of the time, while bee-pollinated plants were accurately predicted 26.7% of the time. Fly-pollinated and beetle-pollinated plants were never accurately predicted. The remaining syndromes were never accurately predicted.

The prediction of pollinators was more successful for some subsections of *Oenothera* had than others. In subsection *Megapterium*, 3 of the 4 taxa were accurately predicted by the traditional pollination syndromes, and 18 of the 28 taxa in subsection *Gaura* were accurately predicted by the traditional pollination syndromes. The one species in subsection *Paradoxus* had its pollination system accurately predicted. The pollination syndromes for subsections *Kneiffia, Gauropsis,* and *Peniophyllum* were never predicted accurately and only 1 of 5 species in section *Hartmannia* had their pollination

system accurately predicted. 1 in 4 taxa of subsection *Xanthocoryne* and 2 in 5 taxa of subsection *Leucocoryne* had accurately predicted pollination systems.

Principle Component Analysis with Floral Measurements

For all 54 *Oenothera* species examined, the floral trait measurement data (floral tube length, floral tube mouth width, corolla span, stamen length, and style length) used in the PCA analyses are given in Table 3-2. The first two PCA axes explained 78.87% and 10.87% of the variance in the data (Fig. 3-4). Although approximately 90% of the data is explained with the first two axes, the PCA is unable to give sufficient resolution to discern any grouping of the *Oenothera* species that might correspond to a pollination syndrome. The eigenvector coefficients of axis 1 are all positive, which suggests an allometric relationship among the variables. The correlations of variables on PCA axes are given in Table 3. Axis 1 shows some differentiation between species with long floral tubes and those without. The species that separate out are in subsection *Megapterium*, which are taxa that all have much longer floral tubes than the other *Oenothera*. Most of the variance for Axis 2 is explained by "corolla throat," however, there is no discernable grouping of species by corolla throat size.

Discussion

The main goal of this study was to assess how accurate pollination syndromes are at predicting pollination systems of *Oenothera*. I defined the pollination syndromes as closely as possible to the traditional floral traits set forth by Faegri and van der Pijl (1979). The idealized syndromes segregated into discernable clusters in the multivariate space. However, the 54 *Oenothera* species did not fall within or near the idealized

syndrome clusters. This is in agreement with recent studies that have also found that real plant species did not fall within pollination syndrome clusters in the phenotypic space (Ollerton et al., 2009). Therefore, I assessed how accurate the predicted pollination syndrome was based on the pollination system nearest to the *Oenothera* species in the multivariate space, and compared that with ecological pollination data collected for each species. I found that less than half the time the predicted pollination syndrome matched the actual dominant pollinator for that species. This is slightly more successful compared to Ollerton et al. (2009), who found that the primary pollinator was successfully predicted by the nearest pollination syndrome one-third of the time. One reason for the higher predictability success with this data set may be that the pollination data were based on both visitation and pollen load; whereas the Ollerton et al. (2009) study used only floral visitor observations to determine pollinators. Visitor observations alone can highly overestimate the number of actual pollinators (Chapter 2), and the more generalized pollination systems are difficult to accurately predict with pollination syndromes. However, both results suggest that pollination syndromes are not a reliable tool to predict a plant's pollination system.

The pollination syndromes differed in predictability. For the *Oenothera* species examined here, butterfly and moth syndromes were predicted most accurately, while fly and beetle were never predicted accurately. The syndromes that were predicted accurately most often differed from Ollerton et al. (2009) who found that bee and fly pollination systems were most predictable, and moth pollination was one of the syndromes that were least often predicted accurately. This difference might be simply a result of the most common pollination syndrome of the species involved in each study. Moth pollinators are

the dominant pollinator for 33 of the 61 *Oenothera* species, and *Oenothera* in general have many floral traits associated with moth syndromes, so it is not surprising that this is the most successfully predicted syndrome. What the pollination syndromes are not capturing is the variability in the pollinators for *Oenothera* that do not use moths, but still have many classic moth-pollinated *Oenothera* floral traits. For instance, several *Oenothera* species have traits that suggest adaptations for night pollinators, but in actuality, these species have a dual pollination system, wherein they utilize both day and night pollinators. *Oenothera* species with more generalized pollination systems were the ones most often predicted inaccurately. This inaccuracy was retained even when I evaluated the pollination syndrome by just looking at the species dominant pollinator type. This finding highlights the problem that pollination syndromes infer a high amount of specialization between plant and pollinator (Fenster et al., 2004). Because so many plant species use multiple pollinators, and because pollination syndromes predict only one type of pollinator, syndromes fail to capture this information.

Some of the subsections of *Oenothera* had higher predictability by the pollination syndromes, namely *Paradoxus*, *Megapterium*, and *Gaura*. This finding is not surprising because each of these subsections have very distinctive floral traits that are also traits used to define traditional pollination syndromes. The taxa in *Paradoxus* and *Megapterium* flower at night and have notably long floral tubes that are associated with moth pollination. These taxa are moth pollinated; however, *O. macrocarpa* is also pollinated in the day by bees, and was inaccurately predicted as butterfly pollinated. Subsection *Gaura* taxa have distinctive morphological traits such that they form a recognizable cluster in the phenotypic space, and many of the species are moth

pollinated. The predictive pollination systems were wrong for those *Gaura* species that had the most generalized pollination systems. The least predictable subsections were those that have many floral traits of *Oenothera*, which suggest moth pollination, but that are pollinated by a different dominant pollinator. For instance, the taxa in subsection *Kneiffia* have many floral traits that suggest classic *Oenothera* moth pollination syndrome; however, they open in the morning and the predicted pollination syndrome was butterfly, which falls closer in the phenotypic space to moth syndrome. In actuality *Kneiffia* are all predominately pollinated by bees. This pattern highlights the problem that pollination syndromes infer a pollinator based on a suite of traits, even though it may be a single trait that is determining the dominant pollinator. The floral traits that match up to the inaccurate pollinator syndrome effectively swamp out this information.

Comparing the predictive power of NMDS to PCA

I used non-metric multidimensional scaling (NMDS) as an analysis tool because it makes no assumptions about the distribution of variables and creates multivariate space in which similar objects are close to each other (McCune and Mefford, 2006). However, many studies use the ordination technique of principle component analyses (PCA) to determine pollination syndromes (DeWitt Smith, 2010; Smith et al., 2008b; Tripp and Manos, 2008; Whittall and Hodges, 2007). While this is common technique, there are concerns associated with it. The first is that PCA is generally performed with quantitative measurements of floral parts, whereas traditional pollination syndromes are based on floral traits that are categorical (Faegri and van der Pilj, 1979). The second concern is that assigning pollinator syndromes to groups of taxa that show clustering in PCA results may not give enough resolution to accurately capture the variability in actual pollination

systems. Finally, inferring pollinators using PCA based on floral part measurements, and then using these results to discuss the evolution of floral traits and pollinator relationships could be circular.

To address the potential problem with PCA, I compared the two ordination techniques using quantitative floral trait measurements and the commonly used PCA and the traditional pollination syndromes and NMDS. For NMDS, when the subsections of *Oenothera* have all the same or nearly same floral traits that were used to define the pollination syndromes, they would obviously form close to a tight cluster or singular point in the phenotypic space; however, they also have to be distinctive enough from other subsections in order to identify them as a separate group. For example, subsection *Megapterium* all cluster very tightly together, and due to the long floral tube, they are separated from all other subsections. Subsection *Gaura* taxa have a zygomorphic floral shape that is different from the more classic *Oenothera* flower, and it is not surprising that they form a recognizable cluster in the phenotypic space. However, given the wide range on pollination systems in subsection *Gaura*, they cannot be assigned to one pollination syndrome.

Unlike the NMDS results, which showed some discernable clustering of the *Oenothera* species, the PCA showed no clustering of species into groups. There was a possible trend for the subsection *Megapterium.* This is not surprising, given that the species in subsection *Megapterium* have very long floral tubes compared to their sister taxa. Broadly, long floral tube plants are pollinated by hawkmoths, and one could assign a pollinator syndrome to this cluster of species; but hawkmoths also pollinate many of the taxa in subsection *Gaura*, which have very short floral tubes. Overall, the PCA does not

provide resolution sufficient to discern groupings of taxa that correspond to pollinator syndromes, and therefore it is not useful as a tool to predict pollinator system.

Although many studies use PCA to group species and then assign pollination syndromes to those groups, this approach would not work for *Oenothera*. PCA results based on quantitative trait measurement data do not take into account traits such as temporal variation that can discern between species with different pollinators. For instance, *O. suffulta ssp. suffulta* and *O. suffulta ssp. nealleyi* are sister taxa that are morphologically the same, however *O. suffulta ssp. nealleyi* is open and pollinated both day and night. The dominant pollinator of *O. suffulta ssp. suffulta* is moths, and the dominant pollinator of *O. suffulta ssp. nealleyi* is bees.

For both PCA and NMDS there is some grouping of taxa by subsection and appearance, but it is not sufficient to accurately infer the dominant pollinator group. NMDS gives better resolution, but is still limited in its predictive power. With either of these ordination techniques, pollination syndromes are not useful as a predictive tool for pollination system.

Conclusions

Overall, we find that pollination syndromes are not appropriate for inferring the current pollination system for a given species of *Oenothera*, and this may also apply to other taxonomic groups. . However, pollination syndromes are very important for our understanding and discussion of the broad trends in angiosperm evolution. While pollinators are important selection forces that influence the development of floral form, there are also multiple factors that have also influenced floral form, such as multiple

dominant pollinator groups, antagonistic interactions, and pleiotropic effects on other plant traits (Reynolds et al., 2009; Strauss and Irwin, 2004). Pollination syndromes are a useful concept for guiding research questions and hypothesis development. They provide a clear example of convergent evolution of floral form due to pollinator mediated selection. Pollination syndromes can help us understand the functional significance of floral trait combinations. However, to determine the current pollination system for a species, direct observation and data collection are still necessary.

Table 3-1. A comparison of the predicted pollinator and the main pollinators for the 54 *Oenothera* species. Predicted pollinators are determined by the closest idealized pollination syndrome to the *Oenothera* species in the multivariate space. The main pollinators are determined by ecological data.

Section	**Species**	**Predicted Pollinator**	**Main Pollinator**
Gauropsis	*O. canescens*	bird	moth/hawkmoth
Hartmannia	*O. deserticola*	butterfly	bee
Hartmannia	*O. platanorum*	bird	bee
Hartmannia	*O. rosea*	butterfly	bee
Hartmannia	*O. speciosa*	moth	moth/hawkmoth
Hartmannia	*O. texensis*	bird	bee
Xanthocoryne	*O. epilobiifolia s. epilobiifolia*	fly	bird
Xanthocoryne	*O. epilobiifolia s. cuphrea*	bird	bird
Xanthocoryne	*O. multicaulis*	fly	bird
Xanthocoryne	*O. seifrizii*	fly	bird
Leucocoryne	*O. dissecta*	moth	moth/hawkmoth
Leucocoryne	*O. kunthiana*	beetle	moth/hawkmoth
Leucocoryne	*O. luciae-julianae*	moth	moth/hawkmoth
Leucocoryne	*O. orizabae*	beetle	moth/hawkmoth
Leucocoryne	*O. tetraptera*	beetle	moth/hawkmoth
Paradoxus	*O. havardii*	moth	moth/hawkmoth
Megapterium	*O. brachycarpa*	moth	moth/hawkmoth
Megapterium	*O. coryi*	moth	moth/hawkmoth
Megapterium	*O. howardii*	moth	moth/hawkmoth
Megapterium	*O. macrocarpa s. macrocarpa*	bird	moth/hawkmoth
Peniophyllum	*O. linifolia*	bird	none/fly/bee
Kneiffia	*O. fruticosa s. fruticosa*	butterfly	bee
Kneiffia	*O. fruticosa s. glauca*	butterfly	bee
Kneiffia	*O. riparia*	butterfly	bee
Kneiffia	*O. perennis*	bird	bee
Kneiffia	*O. pilosella s. pilosella*	butterfly	bee
Kneiffia	*O. pilosella s. sessilis*	butterfly	none
Kneiffia	*O. spachiana*	bird	none
Gaura	*O. anomala*	moth	moth/hawkmoth
Gaura	*O. glaucifolia*	fly	bee/fly/beetle
Gaura	*O. curtiflora*	beetle	none
Gaura	*O. arida*	beetle	moth/hawkmoth
Gaura	*O. suffrutescens*	moth	moth/hawkmoth
Gaura	*O. boquillensis*	moth	moth/hawkmoth

Gaura	*O. cinerea s. cinera*	moth/beetle	moth/hawkmoth
Gaura	*O. cinerea s. parksii*	moth	none
Gaura	*O. calcicola*	moth	moth/hawkmoth
Gaura	*O. filipes*	moth	moth/hawkmoth
Gaura	*O. mckelveyae*	moth	moth/hawkmoth
Gaura	*O. sinuosa*	bee/moth	moth/hawkmoth
Gaura	*O. xenogaura*	moth	moth/hawkmoth
Gaura	*O. coloradoensis s. coloradoensis*	moth	moth/hawkmoth
Gaura	*O. coloradoensis s. neomexicana*	moth	moth/hawkmoth
Gaura	*O. demareei*	bee	bee
Gaura	*O. filiformis*	moth	moth/hawkmoth/bee
Gaura	*O. gaura*	moth	moth/hawkmoth
Gaura	*O. lindheimeri*	bee	bee/butterfly
Gaura	*O. hexandra s. hexandra*	moth	moth/hawkmoth
Gaura	*O. hexandra s. gracilis*	moth	moth/hawkmoth
Gaura	*O. patriciae*	moth	moth/hawkmoth
Gaura	*O. simulans*	beetle	moth/hawkmoth
Gaura	*O. suffulta s. suffulta*	moth	moth/hawkmoth
Gaura	*O. suffulta s. nealleyi*	moth	moth/hawkmoth/bee
Gaura	*O. triangulata*	beetle	moth/hawkmoth

Table 3-2. Mean measurement of flower morphology (in mm) for 54 species of *Oenothera*.

Species	Floral Tube Length	Floral Tube Mouth Width	Corolla Span	Stamen Length	Style Length
O. canescens	12.5	2	17	24.5	7
O. deserticola	7.75	2	25.5	16.5	6.75
O. platanorum	11.5	2.25	23	15.5	6.5
O. rosea	6	2	14	10.25	5
O. speciosa	18.5	4	60	37.5	16
O. texensis	18	3.5	33	28	11
O. epilobiifolia s. epilobiifolia	11	5.25	14.5	10.25	2.25
O. epilobiifolia s. cuphrea	11	5.25	14.5	10.25	2.25
O. multicaulis	5.75	2.75	10.5	7.25	3.25
O. seifrizii	13	4.75	20	18	5.75
O. dissecta	38.5	4.5	60	58.5	13.5
O. kunthiana	19.5	4	26	23	10
O. luciae-julianae	16.5	4	42	25	8.5
O. orizabae	12	4	40	24	5
O. tetraptera	34	4.75	50	43	11.5
O. havardii	52.5	3.85	51	75.5	16.5
O. brachycarpa	165	7.5	103	155	26
O. coryi	87.5	6.5	78	120	21
O. howardii	85	7	100	127.5	31.5
O. macrocarpa s. macrocarpa	105	8	115	147.5	35
O. linifolia	1.5	0.1	8	1.5	1.5
O. fruticosa s. fruticosa	10	1	40	15	10
O. fruticosa s. glauca	12.5	1	35	16	10
O. riparia	13.72	1.95	31.96	13.3	11.08
O. perennis	6.5	1	15	3.5	3.5
O. pilosella s. pilosella	17.5	1	45	15	11
O. pilosella s. sessilis	12.5	1	29.23	11	8
O. spachiana	7	1	19	5	5
O. anomala	3.4	0.5	37	5.25	11.5
O. glaucifolia	9.5	0.1	10	6.5	6.5
O. curtiflora	3.25	0.1	5.5	6	2.25
O. arida	11	0.25	15	20	4
O. suffrutescens	7.5	1	10	15.5	4.75
O. boquillensis	5.75	0.25	14	10.75	3.25
O. cinerea s. cinera	3.5	0.25	21.5	14.25	8
O. cinerea s. parksii	2.75	1	19	12.5	6.75
O. calcicola	6	1	17.5	14.25	5

O. filipes	4.25	0.5	15	13.75	5.75
O. mckelveyae	2.75	0.25	17.5	12.5	7
O. sinuosa	3.75	1.671	21.5	15.25	8
O. xenogaura	9	1	16	19	6.25
O. coloradoensis s. coloradoensis	8	1	20.5	22	7.75
O. coloradoensis s. neomexicana	8	1	24.3	25	7.75
O. demareei	8.5	1	28	24.5	12.5
O. filiformis	8.75	1	21.5	22.75	9
O. gaura	9.25	1.95	18.5	13.5	7.5
O. lindheimeri	6.5	0.25	25.5	21.25	9.75
O. hexandra s. hexandra	6	0.25	11.5	11.75	2.9
O. hexandra s. gracilis	8	1.7	15	15	5
O. patriciae	9.25	0.25	20.5	19.5	6.5
O. simulans	5.5	0.8	12.5	13.25	4.25
O. suffulta s. suffulta	10.25	2	10	24	7.5
O. suffulta s. nealleyi	15	2	13	29	10.5
O. triangulata	4.75	0.25	8.5	9.5	2.75

Table 3-3. Eigenvector coefficients for the morphological characters used in the PCA analysis.

Floral Trait	**Axis 1**	**Axis 2**
Floral tube length	0.467	0.158
Floral tube opening width	0.392	0.801
Corolla Span	0.461	-0.253
Length of Stigma	0.465	-0.093
Length of Stamen	0.448	-0.510

Figure 3-1. Non-metric multidimensional scaling (NMDS) ordination of the 537 idealized pollination syndromes utilizing a modified matrix of traits by Ollerton et al. 2009. Each syndrome has multiple alternatives that group together in a cluster of points that define each of the 10 pollination syndromes phenotypic multivariate space.

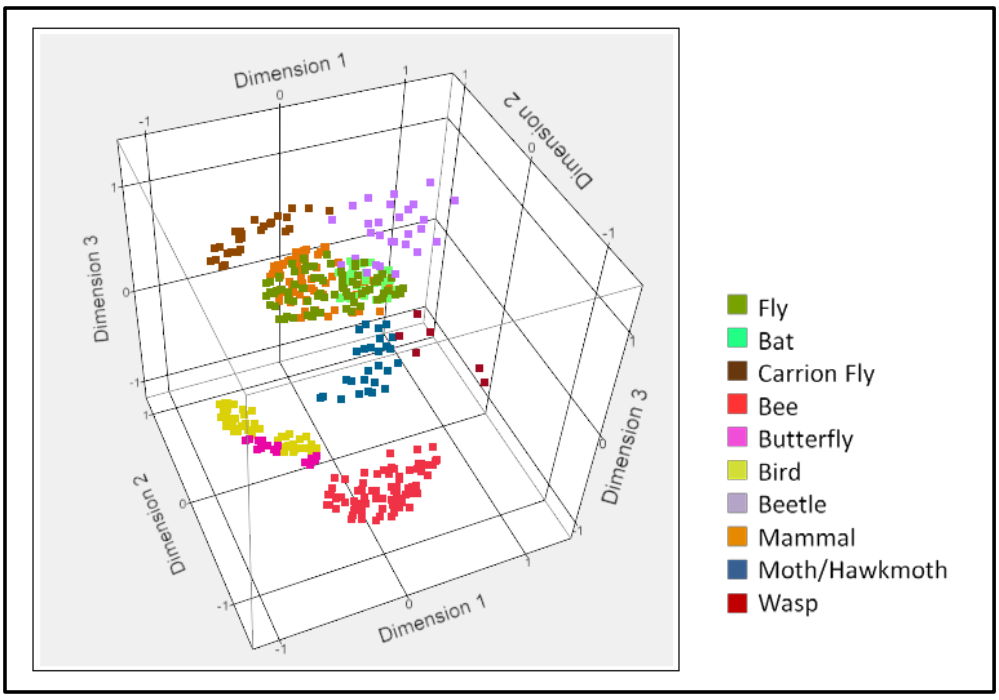


Figure 3-2. The 54 *Oenothera* species mapped in the phenotypic multivariate space of the idealized pollination syndromes.

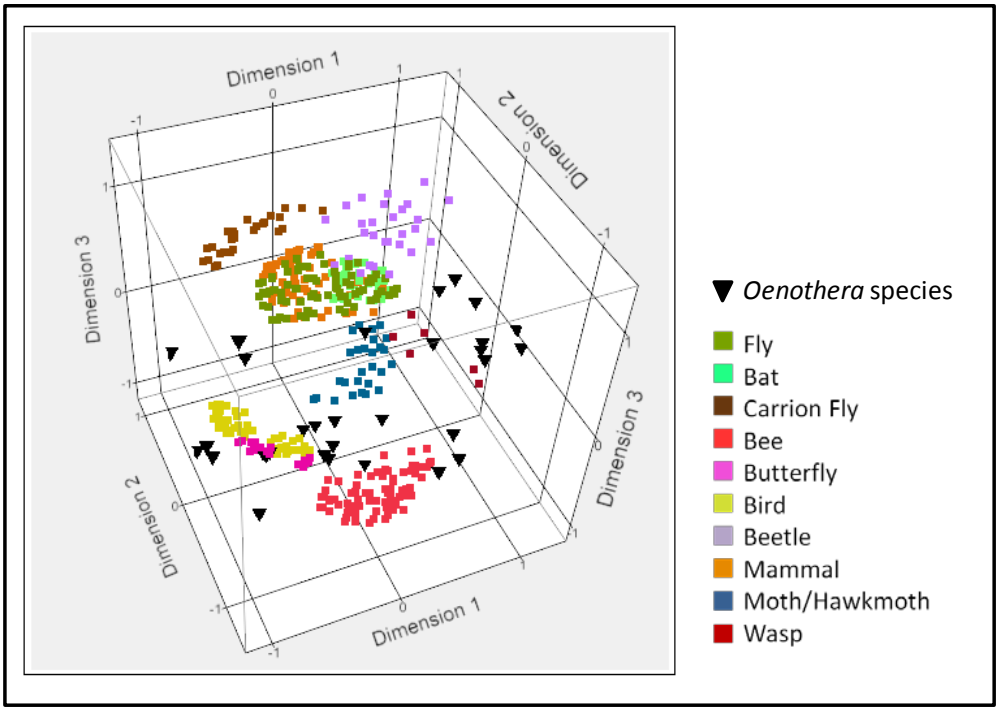


Figure 3-3. NMDS ordination of the 54 *Oenothera* species based on the same 9 floral traits defined by 35 vectors as the previous analyses. Results are color-coded by subsection.

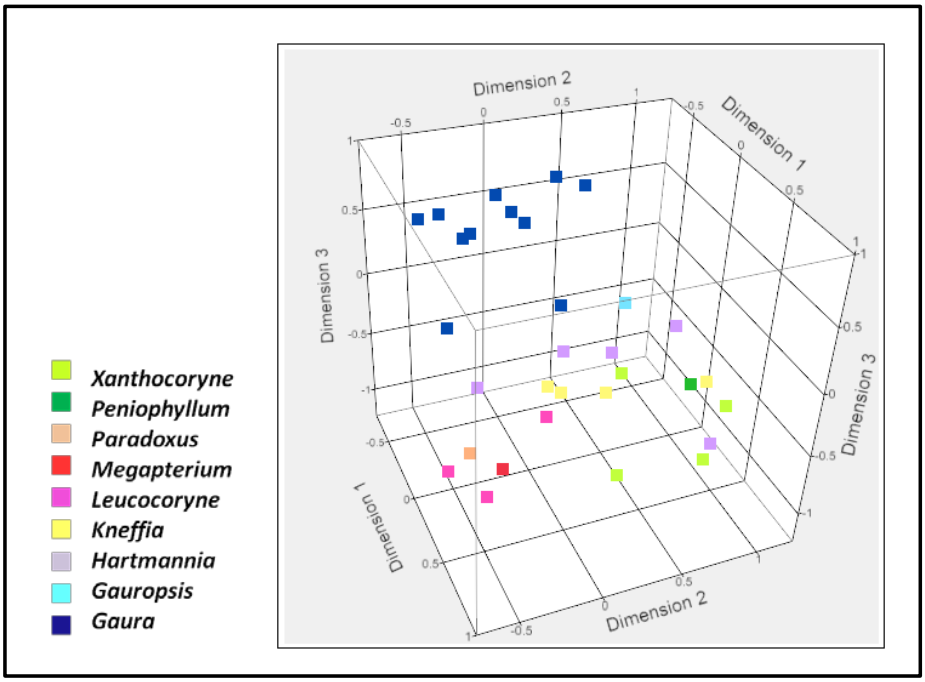
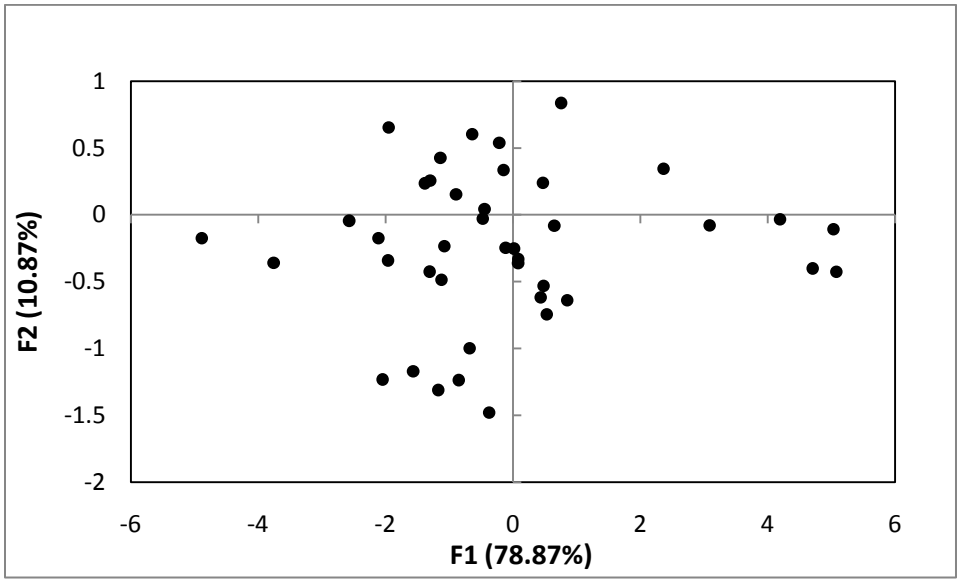


Figure 3-4. Floral morphology of 54 species of *Oenothera* plotted in the two dimensional space defined by principle component analysis (PCA) of 5 floral measurements.



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CHAPTER 4

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MOLECULAR PHYLOGENETICS AND ORIGINS OF SELF COMPATIBILITY IN *OENOTHERA* (ONAGRACEAE)

Introduction

The rapid radiation of angiosperms is due in part to their adaptive relationship with pollinators (Crane et al., 1995; Crepet et al., 2004; De Bodt et al., 2005), which has resulted in an amazing pattern of floral diversity. An important contributor to the origin of this diversity is the repeated evolution of self-compatible breeding systems, because they provide a mechanism of rapid reproductive isolation (Baker, 1955; Barrett, 2002a; Barrett et al., 1996). Although self-compatibility can be detrimental due to the negative impacts of inbreeding, it can also provide advantages such as reproductive assurance during periods of low pollinator availability (Barrett, 2002a; Goodwillie, 1999; Kaliz, 1999; Moeller, 2006; Waser and Ollerton, 2006). The transition from self-incompatibility to self-compatibility is a well-established evolutionary transition in angiosperms (Charlesworth, 2006; Grant, 1981; Igic and Kohn, 2006; Stebbins, 1974).

Here we investigate the phylogenetic history of *Oenothera*, which represents part of a major radiation of tribe Onagreae from Mexico into North America (Katinas et al., 2004). *Oenothera* provides a model system for understanding the evolution of plant reproductive systems (Artz et al., 2010; Clinebell et al., 2004; Evans et al., 2005; Hoch et al., 1993; Hoggard et al., 2004; Johnson, 2010; Johnson et al., 2009a; Moody-Weis and Heywood, 2001; Raguso et al., 2007; Raven, 1988; Theiss et al., 2010; Vilela et al., 2008). Breeding system is thought to have played a key role in the diversification of Onagraceae (Raven, 1979; Raven, 1988). A great diversity of breeding and pollination systems have evolved within *Oenothera*, even among closely related species, but it is not clear whether these differences are due to shared evolutionary history (Freckleton, 2000;

Sanderson and Donoghue, 1996) or reflect repeated independent adaptations to varying ecological conditions.

