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Molecular Phylogenetics and Reproductive Biology of *Oenothera* section *Kneiffia* (Onagraceae)

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Abstract—*Oenothera*, the evening primrose genus, is a model system for studying the evolution of flowering plant reproductive biology. Members of this group vary in the species of pollinator that visit their flowers and in breeding systems, including both self-compatible (SC) and self-incompatible (SI) species. Here, we examine the evolutionary relationships among the six species of *Oenothera* section *Kneiffia* using sequences from two nuclear and four chloroplast genes. Through field studies we describe the effective pollinators for four species that had not been previously reported, and experimentally test for pollen limitation. Three of the six species are SC, and three are SI. The phylogeny strongly supports three separate transitions from SI to SC. Despite the expectation that SC species evolved the ability to self because of pollen limitation, or that pollen limitation evolves among SC species, we found no significant differences in pollen limitation between the SI and SC species in this study. Our results resolve the interspecific relationships within section *Kneiffia,* show that breeding systems can be quite labile, and provide evidence that transitions to self-compatibility do not always coincide with pollen limitation.

Keywords—Breeding system, pollination, pollen limitation, self-compatibility.

Molecular phylogenetics is a powerful tool for understanding 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 led to taxonomic revisions and alteration of many hypotheses about plant species relationships. For instance, flowering plants were long divided into two groups, Dicots and Monocots, while molecular phylogenetic studies have shown that the relationships are more complex and include monocots and eudicots as clades within a diverse basal group of other angiosperms (Bremer et al. 1998; Soltis et al. 1999).

Pollination interactions drive the evolution of floral features, and are often considered as drivers of angiosperm diversification (Crane et al. 1995; Crepet et al. 2004; Fenster et al. 2004; De Bodt et al. 2005;). One of the major elements of these interactions is breeding system, which describes the mechanisms by with plants direct the flow of pollen, including whether a plant is self-compatible (SC) or self-incompatible (SI) (Baker 1955; Barrett et al. 1996; Neal and Anderson 2005). Angiosperms show repeated transitions from SI to SC (Barrett 2002), but almost no reversals back to SI, across diverse taxonomic groups (Schoen et al. 1996; Goodwillie 1999; Charlesworth 2006; Igic et al. 2006; Igic and Kohn 2006; Foxe et al. 2009). Self-compatibility occurs both in strongly outcrossing plant species, where inbreeding may largely or completely absent, and in autogamous (self-pollinating) species, which have genetic mechanisms that allow them to overcome the deleterious effects of inbreeding to varying degrees. If self-pollination is characteristic of the SC species or occurs frequently, it can provide reproductive assurance even in the absence of pollinators, or when their visits are infrequent or inconsistent (Barrett 2002; Kalisz et al. 2004; Moeller 2006; Waser and Ollerton 2006).

Reproductive assurance is not the only hypothesis for the evolution of self-compatibility – for instance, there may be several gene-level selection factors – but it is considered the likely leading factor (Busch and Delph 2012). Self-compatible plants exhibit a gradient from complete autogamy to very strong outcrossing, with differing ecological and genetic consequences associated with the different states. Self-incompatibility often 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). If pollinator services are limited, self-compatibility provides reproductive assurance (Busch and Delph 2012). There can be great diversity of breeding systems within plant groups, and closely-related species may be SI, SC but outcrossing, or autogamous (Brauner and Gottlieb 1987; Macnair et al. 1989; Weller and Sakai 1999). When species with recent shared evolutionary history differ in breeding system, those differences may result from adaptations to variable environmental conditions (e.g. climate), the exploitation of new kinds of habitats, or interactions with insects that visit and pollinate them. We can distinguish between these alternatives by 1) identifying repeated transitions to SC, and 2) using phylogenetically-controlled association tests to determine the relationship between breeding system and pollen limitation (Sanderson and Donoghue 1996; Freckleton 2000; Barrett 2003; Vamosi et al. 2003; Machado and Lopes 2004).

Onagraceae has long served as a model system in which to study the evolution of flowering plant reproductive biology (Raven 1979; Raven 1988; Clinebell et al. 2004; Johnson 2011). *Oenothera*, the evening primrose genus, which has a remarkable and well-documented chromosomal structure, is a particularly important model system (Stebbins 1950; Cleland 1972; Wagner et al. 2007). Analysis of the breeding systems of *Oenothera* reveals extensive variability and no strong relationship between breeding system and life history (Theiss et al. 2010). Several mechanisms for SC have been described (Neal and Anderson 2005; Charlesworth 2006). The specific mechanism is not known for all members of *Oenothera*, but most species exhibit gametophytic SI

(Wagner et al. 2007). Repeated evolution of SC in this group is thought to have played a key role in the diversification of Onagraceae, with autogamy providing a mechanism of rapid reproductive isolation correlated with the ability to expand into new or marginal habitats (Raven 1979; Johnson et al. 2011).

