



## Phylogenetic insights into the correlates of dioecy in meadow-rues (*Thalictrum*, Ranunculaceae)

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### ABSTRACT

Numerous studies have examined the evolution of sexual systems in angiosperms, but few explore the interaction between these and the evolution of pollination mode. Wind pollination is often associated with unisexual flowers, but which evolved first and played a causative role in the evolution of the other is unclear. *Thalictrum*, meadow-rues (Ranunculaceae), provides a unique opportunity to study the evolution of these traits because it contains insect and wind pollination and four sexual systems. We used a phylogenetic approach to reconstruct ancestral states for sexual system, pollination mode, and geographic distribution in *Thalictrum*, and tested for correlations to uncover the factors involved in the evolution of unisexuality and wind pollination. Our results show that dioecy, andro- and gynomonoecy evolved at least twice from hermaphroditism. Wind pollination, unisexual flowers, and New World distribution were all significantly correlated. Wind pollination may have evolved early in the genus, followed by multiple losses and gains, and likely preceded the origin of unisexual flowers in several cases; we found no evidence for unisexual flowers evolving prior to wind pollination. Given a broad scale study showing the evolution of dioecy before wind pollination, our results from a finer scale analysis highlight that different evolutionary pathways are likely to occur throughout angiosperms.

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### 1. Introduction

Flowering plants exhibit a remarkable array of sexual systems and various evolutionary transitions among them have been documented or proposed (Barrett, 2010; Charlesworth, 1993; Mitchell and Diggle, 2005; Sakai and Weller, 1999; Vamosi et al., 2003; Weiblen et al., 2000). While the majority of angiosperms are hermaphroditic (with both male and female functions within a flower), dioecy, the occurrence of separate sexes on different individuals, occurs in approximately 4–6% of angiosperm species (Renner and Ricklefs, 1995; Yampolsky and Yampolsky, 1922). Dioecy has evolved at least 100 times and is distributed among almost half of all angiosperm families (Barrett, 2010; Renner and Ricklefs, 1995; Vamosi et al., 2003). Dioecy can evolve from hermaphroditism, most commonly, via distyly, monoecy or gynodioecy (Barrett and Shore, 1987; Charlesworth, 1999; Lloyd, 1980; Mitchell and Diggle, 2005; Renner and Ricklefs, 1995; Renner and Won, 2001; Weiblen et al., 2000).

In order to identify the ecological factors influencing the evolution of dioecy, several studies have compiled data on its incidence

in floras across a diversity of taxa (Bawa, 1980; Flores and Schemske, 1984; Freeman et al., 1980; Givnish, 1980; Ibarra-Manríquez and Oyama, 1992; Muenchow, 1987; Renner and Ricklefs, 1995; Sakai et al., 1995; Sakai and Weller, 1999; Steiner, 1988; Thomson and Brunet, 1990; Webb et al., 1999). These studies identified “ecological correlates of dioecy,” including wind pollination; fleshy fruit; woodiness; unspecialized, small and inconspicuous flowers and inflorescences; and dry, tropical, or island habitats. Various hypotheses have been proposed to explain these associations, implying causal relationships between one or more of these ecological factors and the evolution of dioecy (Mitchell and Diggle, 2005; Sakai and Weller, 1999; Vamosi et al., 2003).

Wind pollination, one of the ecological correlates of dioecy, has been generally associated with unisexual flowers (Darwin, 1876; de Jong et al., 2008; Friedman and Barrett, 2008; Linder, 1998; Renner and Ricklefs, 1995). In addition to dioecy, other sexual systems with unisexual flowers include andromonoecy, where both male and hermaphroditic flowers occur on an individual, and gynomonoecy, where both female and hermaphroditic flowers occur on an individual. Andromonoecy and gynomonoecy occur in approximately 2% and 3% of angiosperm species, respectively (Yampolsky and Yampolsky, 1922).

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Wind pollination has arisen at least 65 times from animal pollination in angiosperms, occurring in approximately 10% of species (Linder, 1998). In comparison with other reproductive transitions, however, few studies have examined the factors responsible for its evolution (Barrett, 2010; Friedman and Barrett, 2008, 2009). Several ecological correlates of wind pollination have been suggested (Culley et al., 2002; Linder, 1998), and correlated evolution with habitat type, flower sexuality, plant sexuality, flower size, flower showiness, presence or absence of nectar, and ovule number has been shown within a phylogenetic context (Friedman and Barrett, 2008).