Recent molecular phylogenetic analyses have clarified relationships within Onagraceae (Levin et al. 2003; Hoggard et al. 2004; Levin et al. 2004) and have provided the basis for a new classification for the family (Hoggard et al., 2004; Levin et al., 2004; Levin et al., 2003; Wagner et al., 2007). These studies have delimited two well-supported major lineages within *Oenothera* (Levin et al., 2004; Wagner et al., 2007): Subclade A (88% BS), which comprises sections *Oenothera, Kleinia, Anogra, Ravenia, Eremia, Contortae,* and *Pachylophus*; and Subclade B (100% BS), which includes sections *Megapterium, Kneiffia, Paradoxus, Peniophyllum, Hartmannia, Gauropsis, Leucocoryne, Xanthocoryne,* and *Gaura*. This paper focuses on the reproductive evolution of Subclade B.

Subclade B encompasses considerable floral diversity in terms of morphology, breeding system, and pollination systems. Whereas species of Subclade A typically have yellow actinomorphic flowers of varying sizes; those of Subclade B have white, yellow, pink, red, or purple flowers even more variable in size, and the flowers of sect. *Gaura* are zygomorphic, with all of the petals arranged in the upper half of the floral plane and the pistil and stamens in the lower half, a distinctive character state unique to this lineage (Raven and Gregory 1972; Carr et al. 1990). The zygomorphic flowers of sect. *Gaura*, coupled with its indehiscent, mostly stipate fruits containing a reduced number of seeds, led to classifications that consistently placed this group apart from *Oenothera* from the time it was described by Linnaeus in 1753 until 2007 (Wagner et al. 2007).

The recent molecular analyses revealed that not only is sect. *Gaura* nested within *Oenothera,* but the formerly segregate genus *Stenosiphon* is nested within sect. *Gaura* as is *O. glaucifolia* (Hoggard et al. 2004; Levin et al. 2004). Levin et al. (2004) also placed *O. fruticosa* (sect. *Kneiffia*) sister to sect. *Gaura* with weak support, but they found that *O. linifolia* (sect. *Kneiffia* according to Straley 1977) did not form a clade with *O. fruticosa* and now is segregated as sect. *Peniophyllum* (Wagner et al. 2007); they did not further test the monophyly of sect. *Kneiffia*. In fact, Levin et al. (2004) sampled only one or two taxa in all sections in Subclade B, thus, in order to adequately to assess the reproductive evolution of this group, we need a phylogeny based on more comprehensive sampling of the entire clade. In Chapter 5 I report separately on the molecular phylogenetics of sect. *Kneiffia,* but we include those results in the overall analysis here.

In this study, we used the nuclear sequences *ITS* and *ETS* and the chloroplast markers *rps16, ndhF, trnL-F,* and *rbcL* to estimate the phylogeny of 45 species of *Oenothera* Subclade B. *ITS, ETS, rps16,* and *trnL-F* have proved useful in clarifying specific and generic relationships in Onagraceae (Hoggard et al., 2004; Johnson et al., 2009b; Levin et al., 2004), and *rbcL* and *ndhF* in showing deeper node relationships in the Onagraceae (Conti et al., 1993; Hoch et al., 1993; Levin et al., 2003). We use all six markers on a more thorough sampling to taxa to evaluate phylogenetic relationships of *Oenothera* Subclade B.

Our specific objectives were to evaluate the monophyly of the sections *Hartmannia, Leucocoryne, Xanthocoryne, Megapterium, Kneiffia,* and *Gaura,* to verify the relationships within Subclade B hypothesized by Levin et al. (2004), and to map

breeding system onto the phylogeny and test for multiple origins of self-compatibility in *Oenothera*.

Materials/Methods

Taxon Sampling

The tissue used in this study comprises 45 of the 50 species of *Oenothera* in subclade B; we were unable to obtain seed or tissue for the remaining five species (Table 4-1). We used tissue samples from herbarium sheets at the Missouri Botanical Garden Herbarium for two populations each of *O. spachiana, O. howardii,* and *O. coryi*. Rachel Levin (Amherst College) provided the sequence data for *O. canescens, O. rosea, O. speciosa, O. multicaulis, O. tetraptera, O. brachycarpa,* and *O. fruticosa,* which is also available on GenBank (Levin et al., 2004; Levin et al., 2003). Sequence data for all six species of section *Kneiffia* are reported separately (Chapter 5). Sequence data for *O. boquillensis, O. mckelveyae, O. filiformis,* and *O. gaura* were all provided by Gloria and Ron Hoggard (University of Oklahoma) (Hoggard et al., 2004), and is also available on GenBank. For the remaining *Oenothera* species, fresh tissue was obtained from the study sites listed in Table 2. At each site, leaves and floral buds were collected and dried in silica. We used the following taxa as outgroups: *Calylophus lavandulifolius, Calylophus serrulata, O. psammophila, O. lacinata, O. heterophylla,* and *O. albacaulis.* Levin et al. (2003, 2004) and Hoggard et al. (2004) determined these taxa as appropriate outgroups for Subclade B in *Oenothera*. The sequences were provided by Gloria and Rod Hoggard (Hoggard et al., 2004) and Rachel Levin (Levin et al., 2004; Levin et al., 2003), and are available on GenBank.

Breeding Systems

The breeding systems for many of the species of Subclade B have been previously described (Raven and Gregory, 1972; Straley, 1977; Wagner et al., 2007), and are summarized in Table 4-3. To determine and verify the breeding system of the remaining 21 *Oenothera* species, we conducted hand-pollination experiments. These experiments were conducted in the greenhouse for *O. platanorum*. For the other 20 *Oenothera* species, these experiments took place at the described field sites during peak flowering season. The evening or day (depending on flowering time for the species) prior to the experiment we randomly chose ten flowering plants, and placed bags of bridal veil netting over a pair of mature buds on each plant (Lipow et al., 2002). When each flower opened, we pollinated it with either its own or outcrossed-pollen. For the outcrossed flowers, the bag was lifted and the flower's stamens were removed. The stigma was then coated with pollen from a single flower from another plant in the population. Following treatments, the bags were replaced over the flowers for the duration of flowering time. These same protocols were followed for the greenhouse populations of *Oenothera*.

After twenty-four hours, all tested flowers were collected and fixed in a solution of 3:1 95% ethanol:glacial acetic acid for 2 hours. The flowers were then transferred to 70% ethanol for storage and transport. To determine the number of pollen grains on the stigma, and the number of pollen tubes reaching the ovary, the pistil and ovary were dissected from each flower. These tissues were placed in a beaker, covered with a 10% solution of sodium sulfide and heated to 65° C to soften the tissue. They were then washed in de-ionized water and cooled for 15 minutes. Each pistil and ovary was placed on a glass slide, and ovaries were sliced in half and placed face up. Three to five drops of decolorized aniline blue was added to each slide, and the sample was then covered with a

glass coverslip. The softened pistil and ovary tissue was spread by pressing the coverslip in a gentle tapping motion. The completed and labeled slides were refrigerated for a minimum of 24 hours. A Zeiss Univeral microscope with a 100 watt mercury bulb to provide fluorescent light was used to view the pollen tubes.

We recorded the number of pollen grains on the stigma, the number of pollen tubes in the style, and the number of pollen tubes that reached the ovary as a measure of pollination success (Lipow et al., 2002). We performed paired t-tests, assuming equal variance, comparing the selfed vs outcrossed treatment groups for percentage of pollen tubes that reached the ovary. A species was considered self-compatible if there was no significant difference between the two groups.

DNA Isolation, Amplification and Sequencing

DNA was extracted from each species using the Viogene plant DNA isolation kits (www.viogene.com). We amplified 604 bp of the nuclear internal transcribed spacer region (*ITS*), and 1803 bp of the nuclear external transcribed spacer region (*ETS*). We also amplified four cholorplast markers; 966 bp of *trnL-F*, 867 bp of *rps16*, 1054 bp of *ndhF*, and 1268 bp of *rbcL*. PCR reactions contained 25 µL reactions of Promega (www.promega.com) 5x buffer, 2.5 µL of 25 mM MgCl2, 2.5 µL of 0.2 µM dNTPs, 2.5 µL of 0.2 µM of each primer, 0.125 µL (1 unit) of Promega GoTAq DNA polymerase, and 2 µL of template DNA at approximately 5 ng/µL. The PCR program for amplification was 95°C for 3 min, followed by 35 cycles of 95°C for 1 min, annealing temperature for 40 s, and 72°C for 45 s, with a final elongation at 72°C for 7 min. The annealing temperatures and primers are listed in Table 4-4. We used electrophoresis gel techniques to visually examine the PCR results. All PCR products were purified using

Viogene gel purification kits (www.viogene.com). All gene regions were sequenced in both forward and reverse directions on an ABI 3330 at the Washington University Genome Sequencing Center. We manually edited the DNA sequences using SEQUENCHER 4.8 (Ann Arbor, MI) and aligned by hand in GENEDOC.

Phylogenetic Reconstruction

We conducted separate Bayesian analyses for each nuclear gene and for a concatenated dataset of chloroplast genes in MrBayes v3.1.2 (Huelsenbeck et al., 2001; Ronquist and Huelsenbeck, 2003). The cholorplast genes were concatenated because these organelle genes are often inherited as a unit without recombination (Birky, 2001; Reboud and Zeyl, 1994). The models of nucleotide evolution for each of the six gene regions were estimated independently in jModeltest (Posada 2008) by the AIC method. We unlinked parameters across chloroplast loci to allow for independent evolution. The Markov chain Monte Carlo (MCMC) search algorithm of MrBayes was used to reconstruct the evolutionary history of the 45 taxa in Subclade B of *Oenothera* in all three trees (*ETS*, *ITS*, concatenated chloroplast genes). Each search was run on four chains, 3 cold and 1 hot, for 10 million generations with a sampling frequency of 200 generations. When the standard deviation between the log-likelihood scores of two replicate runs was <0.0001, we concluded convergence. Tracer was used to evaluate the convergence of parameters estimated during the analysis, such that each of the 17 model parameters had Effective Sample Sizes (ESS) > 500 and the log–likelihood of the model had reached a plateau. The first 25% of the resulting trees were discarded as “burn-in” after an inspection of the likelihood plots. A majority rule consensus tree was computed using the sumt command in MrBayes and posterior probabilities were averaged across runs.

We compared the individual gene trees from our six data sets using an SH-test (Shimodaira and Hasegawa, 1999) in PAUP* (Swofford, 1999). When all gene tree topologies were deemed compatible (see Results), we concatenated all genes, retained unique models of evolution and unlinked parameters for a final run with the above parameters and 30 million generations.

Independent Origins of Self-Compatibility

We tested the strength of the phylogenetic signal for breeding system (SC or SI) using parsimony in Mesquite (Maddison and Maddison 2004).

The transition from self-incompatibility to self-compatibility is considered the only direction possible in flowering plants, because this transition represents the loss of a complex function (Charlesworth, 2006). Because transition rates between breeding system traits are not equal, ancestral state reconstructions can lead to incorrect conclusions about the evolutionary history of dichotomous traits (Igic et al., 2006; Igic and Kohn, 2006). Accordingly, every SC species was considered to represent an independent origin of selfing unless it was sister to another SC species, in which case the largest clade of 100% SC species was inferred to represent a single origin of self-compatibility. We observed a single instance where there was not enough resolution in the phylogenetic reconstruction to differentiate between 1 and 2 transitions in the clade containing *O. patriciae* (SC), *O. triangulata* (SC), and *O. suffulta ssp. suffulta* (SI). We use topological hypothesis-testing to resolve this ambiguity by comparing the majority rule consensus topology to a topology constrained to keep the two SC species monophyletic. We used BayesFactors (Kass and Raftery, 1995) and a Likelihood ratio

tests to show that the consensus tree is significantly more likely than the 500 trees sampled from the posterior distribution of the topologically constrained trees.

Results

Breeding System

For species that were SC or SI and receiving outcross pollen, pollen grains germinated and pollen tubes entered the style within 24 hours. None of the species tested showed any evidence of late acting self-incompatibility; pollen tubes were either present and reaching the ovary, or did not penetrate the style. The percentage of pollen tubes to successfully reach the ovary for each *Oenothera* species is presented in Table 4-5. Of the 21 species we tested, 12 were SI and 9 were SC. A complete list of all the taxa in Subclade B and their breeding systems is found in Table 4-3.

Phylogenetic Reconstruction

The aligned sequence matrix for both the chloroplast and nuclear regions consisted of 6562 characters. The phylogenetic reconstructions resulted in the consensus tree shown in Fig 4-1. Figure 4-2 is a closer view to show the subsections of section *Gaura.* We refer to all nodes with posterior probabilities above 0.95 as strongly supported. After 30 million generations, the MrBayes analyses reached stationarity between the two runs and all parameters were resolved with ESS values above 500. The nexus file, with the evolutionary models of nucleotide evolution inferred from MrModelTest and the AIC, and the tree file are deposited on TreeBase.org. GeneBank accession numbers are in Table 4-1.

ITS provides support for the monophyly of the major sections in *Oenothera* Subclade B, as well as giving greater resolution for clades in sections *Hartmannia,*

Kneiffia, and *Megapterium.* In addition, *ITS* results in greater tip resolution for the clades in section *Gaura*. *ETS* also contributes greater resolution of the clades in section *Gaura*, and it provides support for the clade containing sections *Leucocoryne* and *Xanthocoryne*. The chloroplast genes *rps16* and *trnL-F* support the monophyly of sections *Gaura* and *Kneiffia*, as well as providing resolution at the species level for sections *Hartmannia*, *Leucocoryne, Xanthocoryne,* and *Megapterium*. The chloroplast genes *ndhF* and *rbcL* sequence data give deeper node support and clarify the position of species in section *Kneiffia*, *Peniophyllum*, and *Paradoxus*. The individual nuclear trees and chloroplast trees are shown in Supplemental Fig. 4-1.

Neither of the nuclear gene trees differ significantly from the concatenated chloroplast gene tree (*ITS, P*= 0.190; *ETS, P*= 0.382); however, the *ETS* and *ITS* individual gene trees are significantly different from each other (Suppl. Fig. 4-1). This disagreement is due to resolution in section *Gaura*, where there are alternative placements for *O. xenogaura*. *Oenothera xenogaura* is of hybrid origin from two species in widely separate lineages, and these alternative placements are consistent with other studies of *Gaura* (Hoggard et al., 2004). Therefore, we exclude the *ITS* region for *O. xenogaura* in the combined analysis. After excluding this species, none of the pairwise S-H tests among regions differed, ensuring that concatenating genes was appropriate for estimating a species tree from a multilocus gene tree.

Independent Origins of Self-Compatibility

There is strong phylogenetic signal for the character of breeding system (*P* = 0.0001, 95% CI 15-22 steps, unordered steps=14). We defined each transition to SC as the most inclusive monophyletic grouping of only SC species. Using this metric, we infer

12 transitions with strong topological support from SI to SC in *Oenothera* Subclade B. A thirteenth and possibly fourteenth transition is inferred for the clade containing *O. patriciae, O. triangulata,* and *O. suffulta ssp. suffulta*. Our topological tests for support of monophyly of the SC species, *O. patriciae* and *O. triangulata,* were inconclusive. The harmonic mean of the two replicate runs for the unconstrained tree was -15285.2 and resulted in separate transitions for *O. triangulata* and *O. patriciae,* which makes our total number of transitions 14. However, when we constrain these two species to be sister to one another, the resulting phylogeny has a harmonic mean -15284.7. Neither BayesFactors nor a log-likelihood ratio test identifies this difference as significant. At present, we are unable to distinguish between 13 and 14 transitions.

Discussion

Phylogenetic structure of Oenothera

The consensus tree of *Oenothera* Subclade B (Fig 4-1) encompasses 45 species and contains all but five species in these sections. Our phylogenetic tree is consistent with (Levin et al., 2004; Wagner et al., 2007) that also show support for the nine sections circumscribed in Subclade B. The additional 26 taxa and 3 markers did not alter the basic structure or weaken support for Subclade B and the sections in it. This new phylogeny does provide greater insight and clarity to the relationships of these *Oenothera*.

Section *Gauropsis* (comprising only *O. canescens*) is strongly supported as sister to section *Hartmannia,* and the clade of those two sections as sister to the rest of Subclade B. In section *Hartmannia*, *O. platanorum* is strongly supported as sister to *O. rosea,* and those species in turn sister to *O. speciosa*. The monophyly of sect. *Hartmannia*

cannot be fully tested until the two missing taxa (*O. deserticola* and *O. texensis*) can be included.

The taxa in sect. *Leucocoryne* and *Xanthocoryne* were included in sect. *Hartmannia* (Munz, 1965; Raven and Parnell, 1970), but recent studies have placed them in their own sections (Wagner et al., 2007). Levin et al. (2004) placed sects. *Leucocoryne* and *Xanthocoryne* in a strongly supported clade (100% BS), but they included only one taxon of each section. Morphological characters, including floral, leaf, and capsule characters, were used to delineate the species into the two sections (Wagner et al., 2007). Our study includes two of the three species in sect. *Xanthocoryne* and three of the five in sect. *Leucocoryne*. We find strong support for a clade with sect. *Xanthocoryne* and *Leucocoryne*; however, within the clade, the taxa sort into the two sections but with inconclusively weak support . We do find that *O. kunthiana* and *O. tetraptera* form a well-resolved clade within sect. *Leucocoryne.*

Section *Megapterium* is strongly supported as monophyletic, with complete sampling of all four taxa. Previous analysis used only a single taxon to represent this section. Within this section, *O. coryi* and *O. macrocarpa* form a strongly supported clade, but relationships with and between *O. brachycarpa* and *O. howardii* are not resolved. These ambiguities may be the result of high levels of polyploidy among these taxa: *O. brachycarpa* and *O. macrocarpa* are diploid (n = 7,), *O. coryi* hexaploid (n = 21), and *O. howardii* tetraploid, hexaploid, and octoploid (n = 14, 21, 28) (Wagner et al., 2007). Additional population sampling and possibly additional gene sequences will be needed to clarify these relationships in sect. *Megapterium*.

The monotypic sections *Peniophyllum* and *Paradoxus* form a weakly supported clade that is sister to either sect. *Megapterium* or sect. *Kneiffia*, and there are as yet no morphological synapomorphies that would clarify these relationships (Wagner et al., 2007). Section *Peniophyllum* consists of only *O. linifolia*, a self-compatible annual formerly placed in sect. *Kneiffia* (Straley, 1977). Section *Paradoxus* consists of only *O. havardii*, a self-incompatible perennial restricted to the Chihuahuan desert in Texas, Arizona and northern Mexico (Wagner et al., 2007). (Wagner et al., 2007) suggested that *O. havardii* might be the sister group to sect. *Gaura*, based on morphological similarities, but our results do not support such a relationship. Further work is needed to clarify these broader relationships between these sections.

Our phylogenetic reconstruction strongly supports section *Kneiffia* as a clade (see also Chapter 4) that is moderately supported as the sister to sect. *Gaura*. Our results support *O. sessilis* as specifically distinct from *O. pilosella* (Chapter 4). Our results also support the specific recognition of *O. riparia*, which forms a strongly supported clade with *O. perennis*.

In agreement with other recent molecular studies (Hoggard et al., 2004; Levin et al., 2004), we find strong support for the monophyly of sect. *Gaura* (Fig. 1). Of the eight recognized subsections, five are monotypic *(Gauridium, Stenisiphon, Schizocarya, Xerogaura,* and *Xenogaura*) (Fig. 2). There is strong support for *O. anomala* (subsect. *Gauridium*) as the sister branch of sect. *Gaura* (Raven & Gregory 1972), and for the strongly supported clade of *O. glaucifolia* (subsect. *Stenosiphon*) and *O. curtiflora* (subsect. *Schizocarya*) as sister to the rest of the section. *Oenothera arida* (subsect.

Xerogaura) is weakly supported as sister to the remainder of the section, which is a strongly supported clade.

The recently re-circumscribed subsect. *Campogaura* (*O. boquillensis* and *O. suffrutescens;* 100% BS; Hoggard et al. 2004) is equally strongly supported in our tree, and sister to a strongly supported clade of subsections *Gaura, Xenogaura* and *Stipogaura*.

In subsection *Stipogaura,* our results clarify that *O. calcicola* and *O. cinera* form a clade with strong posterior probability support that is sister to a weakly supported clade of. *O. filipes* and *O. sinuosa*. Hoggard et al. (2004) found that these four species formed a clade (BS 74%) that was sister to *O. mckelveyae*. Our results agree with this hypothesis, with stronger support (posterior probability.99).

Oenothera xenogaura (subsect. *Xenogaura*) was hypothesized to be of hybrid origin, probably between *O. mckelveyae* (subsect. *Stipogaura*) and *O. suffrutescens* (subsect. *Campogaura*), according to Raven & Gregory (1972) or between *O. mckelveyae* and a species in subsect. *Gaura* related to *O. coloradoensis* or *O. lindheimeri* (Hoggard et al., 2004). Because of this, different markers place *O. xenogaura* in different places on our trees. In our study, we found a consensus for the position of *O. xenogaura* among all the markers except *ITS*, and so we restricted *ITS* for *O. xenogaura* in our final tree. Our results place *O. xenogaura* in subsect. *Gaura* as part of a polytomy, but with strong posterior probability support. This placement is subjective with regards to marker inclusion, and a whole genome analysis might be necessary to clarify the position of subsect. *Xenogaura*.

Subsection *Gaura* forms a clade of 10 species (12 taxa) and combines two previously recognized subsections, *Gaura* and *Pterogaura* (Raven and Gregory, 1972;

Wagner et al., 2007). Within subsect. *Gaura* our results show strong support for the six species that were previously delimited as subsect. *Gaura.* The relationships of these six species agree with earlier studies, and our data strengthen the support for these placements. For instance, Hoggard et al. (2004) found that *O. demareei* and *O. lindheimeri* formed a clade with BS of 61%; whereas we find that these two taxa, which are the day blooming and bee pollinated species of sect. *Gaura,* form a strongly supported clade. This finding provides strong support for the hypothesis that *O. demareei* arose following a hybridization between *O. filiformis* and *O. lindheimeri* (Carr et al., 1990; Wagner et al., 2007) The clade sister to this clade consists of *O. simulans, O. filiformis,* and *O. gaura,* which Hoggard et al. (2004) reported with weak support. However, our results show that *O. gaura* and *O. simulans* are sister taxa with *O. filiformis* sister to them.

The other seven taxa in subsect. *Gaura,* formerly treated as subsect. *Pterogaura,* are markedly paraphyletic. With the exception of *O. xenogaura,* these results are comparable to Hoggard et al. (2004). We also find that *O. patriciae, O. suffulta ssp. suffulta,* and *O. triangulata* form a clade (BS 89%; (Hoggard et al., 2004) and with stronger support (1.0 PP). All three species have overlapping ranges. In sympatric populations of *O. triangulata* and *O. patriciae,* intermediate individuals have been noted, which suggests that suggest these two species can hybridize (personal comm. G. and R. Hoggard, personal comm. P. Raven, personal observations) Therefore, we suggest that *O. triangulata* and *O. patriciae* are sister taxa, and that the total number of transitions to SC in *Oenothera* Subclade B is 13.

Oenothera hexandra ssp. hexandra, *O. hexandra ssp. gracilis*, and *O. suffulta ssp. nealleyi* remain unresolved, and form a polytomy in subsect. *Gaura*. These results are consistent with Hoggard et al. (2004); however, that study did not include the subspecies *O. suffulta ssp. nealleyi* or *O. hexandra ssp. gracilis*. It does not appear that *O. suffulta ssp. nealleyi* is closely related to *O. suffulta ssp. suffulta*, given the placement in the phylogeny, and the difference in scent profiles for these two taxa. *Oenothera suffulta ssp. nealleyi* has a strong sweet scent, characterized by benzaldehyde (almond), cinnamaldehyde, cinnamic alcohol (cinnamon), methyl salicylate and its methyl ether (wintergreen), neral and geranial (citronella), and nerol and geraniol (lemon) (R. Raguso, personal comm.), whereas *O. suffulta ssp. suffulta* does not have a discernable scent. This difference in scent could play a key role in the pollination syndromes for these species. Further work, with sampling across the ranges of both taxa is needed to clarify whether these taxa are truly distinct.

In conclusion, we find that sections *Megapterium, Kneiffia*, and *Gaura* are monophyletic with all species included (100% support). Section *Hartmannia* is monophyletic, but several taxa are not sampled for this study. Sections *Xanthocoryne* and *Leucocoryne* form a monophyletic group together, but within this clade, the species sort only weakly into the two sections; not all species were sampled for these two sections. Sections *Gauropsis*, *Paradoxus* and *Peniophyllum* all contain one species and are, by definition, monophyletic. The relationships of sect. *Gauropsis* are strongly supported, but those of sects. *Paradoxus* and *Peniophyllum* need further clarification. Our results show that sect. *Kneiffia* is strongly supported (1.0 PP) as sister to sect. *Gaura*.

Breeding System Transitions

We find multiple transitions from SI to SC in Subclade B of sect. *Oenothera*. Our results confirm 12 transitions, but our topological tests were unable to clarify whether there are a total of 13 or 14 transitions. This ambiguity is due to the unresolved topology in subsect. *Gaura* of the self-compatible *O. patriciae* and *O. triangulata* and the self-incompatible *O. suffulta ssp. suffulta*. Given the overlapping ranges of these species, and the possibility of hybridization between *O. patriciae* and *O. triangulata*, we make the conservative conclusion that there was only one transition to SC in this clade. A total of 13 transitions to SC in Subclade B, which has 45 species, suggests that breeding system is a highly labile trait. In accordance with previous studies of *Oenothera* (Raven, 1979; Raven, 1988), we also find that sister taxa can differ in breeding system. This kind of lability in breeding system may play a key role in the diversification of *Oenothera* (Raven, 1979). This result concurs with the idea that transitions to SC in breeding system are associated with higher rates of speciation in plants (Barrett, 2010a) .

Table 4-1. Species of Onagraceae included in the phylogenetic analyses. This sampling includes 45 *Oenothera* species and 6 outgroups from Onagraceae. For each gene we indicate the source of the data with either the GenBank accession number or as a species newly sequenced in this study (*). Data not obtained is indicated (-).