Recent molecular phylogenetic analyses have established the delimitation of *Oenothera* and most of the relationships among the major groups within the genus (Levin et al. 2003, 2004; Hoggard et al. 2004; Wagner et al. 2007). The delimitation of *Oenothera* expanded unexpectedly with the addition of the once separate genera *Gaura, Calylophus*, and *Stenosiphon* as sections within a strongly monophyletic *Oenothera* (Wagner et al. 2007). The unexpected sister relationship between sections *Gaura* and *Kneiffia* in the molecular work of Levin et al. (2004) suggested that much insight could be gained by close comparison of the pollination/reproductive biology of sect. *Kneiffia* (day-blooming species with large, actinomorphic yellow flowers) with that of sect. *Gaura* (most species evening-blooming, mostly with smaller, zygomorphic white and sometimes pink flowers). Recent molecular studies used only one species, *Oenothera fruticosa*, to represent section *Kneiffia,* which has six species; *Oenothera linifolia*, previously included in section *Kneiffia*, based on morphological and cytological data (Munz 1937; Straley 1977) is placed in the monotypic section *Peniophyllum* (Levin et al. 2004). Straley included some information on the breeding systems in sect. *Kneiffia*, but without a clear understanding of the relationships in the entire section, even these incomplete data cannot be analyzed in an evolutionary context. 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.*

Species within *Oenothera* section *Kneiffia* are widely distributed in the eastern United States and Canada (Straley 1977). They have bright yellow flowers that open near dawn, vary in size, and are predominately visited and pollinated by bees. The section includes 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 treated as *O. pilosella spp. sessilis* (Pennell) Straley (Straley 1977; Wagner et al. 2007), is a rare species restricted to prairie remnants primarily in eastern Arkansas (Krakos and Hoch in review). *Oenothera riparia* Nutt. 1818, a rare endemic of riparian habitats in the Carolinas, was not recognized by Straley (1977), but was studied cytologically and morphologically by Straley (1982). The molecular data presented here, along with additional morphological and ecological studies, have established that these taxa are best treated as separate species from *O. fruticosa*.

We use molecular data from six gene regions to estimate a species tree for *Oenothera* sect. *Kneiffia* and examine the reproductive biology of its species to test the following hypotheses: 1) Current generic and species level taxonomies reflect evolutionary history; 2) Self-compatibility has evolved only once in section *Kneiffia*; 3) Two self-compatible species in section *Kneiffia* exhibit less pollen limitation than two self-incompatible species.

Materials and 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 Northeast United States. Fieldwork was carried out from April 2007 to August 2010 and included pollination studies, tissue collection, and breeding system experiments. *Oenothera pilosella* Raf. is a native perennial that we found blooming along the roadsides and in the prairie remnants of Illinois in early June. This species typically flowers for only 2–3 wk. 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* L. 1759 is a native perennial common across the eastern US; it flowers 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, where it flowers in May and 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, flowering from 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—All six species of *Oenothera* section *Kneiffia* were sampled for this study. 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 herbarium sheets at the Missouri Botanical Garden Herbarium for *O. spachiana* Torr. & A. Gray. We used published GenBank sequence data for *O. fruticosa* (subspecies *fruticosa*) L. *O. fruticosa* has possibly another subspecies, and multiple varieties, however this study looks at the species level, and we chose the most widespread and common *O. fruticosa*. To account for intraspecific variation relative to interspecific differences, we analyzed an individual from two geographic distinct populations where possible, which was for the species *O. perennis*, *O. riparia*, and *O. pilosella*. We used published GenBank sequence for the outgroups: *Oenothera macrocarpa* Nutt., *Oenothera brachycarpa* A. Gray, *Oenothera lavandulifolius* Torr. & A. Gray and *Oenothera serrulata* Nutt. (Levin et al. 2004). All information on the origin of material, voucher specimens, and GenBank accession numbers are listed in Table 1.

Table 1. Species, locations of samples, voucher numbers and accessions for DNA sequence data for the six *Oenothera* species examined in this study. For each gene we indicate the GenBank accession number. Data not obtained are indicated (–).

Taxon	Location	Voucher	ITS	trnL-F	rps16	ETS	rbcl	ndhF
O. fruticosa	Dane Co, WI	WIS5025	AY271581	AY264569	AY267443	–	AF495771	AF495794
O. riparia	Pender Co. NC	Krakos 1017	KJ135376	KJ135359	KJ135365	KJ135383	KJ135371	KJ135352
	New Hanover Co. NC	Krakos 1018	KJ135377	KJ135360	KJ135366	KJ135384	–	KJ135353
	New Hanover Co. NC	Krakos 1014	KJ135378	–	–	–	–	–
O. perennis	Middlesex Co. MA	Krakos 0817	KJ135379	KJ135364	KJ135367	–	KJ135372	KJ135354
	West Cape, P. E. Island	MTJ85	GU176555	GU176587	–	–	–	–
O pilosella	SE Washington Co. IL	Krakos 0821	KJ135380	KJ135361	KJ135368	–	KJ135375	KJ135357
	Jefferson Co. IL	Krakos 0705	–	–	–	–	–	KJ135358
O. spachiana	Bienville Co. LA	Thomas and Moreland 49150	–	KJ135363	KJ135370	KJ135382	KJ135374	KJ135356
O. sessilis	Prairie Co. AR	Krakos 1006	KJ135381	KJ135362	KJ135369	–	KJ135373	KJ135355

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 nuclear 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*. 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 (Nicholas and Nicholas 1997).