In order to understand whether wind pollination played a role in the evolution of dioecy or vice versa, we need to determine not only whether correlations exist between these traits but, also, which trait originated first (Barrett, 2010; Vamosi et al., 2003). Under one scenario (Hypothesis I, Fig. 1), wind pollination evolves first as a mechanism for reproductive assurance in environments with low pollinator abundance (Culley et al., 2002). Subsequently, dioecy is favored to reduce selfing and the associated inbreeding depression resulting from wind pollination, or as a shift in resource allocation to ensure better transfer of pollen by increasing pollen production in male plants (Charlesworth and Charlesworth, 1978a; Davis, 1997; Friedman and Barrett, 2008; Leppik, 1964; Melampy and Hayworth, 1980; Penny and Steven, 2009). Alternatively (Hypothesis II, Fig. 1), dioecy evolves first as a mechanism to reduce selfing of hermaphrodites and the associated inbreeding depression when pollinators are limited. Wind pollination then arises to facilitate the reproduction of female plants that may be discriminated against by pollinators seeking pollen as a reward in nectarless flowers (Kaplan and Mulcahy, 1971; Weller et al., 1998).

While many authors have argued that adaptations to wind pollination predispose species for the evolution of dioecy (Charlesworth, 1993; Freeman et al., 1997, 1980; Grant, 1951; Melampy and Hayworth, 1980; Renner and Ricklefs, 1995; Sakai et al., 2006; Stebbins, 1951), fewer have argued the opposite pattern (Bawa, 1980; Kaplan and Mulcahy, 1971). However, a large-scale phylogenetic study across angiosperms found the evolution of wind pollination more likely after the establishment of dioecy and unisexual flowers (Friedman and Barrett, 2008), supporting Hypothesis II.

As previously suggested (Barrett, 2010; Friedman and Barrett, 2008; Thomson and Brunet, 1990), studies based on genus-level phylogenies can illuminate the factors that influenced the evolu-

tion of dioecy, other breeding systems with unisexual flowers, and wind pollination by examining correlations between these traits among closely related taxa and determining, more precisely, shifts in traits and relative timing of origins. *Thalictrum* (meadowrue, Ranunculaceae) is an ideal genus to examine the evolution of wind pollination, dioecy, and unisexual flowers because it contains variation in both sexual system and pollination mode. Hermaphroditic, dioecious, andromonoecious, and gynomonoecious species are found within the genus (Fig. 2; Boivin, 1944; Guzmán, 2005). Wind and insect pollination have been reported within the genus (Kaplan and Mulcahy, 1971), and variation in pollination mode even exists within a sexual system (compare Fig. 2a and e; Pellmyr, 1995).

Of the ecological correlates of dioecy discussed above, *Thalictrum* exhibits wind pollination and unspecialized flowers. All *Thalictrum* species have nectarless, apetalous flowers (Leppik, 1964). Many species have small and inconspicuous flowers; and unspecialized pollen-collecting pollinators, such as bee flies, syrphid flies and solitary bees, appear to be vectors for insect-pollinated species (Kaplan and Mulcahy, 1971; Pellmyr, 1995; Steven, 2003). The genus occurs primarily in northern temperate regions (Mabberley, 2008); and the majority of species live in mesic habitats. Thus, dry, island, or tropical habitats typically associated with dioecy do not apply here. However, in the genus, geographic distribution appears to be correlated with dioecy as all dioecious species occur in North America.

The goal of the present study is to develop a phylogenetic framework for the genus *Thalictrum* in order to examine the evolution of sexual systems and pollination modes and their interactions within the genus. Regarding sexual systems, we seek to (1) determine the number of origins of unisexual flowers within the group, and (2) determine whether andromonoecy and gynomonoecy represent steps toward the evolution of dioecy or independent evolutionary events from hermaphroditism. Regarding pollination modes, we seek to (3) determine the number of origins of wind pollination, and (4) determine the number of reversals from wind back to insect pollination. Finally, to better understand whether wind pollination or geography influenced the evolution of sexual systems in this genus, we seek to (5) test for correlations between these traits, and (6) test for the evolutionary order of acquisition of these traits.

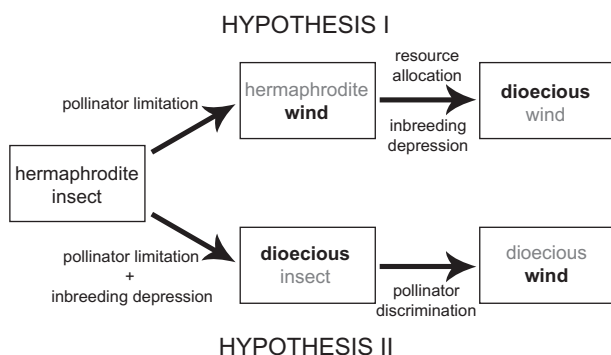
Our study uses a phylogenetic approach to reconstruct ancestral states in the genus and test for correlations and evolutionary sequences among traits. We reconstruct the first comprehensive phylogeny of *Thalictrum* and proceed to show that dioecy, andromonoecy and gynomonoecy each evolved independently and at least twice from hermaphroditism. Multiple gains and losses of wind pollination have occurred, suggesting lability in this trait and a promising area for future research. Furthermore, wind pollination, unisexual flowers, and New World distribution are all significantly correlated in this group.