Section/ Subsection	**Species**	***ITS***	***trnL-F***	***rps16***	***ETS***	***rbcl***	***ndhF***
Gauropsis	*O. canescens*	AY271576	AY264565	AY2674438	–	–	–
Hartmannia	*O. platanorum*	*	–	*	–	*	*
	O. rosea	AY271578	AY264566	AY267440	–	–	–
	O. speciosa	AY271577	AY264565	AY267439	AJ620789	AB516355	–
Xanthocoryne	*O. epilobiifolia s. epilobiifolia*	*	–	–	*	–	*
	O. multicaulis	AY271580	AY264568	AY267442	–	–	–
Leucocoryne	*O. kunthiana*	*	*	*	–	–	*
	O. luciae-julianae	–	*	*	*	*	*
	O. tetraptera	AY271579	AY264567	AY267441	–	–	–
Paradoxus	*O. havardii*	*	*	*	–	*	*
Megapterium	*O. brachycarpa*	AY271572	AY264560	AY267435	–	AF495770	AF495793
	O. coryi	–	–	*	–	–	–
	O. howardii	*	–	*	–	–	*
	O. macrocarpa s. macrocarpa	*	*	*	*	*	*
Peniophyllum	*O. linifolia*	*	*	*	–	*	*
Kneiffia	*O. fruticosa*	AY271581	AY264569	AY267443	–	AF495771	AF495794
	O. riparia	*	*	*	*	*	*
	O. perennis	*	*	*	–	*	*
	O pilosella	*	*	*	–	*	*
	O. sessilis	*	*	*	–	*	*
	O. spachiana	–	*	*	*	*	*

Gaura/ Gauridium	*O. anomala*	*	*	*	*	*	*
Gaura/ Stenisiphon	*O. glaucifolia*	*	*	*	*	*	*
Gaura/ Schizocarya	*O. curtiflora*	*	*	*	*	*	*
Gaura/ Xerogaura	*O. arida*	*	*	*	*	*	*
Gaura/ Campogaura	*O. suffrutescens*	*	*	*	*	*	*
	O. boquillensis	AJ620518	AJ620587	AY267453	AJ620765	–	–
Gaura/ Stipogaura	*O. cinerea s. cinera*	*	*	*	*	–	*
	O. calcicola	*	–	*	*	*	*
	O. filipes	*	*	*	*	–	–
	O. mckelveyae	AJ620529	–	–	AJ620776	–	–
	O. sinuosa	*	*	*	*	*	*
Gaura/ Xenogaura	*O. xenogaura*	–	*	*	*	*	*
Gaura/Gaura	*O. coloradoensis s. neomexicana*	*	*	*	*	–	*
	O. demareei	*	*	*	*	–	–
	O. filiformis	AJ620527	AJ620595	–	AJ620774	–	–
	O. gaura	AJ620517	AJ620586	–	AJ620764	–	–
	O. lindheimeri	AJ620526	AJ620594	–	AJ620773	AM235669	AM235436
	O. hexandra s. hexandra	*	*	*	*	*	*
	O. hexandra s. gracilis	*	*	*	*	*	*
	O. patriciae	*	*	*	*	*	*
	O. simulans	*	*	*	*	*	*
	O. suffulta s. suffulta	*	*	*	*	–	*
	O. suffulta s. nealleyi	*	*	*	–	*	*
	O. triangulata	*	*	*	*	*	*
Outgroups	*Calylophus lavandulifolius*	AJ620543	AJ620603	–	AJ620790	–	–
	Calylophus serrulata	*	*	–	–	–	–
	O. psammophila	AY271571	AY264559	AY267434	*	–	–

O. lacinata	AY271561	AY264549	AY267424	AJ620787	–	–
O. heterophylla	AY271560	AY264548	AY26423	AJ620786	–	–
O. albicaulis	AJ620536	AJ620604	–	AJ620784	–	–

Table 4-2. The taxa, vouchers, and localities for new sequences analyzed in this study. Vouchers are filed at the Missouri Botanical Garden.

Section	Taxon	Location	Voucher/Citation
Hartmannia	*O. platanorum*	Sonora, Mexico	van Devender 2004-563
Xanthocoryne	*O. epilobiifolia s. epilobiifolia*	Merida, Venezuela	van der Werff and Ortiz 5963
Leucocoryne	*O. kunthiana*	Sonora, Mexico	vanDevender 2005-23A
	O. luciae-julianae	El Salto, Durango, Mexico	LM Valenzuela 3-25
Paradoxus	*O. havardii*	Brewster Co., TX, USA	Krakos 0913
Megapterium	*O. coryi*	Crosby Co., TX, USA	Wagner and Butley 3632
	O. howardii	Kane Co., UT, USA	Warren Wagner det. 4506
	O. macrocarpa s. macrocarpa	Gray Summit Co., MO, USA	Krakos 0701
Peniophyllum	*O. linifolia*	Gray Summit Co., MO, USA	Krakos 0903
Kneiffia	*O. riparia*	Pendleton Co., NC, USA	Krakos 1017
	O. perennis	Middlesex Co., MA, USA	Krakos 1010
	O pilosella	SE Washington Co., IL, USA	Krakos 0821
	O. sessilis	Prairie Co., AR, USA	Krakos 1006
	O. spachiana	Bienville Co., LA, USA	Thomas and Moreland 49150
Gaura/ Gauridium	*O. anomala*	Durango, Mexico	Clinebell 3172
Gaura/ Stenisiphon	*O. glaucifolia*	Woodward Co., OK, USA	Krakos 0815
Gaura/ Schizocarya	*O. curtiflora*	Woodward Co., OK, USA	Krakos 0816
Gaura/ Xerogaura	*O. arida*	Brewster Co., TX, USA	Krakos 0935
Gaura/ Campogaura	*O. suffrutescens*	Brewster Co., TX, USA	Krakos 0918
Gaura/ Stipogaura	*O. cinerea s. cinerea*	Union Co., NM, USA	Clinebell 2052
	O. calcicola	Brewster Co., TX, USA	Krakos 0920
	O. filipes	Richland Co., SC, USA	AB Pittman 09059612
	O. sinuosa	Cleveland Co., OK, USA	Krakos 0904
Gaura/ Xenogaura	*O. xenogaura*	Starr Co., TX, USA	Krakos 0908

Gaura/Gaura	*O. coloradoensis s. neomexicana*	Otero Co., NM, USA	Krakos 0926
	O. demareei	McCurtain Co., OK, USA	Krakos 0814
	O. hexandra s. hexandra	Durango, Mexico	Clinebell 3031
	O. hexandra s. gracilis	Brewster Co., TX, USA	Clinebell 2023
	O. patriciae	Rogers Co., OK, USA	Krakos 0801
	O. simulans	New Hanover Co., NC, USA	Krakos 1016
	O. suffulta s. suffulta	Cleveland Co., OK, USA	Krakos 0803
	O. suffulta s. nealleyi	Brewster Co., TX, USA	Krakos 0922
	O. triangulata	Rogers Co., OK, USA	Krakos 0802

Table 4-3. Summary of all breeding systems of *Oenothera* in Subclade B.

Section/ Subsection	Species	Breeding System	Data Source
Gauropsis	*O. canescens*	SC	Wagner, W. L., P. C. Hoch, et al. (2007)
Hartmannia	*O. platanorum*	SC	current study
	O. rosea	SC	Wagner, W. L., P. C. Hoch, et al. (2007)
	O. speciosa	SC	Wagner, W. L., P. C. Hoch, et al. (2007)
Xanthocoryne	*O. epilobiifolia s. epilobiifolia*	SC	Raven, P. H. (1979)
	O. multicaulis	SC	Wagner, W. L., P. C. Hoch, et al. (2007)
Leucocoryne	*O. kunthiana*	SC	Wagner, W. L., P. C. Hoch, et al. (2007)
	O. luciae-julianae	SC	Wagner, W. L., P. C. Hoch, et al. (2007)
	O. tetraptera	SC	Wagner, W. L., P. C. Hoch, et al. (2007)
Paradoxus	*O. havardii*	SI	current study
Megapterium	*O. brachycarpa*	SI	Wagner, W. L., P. C. Hoch, et al. (2007)
	O. coryi	SI	Wagner, W. L., P. C. Hoch, et al. (2007)
	O. howardii	SI	Wagner, W. L., P. C. Hoch, et al. (2007)
	O. macrocarpa s. macrocarpa	SI	current study
Peniophyllum	*O. linifolia*	SC	current study
Kneiffia	*O. fruticosa*	SI	Straley, G. B. (1977).
	O. riparia	SI	Krakos et al. in review
	O. perennis	SC	Krakos et al. in review
	O. sessilis	SC	Krakos et al. in review
	O pilosella	SI	Krakos et al. in review
	O. spachiana	SC	Straley, G. B. (1977).
Gaura/ Gauridium	*O. anomala*	SC	Raven, P. H. and D. P. Gregory (1972)
Gaura/ Stenisiphon	*O. glaucifolia*	SC	current study
Gaura/ Schizocarya	*O. curtiflora*	SC	Raven, P. H. and D. P. Gregory (1972)
Gaura/ Xerogaura	*O. arida*	SI	current study
Gaura/ Campogaura	*O. suffrutescens*	SI	current study

	O. boquillensis	SI	Raven, P. H. and D. P. Gregory (1972)
Gaura/ Stipogaura	*O. cinerea s. cinerea*	SI	Raven, P. H. and D. P. Gregory (1972)
	O. calcicola	SI	current study
	O. filipes	SI	Raven, P. H. and D. P. Gregory (1972)
	O. mckelveyae	SI	Raven, P. H. and D. P. Gregory (1972)
	O. sinuosa	SI	current study
Gaura/ Xenogaura	*O. xenogaura*	SI	current study
Gaura/Gaura	*O. coloradoensis s. neomexicana*	SC	current study
	O. demareei	SI	current study
	O. filiformis	SI	current study
	O. gaura	SC	current study
	O. lindheimeri	SI	current study
	O. hexandra s. hexandra	SC	Raven, P. H. and D. P. Gregory (1972)
	O. hexandra s. gracilis	SC	current study
	O. patriciae	SC	current study
	O. simulans	SC	current study
	O. suffulta s. suffulta	SI	current study
	O. suffulta s. nealleyi	SI	current study
	O. triangulata	SC	current study

Table 4-4. Primers and annealing temperatures used for six molecular markers.

Locus	Primer name	Primer Sequence 5' to 3'	Annealing Temperature	Citation
ITS	*ITS4*	TCC TCC GCT TAT TGA TAT GC	47° C	(Levin et al., 2004)
	ITS5HP	GGA AGG AGA AGT CGT AAC AAG G	47° C	(Levin et al., 2004)
trnL	*trnLf*	ATT TGA ACT GGT GAC ACG AG	50° C	(Levin et al., 2004)
	trnLc	CGA AAT CGG TAG ACG CTA CG	50° C	(Levin et al., 2004)
rps16	*P1839*	TCG GGA TCG CAC ATC AAT TGC AAC	55° C	(Levin et al., 2004)
	P1840	GTG GTA AAA AGC AAC GCG CGA CTT	55° C	(Levin et al., 2004)
ETS	*ETS R2*	AGA AGT CGG GGT TTG TTG C	50° C	(Hoggard et al., 2004)
	ETS F2	ACG ATC GGA TTC GTG ACC TA	50° C	(Hoggard et al., 2004)
rbcL	*P1630*	ATG TCA CCA CAA ACA GAG ACT AAA GC	53° C	(Levin et al., 2003)
	P1782	ATA CTT CAC AAG CAG CAG CTA GTT CC	53° C	(Levin et al., 2003)
ndhF	*P1786*	CCC CGA AAT ATT TGA GAC TTT CT	47° C	(Levin et al., 2003)
	P1785	GTC TCA ACT GGG TTA TAT GAT G	47° C	(Levin et al., 2003)

Table 4-5. Comparative rates of breeding system parameters for *Oenothera* species in hand pollination studies.

Species	Treatment	n (number of flowers)	Number of pollen grains on stigma	Number of pollen tubes reaching ovary	Percent of pollen tubes to reach plant ovary
O. platanorum	Self	8	737.50 (±227.96)	5.0 (±6.0)	50.29 (±.35)
	Cross	8	703.88 (±279.91)	4.0 (±5.32)	38.82 (±.27)
O. havardii	Self	7	109.29 (±70.14)	0	0
	Cross	7	149.0 (±90.81)	2.71 (±2.14)	35.88 (±.31)
O. macrocarpa	Self	12	187.08 (±130.08)	0	0
	Cross	12	142.08 (±123.54)	2.67 (±3.11)	28.44 (±.30)
O. linifolia	Self	7	81.86 (± 84.23)	3.29 (±2.93)	38.43 (±.35)
	Cross	7	85.86 (±96.65)	3.00 (±2.24)	38.33 (±.23)
O. glaucifolia	Self	8	111.38 (±34.80)	1.00 (±1.31)	31.62 (±.36)
	Cross	8	104.25 (±50.75)	1.25 (±1.28)	48.59 (±.67)
O. arida	Self	5	86.0 (±28.15)	0	0
	Cross	5	92.8 (±51.88)	2.4 (±1.82)	30.0 (±.18)
O. suffrutescens	Self	8	71.38 (±49.81)	0	0
	Cross	8	91.38 (±56.13)	2.75 (±1.67)	47.60 (±.28)
O. calcicola	Self	8	112.75 (±47.02)	0	0
	Cross	8	127.63 (±46.93)	1.88 (±1.81)	25.45 (±.20)
O. sinuosa	Self	9	108.13 (±123.83)	0	0
	Cross	9	193.75 (±69.89)	3.13 (±1.36)	13.54 (±.26)
O. xenogaura	Self	9	65.0 (±65.89)	0	0
	Cross	9	88.22 (±51.72)	2.38 (±2.50)	37.73 (±.39)
O. coloradoensis s. neomexicana	Self	11	63.64 (±41.03)	2.73 (±2.90)	30.79 (±.31)
	Cross	11	73.0 (±27.23)	6.09 (±3.30)	49.70 (± .17)
O. demareei	Self	9	126.56 (±67.29)	0	0
	Cross	9	115.56 (±65.45)	3.33 (±1.80)	68.55 (±.32)

O. filiformis	Self	21	164.71 (±89.51)	0	0
	Cross	19	124.79 (±89.60)	1.63 (±1.86)	19.41 (±.24)
O. gaura	Self	10	207.90 (±197.44)	1.60 (±1.17)	45.95 (±.38)
	Cross	10	236.20 (±229.10)	2.30 (±1.70)	61.17 (±.35)
O. lindheimeri	Self	6	53.0 (±26.24)	0	0
	Cross	6	53.67 (±25.84)	.83 (±.98)	17.26 (±.21)
O. hexandra s. gracilis	Self	8	144.86 (±93.14)	1.0 (±1.36)	36.84 (±.38)
	Cross	9	156.0 (±104.70)	1.67 (±2.12)	21.16 (±.29)
O. patriciae	Self	10	381.9 (±225.28)	3.7 (±1.16)	48.0 (±.12)
	Cross	10	488.2 (±67.20)	4.4 (±.84)	60.01 (±.15)
O. simulans	Self	10	51.7 (±35.93)	2.0 (±1.33)	45.83 (±.25)
	Cross	10	31.4 (±17.58)	2.0 (±1.16)	34.25 (±.22)
O. suffulta s. suffulta	Self	17	180.53 (±129.19)	0	0
	Cross	10	101.0 (±52.59)	1.5 (±1.27)	0.6 (±.4295)
O. suffulta s. nealleyi	Self	10	198.2 (±152.16)	0	0
	Cross	10	103.5 (± 37.78)	3.0 (±1.56)	.4658 (±.2526)
O. triangulata	Self	10	227.6 (±204.03)	1.8 (±1.23)	.3724 (±.2764)
	Cross	10	193.0 (±40.57)	5.7 (±2.16)	.6598 (±.1456)

Figure 4-1. Bayesian phylogenetic reconstructions from concatenated mixed model data set for genes *ITS, ETS, rps16, trnL-F, rbcL*, and *nadH*. 30 million runs, SD of .005586, 45 *Oenothera* species. Self-incompatible species are in bold, self-compatible species with an asterisk. Numbers above nodes indicate Bayesian posterior probability values. Subclade B is noted.

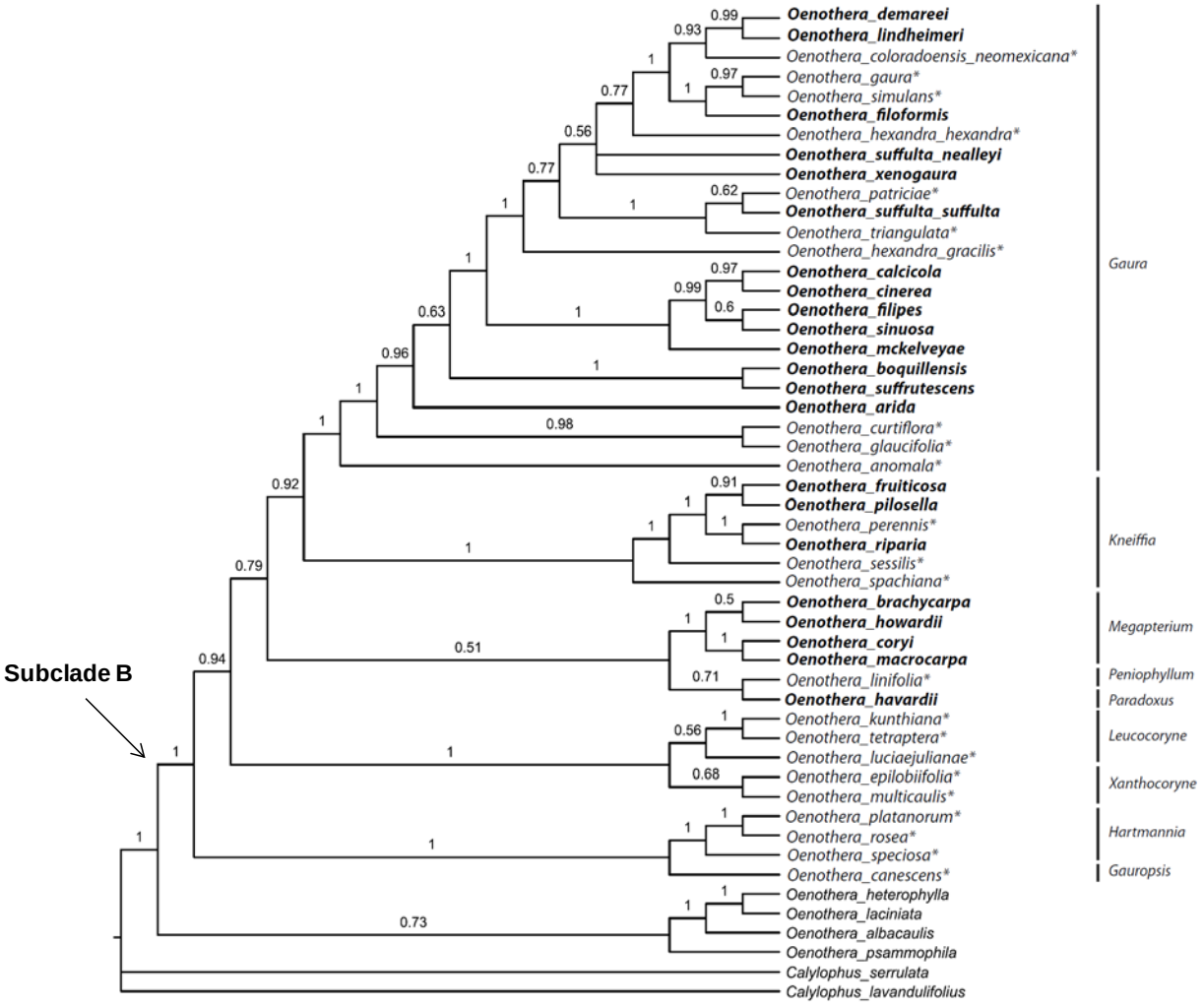
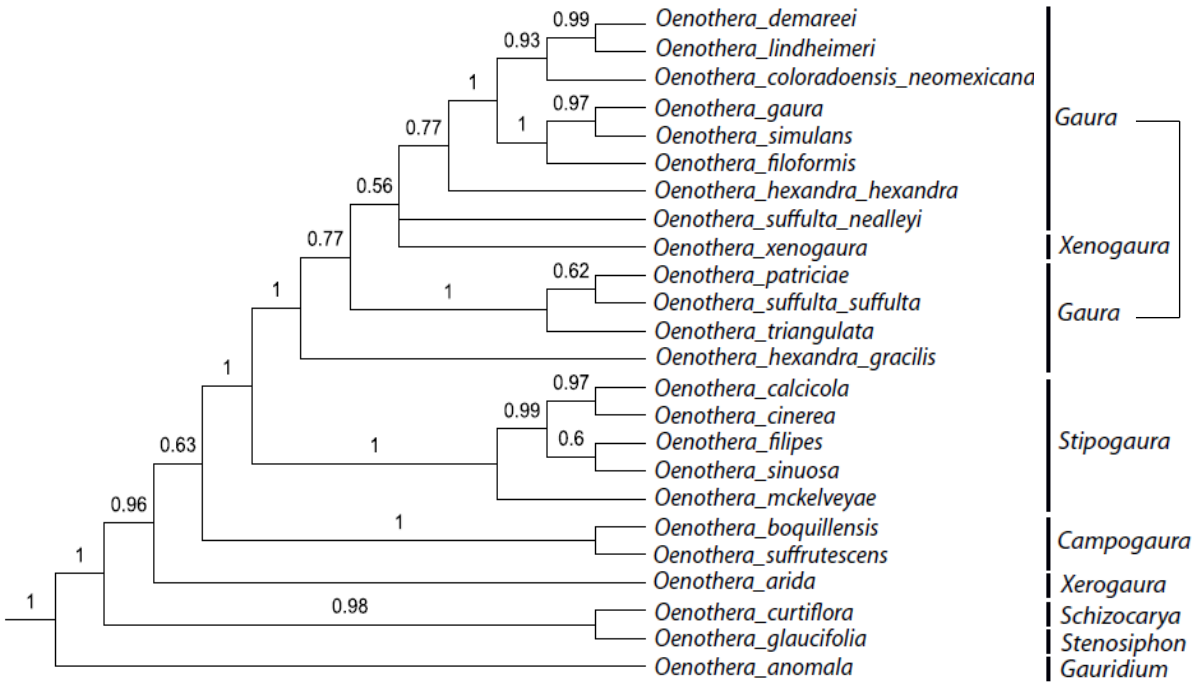


Figure 4-2. The subsections of section *Gaura*.



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CHAPTER 5

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MOLECULAR PHYLOGENETICS AND REPRODUCTIVE BIOLOGY OF *OENOTHERA* SECTION *KNEIFFIA*

Introduction

Molecular phylogenetics is a powerful tool for understanding the evolutionary relationships among plant species (Savolainen and Chase, 2003). Traditional taxonomy classified plant species primarily based on shared morphology, with a strong focus on reproductive traits. In combination with neutral molecular markers, phylogenetic reconstructions have revised taxonomies and altered many hypotheses about plant species relationships. For instance, flowering plants were long classified as Dicots and Monocots; however, molecular phylogenetic studies resulted in a dramatic change in plant classification, where monocots are now grouped with the basal Dicots, and Eudicots as sister to that clade (Bremer et al., 1998; Soltis et al., 1999).

Pollination system is one of several important and potentially interacting aspects of floral reproduction driving angiosperm diversification (Crane et al., 1995; Crepet et al., 2004; De Bodt et al., 2005; Fenster et al., 2004). Breeding system describes whether a plant is self-compatible (SC) or self-incompatible (SI), and is also thought to play a major role in the diversification of plants (Baker, 1955; Barrett et al., 1996). Angiosperms show repeated transitions from SI to SC (Barrett, 2002a), but a lack of reversals back to SI, across diverse taxonomic groups (Charlesworth, 2006; Foxe et al., 2009; Goodwillie, 1999; Schoen et al., 1996). Self-compatibility can provide advantages that outweigh the deleterious effects of inbreeding, such as reproductive assurance when pollinator service is inconsistent (Barrett, 2002a; Kalisz et al., 2004; Moeller, 2006; Waser and Ollerton, 2006). Self-compatibility has been associated with pollen limitation, because a decreased reliance on pollinators to achieve full seed set may be a pre-requisite for the transition to selfing (Larson and Barrett, 2000). A high frequency and/or intensity of pollen limitation

could be a selective force favoring transitions from SI to SC (Weber and Goodwillie, 2009).

There can be great diversity of breeding systems within plant groups, and closely-related species may differ in the level of self-compatibility (Brauner and Gottlieb, 1987; Macnair et al., 1989; Weller and Sakai, 1999). When closely-related species differ in breeding system, those differences may result from adaptations to environmental conditions (e.g., pollen limitation), or simply reflect shared evolutionary history. We can distinguish between these hypotheses by 1) identifying repeated transitions to SC, and 2) phylogenetically controlled associations between breeding system shifts and pollen limitation (Freckleton, 2000; Machado and Lopes, 2004; Sanderson and Donoghue, 1996; Vamosi et al., 2003).

Onagraceae, specifically the genus *Oenothera*, has long served as a model system for the evolution of flowering plant reproductive biology (Clinebell et al., 2004; Hoch et al., 1993; Raven, 1979; Raven, 1988). The repeated evolution of SC in this group is thought to play a key role in the diversification of Onagraceae as a mechanism of rapid reproductive isolation (Raven, 1979). Recent molecular phylogenetic studies have clarified phylogenetic relationships within *Oenothera* (Hoggard et al., 2004; Levin et al., 2004; Levin et al., 2003; Wagner et al., 2007), placing the once segregate genera *Gaura*, *Calylophus* and *Stenisiphon* now within a monophyletic *Oenothera* (Carr et al., 1990; Raven, 1988; Raven and Gregory, 1972). These sections form a clade with sections *Kneiffia, Megapterium, Peniophyllum, Paradoxus* and *Gaura*. These recent studies used one species, *Oenothera fruticosa*, as representative of section *Kneiffia* which has six species. The new phylogenies also show that *Oenothera linifolia*, previously included in

section *Kneiffia*, is now in section *Peniophyllum* (Levin et al. 2004). In Straley's 1977 treatment of section *Kneiffia*, a hypothesis of the evolutionary relationships is given based on morphological and cytological data. The 1977 treatment studied some of the *Kneiffia* breeding systems and gave preliminary pollination observations. Since that study, section *Kneiffia* has not been surveyed. In addition, a molecular phylogenetic study has never been conducted for the full species set of section *Kneiffia*, and so relationships among these species are unknown.

Species within *Oenothera* section *Kneiffia* are widely-distributed in eastern North America (Straley, 1977). They have bright yellow flowers that vary in size and are predominately bee pollinated. There are both annual and perennial species, and both SC and SI species. In this study, we recognize six species of *Oenothera* in sect. *Kneiffia*. *Oenothera sessilis*, previously known as *O. pilosella spp. sessilis* (Krakos et al. in review), is a rare species restricted to prairie remnants primarily in eastern Arkansas. *Oenothera riparia* is also a rare species found only in the riparian habitats of the Carolinas. Both of these rare *Kneiffia* taxa were not recognized at the species status by Straley (1977). A molecular phylogeny establishing the species level for these rare taxa will help in conservation efforts and in understanding the evolution of reproductive traits in this group.

In this study we present the first phylogenetic reconstruction for section *Kneiffia* that includes all taxa. We examine the reproductive biology of these species and use this phylogeny to test the following hypotheses: 1) Current generic and species level taxonomies reflect evolutionary history; 2) Self-compatibility has evolved once in section *Kneiffia*; 3) Two self-compatible species in section *Kneiffia* exhibit less pollen limitation

than two self-incompatible species. This study establishes relationships among *Kneiffia* species, identifies transitions to self-compatibility, and examines pollen limitation as a potential force affecting those transitions.

Materials/Methods

Study Sites

To assess levels of pollen limitation in SI and SC species, we conducted field studies on four species of section *Kneiffia* in sites throughout the Midwest and North East areas of North America. Fieldwork was carried out from April 2007 to August 2010 and included pollination studies, tissue collection, and breeding system experiments. *Oenothera pilosella* is a native perennial found blooming along the roadsides and in the prairie remnants of Illinois in early June. This species typically blooms for only 2-3 weeks. Our focal populations of *O. pilosella* were located in SE Washington Co. IL, 3 miles south of Posen, IL (38° 15.508 N, 89° 18.214 W), and Jefferson Co., IL along Rt. 15 (38 ° 15.849 N, 89° 02.396 W). *Oenothera perennis* is a native perennial common across the eastern US and blooms from mid-July through August. Our focal population was located in Middlesex Co, MA at the Great Meadows National Wildlife Refuge (42°23’32.6 N, 71° 22’ 55.1 W). *Oenothera sessilis* is a native annual found in prairie remnants of Arkansas that blooms May-June. Our focal populations were located in Prairie Co., AR at Downs Prairie Natural Area (34° 46’ 43” N, 91° 21’ 44” W) and Railroad Prairie Natural Area (34° 46’ 59” N, 91° 29’ 44” W). *Oenothera riparia* is a native perennial endemic to the riparian habitats of North and South Carolina and blooms mid-June through July. Our focal populations were located in New Hanover Co., NC on the banks of Island Creek (N 34° 22’ 02”, W 77° 48’ 54”), Pender Co., NC (34° 14’ 40”

N, 78° 00’ 59” W), and New Hanover Co., NC along the banks of Upper Smith Creek (34° 15’44 N, 77° 53’ 15” W).

Tissue Collections

The tissue used in this study comprises all six species of *Oenothera* in section *Kneiffia*. We used fresh tissue for the species *O. sessilis*, *O. riparia*, *O. pilosella*, and *O. perennis* from the study sites listed above. We used tissue samples from two locations from herbarium sheets at the Missouri Botanical Garden Herbarium for *O. spachiana*. We used published GenBank sequence data for *O. fruticosa*. Because the species status of *O. riparia* and *O. sessilis* were suspect, we used two samples of each species, each from a separate locality (see above). We report on a single sequence in the phylogeny because intraspecific variation (e.g., the number of nucleotide differences) was universally less than interspecific variation. All information on the origin of material, voucher specimens, and GenBank accession numbers are listed in Table 5-1.