Phylogenetic Reconstruction—We estimated models of nucleotide evolution for each of the six gene regions independently in jmodeltest (Posada 2008). We employed two approaches to phylogenetic reconstruction. First, a gene tree was computed for each of the two nuclear markers and for all chloroplast markers combined. Because these gene trees largely agreed with one another, we combined them in a concatenated analyses in MrBayes (Ronquist and Huelsenbeck 2003). We used thirty million generations with a sampling frequency of 200 generations and the standard one hot and three cold chains. 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 less than 0.0001. We examined 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 majority-rule consensus tree using the sumt command in MrBayes. Second, all six gene regions were used to estimate a species tree that accounted for uncertainty among gene regions using the Bayesian Markov-Chain Monte-Carlo search algorithm of *BEAST (Heled and Drummond 2010). The outgroup taxa were not identified a priori by grouping species sets. Each gene region was given the model of evolution determined by the AIC method in jmodeltest. The species tree prior was set to a Yule Process following author recommendations. The Markov Chain was run for 400 million generations with parameters logged every 1000 generations. Convergence in the run was estimated by examining log-likelihood and ESS values using Tracer (Rambaut and Drummond 2007), wherein each model parameter 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 maximum clade credibility tree using TreeAnnotator (Drummond and Rambaut 2007). For both approaches to phylogenetic reconstruction, we rooted our trees using four outgroup species, *Oenothera brachycarpa* and *O. macrocarpa* (sect. *Megapterium*), *O. lavandulifolia* (sect. *Calylophus* subsect. *Salpingia*), and *O. serrulata* (sect. *Calylophus* subsect. *Calylophus*).

Determining Breeding System and Pollen Limitation—The breeding system and pollination data for *O. spachiana* and *O. fruticosa* were previously described (Straley 1977), and we did not test these two species. To determine and/or verify the breeding system of the other four *Kneiffia* species, we conducted experiments in both the field and the greenhouse. For each study site, during peak flowering season, we randomly chose ten flowering plants by assigning every plant within a 10 m quadrant a number and choosing plants via a random number generator. 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. Flowers 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 were 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. Some *Oenothera* species, especially those with permanent translocation heterozygote (PTH) chromosome patterns (Johnson et al. 2009; Johnson et al. 2010) have anthers that generally dehisce prior to the flower opening. The anthers of *O. perennis*, which is a PTH species, did not dehisce prior to opening. 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.

Twenty-four hours after each treatment, all pairs of flowers were collected and fixed in a 3:1 ethanol-glacial acetic acid mixture for 2 hr. They were then transferred to a 70% ethanol 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 hr. A Zeiss Universal microscope with a 100 W 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. All statistical analyses were executed in JMP v.7 (SAS Institute Inc.). No statistical difference between the pairs indicates that the species is self-compatible.

To assess whether SI *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 as described previously. Before the onset of flowering (predawn), we marked two flowers per plant with yarn tied at the base of the flower and assigned each to a treatment group. Group one, the control, was left open to natural pollinators throughout the flowering period (one day). Group two, the supplementation treatment, was left open to natural pollinators and in addition, individuals 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 hr, all pairs of flowers 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: $L = 1 - \frac{T_c}{T_s}$, 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 tested for significant associations between pollen limitation (L) and species or breeding system with a phylogenetic ANOVA (Garland et al. 1993) using the Geiger (Harmon et al. 2008) module in R (R Core Development Team 2009), iterated over 1000 equally likely trees drawn from the posterior distribution estimated in *BEAST.

Determining Pollination System—Pollination system was determined by recording visitation by animals, pollen load, and stigma contact. For each population of *Oenothera* studied, we conducted 20 min observations of multiple randomly chosen flowers and recorded the total number of visits, type of visitor, and behavior of visitors. Random flowers were chosen by assigning each flower a number and using a random number generator to choose individuals. 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 two weeks. Observations 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 examined 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 plants flowering at each study site. Dehiscent stamens were placed on glass slides. The pollen was teased out with probes, stained with Calbera's fluid to make a semi-permanent mount (Goldblatt et al. 1998; Bernhardt et al. 2003), 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. Insects were identified and grouped into one of five functional groups including the larger bumble bees (*Bombus*) and carpenter bees (*Xylocopa*), midsized megachilid bees (*Megachile*), and small and medium halictid bees (*Lassioglossum*). The latter two groups are distinguished by maximum thorax lengths of 1 cm and 3 cm, respectively. The pollen on the slide was stained with Calbera's fluid and a cover slip was applied to the surface of the drop. Pollen was identified under light microscopy by comparison to the pollen library. The type and amount of pollen on the legs, thorax, and proboscis was recorded separately.

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

$$\sum(VR_x * PL_x)$$

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

Independent Origins of Self-Compatibility—Because three species proved to be SC, we needed to test for the number of independent or shared transitions from SI to SC. First, we sampled 10,000 equally likely species trees from the posterior distribution estimated in *BEAST. Next, 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 alternate configurations of two origins of SC, or three separate origins of SC. Using PAUP (Swofford 2003), we queried the set of 10,000 trees for the number of trees conforming to each configuration. We assumed that if more than 500 trees (corresponding to $\alpha = 0.05$) satisfied a given topological constraint, that we could not reject that relationship. As a second test, we compared our unconstrained topology generated in *BEAST and including three independent origins to four constrained topological alternatives, and used Bayes Factors (Kass and Raftery 1995) to test whether the maximum clade credibility tree of the unconstrained analysis was significantly more likely (Bayes Factors greater than 10; Kass and Raftery 1995) than 1000 trees sampled from the posterior distributions of the four topologically constrained reconstructions.

Results

Phylogenetic Reconstruction—The species tree estimated from our phylogenetic reconstruction in *BEAST is shown in Fig. 1. The analysis reached convergence after 100 million generations, but was allowed to run for all 400 million generations. All parameters were resolved with ESS values above 500. The results of the MrBayes analysis was strongly congruent and posterior probabilities for each node from this analysis are also presented in Fig. 1. The tree file has been deposited on TreeBASE.org (accession # 15123) and GenBank accession numbers are in Table 1. The xml 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 hr of pollination, pollen tubes growing from the SC flowers or the SI flowers that received outcross pollen had entered the style. As is the case in other SC Onagraceae, 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 of swollen pollen tube tips. Breeding system and pollination system differed among species (Table 2 and Fig. 2). All of the species were pollinated by bees in the morning, but they differed in which pollinator functional groups were responsible for the majority of pollen (Fig. 2). The pollinator

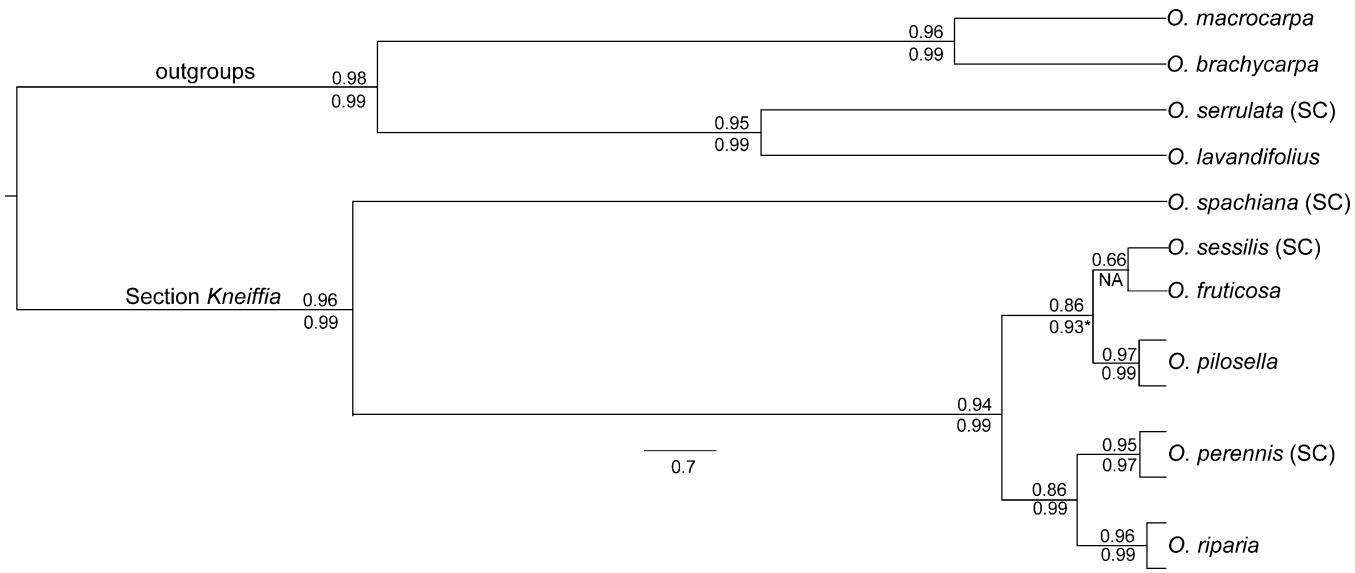

Fig. 1. Bayesian phylogenetic reconstructions from species tree analysis in *BEAST for the genes *ITS, ETS, rps16, trnL-F, rbcL,* and *nhdF*. All species are self-incompatible unless labeled as self-compatible (SC). *O. macrocarpa, O. brachycarpa, C. lavandulifolius,* and *C. serrulata* are the outgroup species to section *Kneiffia*. Numbers above branches at nodes represent posterior probabilities from species tree, while numbers below branches represent posterior probabilities from concatenated gene tree analysis in MrBayes. Topologies were identical except for the placement of *O. sessilis*, which in the gene tree was sister to a clade including *O. fruticosa*, *O. pilosella*, *O. perennis*, and *O. riparia*. An asterisk denotes support for this node with this topological difference.

TABLE 2. Results from the hand pollination studies used to determine the breeding system for *Oenothera* species. Because there was no significant difference between the greenhouse and field population experiments, results from these locations are pooled for each species.

Species	Treatment	# flowers	# pollen grains on stigma	% pollen tubes to reach plant ovary
O. riparia	Self	13	390.0 (± 256.4)	0
	Cross	16	452.0 (± 370.6)	33.5 (± 24.0)
O. pilosella	Self	8	800.0 (± 392.8)	0
	Cross	10	890.0 (± 272.6)	41.5 (± 13.4)
O. sessilis	Self	20	279.7 (± 189.9)	26.0 (± 29.2)
	Cross	20	325.7 (± 165.7)	39.8 (± 33.1)
O. perennis	Self	9	477.8 (± 83.3)	26.3 (± 19.9)
	Cross	9	522.2 (± 84.2)	36.4 (± 23.6)

species and their average visitation rates and pollen loads are listed in Table 3. *Oenothera* species did not differ significantly in their degree of pollen limitation, *L* (Fig. 3; $F_{3,48}$ = 1.146, p = 0.34) and there was no association between breeding system and pollen limitation ($F_{1,50}$ = 1.42, p = 0.24). Results of the phylogenetic ANOVA confirmed with phylogenetic controls that there was no association between breeding system and pollen limitation, with an average $p > 0.01$ across 1000 trees sampled from the prior distribution estimated in *BEAST.