DNA Isolation, Amplification and Sequencing

We isolated DNA using Viogene plant DNA isolation kits (www.viogene.com) according to the manufacture’s protocols. We amplified 604 bp of the chloroplast internal transcribed region (ITS), 966 bp of chloroplast marker trnL, 1803 bp of the nuclear external transcribed region (ETS), 867 bp of chloroplast marker rps16, 1054 bp of chloroplast marker ndhF, and 1268 bp of chloroplast marker rbcL. Primer combinations and annealing temperatures are listed in Table 5-2. Polymerase chain reactions (PCR) were performed in 25 µL reactions of Promega (www.promega.com) 5x buffer, 2.5 µL of 25 mM MgCl2, 2.5 µL of 0.2 µM dNTPs, 2.5 µL of 0.2 µM of each primer, 0.125 µL (1 unit) of Promega GoTAq DNA polymerase, and 2 µL of template DNA at approximately

5 ng/μL. The PCR thermal profile included 95°C for 3 min, followed by 35 cycles of 95°C for 1 min, annealing temperature for 40 s, and 72°C for 45 s, with a final elongation at 72°C for 7 min. PCR products were visualized through agarose-gel electrophoresis and purified using Viogene gel purification kits (www.viogene.com). Sequences were generated at the Washington University Genome Sequencing Center on an ABI 3330. All gene regions were sequenced in both the forward and the reverse directions. DNA sequences were manually edited using SEQUENCHER 4.8 (Ann Arbor, MI) and aligned by hand in GENEDOC.

Phylogenetic Reconstruction

We estimated models of nucleotide evolution for each of the six gene regions independently in jmodeltest (Posada, 2008). We generated a phylogenetic tree from a concatenated data set of the four chloroplast genes, and compared this to separate phylogenetic trees generated for each nuclear marker. These phylogenetic trees largely agreed, and so all six markers were concatenated and used to generate a single phylogenetic tree. The evolutionary history of the six taxa was reconstructed using Bayesian Markov-Chain Monte-Carlo search algorithm of MrBayes (Ronquist and Huelsenbeck, 2003). We used thirty million generations with a sampling frequency of 200 generations and the standard 3 cold and 1 hot chain. Each partition was given the model of evolution determined by the AIC method in jmodeltest and we unlinked all parameters across loci to allow them to evolve independently. Convergence in two replicate analyses was determined when the standard deviation between the log-likelihood scores of the two runs was < 0.0001. Parameters estimated during the analysis were evaluated for parameter estimate convergence using Tracer (Rambaut and

Drummond, 2007), wherein each of the 17 model parameters had Effective Samples Sizes (ESS) > 500 and the log-likelihood of the model had reached a plateau. We discarded 25% of the resulting trees as a burn-in and computed a consensus tree using the sumt command in MrBayes. We rooted our trees using four outgroup species, *Oenothera macrocarpa, Oenothera brachycarpa, Calylophus lavandulifolius*, and *Calylophus serrulata.*

Determining Breeding System and Pollen Limitation

The breeding system and pollination data for *O. spachiana* and *O. fruticosa* have been previously described (Straley, 1977), and we did not test these two species. To determine and verify the breeding system of the other four *Kneiffia* species, we conducted hand-manipulated experiments in both the field and in the greenhouse. For each study site, during peak flowering season, we randomly chose ten flowering plants. The evening prior to the experiment, we chose pairs of mature buds on each plant and bagged them in bridal veil netting following Lipow et al. (2002) protocols. When the flower opened the next morning it received one of two treatments. For group one, the Self-Pollen treatment, when the flower opened in the morning, the bag was removed and pollen from the flowers own stamens was applied to the stigma. The bag was then placed back over the flower for the duration of flowering time. For group two, the Cross-pollen treatment, when the flower opened, the bag was removed and all stamens were removed. The stigma was then manually pollinated with the pollen from a single flower from a distant plant in the population. Pollen was applied with a paintbrush until the stigmatic surface was coated. The bag was then placed back over the flower for the duration of the experiment. These same protocols were repeated with greenhouse populations to verify the breeding

system of all four *Kneiffia* species without any potential confounding variables such as pollinator contamination. In the greenhouse we grew between 20 and 30 plants of each species, representing all populations.

Twenty-four hours after each treatment, all pairs of inflorescences were collected and fixed in a 3:1 EtOH: glacial acetic acid mixture for 2 hours. They were then transferred to a 70% EtOH solution and stored. To count the number of pollen tubes present and reaching the ovary, the pistil and ovary were dissected from each flower and placed in a small beaker. The specimens were covered with a 10% solution of sodium sulfide and incubated at 65 degrees until the tissue was soft. The specimens were then covered with de-ionized water for 15 minutes. Each pistil and ovary was placed on a separate glass slide, covered in 3-5 drops of decolorized aniline blue, and covered with a cover slip. The softened tissue was spread by tapping the coverslip with a probe. Ovaries were sliced in half and placed face up prior to tissue spreading. The labeled slides were refrigerated for a minimum of 24 hours. A Zeiss Universal microscope with a 100 watt mercury bulb to give fluorescent light was used to view the pollen tubes. The number of pollen grains on the stigma, the number of pollen tubes in the style, and the number of pollen tubes that reached the ovary were all counted to determine successful rates of pollination (see Lipow et al. 2002).

To determine if the species was self-compatible, we performed a paired *t*-test, assuming equal variance, comparing Self vs. Cross percentage of pollen tubes that reached the ovary. No statistical difference between the pairs indicates that the species is self-compatible.

To assess whether self-incompatible *Kneiffia* species exhibit greater pollen limitation than SC species, we performed supplementary pollination experiments in the study populations of all four species. In each population we chose 10 random flowering plants. Before the onset of flowering (predawn), we marked two inflorescences per plant with yarn tied at the base of the inflorescence and assigned each to a treatment group. Group one, the control, were left open to natural pollinators throughout the flowering period (one day). Group two, the supplementation treatment, were left open to natural pollinators and in addition were manually pollinated with a mixture of pollen from five distant plants in the population. Pollen was applied to the stigma with a paintbrush three times during the period of stigma receptivity. After 24 hours, all pairs of inflorescences were collected, fixed, and pollen tube counts obtained by the same methods described above for the breeding system experiments.

For each pollen supplementation and control pair, the degree of pollen limitation, L, was calculated by: $L = 1 - \frac{Tc}{Ts}$, where T_s is the number of pollen tubes that reached the ovary in the supplementation treatment, and T_c is the number of tubes that reached the ovary in the control treatment. $L \approx 0$ indicates that there is no pollen limitation for that population of the species. (Larson and Barrett 2000). Therefore, if a species has a positive L value, and the 95% CI does not include 0, it is to be considered pollen limited. We used restricted maximum likelihood (REML) to estimate the variance. We followed this analysis with a post-hoc test (Tukey-Kramer HSD) to determine whether L differed significantly among the species. We also conducted a phylogenetic ANOVA (Garland et al., 1993) using the Geiger module in R (Harmon et al., 2008) to test for significant associations pollen limitation (L) and species or breeding system.

Determining Pollination System

Pollination system was determined by measuring visitation by animals, pollen load, and stigma contact. For each population of *Oenothera*, we conducted 20 min observations of multiple randomly chosen inflorescences and recorded the total number of visits, type of visitor, and behavior of visitors. We recorded observation of physical contact between an insect and the receptive stigma, as well as duration of visit, and which plant species the insect visited next. Observations began in the second week of flowering for each population and continued for 2 weeks. They were conducted during times of peak pollinator activity, which began pre-dawn and continued until early afternoon.

Insect visitors to the flower were collected using a net and a killing jar charged with ethyl acetate. Insects were pinned and taken to the lab to quantify the amount and location of pollen carried. To assess the identity and number of pollen grains carried by each visitor to *Oenothera* we made a library of pollen grains from flowering plants at each study site. Dehiscent stamens were placed on glass slides. The pollen was teased out with probes, stained with 1-2 drops of Calbera's fluid to make a semi-permanent mount (Bernhardt et al., 2003; Goldblatt et al., 1998a), and labeled to species for future reference known as a "pollen library."

We counted and identified the pollen carried by the insect visitors. Each euthanized insect collected on the *Oenothera* species was placed on a separate glass slide and washed in a few drops of ethyl acetate. The insect specimen was removed from the slide and the slide was allowed to air dry. Washed insect specimens were then dried, pinned, and saved for identification by regional entomologists. Insects were identified and grouped into one of five functional groups based on major genera, type and size.

These groups were bumble bees (*Bombus*), carpenter bees (*Xylocopa*), megachilid bees (*Megachile*), and small and medium halictid bees (*Lassioglossum*). The pollen on the slide was stained with one-two drops of Calbera's fluid (Goldblatt et al., 1998a) and a cover slip was applied to the surface of the drop. All pollen identified under light microscopy was compared to the pollen library. The type and amount of pollen on the legs, thorax, and proboscis was recorded.

The pollen flow, *P*, was calculated for each *Oenothera* species by

$$\sum (VR_x * PL_x)$$

where VR is the frequency of an insect visitor, *x*, and PL is the average pollen load carried by that insect species. All insect visitors and their % contribution to total pollen flow were recorded and the main pollinator systems for each plant species was determined as the pollinator functional groups that accounted for 95% of the total pollen flow.

Independent Origins of Self-Compatibility

We identified three self-compatible species. The consensus tree of our concatenated dataset will identify whether or not these three species represent a single or multiple transitions to self-compatibility. However, the consensus topologies may only be negligibly more likely than alternative topologies. We used topological hypothesis testing to identify the number of origins of self-compatibility. Given three SC species (see Results), there are five possible topologies: all three form a clade (single origin of SC), three configurations of two origins of SC, or three separate origins of SC. We compared our unconstrained topology of three independent origins to four constrained topological alternatives: all three SC species form a clade, and three additional constraints

representing all possible alternative configurations of two independent origins of SC (e.g., 1,2 and 3; 1,3 and 2; and 2,3 and 1), and used a likelihood ratio test and Bayes Factors (Kass and Raftery, 1995) to test whether the consensus tree of the unconstrained analysis with three independent origins is significantly more likely than 500 trees sampled from the posterior distributions of the four topologically constrained trees.

Results

Phylogenetic Reconstruction

The consensus tree resulting from our phylogenetic reconstruction is shown in Figure 5-1. The duplicate analyses in MrBayes converged after 10 million generations, and all of 17 parameters were resolved with ESS values above 500. The tree file has been deposited on TreeBase.org and genbank accession numbers are in Table 5-1. The nexus file is also available on TreeBase and contains evolutionary models of nucleotide evolution inferred from jmodeltest and the AIC.

Breeding System, Pollination System, and Pollen Limitation

Within 24 hours of pollination, pollen tubes growing from the SC flowers or the SI flowers that received outcross pollen had entered the style. There was no obvious evidence of late-acting self-incompatibility mechanisms such as pollen tubes that extend down the style, but then turn and grow upward, or swollen pollen tube tips. Breeding system and pollination system differed among species (Table 5-3. and Fig. 5-2.). The *Oenothera* species all had morning bee pollination systems, but varied with regards to what percentage of the pollen flow different pollinator functional groups were responsible for (Fig. 5-2). A full set of pollinator species and their average visitation rate and pollen load are listed in Table 5-5. *Oenothera* species did not differ significantly in

their degree of pollen limitation, *L* (F = 1.146, *P* = 0.34) and there was no association between breeding system and pollen limitation (F = 1.42, *P* = 0.24).

For *O. riparia*, in both field and greenhouse experiments (n = 22 pairs), no pollen tubes germinated from self-pollen, and 50% of the pollen tubes from cross-pollen reached the ovary. We determined that *O. riparia* has a self-incompatible breeding system and is pollinated by large bees (82% of pollen flow) and megachilids (13%) in 185 observations. *Oenothera riparia* had an *L* value of 0.371 ± 0.116 (n = 14 pairs), indicating that it is not pollen limited in these populations.

In both field and greenhouse experiments of *O. sessilis* (n = 20 pairs), there was no significant difference between the number of pollen tubes reaching the ovary for cross or self-pollen (*P* = 0.17 for field experiments, *P* = 0.23 for greenhouse experiments). Therefore, *O. sessilis* has a self-compatible breeding system. This species is not visited by pollinators (n = 137 observations), and is designated as autogamous. We calculated an *L* value of 0.3208 ± 0.137 (n = 10 pairs), indicating that these populations are not pollen limited.

For *O. perennis*, in both field and greenhouse experiments (n = 18 pairs), there was no significant difference between the number of tubes reaching the ovary for cross vs. self pollen (*P* = 0.34 for field experiments, *P* = 0.28 for greenhouse experiments), indicating that it has a self-compatible breeding system. *Oenothera perennis* (n = 236 observations) is pollinated by small halictid bees (76% of pollen flow), bumble bees (16%), and medium bees (8%). All six insect pollinator species are listed in Supplemental Table 5-5. *Oenothera perennis* has an *L* value of 0.0465 ± 0.145 (n = 9 pairs), indicating that these populations are not pollen limited.

For *O. pilosella*, in both field and greenhouse experiments (n=19 pairs), no pollen tubes germinated from self-pollen, and 42% of the pollen tubes from the cross-pollen reached the ovary. This pattern indicates that *Oenothera pilosella* has a self-incompatible breeding system. Through 185 observations we determined that *O. pilosella* is pollinated by medium bees (53% of pollen flow), megachilids (36.7%), and small halictids (8.9%). All seven species of pollinator are listed in Supplemental Table 1. *Oenothera pilosella* has an *L* value of 0.3167 ± 0.099 (n = 11 pairs), indicating that it is not pollen limited.

Oenothera spachiana is already known to be autogamous (Straley, 1977). *Oenothera fruticosa* has a self-incompatible breeding system and is described as having a bee pollination system and is most likely very similar to *O. pilosella* (Straley, 1977).

Independent Origins of Self-Compatibility

The phylogenetic reconstruction depicted in Figure 5-1 is highly resolved and shows three independent origins of SC. There are five potential patterns in the evolution of self-compatibility in this clade, and the consensus tree clearly supports three independent origins. Topological hypothesis testing provides the statistical framework for evaluating this hypothesis by refuting all possible topological configurations that are different from the consensus tree pattern of three independent origins. All four alternative topologies were refuted (Table 5-4) according to a log-likelihood ratio test at an $\alpha = 0.05$, and according to Bayes Factors with values greater than 50 in favor of three independent origins over all possible alternatives (values of greater than 10 are considered decisive).

Discussion

Phylogenetic structure of Oenothera sect. Kneiffia

Our study examined all six species of *Oenothera* in section *Kneiffia* (Fig. 5-4). The *Oenothera* section *Kneiffia* consensus tree (Fig. 5-1) is the first phylogenetic tree based on molecular data that includes all species in this section. The relationships among these *Oenothera* species were previously based on morphological and cytological data. This study clarifies the evolutionary relationships within section *Kneiffia*, and has several striking changes from previous assumptions and work. Straley (1977) presented a hypothetical tree based on morphology and cytological data. He recognized two major subsections in *Kneiffia*, one containing the small flowered annual *O. linifolia*, and a second subsection that contained *O. pilosella*, *O. perennis*, *O. fruticosa*, and *O. spachiana*, all of which are perennials with larger yellow flowers. Although Straley (1977) included the species *O. linifolia* as a basal species in *Kneiffia*, molecular phylogenetic studies in *Oenothera* showed it to be outside of section *Kneiffia* in a closely-related section *Peniophyllum* (Levin et al., 2004). Our analyses agree with Levin (2004), and place *O. linifolia* outside of section *Kneiffia* (Chapter 4). Our study agrees with Straley and places the self-compatible annual *O. spachiana* as basal in section *Kneiffia*.

Within the second subsection, Straley grouped the self-incompatible *O. fruticosa* and *O. pilosella* together, and as sister to *O. perennis*. He does not include the self-incompatible *O. riparia*, which Straley described as a subspecies of *O. fruticosa* (Straley, 1982), but was recognized as a species in later studies (Wagner et al., 2007). It is a reasonable assumption to group all the self-incompatible species together a single transition to self-compatibility. However, with the inclusion of *O. riparia* and *O. sessilis*, the self-incompatible species no longer form a monophyletic group. We find a strong

posterior probability support (1.0) for a sister clade to the *O. fruticosa* and *O. pilosella* clade that contains the self-incompatible *O. riparia* and the self-compatible *O. perennis*.

Oenothera sessilis is a day flowering yellow perennial *Oenothera* hypothesized by Straley (1977) to be a subspecies of *O. pilosella* based on cytological and morphological data. Previously it was recognized and described as a species by Munz 1965, called *Oenothera sessilis,* and earlier by Pennell 1919 as *Kneiffia sessilis.* Straley does recognize the distinct morphological differences in height and flower size between the two taxa, however, based on the same chromosome count (n = 28) and an incorrect determination that both taxa were self-incompatible; Straley placed *O. sessilis* as a subspecies of *O. pilosella*. However, our data show that in fact *O. sessilis* is not self-incompatible. Our breeding system experiments confirm that *O. sessilis* is self-compatible and may in fact be entirely autogamous. Our pollination studies did not document any insect visitors during the peak flowering season, yet *O. sessilis* achieved full seed set. Potential pollinators were present and active on other prairie species co-blooming with *O. sessilis*. Based on these current field studies, breeding experiments, and molecular phylogenetic data, there has been a revision of the nomenclature and *O. sessilis* (previously *O. pilosella ssp. sessilis*) is now recognized at the species level (personal communication P. Raven). Our phylogenetic reconstruction supports *O. sessilis* as sister to the clade containing *O. riparia* and *O. perennis* as sister taxa, and *O. fruticosa* and *O. pilosella* as sister taxa (Figure 5-1).

The classification of sect. *Kneiffia* is still unclear with regards to the subspecies of *O. fruticosa*. This species has been subject to numerous revisions and regrouping of taxa due to the wide range and morphological variation of this species. Wagner et al. (2007)

agree with the conservative approach taken by Straley (1977) and delineating two subspecies, *O. fruticosa ssp. glauca* and *O. fruticosa ssp. fruticosa*. In this study, we used *O. fruticosa ssp. fruticosa* and we did not address this issue. Further work is needed to determine the proper species status and placement of these taxa based on molecular data.

Pollination Systems and Pollen Limitation

The *Oenothera* species we studied that used out-cross pollen were all bee and small fly pollinated, as has been previously noted for this section (Straley, 1977). However, this is the first study that closely examined the pollination systems of these species with details such as visitation rates, pollen loads, and stigma contact. We show that while all three use similar functional groups, the percentage of pollination due to each functional group varies among species. While the pollination systems of section *Kneiffia* are broadly similar, at a functional group and genus level, they are more specialized. We also show that *O. perennis*, previously described as autogamous, actually has a pollination system that consists of small and medium bees of the family Halictidae and the genus *Bombus*.

A long-held hypothesis is that pollen limitation leads to the evolution of self-compatible breeding systems in plants (Lloyd, 1979), and is reflected in the tendency for plant species that have reduced reproductive traits such as smaller flowers to be autogamous (Stebbins, 1974). Alternatively, plants that are SC may evolve reduced reproductive traits and tend to be pollen limited. However, we find in section *Kneiffia* of *Oenothera*, that there is no statistical correlation between breeding system and pollen limitation. None of the species we studied experienced significant pollen limitation, regardless of the breeding system (Fig. 5-3). While floral traits may correlate with

breeding system for the *Oenothera*, the functional pollination systems do not. Both of the self-compatible species have the smaller flowers and reduced size associated with self-compatibility; however *O. sessilis* is completely autogamous, while *O. perennis* utilizes pollinators to set seed. The addition of cross pollen did have a larger effect on the two self-incompatible species, *O. riparia* and *O. pilosella*, but with such a small number of species being examined this result is only a trend. Broader sampling is needed to determine if the impact of pollen addition has a larger effect on self-incompatible species than self-compatible species.

Breeding Systems and Transitions to Self-compatibility

Our study verified the breeding system of *O. pilosella* as self-incompatible and *O. perennis* as self-compatible. We corrected previous incorrect assumptions and show *O. sessilis* to be self-compatible. We clarified that the breeding system of *O. riparia* is self-incompatible. The topological tests clearly demonstrate exactly three transitions to self-compatibility within section *Kneiffia*.

Table 5-1. Species, locations of samples, voucher numbers and accessions for DNA sequence data for the six species examined in this study. For each gene we indicate the source of the data with either the Genback accession number or as a species newly sequenced in this study (*). Data not obtained is indicated (-).

Taxon	Location	Voucher	ITS	trnL-F	rps16	ETS	rbcl	ndhF
O. fruticosa	Dane Co, WI	WIS5025	AY271581	AY264569	AY267443	-	AF495771	AF495794
O. riparia	Pendleton Co. NC	Krakos 1017	*	*	*	*	*	*
O. perennis	Middlesex Co., MA	Krakos1010	*	*	*	-	*	*
O pilosella	SE Washington Co. IL	Krakos0821	*	*	*	-	*	*
O. spachiana	Bienville Co., LA	Thomas and Moreland 49150	-	*	*	*	*	*
O. sessilis	Prairie Co., AR	Krakos 1006	*	*	*	-	*	*

Table 5-2. Primers used for six molecular markers.

Locus	Primer name	Primer Sequence 5' to 3'	Annealing Temperature	Citation
ITS	ITS4	TCC TCC GCT TAT TGA TAT GC	47° C	(Levin et al., 2004)
	ITS5HP	GGA AGG AGA AGT CGT AAC AAG G	47° C	
trnL	trnLf	ATT TGA ACT GGT GAC ACG AG	50° C	(Levin et al., 2004)
	trnLc	CGA AAT CGG TAG ACG CTA CG	50° C	
rps16	P1839	TCG GGA TCG CAC ATC AAT TGC AAC	55° C	(Levin et al., 2004)
	P1840	GTG GTA AAA AGC AAC GCG CGA CTT	55° C	
ETS	ETS R2	AGA AGT CGG GGT TTG TTG C	50° C	(Hoggard et al., 2004)
	ETS F2	ACG ATC GGA TTC GTG ACC TA	50° C	
rbcL	P1630	ATG TCA CCA CAA ACA GAG ACT AAA GC	53° C	(Levin et al., 2003)
	P1782	ATA CTT CAC AAG CAG CAG CTA GTT CC	53° C	
ndhF	P1786	CCC CGA AAT ATT TGA GAC TTT CT	47° C	(Levin et al., 2003)
	P1785	GTC TCA ACT GGG TTA TAT GAT G	47° C	

Table 5-3. Comparative rates of breeding system parameters for *Oenothera* species in hand pollination studies. Because there was no significant difference between the greenhouse and field population experiments, results from these locations are pooled for each species.

Species	Treatment	Number of flowers	Number of pollen grains on stigma	Number of pollen tubes reaching ovary	Percent of pollen tubes to reach plant ovary
O. riparia	Self	13	390.0 (± 256.44)	0	0
	Cross	16	452.0 (±370.57)	12.64 (±11.70)	33.52 (±24.01)
O. pilosella	Self	8	800.0 (±392.79)	0	0
	Cross	10	890.0 (±272.64)	8.1 (±4.65)	41.5 (±13.4)
O. sessilis	Self	20	279.65 (±189.92)	2.5 (± 2.76)	26.03 (± 29.22)
	Cross	20	325.7 (±165.66)	3.4 (±3.15)	39.79 (±33.07)
O. perennis	Self	9	477.78 (±83.33)	6.44 (±4.19)	26.31 (±19.87)
	Cross	9	522.22 (±84.22)	7.56 (±3.40)	36.35 (±23.61)

Table 5-4. Results of the topological hypothesis testing of the number of origins of self-compatibility in section *Kneiffia*. The topology is listed as a single origin with all three SC species forming a clade, and the following two-origin scenarios in standard Newick format: Two Origins[1]: ((*O. sessilis. O. perennis*), *O. spachiana*); Two Origins[2]: ((*O. perennis*, *O. spachiana*), *O. sessilis*); Two Origins[3]: ((*O. sessilis*, *O. spachiana*), *O. perennis*). The results of the Bayes Factor tests are given (values greater than 10 are considered decisive) based on the harmonic Mean of the log-likelihoods of the two independent MrBayes runs, and the D-value for the log-likelihood ratio test are given based on the arithmetic mean of the log-likelihoods of the two runs (LnL), with asterisks denoting significance at an $\alpha = 0.001$. The consensus topology without any topological constraints (†) represents three independent origins of SC, and is favored over all other alternative topologies.

Topology	**LnL**	**Harmonic Mean**	**Bayes Factors**	***D***
Single Origin	-9247	-9290	144	118*
Two Origins[1]	-9211	-9239	42	46*
Two Origins[2]	-9230	-9273	110	84*
Two Origins[3]	-9219	-9264	92	62*
Three Origins†	-9188	-9218	-	-

Table. 5-5. The visitation rate and average pollen load of pollinators to the *Oenothera* species.

Species	Insect Species	Visitation Rate (visits/flower/20 min)	Average Pollen Load
O. riparia	*Bombus pennsylvanicus* DeGeer (female)	0.089	120.00
	Xylocopa virginica Linn. (female)	0.098	108.50
	Megachile xylocopoides Say (female)	0.223	15.00
	Lassioglossum ssp.	0.062	14.00
	brown moth	0.054	6.00
	black moth	0.036	6.00
O. pilosella	*Agapostemon virescens* Fab. (female)	0.532	458.33
	Megachile montivaga Cresson (female)	0.389	418.33
	Lasioglossum versatum Robertson (female)	0.663	17.75
	Augochlorella purae Smith. (female)	0.856	11.00
	Apis mellifera Linn. (female)	0.011	500.00
	dull brown moth	0.011	25.00
	Syrphidae sp.	0.151	1.25
O. sessilis	none	na	na
O. perrenis	*Augochlorella aurata* Smith. (female)	0.146	500.00
	Lasioglossum versatum Robertson (male)	0.250	268.18
	Bombus impatiens Cresson (female)	0.056	500.00
	Agapostemon virescens Fab. (female)	0.031	500.00
	Lasioglossum oceanicum CK II. (female)	0.031	5.00
	Syrphidae ssp.	0.170	0.10

Figure 5-1. Bayesian phylogenetic reconstructions from concatenated mixed model data set for genes *ITS, ETS, rps16, trnL-F, rbcL,* and *nhdF*. Self-incompatible species in bold, self-compatible species with an asterisk. *O. macrocarpa, O. brachycarpa, C. lavandulifolius,* and *C. serrulata* are the outgroup species to section *Kneiffia*.