For *Oenothera 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 both large bee functional groups, *Bombus* and *Xylocopa*, (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 for the population included in this study. This species is not visited by pollinators (N = 137 observations), and is designated in this study 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

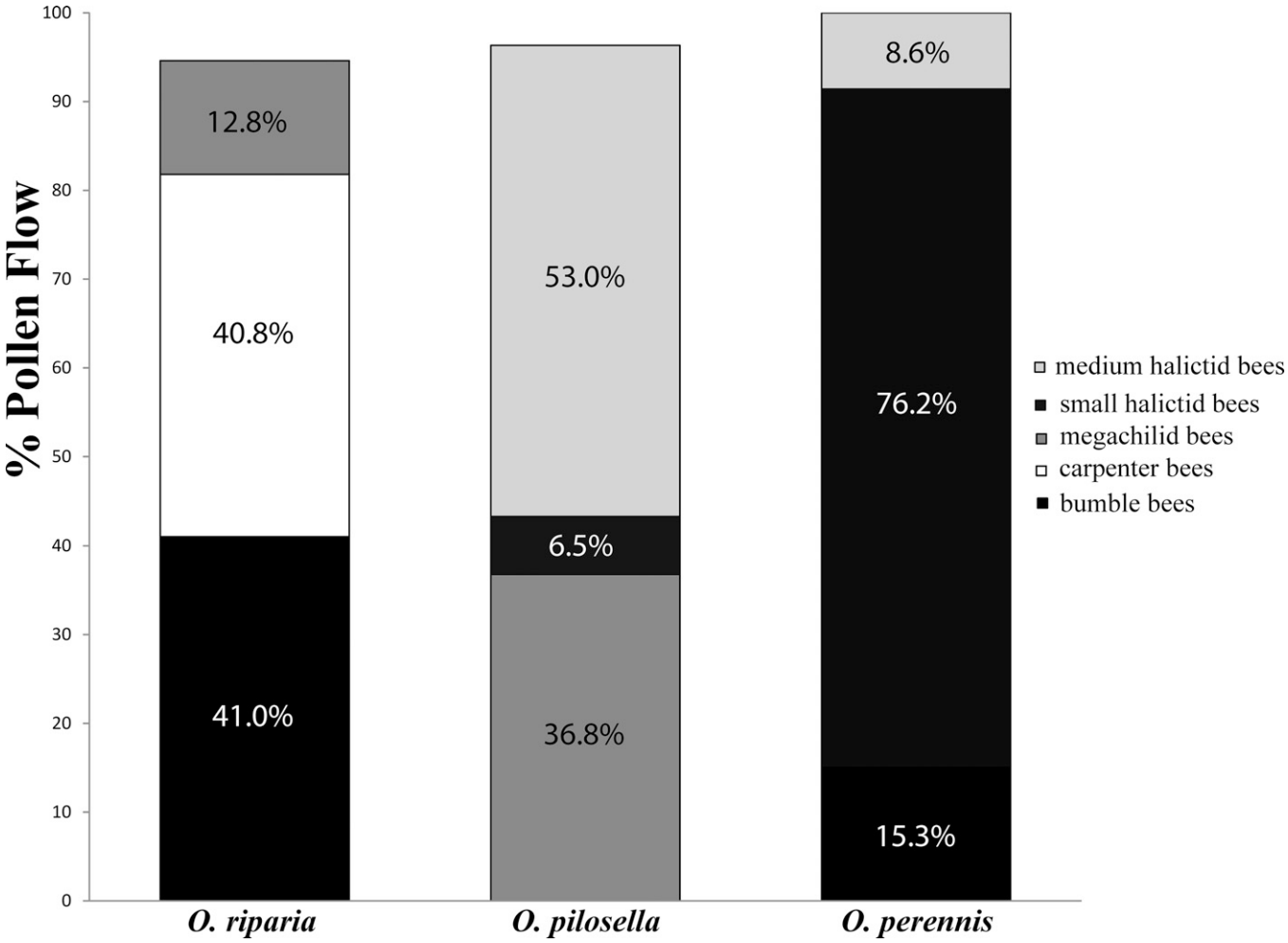

FIG. 2. The major pollinator functional groups that account for 95% of pollen flow for the three species of *Oenothera* sect. *Kneiffia* that we studied. *Oenothera sessilis* is not listed because no pollinators were observed (see Results).

TABLE 3. The visitation rate (visits per flower per 20 minute observation) and average pollen load (number of grains) of pollinators to the *Oenothera* species.

Species	Insect Species	Visitation Rate	Pollen Load
O. riparia	*Bombus pennsylvanicus* DeGeer (female)	0.089	120
	Xylocopa virginica Linn. (female)	0.098	109
	Megachile xylocopoides Say (female)	0.223	15
	Lassioglossum ssp.	0.062	14
	Zale ssp.	0.054	6
	Parallelia ssp.	0.036	6
O. pilosella	*Agapostemon virescens* Fab. (female)	0.532	458
	Megachile montivaga Cresson (female)	0.389	418
	Lasioglossum versatum Robertson (female)	0.663	18
	Augochlorella purae Smith. (female)	0.856	11
	Apis mellifera Linn. (female)	0.011	500
	Caenurgina ssp.	0.011	25
	Syrphidae ssp.	0.151	1
O. sessilis	none	n. a.	n. a.
O. perennis	*Augochlorella aurata* Smith. (female)	0.146	500
	Lasioglossum versatum Robertson (male)	0.250	268
	Bombus impatiens Cresson (female)	0.056	500
	Agapostemon virescens Fab. (female)	0.031	500
	Lasioglossum oceanicum CK II. (female)	0.031	5
	Syrphidae ssp.	0.170	1

breeding system. However, *O. perennis* had a high level of pollen flow which could lead to outcrossing (N = 236 observations) and is pollinated by small halictid bees (76% of pollen flow), bumble bees (16%), and medium halictid bees (8%). All six insect pollinator species are listed in Table 3. *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 *O. pilosella* has a self-incompatible breeding system. Through 185 observations we determined that *O. pilosella* is pollinated by medium halictid bees (53% of pollen flow), megachilid bees (36.7%), and small halictid bees (8.9%). All seven species of pollinators are listed in Table 3. *Oenothera pilosella* has an *L* value of 0.3167 ± 0.099 (N = 11 pairs), indicating that it is not pollen limited. However, further studies based on both pollen tube results and viable seed set are needed to determine if this species is pollen limited.