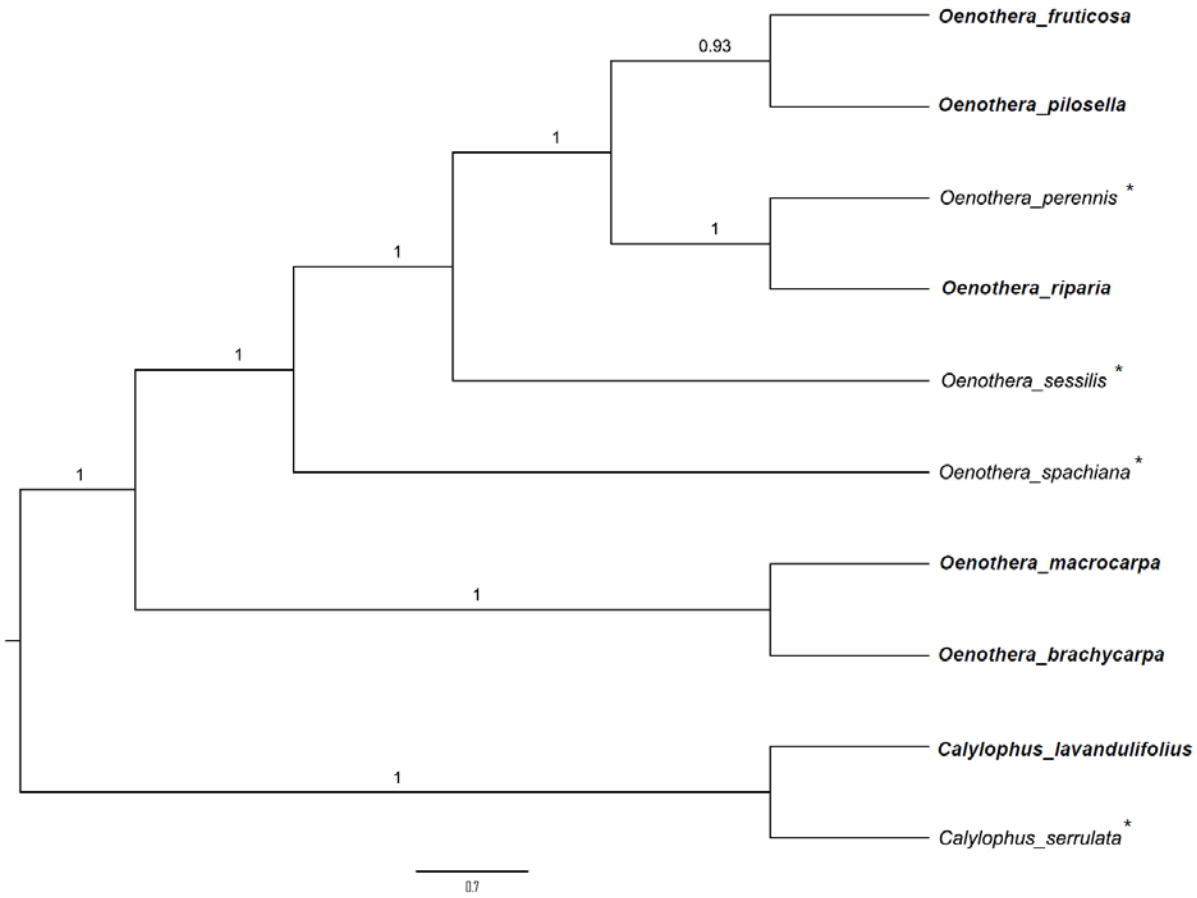


Figure 5-2. The major pollinator functional groups that account for 95% of pollen flow for *Oenothera* species. *Oenothera sessilis* is not listed because no pollinators were observed (see Results).

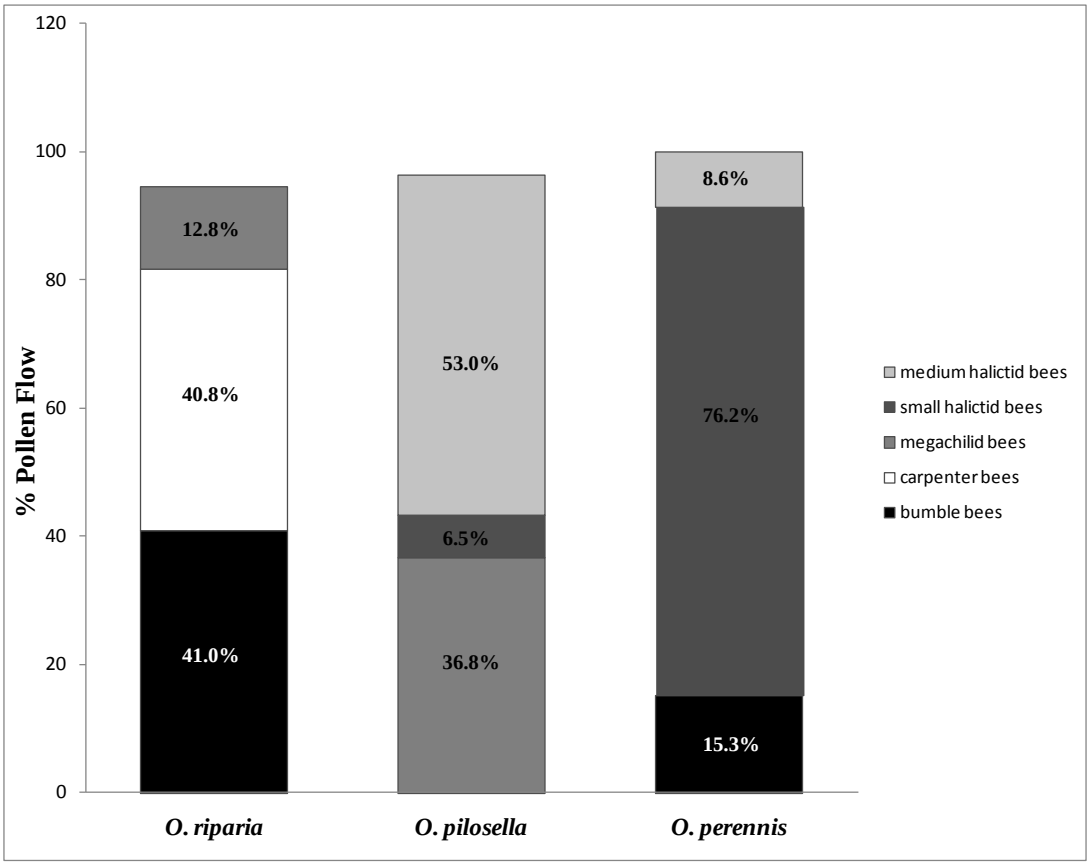


Figure 5-3. The mean degree of pollen limitation, *L*, ±SE for the *Oenothera* species based on supplement and control treatments tested at field sites. There are no significant differences either by species or by breeding system.

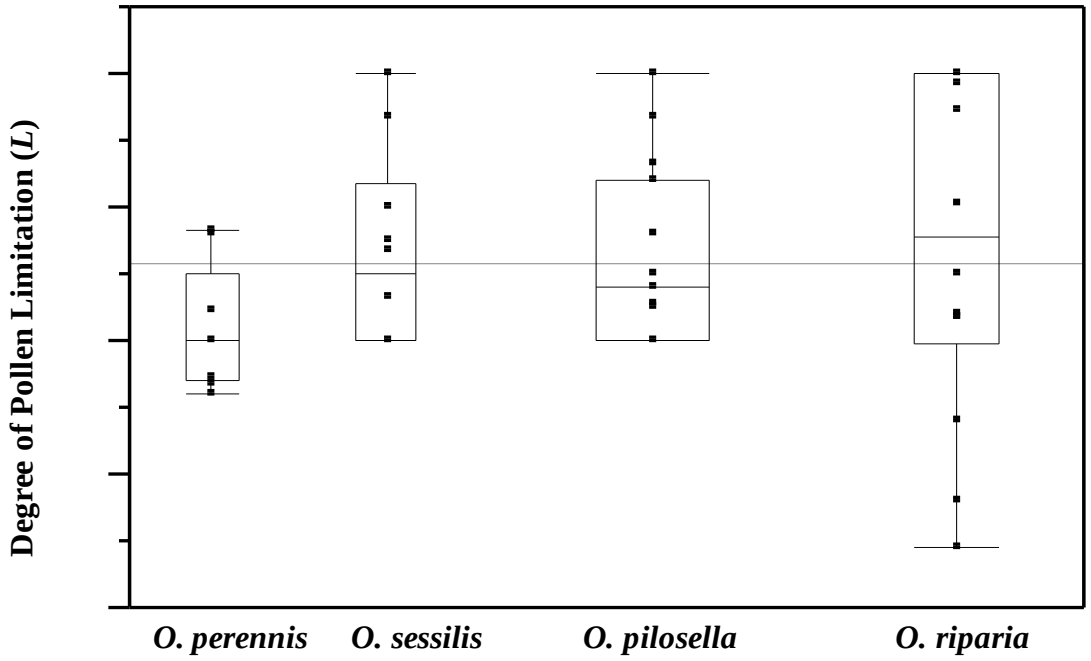
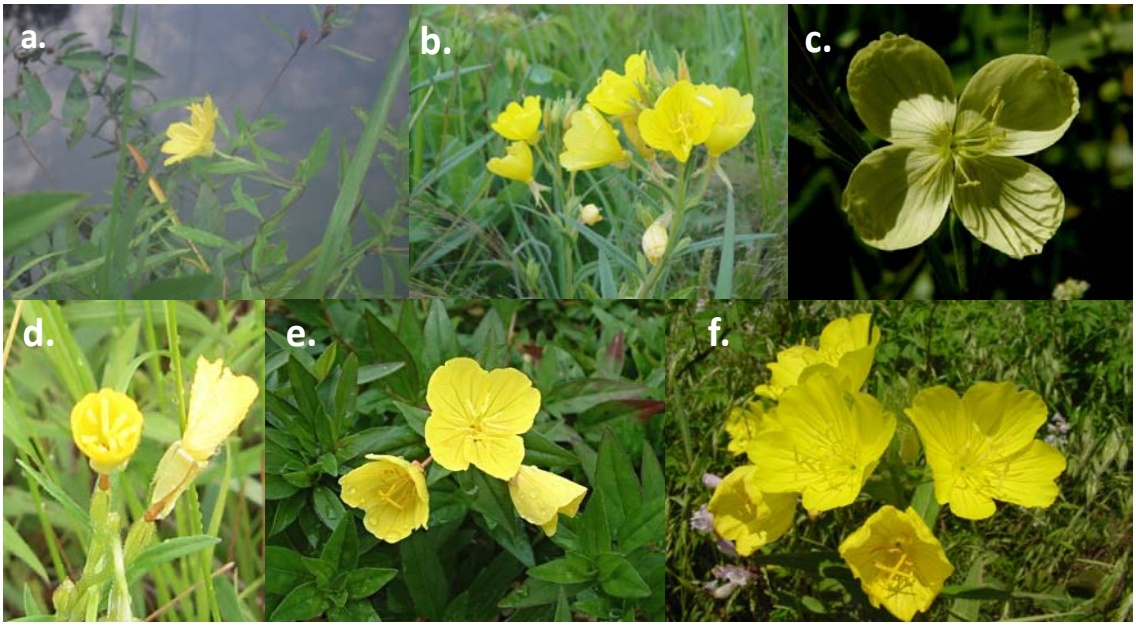


Figure 5-4. Photos of flowering *Oenothera* species: **a.** *O. riparia* **b.** *O. sessilis* **c.** *O. spachiana* **d.** *O. perennis* **e.** *O. fruticosa* **f.** *O. pilosella*. Photo credit: a.,b.,d.,f. K. N. Krakos; c. Charles Llewallen; e. G. L. Deeproot



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CHAPTER 6

PAGES 138-188

EVOLUTIONARY TRANSITIONS IN THE REPRODUCTIVE TRAITS OF *OENOTHERA*

Introduction

"The rapid development as far as we can judge of all the higher plants within recent geological times is an abominable mystery."

---Darwin, C.R., Letter to J.D. Hooker, July 22nd 1879. (Darwin and Seward, 1903)

The angiosperms arose in the early Cretaceous, approximately 125 million years ago, and showed rapid diversification, such that every major flowering plant lineage is present in the fossil record within 10-12 million years thereafter (Crane et al., 1995; De Bodt et al., 2005). The origin and radiation of angiosperms is traditionally attributed in large part to co-evolutionary relationships between plants and their pollinators (Crepet et al., 2004; De Bodt et al., 2005; Grimaldi, 1999; Sapir and Armbruster, 2010), specifically the idea that reproductive specialization has repeatedly lead to speciation. However, previous studies have yielded conflicting results (Armbruster and Baldwin, 1998; Goldblatt et al., 1995) regarding the association between diverisification and pollinator specialization (citations). These conflicting results may be in part due to the inaccurate metrics of reproductive specialization. Two main reproductive traits involved are the pollination system, which includes both biotic and abiotic interactions (Waser et al., 1996); and breeding system, which determines whether a plant species is self-compatible (SC) or self-incompatible (SI) (Baker, 1955; Barrett, 1998; Barrett, 2002a). These two systems, breeding and pollination, are assumed to correlate such that they promote outcrossing, while still maintaining reproductive assurance when necessary (Barrett, 2002a; Barrett, 2003).However, the assumption that specialized pollination systems are a trait strongly associated with SI is not always supported (Barrett, 2003; Fenster and Marten-Rodriguez, 2007; Perez et al., 2009). For example, the radiation of *Dalechampia*

is attributed to pollination system shifts between resin collecting and pollen collecting bees. These are both very specialized pollination systems, and it would be expected that plants with these very specialized pollination systems would also be self-incompatible (SI). However, many species of *Dalechampia* with these specialized pollination systems are self-compatible (SC). (Armbruster, 1988; Armbruster, 1994).

Pollination and breeding systems are evolutionarily labile and lineages frequently shift between character states (Fenster et al., 2004; Grant and Grant, 1965; Smith et al., 2008b; Weller and Sakai, 1999; Whittall and Hodges, 2007). Shifts in reproductive traits may be a first step in reproductive isolation and subsequent speciation of a plant lineage (Fenster et al., 2004; Kay and Sargent, 2009b; Smith et al., 2008a; Van der Niet et al., 2006; Weller and Sakai, 1999). Plant breeding systems shift uni-directionally from being SI to a state of being SC, and then may become autogamous or remain highly to moderately outcrossing (Charlesworth, 2006; Igic et al., 2006; Igic and Kohn, 2006), and are associated with speciation (Foxe et al., 2009; Theiss et al.; Theiss et al., 2010). Reversals from SC back to SI are thought to be impossible (Barrett, 2003; Igic and Kohn, 2006) because of the genetic complexity of the SI systems. The most common transition for pollination systems is from specialist (a plant species with 1 or 2 pollinator species) to generalist (a plant species with 3 or more major pollinator species) or between alternative specialized pollinator groups (Armbruster and Baldwin, 1998; Fenster et al., 2004; Marten-Rodriguez et al., 2010; Tripp and Manos, 2008); however, a few studies document a shift from a generalized pollination system to a specialized one (Marten-Rodriguez et al., 2010). Shifts in pollination systems can impact angiosperm speciation (Alcantara and Lohmann, 2010; Cozzolino and Widmer, 2005; Kay and Sargent, 2009b;

Sanderson and Donoghue, 1996). Grant and Grant (1965) hypothesized that repeated shifts in pollination system were key to the floral radiation of Polemoniaceae. Shifts in breeding system and/or pollination system are also associated with floral evolution (Anderson et al., 2002; Armbruster and Muchhala, 2009; Foxe et al., 2009; Perez et al., 2009; Van der Niet et al., 2006; Whittall and Hodges, 2007).

Floral reward traits, such as nectar and scent composition, play a key role in promoting specialization and may be indicative of specialized pollination systems (Raguso et al., 2007). These reproductive traits of angiosperms both promote out-crossing via pollinator-mediated selection (Goldblatt et al., 2001; Grimaldi, 1999; Stebbins, 1970), and limit self pollination to avoid the deleterious consequences of inbreeding (Barrett, 2002b; Darwin, 1876; Holsinger, 1996; Yang and Hodges, 2010). For instance, flower size in *Amsinckia* correlates with the degree of outcrossing; the predominantly selfing *A. vernicosa* is very small-flowered compared to its sister taxon, *A. furcata*, which is large-flowered and outcrossing (Schoen et al., 1996). Similarly, two autogamous species of *Oenothera* exhibited reduced investment in floral display, nectar, and scent, when compared to outcrossing congeneric species (Raguso et al., 2007). Conversely, the elaborate floral traits of *Tacca chantrieri* suggest a substantial investment in outcrossing by flies, yet the species is highly selfing (Zhang et al., 2005). The lack of precise correlation between floral traits and breeding system is a puzzle that perplexed Darwin (Darwin, 1877) and many others (Barrett, 2003; Barrett et al., 1996; Theiss et al., 2010; Yang and Hodges, 2010).

To investigate the associations between pollination and breeding systems and floral traits, reproductive characters must be clearly defined. Pollination system must be

determined from detailed pollination ecology data, and not inferred from pollination syndromes, which do not have sufficient predictive power (Knapp, 2010; Mitchell et al., 2009; Wilson et al., 2004). Correlations among the reproductive traits (pollination system, breeding system, and floral traits) may simply reflect the shared evolutionary history of a group and are not necessarily the product of selective forces. Therefore, a well-resolved phylogenetic tree is needed for comparative studies that can control for any shared evolutionary history (Freckleton, 2000; Machado and Lopes, 2004; Nosil and Mooers, 2005; Sanderson and Donoghue, 1996; Vamosi et al., 2003).

Identifying transitions in floral traits allows us to determine when evolutionary changes took place in relation to the transitions in pollination and breeding system (Alcantara and Lohmann, 2010; Armbruster and Baldwin, 1998; DeWitt Smith, 2010). Previous studies in the evening primroses (genus *Oenothera*) suggested that shifts in reproductive traits played a key role in floral diversification and increased species richness (Raven, 1979). Here, we identify the phylogenetic placement and directionality of shifts in the reproductive traits (pollination system, breeding system, and floral reward traits) for 45 of the 50 species within a well-supported clade of *Oenothera* that includes sections *Kneiffia*, *Paradoxus*, *Megapterium*, *Peniophyllum*, and *Gaura*. This allows us to address the following hypotheses: (1) Reproductive floral traits, breeding system and pollination system evolve independently of the evolutionary history in these *Oenothera* species; and (2) Floral traits, breeding system, and pollination system show strong patterns of correlated trait evolution. Finally, we discuss the possible association between reproductive trait lability and species richness.

Methods/Materials

Study System

Onagraceae, specifically the genus *Oenothera*, is a model system for studying the evolution of flowering plant reproductive biology (Artz et al., 2010; Clinebell et al., 2004; Evans et al., 2005; Hoch et al., 1993; Johnson, 2010; Moody-Weis and Heywood, 2001; Raven, 1979; Raven, 1988; Theiss et al., 2010; Vilela et al., 2008; Wagner et al., 2007). Our understanding of the phylogenetic relationships within section *Oenothera* has advanced due to recent molecular phylogenetic studies (Hoggard et al., 2004; Levin et al., 2004; Levin et al., 2003; Wagner et al., 2007). The most notable change is that the once segregate genera *Gaura, Calylophus* and *Stenisiphon* now appear within a monophyletic *Oenothera* (Carr et al., 1990; Raven, 1988; Raven and Gregory, 1972). Furthermore, the diversity of pollination and breeding systems within *Oenothera* make it ideal for examining hypotheses regarding their character-state evolution and their effects on diversification. This study uses 45 of the 50 species in sections *Kneiffia, Megapterium, Peniophyllum, Paradoxus* and *Gaura*, which comprise a single well-supported clade within the genus *Oenothera.*

The species of this clade have a broad geographic distribution throughout North America and Mexico in diverse habitats that range from sand dunes, prairie remnants and riparian areas (Raven, 1979; Raven and Gregory, 1972; Straley, 1977; Wagner et al., 2007). These species exhibit a diversity of floral form that includes both diurnal and nocturnal flowering times, as well as a broad array of pollinators including the traditional pollinator associated with *Oenothera*, the hawkmoth. *Oenothera* also have species with pollination systems that include noctuid moth, antlion, bee, fly, wasp, butterfly (Raven and Gregory 1972, Straley 1977, Raven 1979, Nonnenmacher 1999, Moody-Weis 2001,

Clinebell et al. 2004, Krakos unpubl.). Twenty-three of the species sampled are SC, and at least three are fully autogamous. Bee-pollination is most likely the ancestral state to the family of Onagraceae, with hawkmoth pollination as a derived state that has arisen multiple times (Raven, 1979; Raven and Gregory, 1972) and is ancestral to section *Oenothera*. The subsections *Gaura* and *Kneiffia* are hypothesized to have clades with independent transitions back to bee-pollination (Raven, 1979; Raven and Gregory, 1972). Raven (1979) suggested repeated shifts to obligate selfing from bee, fly, noctuid and hawkmoth pollinated species.

Phylogeny

A well-supported phylogeny for these 45 *Oenothera* species recently clarified several relationships in this monophyletic group (Chapter 4), and we use this phylogeny for our comparative analyses. This phylogeny uses 2 nuclear gene regions, *ITS* and *ETS*, and 4 chloroplast genes, *rps16, rbcL*, *ndhF*, and *trnL-F*. We use the Bayesian 50% majority-rule consensus tree of 45 taxa for the analyses in this study (Fig. 6-1). In addition, we pruned the tree to the 26 taxa for which we have pollination specialization scores (Fig. 6-2). This second tree was used in analyses that included measurements pertaining to the level of specialization in a pollination system.

Pollination Systems, Breeding Systems, and Floral Reward Traits

Details of the level of pollination system specialization and the main pollinating functional groups were obtained from field studies as described in Chapter 2 and data on *Oenothera* pollination that has been collected and stored at the Missouri Botanical Garden, specifically the collections of R. Clinebell and D. Gregory and P. Raven. The

breeding systems for these *Oenothera* species are described in Chapter 3 and 4. For this study we described the following floral traits: shape, nectar presence, scent presence, floral tube length, corolla type, anthesis time, brightness, color, and if a species was a complex heterozygote (PTH, or permanent translocation heterozygote) (see Table 6-1). These traits were described in detail in Chapter 3. These data were compiled from greenhouse and field study measurements and from published records. The PTH species were determined from the literature and personal comm. with M. Johnson (Johnson et al., 2009).

Analyses

Phylogenetic reconstruction using Bayesian methods were conducted in MrBayes v3.04 (Huelsenbeck et al., 2001; Ronquist and Huelsenbeck, 2003). We are interested in understanding the evolutionary history of breeding system, pollination system and floral trait characters, and any evidence for non-random combinations of characters. Examining character evolution without accounting for correlations among characters based on shared evolutionary history can introduce known biases (Felsenstein, 1985). Accordingly, we estimated the degree to which evolutionary history affected the distribution of pollination system and floral characters by estimating phylogenetic signal for each of the five characters listed in Table 6-2. We estimated lambda (λ) (Pagel, 1999) which is an estimate of phylogenetic signal where a λ of 0 means no phylogenetic signal and a λ of 1 means strong phylogenetic signal. We estimated λ using the fitContinuous command in the Geiger module (Harmon et al., 2008) of R (R Core Development Team, 2009). We compared AICc scores to demonstrate that the estimate of lambda obtained was a better fit by comparing the AICc score of our estimate of lambda to the AICc score for a

lambda of 0, where a ΔAICc of greater than 4 is considered support for one model over another (Burnham and Anderson, 2002).

Most characters showed strong phylogenetic signal, and thus we needed to account for phylogenetic history when estimating correlations among characters. Ancestral states for breeding system were inferred using the phylogeny generated above, where ancestral states at nodes were assumed to be SI unless that node comprised a clade of 100% SC species. This is necessary because breeding systems shift unidirectionally from SI to SC. We reconstructed ancestral states for discrete characters of pollination systems and the floral traits in *Oenothera* to identify the topological placement of transitions between states. The traits and their states are given in Table 6-1. We estimated ancestral states using stochastic character mapping as implemented in the program SIMMAP 1.5 (Bollback, 2006). The stochastic character mapping analyses were based on 500 post burn-in trees sampled from the posterior distributions estimated in MrBayes (Chapter 4). This approach accounts for phylogenetic uncertainty in the reconstruction process. We conducted 20 realizations per tree for a total of 10,000 simulated character histories. Stochastic character mapping estimates ancestral states stochastically along branches based on given terminal states, a transition rate prior that for our purposes was based on rescaled branch lengths, and a bias parameter that was a flat prior. This method allows for mid-branch transitions rather than restricting transitions between states of a character to occur only at nodes. However, the ancestral state at a given node was determined by compiling 10,000 stochastic character maps and interpreting the proportion of maps for which a node was inferred to be in a given state. We recorded the states for nodes when the probability was above 0.75. Most of the nodes had values

above 0.90. This method also allows for investigation of the mean number and directionality of trait shifts, as well as the mean transition rate for all possible character changes summed across all stochastic reconstructions (Bollback, 2006; DeWitt Smith, 2010; Huelsenbeck et al., 2003; Marten-Rodriguez et al., 2010). We reconstructed the ancestral states for both a complete phylogeny of 45 taxa and the characters: pollination system, breeding system, and 9 floral traits, and again for subset of taxa corresponding to 26 species and the following characters: specialized vs. generalized pollination systems and the 9 floral traits (Table 6-1). We used the smaller tree for investigating the relationship of generalist vs. specialist pollination systems to floral traits because we have detailed pollination data that included both visitation rate and pollen load analysis for these 26 taxa. We ensured that reconstructions for the pruned tree of 26 species did not differ in any way from states estimated for the full 45 species tree with respect to breeding system and pollination system and 9 floral traits (these are the characters for which data was available for all species).

Stochastic character mapping was also used to estimate correlation among floral traits, pollination system, and breeding system. This approach uses the posterior probabilities from the stochastic mapping and samples the character histories across the trees to create a distribution that accounts for phylogenetic relatedness (Bollback, 2006). The association of two characters is the product of the frequency of those two characters. This approach has the benefit of being able to detect associations between characters even if an evolutionary transition is rare (Bollback, 2006; DeWitt Smith, 2010; Huelsenbeck et al., 2003; Marten-Rodriguez et al., 2010).

Results

Phylogenetic Reconstructions

Our results suggest that pollination system and floral traits are not independent of evolutionary history. The λ value for each of the floral traits showed statistically significant phylogenetic signal (Table 6-2). For some traits, flower shape, color, floral tube, orientation, and anthesis time, the signal was very strong. For the other traits, the signal was weaker, but still present. Breeding system is a trait that only shifts from SI to SC and therefore, each origin of SC is independent. The presence of a phylogenetic signal means that our further analyses need to account for phylogenetic history.

Associated Character Evolution

Significant statistical associations between the dominant pollinator of the pollination system and the pollination traits are summarized in Table 6-3. The D statistic is reported for all associations that were statistically significant ($P < 0.05$). Yellow flower color, long floral tubes, an upright orientation, and an actinomorphic floral shape were all significantly associated with hawkmoth pollination. White flower color, short floral tubes, a horizontal orientation, night anthesis, the presence of scent, the presence of nectar, a zygomorphic shape, and not being PTH are all significantly associated with moth pollination. The significant statistical associations between the pollination traits and whether a pollination system was generalist, specialist or not dependent on pollinators are summarized in Table 6-4. We find that a specialized pollination system is significantly associated with long floral tubes, an upright orientation, and zygomorphic floral shape. A generalized pollination system is significantly associated with white flower color, short floral tubes, a horizontal orientation, and an actinomorphic floral shape. Having no

pollinators or having a generalized pollination system both show a significant negative association with moth pollination.

Evolution of floral characters and pollination systems

For the full tree of 45 *Oenothera* species, we find for pollination system, the dominant pollinator group that is the ancestral state for the entire clade is hawkmoths (Fig. 6-3). Within this section, we find that the dominant pollinator group is a labile trait that transitions directionally away from hawkmoth pollination. Posterior transition expectations (mean) for pollination system transitions were: hawkmoth to moth (1.17), hawkmoth to bee (1.52), hawkmoth to no pollinators (1.12), and hawkmoth to bird (1.40). The posterior expectations for transitions between the more derived state pollination systems were: moth to bee (1.95), and moth to no pollinator (2.19). All other transitions between major pollinator groups had posterior expectations below 1.0. These results suggest that directional shifts away from hawkmoth pollination most commonly follow a pattern of hawkmoth to moth to bee, but that transitions also occur directly from hawkmoth to bee, to bird, to fly, or to no pollinator. The most common transition is moth-pollinated to autogamy. The ancestral state for dominant pollinator at the base of subsection *Gaura* is moth pollination, with both bee pollination and autogamy as derived states within the clade. The ancestral state for dominant pollinator for subsection *Kneiffia* is autogamy, with bee pollination as a derived state developing within the subsection (Fig. 6-3). Bird pollination is the ancestral state for subsection *Xanthocoryne*.

Breeding system is a highly labile trait that only transitions directionally from self-incompatible to self-compatible. By definition, the ancestral state for breeding

system is self-incompatibility with 13 transitions to self-compatibility within this clade (Chapter 4). These transitions do not occur in a concerted fashion with the transitions in pollination system, which shows a total of 10 transitions (Fig. 6-3).

Our analyses show a high lability in the floral traits color, brightness, scent, floral tube length, and nectar (Table 6-5). We combined the results for color and brightness because they showed a perfect correlation. Ancestral reconstructions for these traits indicate that the ancestral phenotype for this clade was a vivid yellow flower, with a short floral tube that had nectar but no scent. For color the posterior transition expectations (mean) were: yellow to white (1.99), yellow to pink (1.13), and white to pink (2.26). All other transitions had posterior expectations below 1.0. Ancestral trait reconstructions do show that one transition from yellow to red occurs at the base of subsection *Xanthocoryne*, but this is not a common transition. The ancestral flower at the base of subsection *Gaura* is yellow, with transitions to white and pink as derived states within the clade (Fig. 4). Posterior transition expectations (mean) for floral tube transitions were: short floral tube to long floral tube (6.8) and long floral tube to short floral tube (4.12). The transitions in floral tube did not occur consistently with transitions in any other trait. Posterior transition expectations (mean) for scent were: no scent to scent (11.54) and scent to no scent (9.68). Posterior transition expectations for nectar were: no nectar to nectar (5.10) and nectar to no nectar (12.3). These results indicate that the most common transitions are to gain scent and lose the presence of nectar (Table 6-5). These transitions did not correlate to transitions in pollinator type, breeding system, or other floral traits.

For the floral traits orientation, anthesis, and shape our results show high lability, but that the shifts are directional in that they always shift from one specific state to

another, without reversals (Table 6-5). Ancestral reconstructions for these traits indicate that the ancestral flower for this clade was upright, actinomorphic and opened at night. Posterior transition expectations for these traits were: upright to horizontal (2.09), night opening to day-opening (9.83), and actinomorphic to zygomorphic (2.12). The floral traits of orientation and shape each had only one shift in the phylogenetic tree. The transition to horizontal flowers is basal to subsection *Gaura*, while the transition to zygomorphic flowers occurs within subsection *Gaura* (Fig. 6-4). There are four transitions to day-opening flowers, one is basal to subsection *Kneiffia*, and the second is in subsection *Gaura*, as the ancestral state of the taxa *O. demareei* and *O. lindheimeri*. The third transition to day anthesis subtends a clade that contains the subsections *Hartmannia* and *Gauropsis*. The final transition is *O. linifolia*, which is a single species shift to day anthesis. The transitions in orientation, anthesis, and shape did not occur in a concerted fashion with pollination system, breeding system or other floral traits.

For the tree of 26 *Oenothera* species, stochastic mapping of specialization of pollination systems indicated a high lability for this trait (Table 6-5). Posterior expectations (mean) for specialization were: generalist to specialist (4.10), specialist to generalist (7.41), generalist to no pollinators (3.03), and specialist to no pollinators (4.54). While the most common transition was from specialist to generalist pollination systems, we do see a reversal back to a specialist pollination system within subsection *Gaura* (Fig. 6-5) at the base of the clade that includes *O. demareei*, *O. lindheimeri*, and *O. coloradoensis.* ssp. *neomexicana*. The transitions in specialization do not occur in concert with breeding system, pollinator group, or the other floral traits.

For the full tree of 45 species, ancestral trait reconstruction was not clear on the dominant pollinator for the clade consisting of both subsections *Gaura* and *Kneiffia*. The ancestral state for the pollination system of these two subsections was either moth pollination or autogamy; however this ambiguity does not impact the total number of transitions in the phylogenetic tree. If the ancestral state was moth, then a transition to autogamy happened on the branch to subsection *Kneiffia*, and if the ancestral state was autogamy, then the transition to moth pollination happened on the branch *Gaura*. Either scenario results in a total of 10 transitions in pollination system for this clade.

For the tree of 26 species, ancestral trait reconstructions were not clear for dominant pollinator and specialization of pollination system for the clade that encompasses *O. curtiflora* and *O. glaucifolia* (Fig. 6-5). The ancestral state of the dominant pollinator group for this clade was either bee or no pollinator, and with an ancestral state of fly or moth at the node immediately preceding this clade. Likewise, for specialization the ancestral state for this clade is either generalist or specialist, and the immediate ancestor for these two *Oenothera* is either generalist pollinated or has no pollinator. In both cases, the state of the trait does not alter the overall number of transitions.

Overall, our results to do not show that transitions in any floral traits are consistently occurring in a concerted fashion. However, our results do show the order of the transitions and thus reveal which floral traits occurred prior and post a transition in pollination system (Fig. 6-4). Flowers had short floral tubes and scent prior to the transition to moth pollination; however the shift from yellow to white flowers happened after this transition. The transition from actinomorphic shape to zygomorphic flowers

also occurs after the transition to moth pollination. In subsection *Gaura*, the shift to bee pollination occurs in concert with a shift to pink, day opening flowers, but not in tandem with a shift in scent. While in subsection *Kneiffia*, the transition to day opening occurs with a transition to no pollinators, and a later transition to bee pollination occurs in tandem with a transition to having scent. There is no transition in color for subsection *Kneiffia*. A transition to bee pollination in subsection *Hartmannia* does not occur in concert with a shift in any floral trait, but is preceded by a transition to day opening and long floral tubes for the clade that encompasses both *Hartmannia* and *Gauropsis*. The transition to bird pollination in subsection *Xanthocoryne* happens concurrent with a shift to short tubes, but the transition to red colored flowers occurs at an earlier ancestral node that encompasses the subsections *Xanthocoryne* and *Leucocoryne* and is not associated with a pollinator shift. *O. linifolia* transitions to fly pollination and day opening at the same time.

In the tree with 26 *Oenothera* species, we see 9 transitions in specialization of pollination system and 8 transitions in dominant pollinator group; however these transitions are not correlated. The transitions in specialization do not correlate to breeding system or other floral traits. A transition back to specialist pollination occurs prior to the transition to bee pollination in subsection *Gaura*, however this transition in specialist pollination is not seen as an exclusive precursor to other bee pollination transitions (Fig. 6-5).

Discussion

In a very general way, we can identify two morphological "types" of flowers in this phylogeny (Fig. 6-6). The first is the traditional "*Oenothera*-type" flower, which is most commonly yellow and actinomorphic. The second is what we refer to as the "*Gaura*-type", which has a white, zygomorphic flower. Until the most recent phylogenetic hypotheses for these species, subsection *Gaura* was recognized at the genus level (Levin et al., 2004; Wagner et al., 2007). The distinct *Gaura*-type inflorescence shape is easily recognized. *Oenothera anomala*, which is the basal species in subsection *Gaura*, is clearly a transitional species between the two types, with its yellow color, but the beginnings of the *Gaura*-type shape. It is dominantly moth pollinated; however, hawk moths have been seen occasionally visiting the flower (personal comm. P. Raven). This is consistent with its place as a transitional species between the traditional actinomorphic *Oenothera*-type flower and the derived zygomorphic, white "*Gaura*-type" flower. This very distinctive transition in flower type within section *Oenothera* prompted the questions for this study.

Breeding and Pollination systems

We first focused on whether breeding system and pollination system traits were correlated in the phylogenetic tree. Breeding system and pollination system have usually been studied separately, despite the relationship between these two aspects of plant reproductive biology (but see Fenster and Marten-Rodriguez, 2007; Perez et al., 2009). The need for studies of plant reproductive studies to address these two systems simultaneously has been emphasized (Barrett, 2003; Fenster and Marten-Rodriguez, 2007; Holsinger, 1996). Our results showed no association between these two systems. In addition, ancestral trait reconstructions show that these two systems do not transition in a

concerted fashion. These results provide strong statistical support that the evolutionary correlation between breeding system and pollination system may be under different and unlinked selective forces (Armbruster, 1994; Fenster and Marten-Rodriguez, 2007).

Evolution of floral characters and pollination systems

The correlation between a suite of floral traits and a pollinator has been tested by numerous studies assessing the pollinator syndrome concept (Consiglio and Bourne, 2001; Fenster et al., 2004; Hingston and Mc Quillan, 2000; Ley and Classen-Bockhoff, 2009; Muchhala, 2006; Ollerton et al., 2009; Reynolds et al., 2009). When assessing this relationship, there are two important factors that could lead to erroneous conclusions. First, the pollination system must be assessed from detailed pollination ecology data that takes into account not only visitation, but also pollen flow (Chapter 2). This is to ensure correct identification of the major pollinators, and not mistake visitors as pollinators, and is a need that has been recently discussed (Fenster et al., 2004; Johnson and Steiner, 2000; Tripp and Manos, 2008). In this study, we determined the pollination system from detailed pollination ecology that was collected for these *Oenothera* species (Chapter 2). Second, these correlations may need to be addressed in a phylogenetic context (Smith, 2010). A test for phylogenetic signal of a trait can elucidate whether this context is necessary to avoid misinterpretation of the data. If a strong signal is present, and correlations are conducted without controlling for phylogenetic history, the results could be skewed, and erroneously suggest that a tight correlation exists between certain traits, when in reality, the correlation is entirely due to ancestry. This could also lead to improper conclusions about pollination syndromes. In our study we tested for phylogenetic signal in the floral traits being addressed. Our results found a phylogenetic

signal, ranging from weak to very strong, for all of the traits (Table 6-2). Therefore, we tested for associations between these floral traits and the pollination systems in a phylogenetic context. When evolutionary history was taken into account, we found that the associations between pollinator and floral traits do not give sufficient information to support the presence of clear pollination syndromes (Table 6-3). Bee, Bird, and Fly pollinator groups do not have significant associations with enough traits to be meaningful. Moth pollination is associated with zygomorphic, white flowers with short tubes and hawkmoth with actinomorphic, yellow flowers with long tubes, but these traits are not exclusive to these pollination groups. For instance, all the taxa in section *Kneiffia* have yellow, actinomorphic flowers, but none of them are pollinated by hawkmoths.

A specific floral trait repeatedly evolving across a phylogeny in a correlated fashion with a pollination system is used as evidence for adaptation (Pagel, 1999). Several studies have looked at how changes in a specific floral trait play a key role in the diversification process (Kadereit and von Hagen, 2003; Smith et al., 2008b; von Hagen, 2007; von Hagen and Kadereit, 2003; Whittall and Hodges, 2007), as an example of the idea of "key innovations," which is a morphological character that is responsible for higher diversification rates (Maynard and Szathmary, 1995). The ancestral state reconstructions of the pollination systems and reproductive reward traits in *Oenothera* give a much more detailed picture of the evolutionary history and possible selective pressures involved (Fig. 6-4). The identification of when specific shifts took place in pollination system, breeding system, and floral traits allows us to establish an order for significant evolutionary changes.

For many species, color seems to be a major selective pressure on the interaction of a plant with its pollinator. For example, in *Ruellia*, authors found that a shift from red to purple flowers was concurrent with a switch from hummingbird pollination to bee and butterfly pollination and that a shift from purple to white coincided with a shift to moth pollination (Tripp and Manos, 2008). In *Iochroma* (Solanaceae), authors found that a shift in flower color was a preadaptation for subsequent pollinator system shifts (Smith et al., 2008b). In this study, we find that floral color is not always correlated with a pollination system. The major transition from hawkmoth pollination to moth pollination occurs prior to the shift from yellow to white flowers (Fig. 4). However, the shift to bird pollination in section *Xanthocoryne* happens in a concerted fashion with a shift to red flowers. The shift to bee pollination in section *Kneiffia* is not associated with any shift in color, but the shift to bee pollination in section *Gaura* coincides with a shift to pink flowers. Therefore, we cannot make a judgment that color is a selective pressure (Baum and Larson, 1991).

Floral traits that relate to a pollinator's ability to reach a reward such as nectar have also been focused on as a driving reason for pollinator shifts and floral diversification (Johnson et al., 1998). For example, in *Dianthus*, butterflies with shorter proboscides correlated to shifts in the floral tube length (Bloch and Erhardt, 2008). And Whittall and Hodges (2007) found that shifts towards pollinators with longer tongues were correlated to increased nectar spur length in *Aquilegia*. Our results indicate that a shift to longer floral tubes occurs after the transition to hawkmoth pollination, which gives evidence that pollinators are a selective pressure for longer floral tubes. Subsequent reversals back to short tubes, such as in *O. linifolia*, correlate with a transition to fly or

small bee pollination. This same concerted shift in pollination and floral tube length is seen in subsection *Hartmannia* where a shift to bird pollination is concurrent with a shift to short floral tubes. However, the major transition from hawkmoth pollination system to moth pollination occurs without a transition in floral tube length. This finding indicates that hawkmoths select for long floral tubes, but moths do not necessarily select for short or long floral tubes.

Specialization and Pollination systems

The role of specialization in pollination systems and angiosperm evolution has been the subject of many recent studies (Fenster et al., 2004; Johnson and Steiner, 2000; Larsson, 2005; Marten-Rodriguez et al., 2010; Muchhala, 2006; Nosil and Mooers, 2005; Tripp and Manos, 2008; Weller and Sakai, 1999). Our study included detailed pollination ecology data, based on visitation rate and pollen load analysis, in order to accurately characterize the specialization of the pollination systems. The use of functional groups (Fenster et al., 2004), provides a more accurate measurement of specialization of the pollination systems. We were specifically testing for associations between pollinator specialization and breeding system, dominant pollination group, and specific floral traits. Historically, the association of specialization in pollination system with breeding system has not been found to have a clear pattern (Fenster and Marten-Rodriguez, 2007). Here we evaluate this problem by simultaneously taking into account evolutionary history and precise measurements of pollination biology. Our results also do not find a clear association between specialization of pollination system and breeding system. With regards to an association between specialization and dominant pollinator group, for all of the pollinator groups, except moth, our results do not show an association. For example,

bee pollination in subsection *Kneiffia* does appear to correlate to specialist pollination; however, the transition to specialist pollination happened much earlier in the clade and encompasses both moth and hawkmoth pollination systems (Fig. 6-5). In addition, *O. glaucifolia* is bee-pollinated, but has a generalist pollination system. There is a negative correlation between generalist pollination and moth pollination, which suggests that species that are predominantly pollinated by moths are not generalist pollinated. This makes sense because although multiple species of moth visit *Oenothera*, they are usually grouped as one functional group defined by size and tongue length.

Pollination syndromes, or the concept that floral traits, or suites of floral traits correspond to a specific pollinator (Faegri and van der Pilj, 1979), are inherent to the idea of specialization in pollination systems. There are many differing opinions and results regarding the pollination syndrome concept, and its reality in nature (Fenster et al., 2004). Our results do not show a clear association of a suite of floral traits with specialist or generalist pollination systems (Table 6-4). However, for these *Oenothera* species, we do see an association between generalist pollination and flowers that are white, horizontal, actinomorphic, and with short floral tubes. In addition, we see an association between specialist pollination systems and upright, zygomorphic flowers with long floral tubes. Long floral tubes are a trait that excludes most pollinator groups, and is expected to be associated with specialization. Zygomorphy is a floral trait that has been associated with specialization of pollination systems in other species (Fenster et al., 2004; Fenster and Marten-Rodriguez, 2007).

Transitions in pollination system have occurred most often between specialist systems (Kay and Sargent, 2009a; Whittall and Hodges, 2007; Wilson et al., 2007), and

from generalist to specialist (Thomson and Wilson, 2008; Tripp and Manos, 2008), with limited examples of reversals back to a specialized pollination system from a generalized pollination system (Marten-Rodriguez et al., 2010). Our data most often show a transition towards specialization, or that the level of specialization is maintained when a transition to a new dominant pollinator occurs. However, we do add another example of a shift to generalist pollination followed by a reversal back to a specialist pollination system (Fig. 6-5). What is striking is that the clade in which the reversal to specialist pollination occurs contains two transitions to two different pollinator groups. This is in agreement with our result that finds no association between a specific pollinator group and having a specialist pollination system.

It is not well understood what prompts a shift in pollination system (Campbell, 2008), but one compelling argument is that transitions happen when the plant habitat alters in a way that affects pollinator service (Kay and Sargent, 2009b). Shifts between pollinators or use of multiple pollinator taxa can provide reproductive assurance for a plant species. The floral traits of a plant species may reflect adaptation for a particular type of pollinator, yet the plant species may still utilize multiple pollinator taxa as a bet-hedging strategy. For instance, the evening-opening, hawkmoth pollinated *Oenothera macrocarpa* is also pollinated by bees in the early morning (Moody-Weis and Heywood, 2001; Nonnenmacher, 1999).

Breeding System Lability and Species Richness

The role of pollinators is central to the radiation of angiosperms (Brown, 2002; Solds et al., 2008), and recent studies have focused specifically on how transitions in

pollination systems influence plant diversification (Campbell, 2008; DeWitt Smith, 2010; Kay and Sargent, 2009b). The shifts in pollination systems are thought to affect rates of angiosperm speciation (Cozzolino and Widmer, 2005; Sanderson and Donoghue, 1996; Smith et al., 2011). Transitions toward more specialized pollination systems have been shown to correlate with increased species richness (Armbruster and Muchhala, 2009; Schiestl and Schlater, 2009). While we did not test the effect of pollination system directly on diversification rate, our results do not show a correlation between a pollination system, specialization, or a specific floral trait, but do suggest that the high lability of a trait is associated with higher species richness in *Oenothera* evolution. However, it is most likely the lability of breeding system that influences species richness the most. We look at the example of the high number of annual species to illustrate this concept.

The hypothesis that breeding system and life history are associated predicts that a high number of annual plants would be self-compatible (Barrett et al., 1996), because annuals have only one season of reproduction, and SC would provide reproductive assurance. Although it is an expected pattern seen in other species (Barrett, 2010b), these 45 *Oenothera* species show no association between breeding system and life history. Many of these *Oenothera* are annual species that can be either SC or SI, and several of the perennial species are SC. However, this *Oenothera* clade does have a high number of transitions to SC, and breeding system is one of the key traits of annual plants that is associated with their high rate of speciation (Barrett, 2010b). Clades with numerous transitions to SC, are by definition highly labile for this trait, because it is a directional transition without the ability to transition back to SI (Igic et al., 2006). Therefore, annual

plants have highly labile breeding systems and transition with more frequency to SC than perennial plants. Perennial plants have a higher association with SI breeding systems, and this is considered one of the reasons for the disparity in species numbers between the two groups (Barrett, 2010b; Barrett et al., 1996). The higher number of species in the annual plants, compared to the perennials is driven by the lability of breeding system. In this *Oenothera* clade, the high lability of breeding system could lead to increased rates of diversification. In comparison to other lineages of the same age and distribution, but that are not associated with as many transitions to SC, we would expect to see fewer number of species. As more studies define the breeding system of species with well-resolved phylogenies, this can be tested further. It might not be the specific state of a trait that is important to diversification, but rather, the ability to transition between different states.

Conclusions

In conclusion, stochastic mapping of multiple floral traits and pollination system show that even within this clade of 45 species, the interactions between plants and their pollinators are complex and diverse. The set of circumstances that lead to shifts in pollination system, breeding system and morphological diversification vary for each clade. There is no one trait exerting selective pressure on the plant or the pollinators that is responsible for the evolutionary patterns and transitions in these *Oenothera*. The placement of floral trait transitions with regards to pollinator shifts suggests selective pressures in floral traits that are predictable and follow transitions to novel dominant pollinator groups, rather than a change in pollination system. We speculate that the lability of breeding system, rather than the frequency of specific breeding system traits, is consistent with higher levels of lineage diversification of these *Oenothera*. Our next step

will be to test for an association between specialization and diversification rates in this phylogenetic tree. An analysis that simultaneously addresses diversification rates and character evolution (e.g, Binary State Speciation and Extinction models:(Maddison et al., 2007; Smith, 2010), could be used to test the effect of floral traits, breeding system and pollination system on diversification rates (Kay and Sargent, 2009b; Smith, 2010).

Tables

Table 6-1. The nine floral traits, and the reproductive systems, and the character states for each that were used in the phylogenetic analyses for both the full phylogenetic tree of all 45 *Oenothera* species and the trimmed tree of 26 *Oenothera* species.

Reproductive Systems and Floral Traits	
Major Pollinator Group	**Breeding System**
Moth	Self-compatible
Bee	Self-incompatible
Bird	**Specialization**
Fly	Generalist
Hawkmoth	Specialist
None	No Pollinators
Color at Anthesis	**Time of Anthesis**
Yellow	Day
White	Night
Red	**Scent**
Pink	Present
Brightness	Absent
Vivid	**Shape**
Drab	Actinomorphic
Floral Tube	Zygomorphic
Short	**Nectar**
Long	Present
Orientation	Absent
Upright	**PTH**
Horizontal	Present
	Absent

Table 6-2. Estimation of the phylogenetic signal (λ) conducted in SIMMAP 1.0 (Bollback, 2006) for all reproductive systems and floral traits.

Character	**Signal**	**Estimate of Signal (λ)**
Breeding System	No*	na
Major Pollinator	Yes	0.998
Dual Pollination	Yes	0.472
Color	Yes	1
Brightness	Yes	1
Scent	Yes	0.974
Floral Tube	Yes	1
Orientation	Yes	1
Anthesis	Yes	1
Shape	Yes	1
Nectar	Yes	0.995
PTH	Yes	0.579

* Breeding system is excluded from these results because a distribution of the two character states is is not possible given the rules of directional transitions for self-compatibility in plants.

Table 6-3. The statistic *D* for tests of association between the main pollinator functional groups and the floral traits generated by SIMMAP 1.0 (Bollback, 2006). Associations for all *P*-values less than 0.05 are reported. Negative associations are indicated by a minus sign, ns means there is no significant association between the two groups.

	***D*-value**					
	Hawkmoth	**Moth**	**Bee**	**Bird**	**Fly**	**None**
Color						
Yellow	0.045	-0.072	ns	ns	ns	ns
White	-0.068	0.117	ns	ns	ns	-0.013
Red	ns	ns	ns	ns	ns	ns
Pink	ns	-0.03	ns	ns	ns	ns
Floral Tube					ns	ns
Short	-0.071	0.052	ns	ns	ns	ns
Long	0.071	-0.052	ns	ns	ns	ns
Orientation						
Upright	0.1	-0.142	ns	ns	0.01	0.01
Horizontal	-0.1	0.142	ns	ns	-0.01	-0.01
Anthesis						
Day	ns	-0.062	0.039	0.02	ns	ns
Night	ns	0.062	-0.039	-0.02	ns	ns
Scent						
Present	ns	0.038	ns	ns	ns	ns
Absent	ns	-0.038	ns	ns	ns	ns
Shape						
Actinomorphic	0.066	-0.108	ns	ns	ns	ns
Zygomorphic	-0.066	0.108	ns	ns	ns	ns
Nectar						
Present	ns	0.041	ns	ns	ns	ns
Absent	ns	-0.041	ns	ns	ns	ns
PTH						
Present	ns	-0.027	ns	ns	ns	ns
Absent	ns	0.027	ns	ns	ns	ns

Table 6-4. The statistic *D* for tests of association between the pollination system specialization state and the dominant pollinator or the floral traits conducted in SIMMAP 1.0 (Bollback, 2006). Associations for all *P*-values less than 0.05are reported. Negative associations are indicated by a minus sign, ns means there is no significant association between the two groups.

	***D*- value**		
	Specialist	**Generalist**	**None**
Color			
Yellow	ns	-0.022	ns
White	ns	0.021	ns
Pink	ns	ns	ns
Floral Tube			
Short	ns	0.033	ns
Long	0.025	ns	ns
Orientation			
Upright	0.011	-0.019	ns
Horizontal	-0.011	0.019	ns
Shape			
Actinomorphic	-0.022	0.021	ns
Zygomorphic	0.022	-0.021	ns
Main Pollinator			
Hawkmoth	ns	ns	ns
Moth	ns	-0.019	-0.011
Bee	ns	ns	ns
Bird	ns	ns	ns
Fly	ns	ns	ns

Table 6-5. A summary of the key reproductive trait character state transitions as generated in SIMMAP 1.0 (Bollback, 2006).

Trait	Lability	Pattern	Transition Rate (Posterior Expectations)
Major Pollinator	directional	Hawkmoth > Moth > Bee	2.91
Breeding System	na	SI > SC	16.09
Pollination Specialization	high	Specialist > Generalist	7.41
		Generalist > Specialist	4.1
		Specialist > No Pollinator	4.54
		Generalist > No Pollinator	3.03
Color	high	Yellow > White	1.99
		White > Pink	2.66
		Yellow > Pink	1.13
Scent	high	No scent > Scent	11.54
		Scent > No Scent	9.68
Floral Tube	high	Short > Long	6.8
		Long > Short	4.12
Orientation	directional	Upright > Horizontal	2.09
Anthesis	directional	Night > Day	9.83
Shape	directional	Actinomorphic > Zygomorphic	2.12
Nectar	high	Nectar > No nectar	12.3
		No nectar > Nectar	5.1
PTH	na	No PTH > PTH	8.9

Figures

Figure 6-1. Bayesian phylogenetic reconstructions of 45 *Oenothera* species from concatenated mixed model data set for genes *ITS*, *ETS*, *rps16*, *trnL-F*, *rbcL*, and *ndhF* (Chapter 4). Numbers above the branches indicate posterior probabilities for branch support.

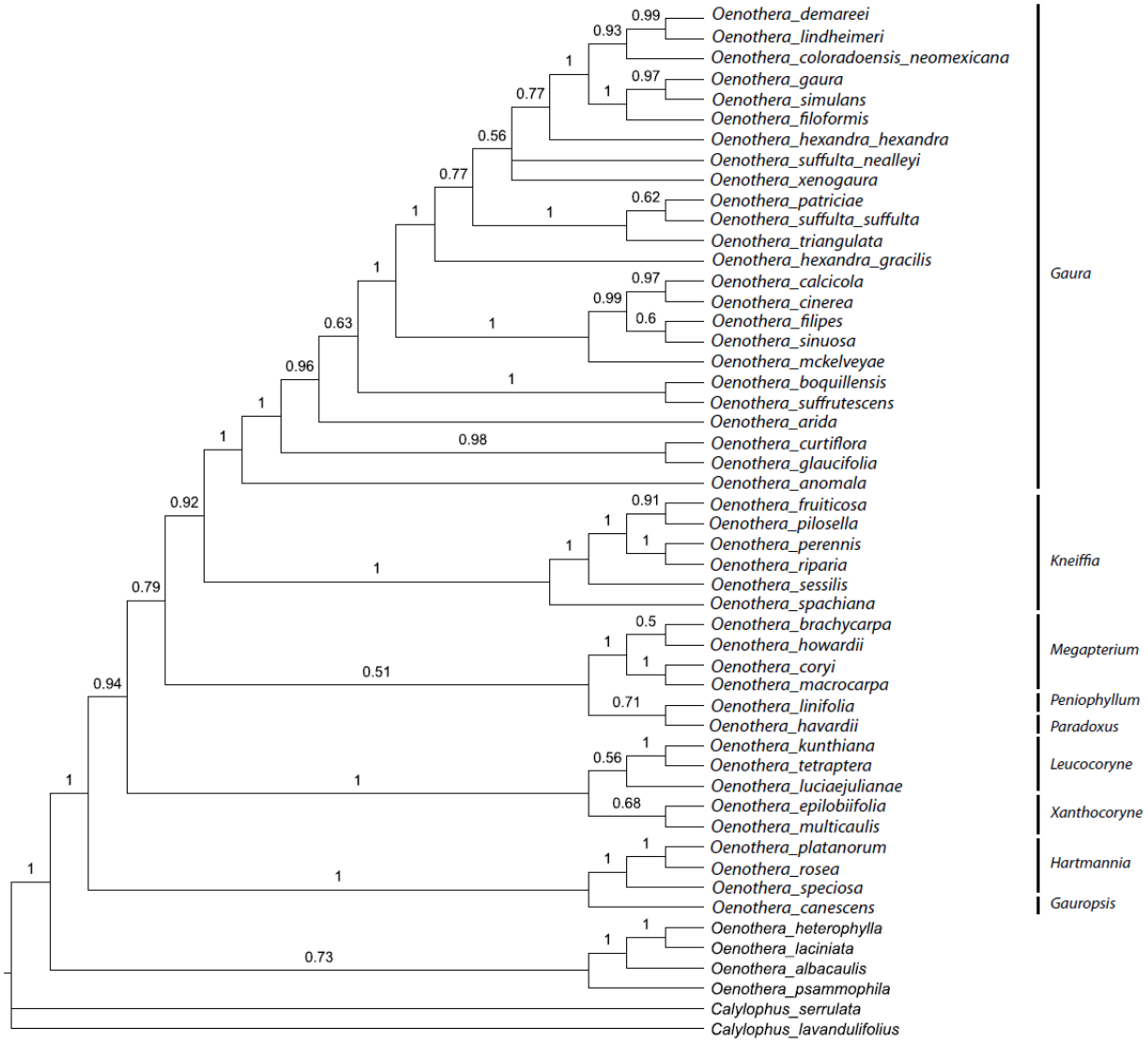


Figure 6-2. Bayesian phylogenetic reconstructions from concatenated mixed model data set for genes *ITS, ETS, rps16, trnL-F, rbcL*, and *ndhF*, 26 *Oenothera* species.