The only annual species of sect. *Kneiffia, O. spachiana*, is reported to be autogamous (Straley 1977). The polymorphic and widespread *O. fruticosa* has a self-incompatible breeding system and was documented to have a bee pollination system similar to that of *O. pilosella* (Straley 1977).

Independent Origins of Self-Compatibility—The phylogenetic reconstruction depicted in Fig. 1 is highly resolved and demonstrates that there have been three independent origins of SC in the group. While uncertainty surrounding each node increased in the species tree (*BEAST) relative to the concatenated gene tree (MrBayes), the species tree better reflects uncertainty within and across independent gene regions. Despite this uncertainty, both tests confirm significant support for three independent origins of SC in sect. *Kneiffia*. Table 4 depicts the number of possible species tree reconstructions that included relationships with fewer than three independent origins of SC, none of which occurred frequently enough to meet our criteria

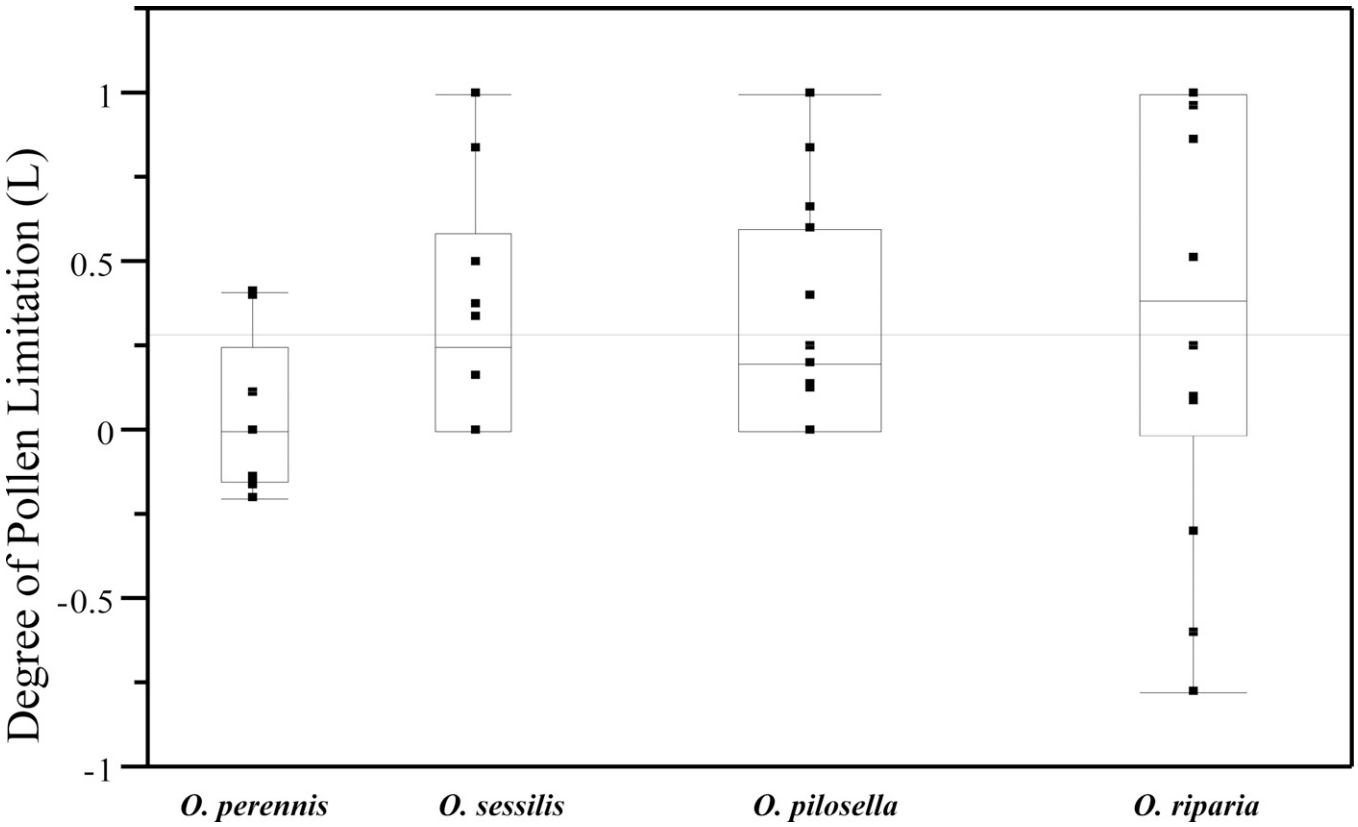

FIG. 3. A box plot of the mean degree of pollen limitation, *L*, ± standard error, for the *Oenothera* species based on supplement and control treatments tested at field sites. There are no significant differences either by species or by breeding system. The horizontal line represents the mean across all species.

TABLE 4. Results of the topological hypothesis testing of the number of origins of self-compatibility in section *Kneiffia*. Alternative topologies considered were 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 constrained trees. A search of topologies consistent with each scenario out of 10,000 trees sampled from the posterior distribution of trees (PDT) in the species tree analysis are also given, with resounding support for three independent origins(†) of SC.