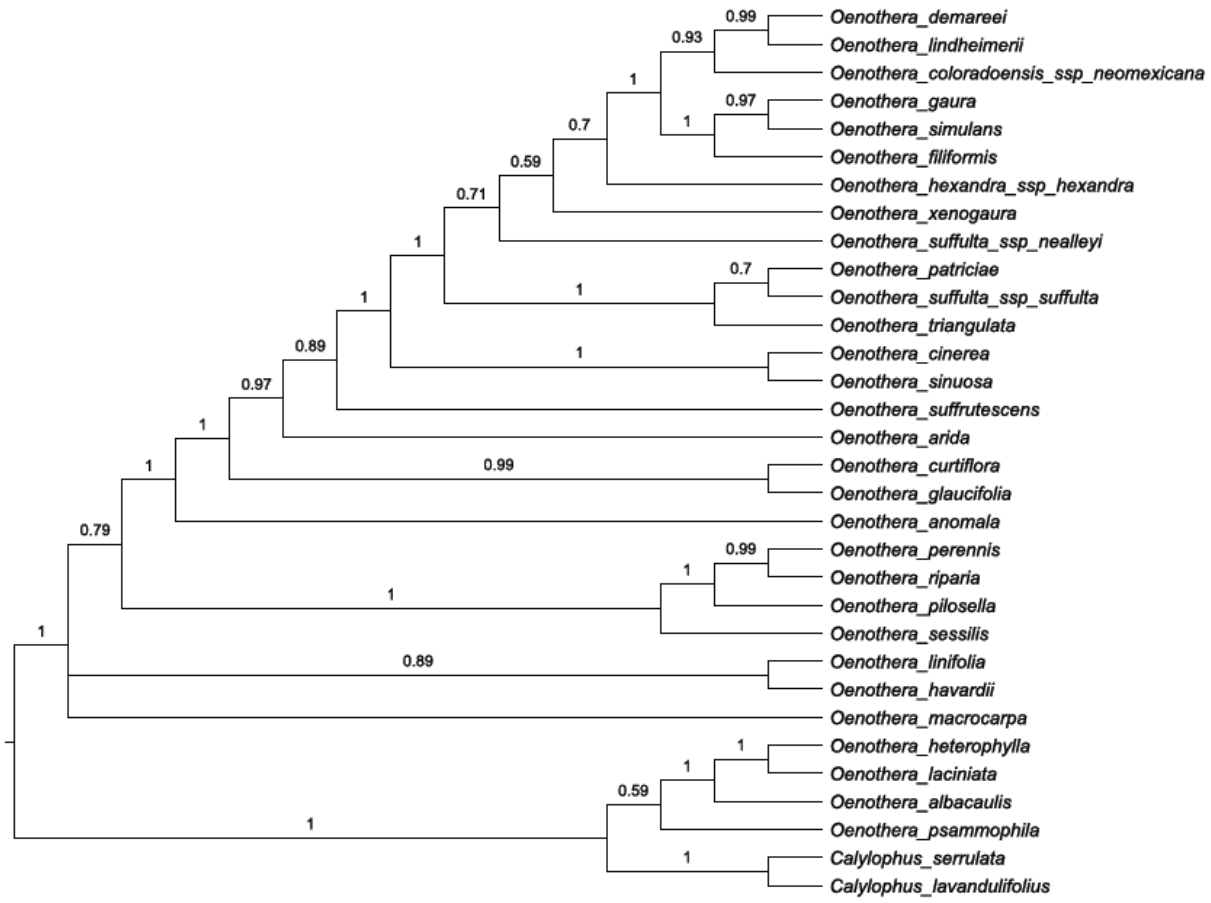


Figure 6-3. A comparison of the evolution of the pollination and breeding systems of 45 *Oenothera* species under Bayesian stochastic character mapping. On the left is pollination system and on the right is breeding system. Transitions between character states are indicated by black vertical bars.

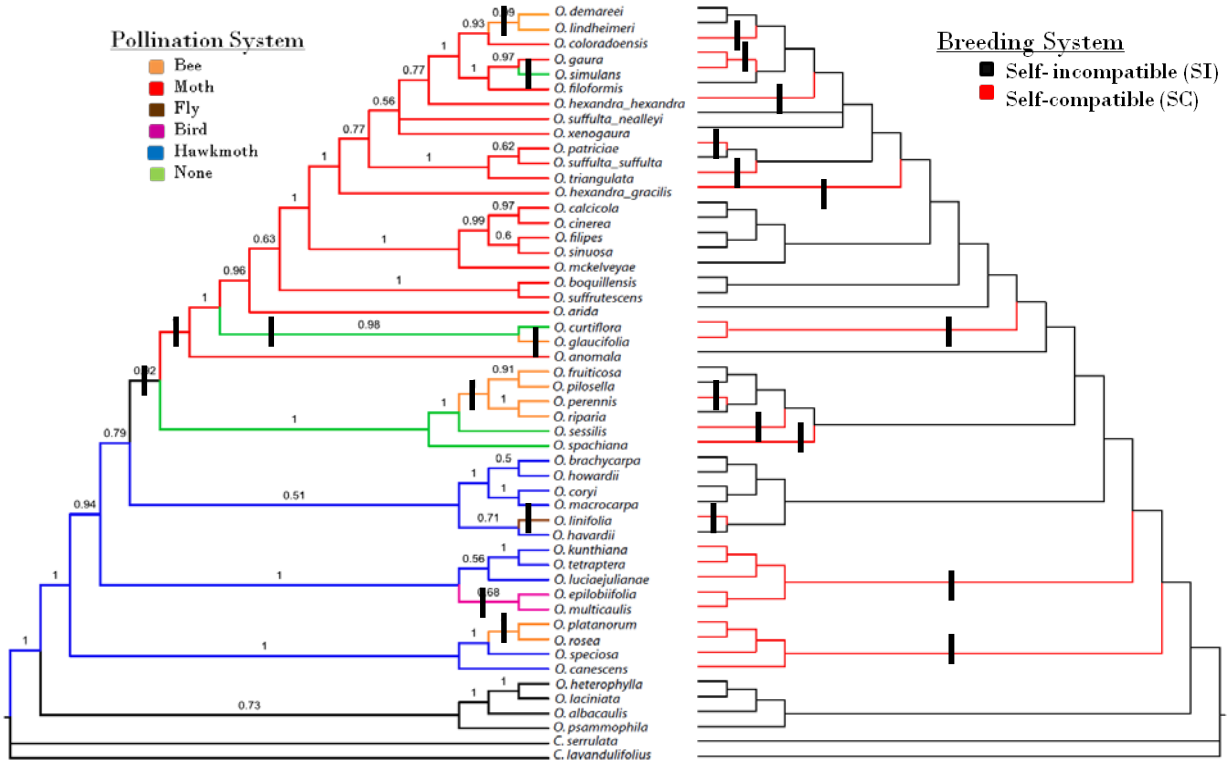


Figure 6-4. A summary of the evolution of the timing of key points of transition for reproductive traits as determined by stochastic character mapping. Pollination system is indicated by color changes along the branches. The character states in the box are those of the ancestor of the clade as determined by the stochastic character mapping. The point of transition for the floral traits, as inferred from the ancestral trait reconstructions are indicated with arrows and labels.

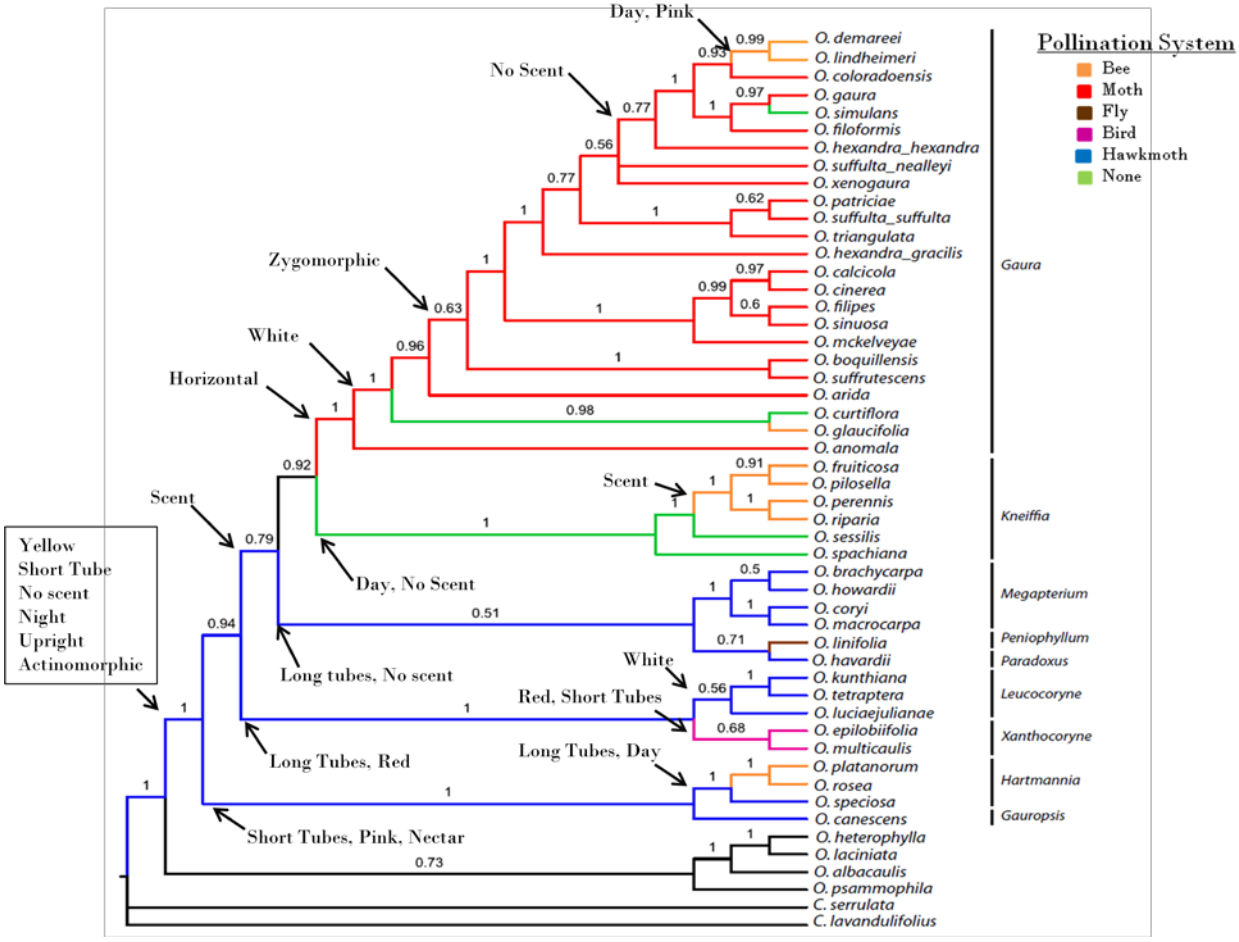


Figure 6-5. A comparison of the evolution of timing of transitions in pollination system and level of specialization under Bayesian stochastic character mapping using the 26 *Oenothera* taxa phylogenetic tree. On the right is the specialization of the pollination system and on the left in the main pollinator group. Transitions between character states are indicated black vertical bars.

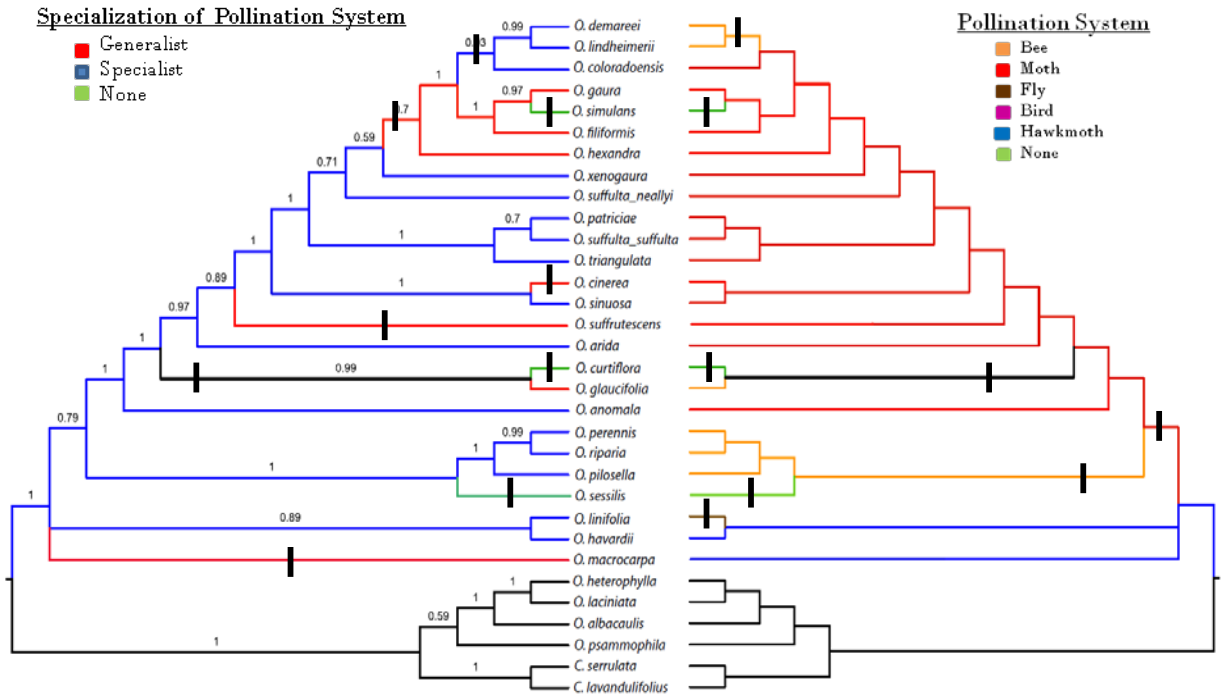


Figure 6-6. Representative taxa of the two morphologically distinct types of *Oenothera* within the clade. A. The "*Gaura*" type is represented by *O. demareei*. B. The traditional "*Oenothera*" type is represented by *O. pilosella* photo credits: K. N. Krakos.

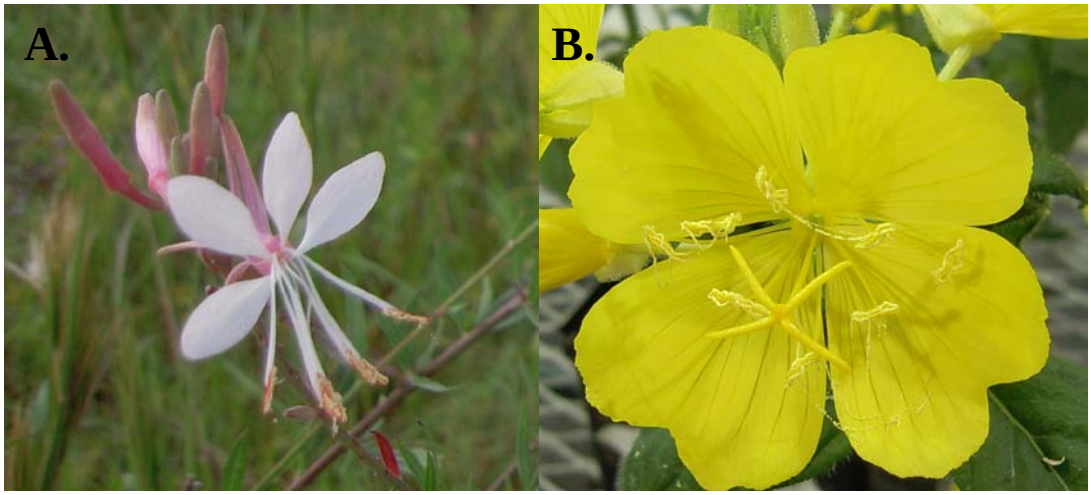
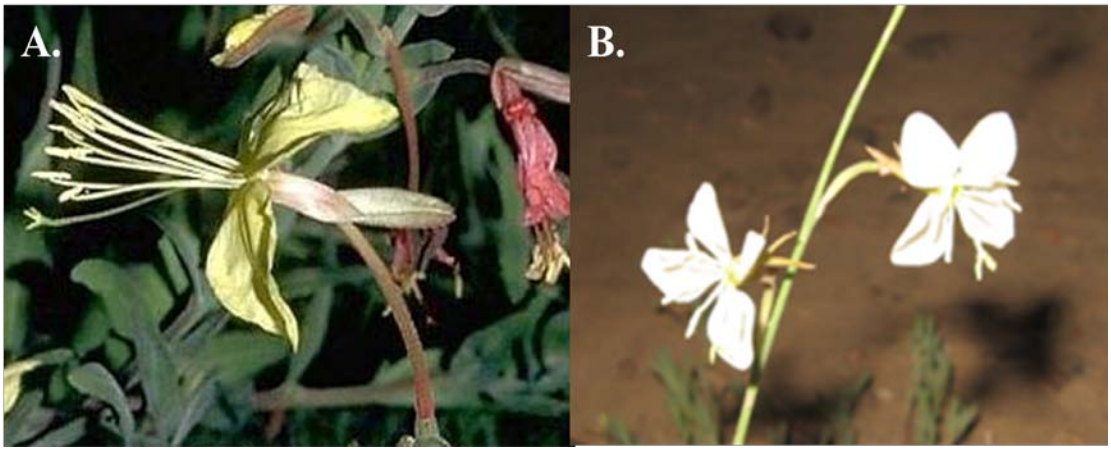


Figure 6-7. Taxa showing transitional floral traits between the two types of *Oenothera* within the clade. A. *O. anomala* photo credits: W. Wagner B. *O. arida* photo credits: K. N. Krakos



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CHAPTER 7

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CONCLUSIONS AND FUTURE WORK

The goal of this work is to describe the relationship between pollination and breeding system in *Oenothera,* with an integration of both ecological and phylogenetic approaches. First, I collected detailed data describing the pollination systems, breeding systems, and floral traits associated with pollinator rewards; and second I determined the phylogenetic structure, evolutionary history and relationships among these species. Finally, in that phylogenetic context, I examined the timing and position of transitions in the reproductive traits and how these traits are associated with pollination and breeding systems. My results confirm that plant-pollinator interactions play an important role in the diversification of floral form, but also offer new insights regarding the specialization of pollination systems, the predictive power of pollination syndromes, and how the lability of pollination and breeding systems impacts the evolution of *Oenothera*. My results also clarified phylogenetic relationships in the genus *Oenothera*, and provided the first phylogenetic tree for subsection *Kneiffia*.

One of the major themes in pollination biology is generalization and specialization in pollination systems (Waser and Ollerton, 2006). Floral trait evolution has long been attributed to the co-evolution of plants with their animal pollinators (Kay and Sargent, 2009). This evolutionary history suggests that plant-pollinator interactions are highly specialized (Fenster et al., 2004); however, in the last two decades research has shown that the pollination ecology of most plants is highly generalized (Ollerton and Coulthard, 2009; Waser et al., 1996). My goal was to address this apparent paradox by using detailed measurements of the *Oenothera* plant-pollinator interactions to show that visitation alone does not accurately describe the specialization of a pollination system. My results did show that visitation alone highly overestimated the number of pollinators,

but it also highlighted that visitation and pollination are not necessarily correlated. This was an unexpected result and highly relevant to how future studies interpret and draw inferences from visitation data. My pollination ecology work also agreed with the work of Fenster (Fenster et al., 2004), who also found that when pollinators were placed into functional groups, most pollination systems used only one or two pollinator groups.

Another major approach in understanding the role of specialization in pollination systems is to re-examine the concept of ”pollination syndromes” and look at how these interactions are affecting broader evolutionary patterns. My goal was to evaluate the predictive power of pollination syndromes by comparing the predicted pollinators with the observed pollinators. My results agreed with recent work by Ollerton (2009), in that I also found pollination syndromes were not a reliable substitute for determining a plant's pollination system. In addition, I evaluated the accuracy of the current methods of determining pollination syndromes, and found that these methods were inadequate for these *Oenothera*. While floral measurements alone may be enough to delineate pollination groups for some species, this work highlights the need to address that assumption carefully.

One of the primary taxonomic revisions resulting from this work relates to the subsection *Kneiffia*. These results provide the first phylogenetic tree for subsection *Kneiffia*, and clarifies relationships of these species, which have not been directly addressed since the work by Straley (Straley, 1977). My work found two new results pertaining to this group. First, based on molecular data and the new information about its reproductive biology, the taxon known currently as *Oenothera pilosella subsp. sessilis* (Pennell) Straley is in the process of being reclassified as a separate species, *Oenothera*

sessilis (Pennell) Munz (Munz, 1965). Second, the phylogenetic data showed a hundred percent support for O. riparia and O. perennis as sister taxa, and for *O. fruticosa* and *O. pilosella* as sister taxa. This is in contrast to Straley's hypothesis that the self – incompatible species would all group together. My results show at least two independent transitions to self-compatibility.

The new phylogeny provides greater insights and clarity to the relationships of these 45 *Oenothera in* Subclade B. Many of the relationships among taxa that are suggested by earlier studies (Hoggard et al., 2004; Levin et al., 2004), now, with this broader taxonomic sampling, are stronger. Sections *Megapterium*, *Kneiffia*, and *Gaura* are all monophyletic, with all species included. Although section *Hartmannia* was not fully sampled, the taxa included did form a strongly supported clade. Within subsection *Gaura, O. suffulta* and *O. hexandra* both consist of two subspecies that fail to group together in the phylogeny. Our results suggest that *O. suffulta ssp. nealleyi* and *O. hexandra ssp gracilis,* based on both molecular data and reproductive traits, need to be taxonomically reassessed. The breeding system data verified the compatibility of several of the *Oenothera,* and gave new information for several species. The results demonstrate the lability of breeding system in this clade, and in accordance with previous studies (Raven, 1979; Raven, 1988), also shows that sister taxa can differ in breeding system.

Finally, this work examined the patterns of correlated trait evolution among the pollination systems, breeding systems, and floral traits within this clade of 45 *Oenothera* species. Breeding system and pollination system were not associated for these taxa, and did not transition between character states in a concerted fashion. In addition, no one floral trait was responsible for the transitions in pollination system; rather, the

interactions between the plant species and their pollinators are complex. Floral trait transitions within these *Oenothera* are sometimes in response to a pollinator group, and sometimes unaffected by a transition in pollinator system. The floral traits that shift after a transition in pollination system are most likely experiencing selective pressure to a new dominant pollinator group, rather than selective pressure to shift to a different pollination system. These results highlight the importance of order and timing of transitions in floral traits and pollination system to understanding the evolutionary history of these *Oenothera*.

Future Work

My pollination ecology work was a "snapshot" approach, in that we usually had only one blooming season per species, and certainly multiple seasons would give a more complete picture of the stochasticity of the pollination systems. Future work will now look at the pollination systems across a species range and for multiple years in order to assess the variation of the pollination systems in time and space. I would also suggest that future pollination ecology studies focus on subsection *Hartmannia.* These taxa have not had as focused a pollination study as other *Oenothera* species, and our understanding of the evolutionary history of their floral traits is incomplete.

A more complete sampling of sections *Hartmannia, Leucocoryne,* and *Xanthocoryne* is still needed to clarify the relationships of these clades. In this clade, there are many subspecies that we did not include in this study. The six markers used for the phylogenetic reconstructions yielded strong results; however, new markers should be developed. This would be especially useful in clarifying the subspecies' evolutionary

relationships within this clade. In particular, the many subspecies of *O. fruticosa* and *O. macrocarpa* need to be included in a phylogenetic study, as well as an assessment of the subspecies range. It is unclear which of the subspecies for these two species should still be recognized. However, *O. hexandra ssp. hexandra* and *O. hexandra ssp. gracilis*, most likely will not group together, even with additional markers, and are potentially an example of convergent evolution on trimery.

I suggest the next step is to test for an association between specialization and diversification rates in this phylogenetic tree. A BiSSE analysis (Binary State Speciation and Extinction models:(Maddison et al., 2007; Smith, 2010), which describes the association of a trait with rates of diversification, could be used to test the effect of floral traits, breeding system and pollination system on diversification rates (Kay and Sargent, 2009; Smith, 2010).