Topology	Harmonic Mean LnL	Bayes Factors	*PDT*
Single Origin	–7684	171	0
Two Origins[1]	–7570	57	4
Two Origins[2]	–7636	123	0
Two Origins[3]	–7625	112	0
Three Origins†	–7513	–	9996

for significant support for those relationships. In addition, Bayes Factors rejected phylogenetic configurations with fewer than three independent origins of SC (Table 4).

DISCUSSION

Phylogenetic structure of Oenothera sect. Kneiffia—Our study examined all six species of *Oenothera* sect. *Kneiffia* (Fig. 4). The species tree estimated for this group (Fig. 1) is based on new molecular data combined with the GenBank sequences. This phylogeny clarifies the evolutionary relationships within section *Kneiffia*, and indicates several striking differences from the previous classification. Straley (1977) grouped the self-incompatible *O. fruticosa* and *O. pilosella* together, and considered *O. perennis* to comprise a separate lineage arising more recently from those SI species. With the inclusion of *O. riparia* and *O. sessilis*, the self-incompatible species no longer form a monophyletic group. We find strong posterior probability support for one clade including *O. pilosella*, *O. fruticosa*, and *O. sessilis* (SC), and this clade is sister to another clade that includes *O. perennis* and *O. riparia* (SC). One differences between the concatenated gene tree and the species tree was that in the former, *O. sessilis* was an outgroup to these two clades, whereas in the species tree, it is included in the *O. pilosella*/*O. fruticosa* clade. Irrespective of this relationship, there is still strong support for separate evolution of SC in *O. sessilis* and *O. riparia*. Overall, our results highlight how molecular data reveals the dynamic nature of breeding system within this section.

Oenothera sessilis is a day-flowering yellow perennial *Oenothera* that Straley (1977), unlike previous authors, treated as a subspecies of *O. pilosella*. Straley recognized the distinct morphological differences in pubescence and ovary length separating the two taxa, but was impressed by the fact that both are octoploids (n= 28). In addition, he incorrectly suspected that because *O. pilosella* was SI, its subspecies, *O. sessilis*, would also be self-incompatible. Our data for the populations included in this study, show that *O. sessilis* is self-compatible and may in fact be autogamous. However, further breeding system studies at populations of *O. sessilis* across its range are needed. We recorded no insect visitors during the peak flowering season, and yet all *O. sessilis* examined achieved full seed set. Potential pollinators were present and active on other prairie species co-blooming with *O. sessilis* both populations. However, this lack of pollinators could be due to a depauperization of the native pollinator community caused by the fragmentation of the prairie habitat inhabited by *O. sessilis*. Based on these current field studies, breeding experiments, and molecular

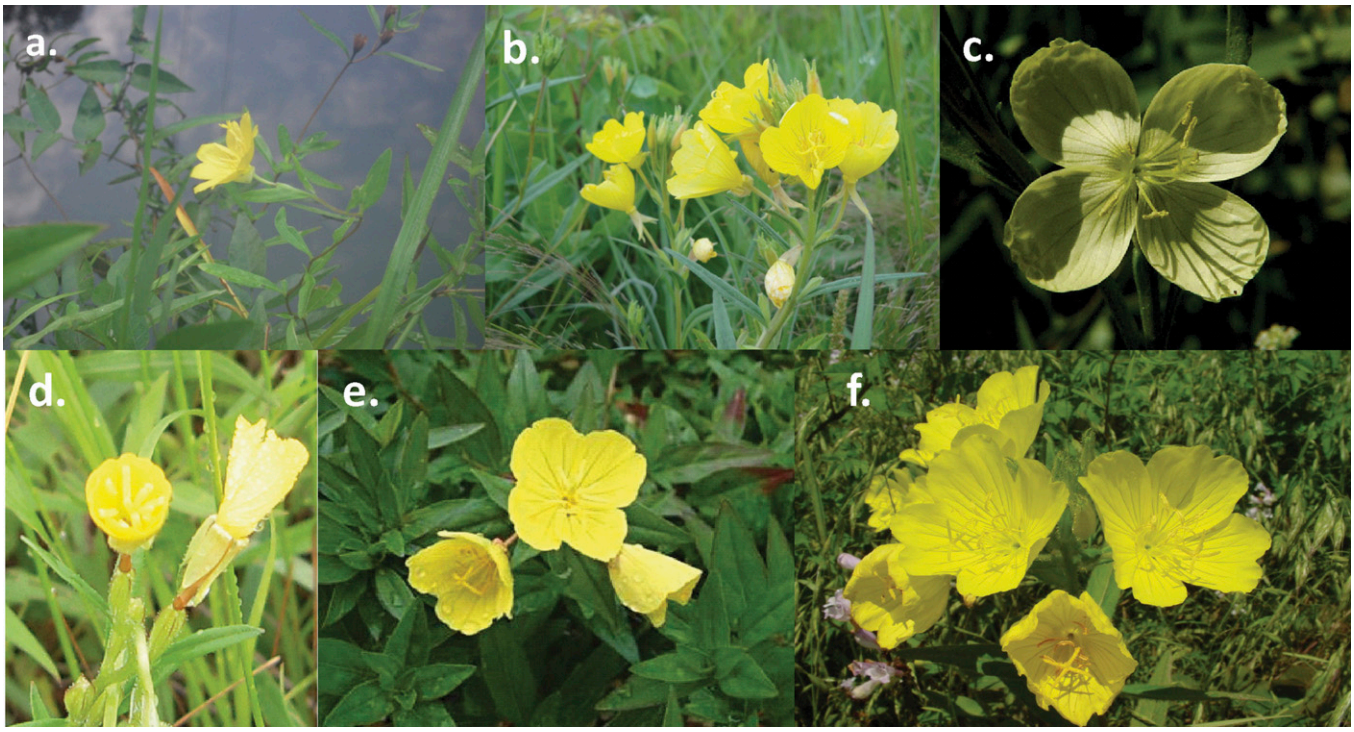

FIG. 4. Photos of flowering *Oenothera* species: a. *O. riparia* b. *O. sessilis* c. *O. spachiana* d. *O. perennis* e. *O. fruticosa* f. *O. pilosella*. Photo credit: a, b, d, f. K. N. Krakos; c. Charles Llewallen; e. G. L. Deeproot.

phylogenetic data, we consider that *O. sessilis* is best recognized as a distinct species.