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APPENDIX A: SUPPLEMENTARY MATERIAL

Tables

Supplemental Table 2-1. A summary of the 144 records from the literature search that were used to characterize pollination data collection. (see electronic file)

Supplemental Table 2-2. A summary of all the pollinator taxa for each of the 26 *Oenothera*. (see electronic file)

Supplemental Table 3-1. Matrix for 10 Idealized Pollination Syndromes defined by 37 floral traits for *Oenothera* (see electronic file)

Supplemental Table 3-2. A matrix of the 37 floral traits x 10 idealized syndrome combinations used to generate the idealized syndrome phenotype space. (see electronic file)

Supplemental Table 6-1. The ancestral trait reconstructions for all 45 *Oenothera* species conducted in SIMMAP. Sites are as follows: 1. Breeding system, 2. Major pollinator group, 3. Dual pollination, 4. Color, 5. Brightness, 6. Scent, 7. Floral tube length, 8. Orientation, 9. Time of anthesis, 10. Flower shape, 11. Nectar, 12. PTH. The character states for each trait are listed in the first column. (see electronic file)

Supplemental Table 6-2. The ancestral trait reconstructions for the 26 *Oenothera* species in the smaller phylogenetic tree conducted in SIMMAP. Sites are as follows: 1. Specialization in the pollination system, 2. Breeding system, 3. Major pollinator group, 4. Dual pollination, 5. Color, 6. Brightness, 7. Scent, 8. Floral tube length, 9. Orientation,

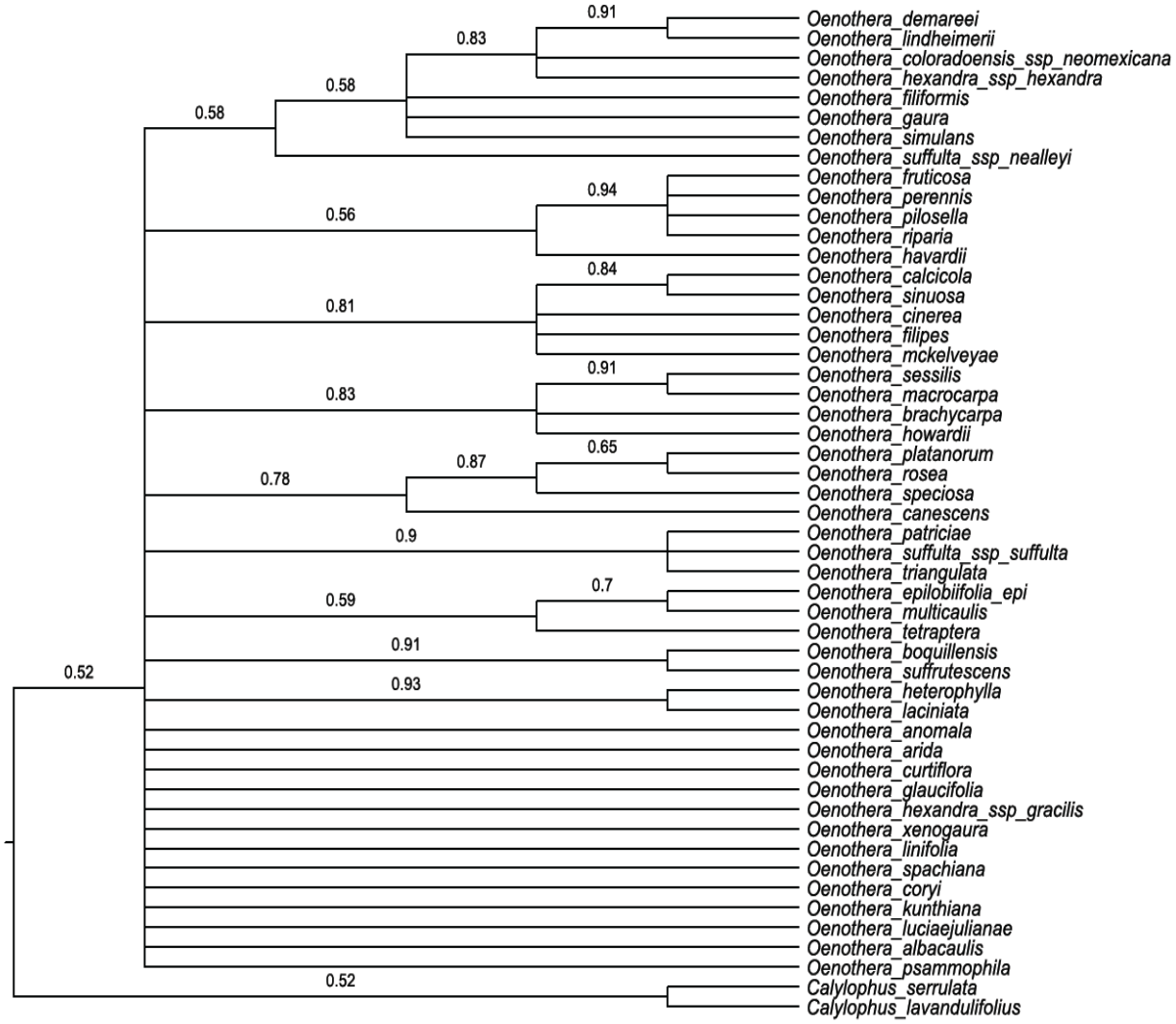
10. Time of anthesis, 11. Shape, 12. Nectar, 13. PTH. The character states for each trait are listed in the first column. (see electronic file)

Supplemental. Table 6-3. Transition rates for all reproductive traits generated in SIMMAP for both the full phylogenetic tree and the smaller 26 taxa phylogenetic tree. (see electronic file)

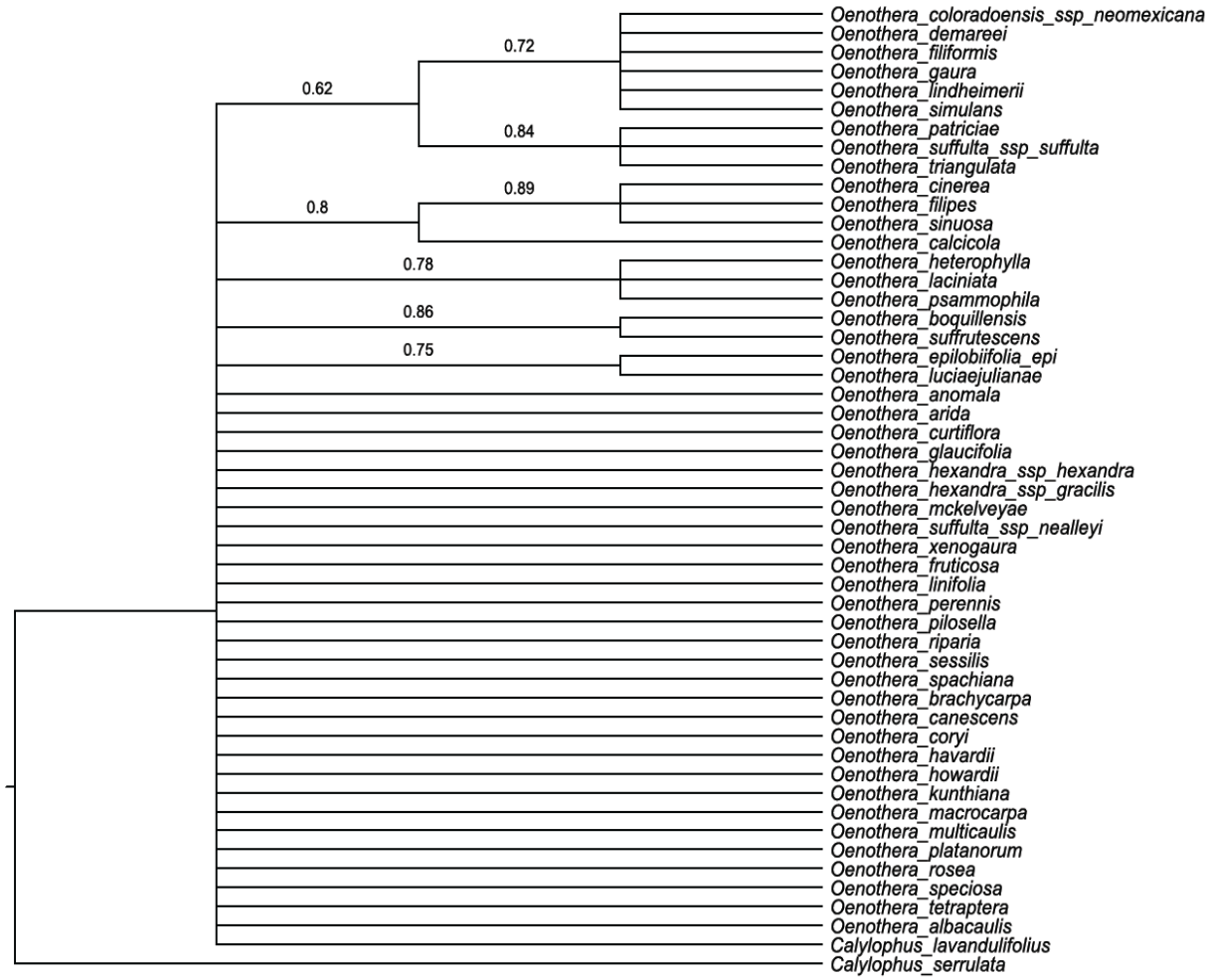
Figures

Supplemental Figure 4-1. Bayesian phylogenetic reconstructions for genes *ITS, ETS, rps16, trnL-F, rbcL,* and *nadH*. Bayesian phylogenetic reconstruction for all four cholorplast genes concatenated. Numbers above nodes indicate Bayesian posterior probability values. a. *ITS*, b. *ETS*, c. *rps16*, d. *trnL-F*, e. *rbcL*, f. *ndhF*, e. Chloroplast phylogenetic tree.

a. *ITS*



b. *ETS*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0.62
0.84
0.89
0.8
0.78
0.86
0.75
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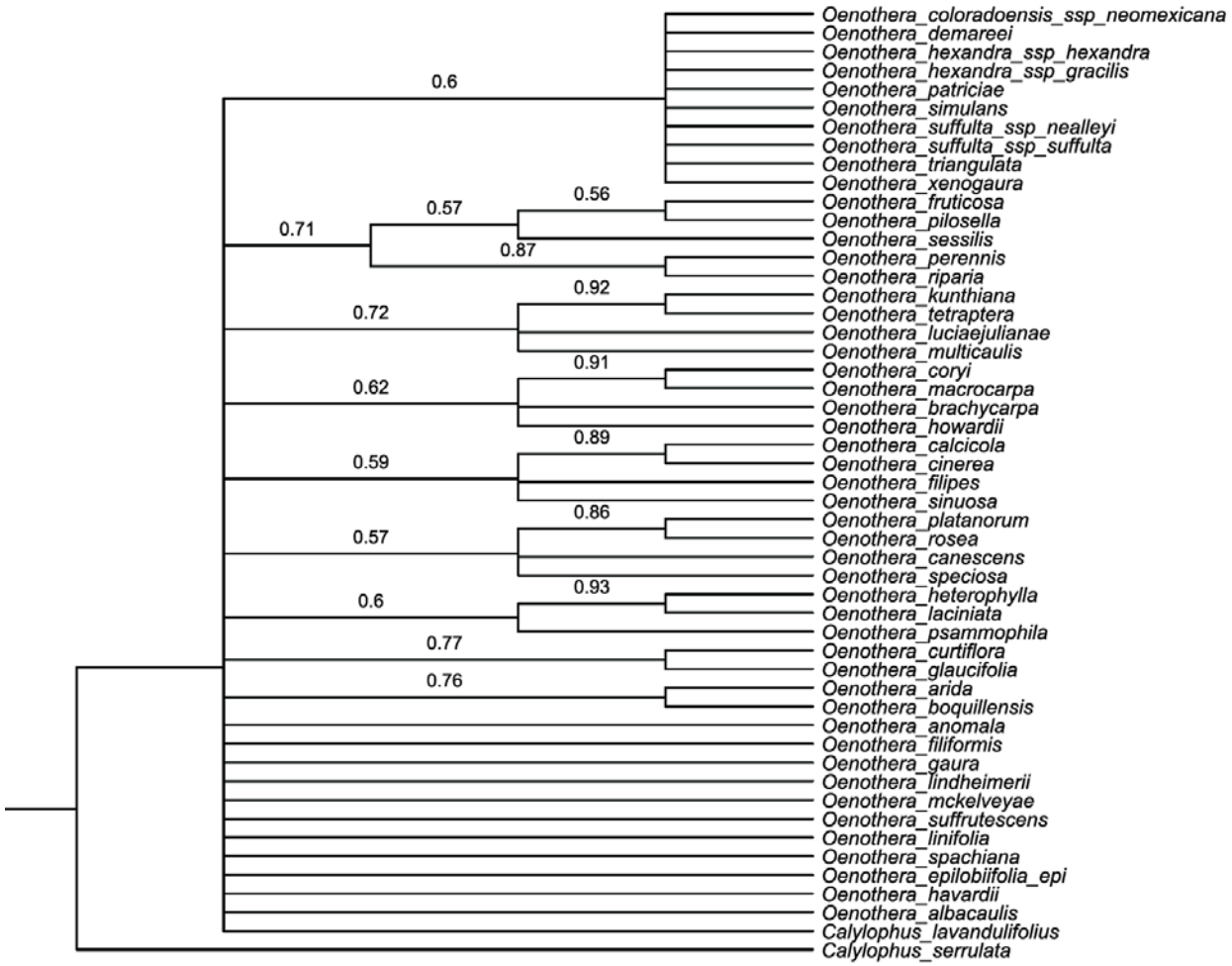
Oenothera_demareei
Oenothera_filiformis
Oenothera_gaura
Oenothera_lindheimerii
Oenothera_simulans
Oenothera_patriciae
Oenothera_suffulta_ssp_suffulta
Oenothera_triangulata
Oenothera_cinerea
Oenothera_filipes
Oenothera_sinuosa
Oenothera_calcicola
Oenothera_heterophylla
Oenothera_laciniata
Oenothera_psammophila
Oenothera_boquillensis
Oenothera_suffrutescens
Oenothera_epilobiifolia_epi
Oenothera_luciaejulianae
Oenothera_anomala
Oenothera_arida
Oenothera_curtiflora
Oenothera_glaucifolia
Oenothera_hexandra_ssp_hexandra
Oenothera_hexandra_ssp_gracilis
Oenothera_mckelveyae
Oenothera_suffulta_ssp_nealleyi
Oenothera_xenogaura
Oenothera_fruticosa
Oenothera_linifolia
Oenothera_perennis
Oenothera_pilosella
Oenothera_riparia
Oenothera_sessilis
Oenothera_spachiana
Oenothera_brachycarpa
Oenothera_canescens
Oenothera_coryi
Oenothera_havardii
Oenothera_howardii
Oenothera_kunthiana
Oenothera_macrocarpa
Oenothera_multicaulis
Oenothera_platanorum
Oenothera_rosea
Oenothera_speciosa
Oenothera_tetraptera
Oenothera_albacaulis
Calylophus_lavandulifolius
Calylophus_serrulata

c. *rps16*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Oenothera_patriciae
Oenothera_simulans
Oenothera_suffulta_ssp_nealleyi
Oenothera_suffulta_ssp_suffulta
Oenothera_triangulata
Oenothera_xenogaura
Oenothera_fruticosa
Oenothera_pilosella
Oenothera_sessilis
Oenothera_perennis
Oenothera_riparia
Oenothera_kunthiana
Oenothera_tetraptera
Oenothera_luciaejulianae
Oenothera_multicaulis
Oenothera_coryi
Oenothera_macrocarpa
Oenothera_brachycarpa
Oenothera_howardii
Oenothera_calcicola
Oenothera_cinerea
Oenothera_filipes
Oenothera_sinuosa
Oenothera_platanorum
Oenothera_rosea
Oenothera_canescens
Oenothera_speciosa
Oenothera_heterophylla
Oenothera_laciniata
Oenothera_psammophila
Oenothera_curtiflora
Oenothera_glaucifolia
Oenothera_arida
Oenothera_boquillensis
Oenothera_anomala
Oenothera_filiformis
Oenothera_gaura
Oenothera_lindheimerii
Oenothera_mckelveyae
Oenothera_suffrutescens
Oenothera_linifolia
Oenothera_spachiana
Oenothera_epilobiifolia_epi
Oenothera_havardii
Oenothera_albacaulis
Calylophus_lavandulifolius
Calylophus_serrulata
0.6
0.56
0.57
0.71
0.87
0.92
0.72
0.91
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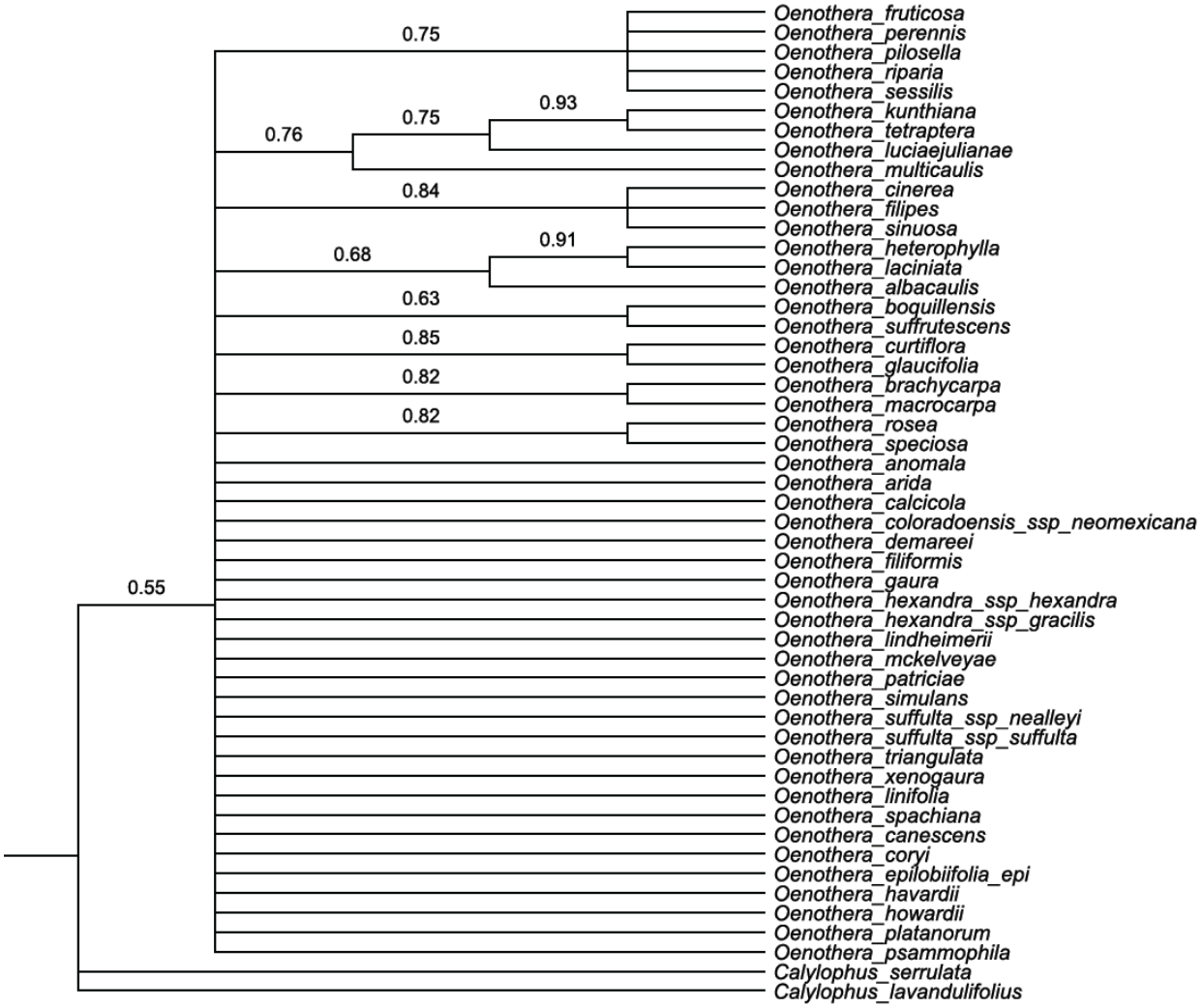
0.59
0.86
0.57
0.93
0.6
0.77
0.76

d. *trnL-F*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0.63
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0.82
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0.55
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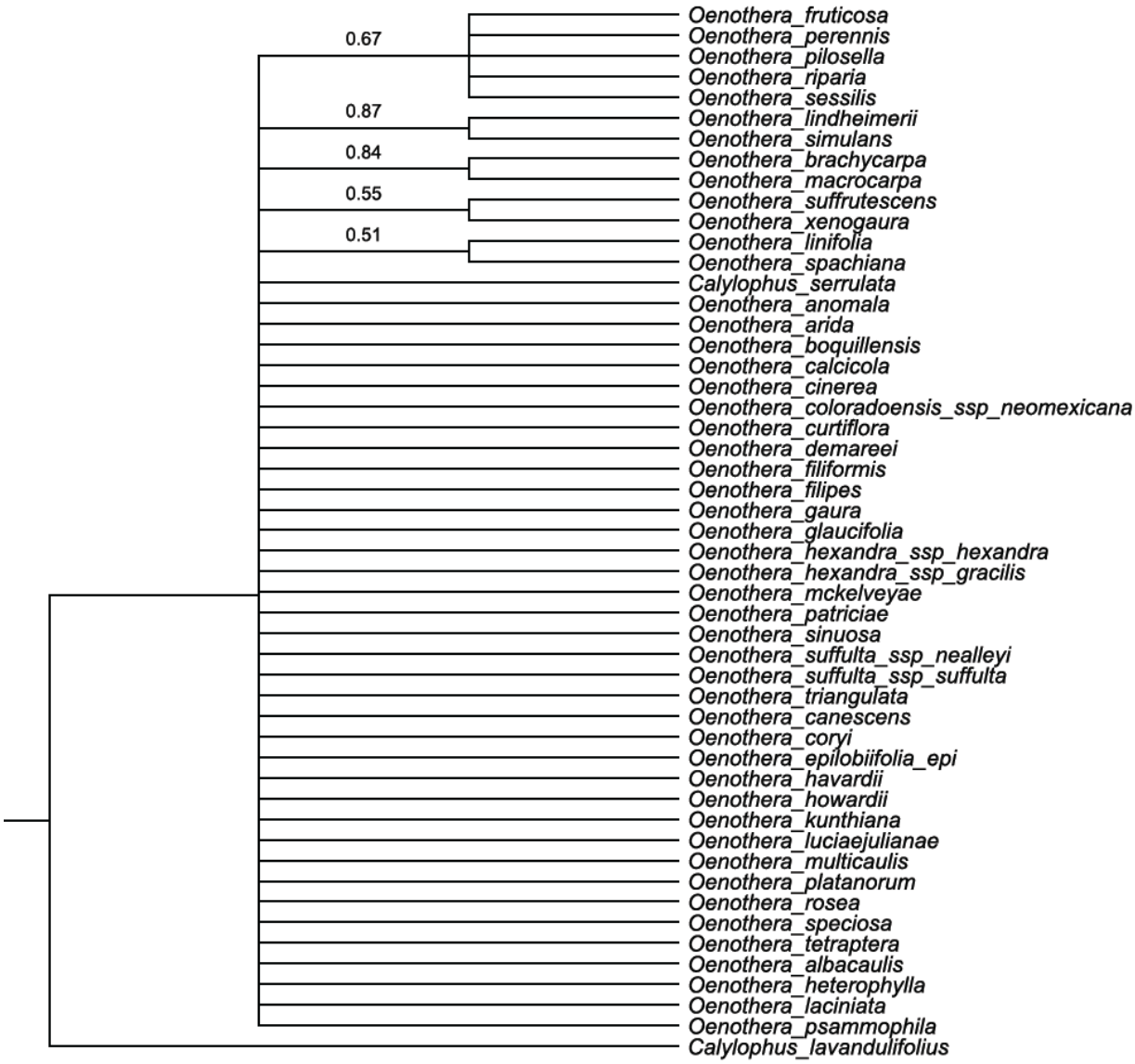
Oenothera_perennis
Oenothera_pilosella
Oenothera_riparia
Oenothera_sessilis
Oenothera_kunthiana
Oenothera_tetraptera
Oenothera_luciaejulianae
Oenothera_multicaulis
Oenothera_cinerea
Oenothera_filipes
Oenothera_sinuosa
Oenothera_heterophylla
Oenothera_laciniata
Oenothera_albacaulis
Oenothera_boquillensis
Oenothera_suffrutescens
Oenothera_curtiflora
Oenothera_glaucifolia
Oenothera_brachycarpa
Oenothera_macrocarpa
Oenothera_rosea
Oenothera_speciosa
Oenothera_anomala
Oenothera_arida
Oenothera_calcicola
Oenothera_coloradoensis_ssp_neomexicana
Oenothera_demareei
Oenothera_filiformis
Oenothera_gaura
Oenothera_hexandra_ssp_hexandra
Oenothera_hexandra_ssp_gracilis
Oenothera_lindheimerii
Oenothera_mckelveyae
Oenothera_patriciae
Oenothera_simulans
Oenothera_suffulta_ssp_nealleyi
Oenothera_suffulta_ssp_suffulta
Oenothera_triangulata
Oenothera_xenogaura
Oenothera_linifolia
Oenothera_spachiana
Oenothera_canescens
Oenothera_coryi
Oenothera_epilobiifolia_epi
Oenothera_havardii
Oenothera_howardii
Oenothera_platanorum
Oenothera_psammophila
Calylophus_serrulata
Calylophus_lavandulifolius

e. *rbcL*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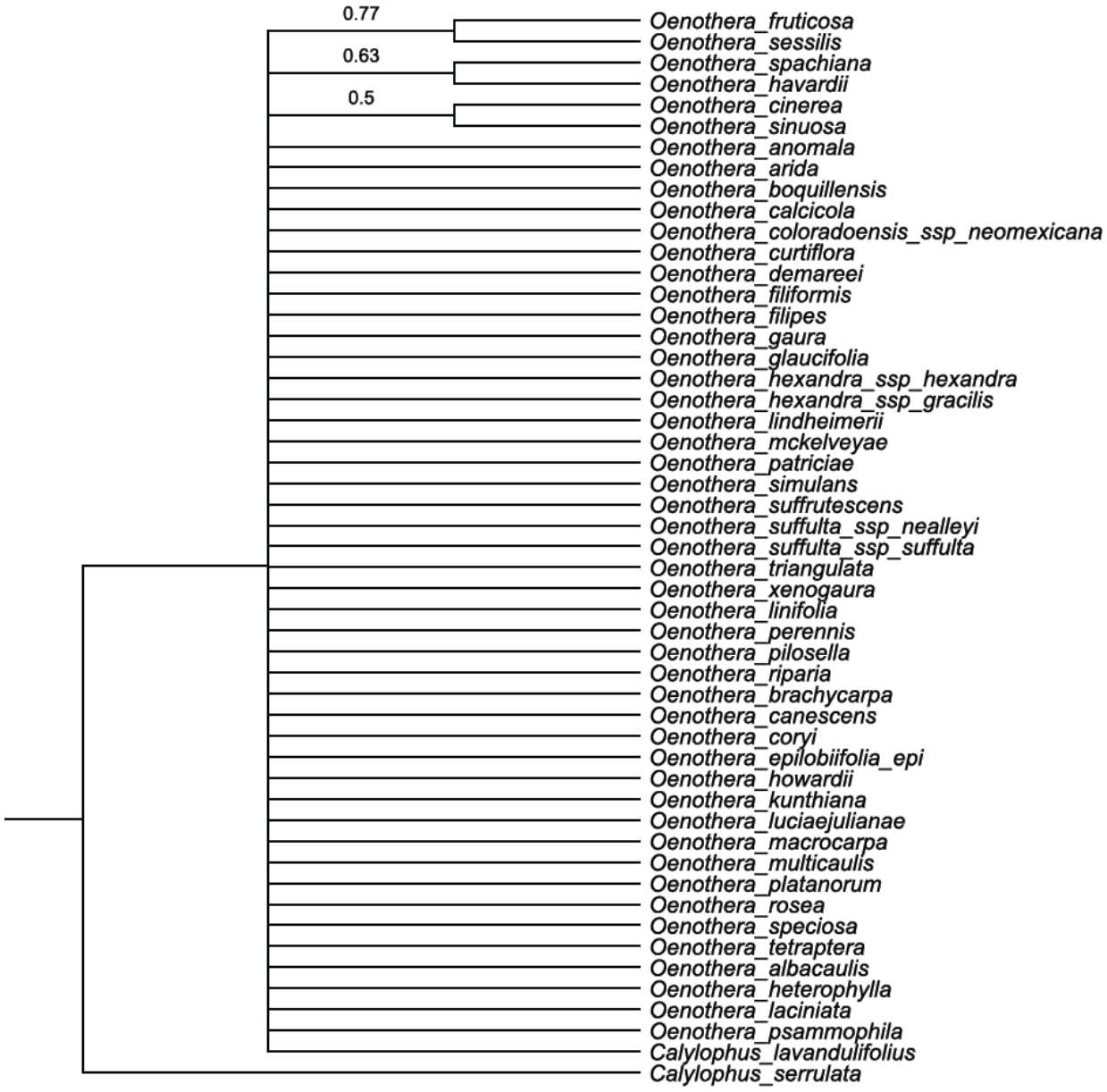
Oenothera_sessilis
Oenothera_lindheimerii
Oenothera_simulans
Oenothera_brachycarpa
Oenothera_macrocarpa
Oenothera_suffrutescens
Oenothera_xenogaura
Oenothera_linifolia
Oenothera_spachiana
Calylophus_serrulata
Oenothera_anomala
Oenothera_arida
Oenothera_boquillensis
Oenothera_calcicola
Oenothera_cinerea
Oenothera_coloradoensis_ssp_neomexicana
Oenothera_curtiflora
Oenothera_demareei
Oenothera_filiformis
Oenothera_filipes
Oenothera_gaura
Oenothera_glaucifolia
Oenothera_hexandra_ssp_hexandra
Oenothera_hexandra_ssp_gracilis
Oenothera_mckelveyae
Oenothera_patriciae
Oenothera_sinuosa
Oenothera_suffulta_ssp_nealleyi
Oenothera_suffulta_ssp_suffulta
Oenothera_triangulata
Oenothera_canescens
Oenothera_coryi
Oenothera_epilobiifolia_epi
Oenothera_havardii
Oenothera_howardii
Oenothera_kunthiana
Oenothera_luciaejulianae
Oenothera_multicaulis
Oenothera_platanorum
Oenothera_rosea
Oenothera_speciosa
Oenothera_tetraptera
Oenothera_albacaulis
Oenothera_heterophylla
Oenothera_laciniata
Oenothera_psammophila
Calylophus_lavandulifolius

f. *ndhF*


0.77
0.63
0.5
Oenothera_fruticosa
Oenothera_sessilis
Oenothera_spachiana
Oenothera_havardii
Oenothera_cinerea
Oenothera_sinuosa
Oenothera_anomala
Oenothera_arida
Oenothera_boquillensis
Oenothera_calcicola
Oenothera_coloradoensis_ssp_neomexicana
Oenothera_curtiflora
Oenothera_demareei
Oenothera_filiformis
Oenothera_filipes
Oenothera_gaura
Oenothera_glaucifolia
Oenothera_hexandra_ssp_hexandra
Oenothera_hexandra_ssp_gracilis
Oenothera_lindheimerii
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Oenothera_patriciae
Oenothera_simulans
Oenothera_suffrutescens
Oenothera_suffulta_ssp_nealleyi
Oenothera_suffulta_ssp_suffulta
Oenothera_triangulata
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Oenothera_brachycarpa
Oenothera_canescens
Oenothera_coryi
Oenothera_epilobiifolia_epi
Oenothera_howardii
Oenothera_kunthiana
Oenothera_luciaejulianae
Oenothera_macrocarpa
Oenothera_multicaulis
Oenothera_platanorum
Oenothera_rosea
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Oenothera_tetraptera
Oenothera_albacaulis
Oenothera_heterophylla
Oenothera_laciniata
Oenothera_psammophila
Calylophus_lavandulifolius
Calylophus_serrulata

g. Concatenated chloroplast gene tree (*rps16*, *rbcL*, *trnL-F*, *ndhF*)