Our phylogenetic reconstruction supports *O. sessilis* as sister to the clade containing *O. riparia* and *O. perennis* for the concatenated gene tree, but as nested within a clade containing all three species for the species tree analysis (though there is still not strong evidence to suggest that *O. sessilis* is nested within *O. pilosella*). While we were only able to sample a single individual of *O. sessilis*, two individuals of *O. pilosella* from distinct populations were sampled, found to be strongly monophyletic, and intraspecific variation within *O. pilosella* was less than half of the interspecific distance (Fig. 1). Implicit in this relationship is the fact that if *O. pilosella* and *O. sessilis* are not sister taxa, then they also represent independent polyploid events. Given the morphological traits and similar habitat, life history, and plant size, it was interesting that *O. fruticosa* did not group with *O. perennis*; and surprising that *O. riparia*, with its restricted riparian distribution that is unique within section *Kneiffia* was sister to *O. perennis*. Given that *O. fruticosa* is a polymorphic and widespread species with potential subspecies and numerous possible varieties that have not been evaluated with molecular studies, further studies are needed that focus on the relationships and ancestral distributions of section *Kneiffia*.

Other species in section *Kneiffia* may require further subdivision following additional studies. For example, the polymorphic *O. fruticosa*, was divided by Straley (1977) into two subspecies, *O. fruticosa* ssp. *glauca* and *O. fruticosa* ssp. *fruticosa*. However, these divisions have not been evaluated with molecular studies, or with current documentation of the subspecies ranges.

Pollination Systems and Pollen Limitation—The outcrossing *Oenothera* species we studied were all pollinated by bees, as previously noted (Straley 1977). We closely examined the pollination systems of these species and recorded visitation rates, pollen loads, and stigma contact. We show that while all sect. *Kneiffia* use similar functional groups of pollinators, the percentage of pollination due to each functional group varies among species (Fig. 2). While the pollination systems of species in sect. *Kneiffia* are broadly similar, at the level of functional group and compared to other sections within the genus, they do show differences. We also show that the self-compatible, relatively small-flowered *O. perennis*, previously described as autogamous (Straley 1977), actually has a pollination system involving small and medium bees of the family Halictidae and the genus *Bombus*. This is a surprising result because *O. perennis* is well known for having a permanent translocation heterozygote (PTH) genetic system (Johnson et al. 2009; Johnson et al. 2010). Typically, PTH species self-pollinate prior to the flowers opening, and therefore, any visitors are not important or effective pollinators (Johnson et al. 2009; Johnson et al. 2010; Brown and Levin 2011). However, for the populations of *O. perennis* included in this study, we observed that the receptive stigmas, without pollen on their surface, were exserted out of the opening buds of the flowers. Further studies are needed to determine if this stigma exsertion is a unique variation or is seen across the range of *O. perennis*.

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 that in *Oenothera* sect. *Kneiffia* 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. 3). Of course regardless of whether a plant has a self-compatible breeding system, the amount of self-fertilization can vary greatly within and among populations (Goodwillie et al. 2005). For this study, we focused on correlations between pollination and breeding system. Future studies could exam relationships between pollination and the percentage of seed set that is due to self-fertilization. While floral traits may correlate to some degree with breeding system for the *Oenothera*, the functional pollination systems for the *Oenothera* do not. The self-compatible species have the smaller flowers and reduced size associated with self-compatibility; however, the populations of *O. sessilis* in this study are functionally autogamous, while the data showing high levels of pollen flow suggests that *O. perennis* utilizes pollinators to set seed. The addition of cross pollen did have a larger effect on the two SI species, *O. riparia* and *O. pilosella*, but broader sampling is needed to determine if the impact of pollen addition has a larger effect on self-incompatible species than self-compatible species. It is possible that as species evolve to rely increasingly on self-pollination, their flowers may become smaller overall and their supply of pollen more limited. Such species may outcompete their SI or outcrossing competitors in small or more isolated habitats because of their ability to colonize new areas with low numbers of individuals (Baker 1955; Barrett et al. 1996; Barrett 2003).

Breeding Systems and Transitions to Self-compatibility—Our study verified *O. pilosella* as self-incompatible and *O. perennis* as self-compatible. We corrected a previous incorrect assumption and show *O. sessilis* to be self-compatible. We determined that *O. riparia* is self-incompatible. The topological tests of both a species tree and a concatenated gene tree clearly demonstrate three independent transitions to self-compatibility within section *Kneiffia*.

Overall, the results of our study provide a robust phylogeny with evidence for multiple independent transitions to self-compatibility within *Oenothera* sect. *Kneiffia*. We describe the pollination biology of this section, and find no consistent differences in pollinators between SC and SI species. Contrary to our expectations, we find no evidence that selfing species are more pollen limited than outcrossing species, although broader taxonomic sampling may provide greater resolution to this question.

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