## 2. Materials and methods

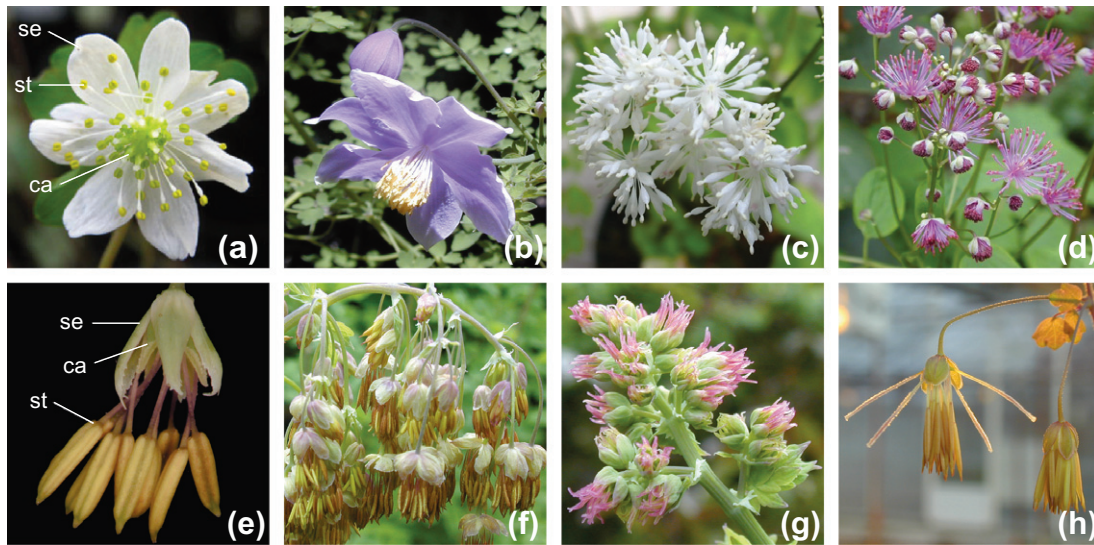
### 2.1. Sampling methods

In the only complete, modern synopsis of the genus *Thalictrum*, Tamura (1995) recognized 190 species of *Thalictrum*. We verified the taxonomic status of 901 published species names from Index Kewensis (1997) against *Thalictrum* monographs and revisions (Boivin, 1944; Emura, 1972; Lecoyer, 1885; Lourteig, 1956) and in nine current floras covering the geographic range of the genus. This resulted in 196 non-synonymized species of *Thalictrum* (Di Stilio and Liston, 2011), which is in agreement with Tamura (1995).

To date, we have sampled 63 species from 13 of the 14 sections (Tamura, 1995), to represent the breadth of taxonomic classification



**Fig. 1.** Two hypotheses for the evolutionary sequence of wind pollination and dioecy from hermaphroditic, insect-pollinated angiosperms. Hypothesis I suggests that wind pollination evolved first due to low pollinator abundance; dioecy evolved second due to a shift in resource allocation or due to increased selfing and inbreeding depression. Hypothesis II suggests that dioecy evolved first due to low pollinator abundance followed by increased selfing and inbreeding depression; wind pollination evolved second due to pollinator discrimination against females.



**Fig. 2.** Floral diversity of sexual systems and pollination modes in *Thalictrum* (Ranunculaceae). (a–d) Hermaphroditic, insect-pollinated species. (a) *T. thalictroides*; (b) *T. diffusiflorum*; (c) *T. filamentosum*; (d) *T. aquilegifolium*. (e–h) Wind-pollinated species. (e) *T. alpinum*, hermaphroditic; (f) *T. polycarpum* male, dioecious; (g) *T. polycarpum* female, dioecious; (h) *T. guatemalense*, andromonoecious: hermaphroditic (left) and staminate (right) flowers shown. (ca = carpel, se = sepal, st = stamen).

and geographic distribution (Supplementary Tables S1–S2), and four outgroups: *Aquilegia alpina*, *A. vulgaris*, *Leptopyrum fumaroides*, and *Paraquilegia microphylla*, which were identified from previous work on the molecular phylogeny of the family (Cai et al., 2010; Hoot, 1995; Hoot et al., 2008; Johansson, 1995; Johansson and Jansen, 1993; Kosuge et al., 1995; Ro et al., 1997; Ro and McPheron, 1997; Wang and Chen, 2007; Wang et al., 2009).

## 2.2. Molecular methods

We used the nuclear ribosomal internal transcribed spacer (ITS) region (ITS1, ITS2, and 5.8S) and the chloroplast (cpDNA) *rpl16* intron for phylogenetic reconstruction. The popularity of the ITS region for infrageneric studies within angiosperms is well known (Baldwin et al., 1995; Hughes et al., 2006; Mort et al., 2007), as well as its limitations (Alvarez and Wendel, 2003). Levels of ITS sequence divergence within *Thalictrum* are relatively high; and the phylogenetic utility of this locus in the genus was demonstrated by Ro and McPheron (1997). Interspecific cpDNA sequence divergence is generally less than ITS (Hughes et al., 2006; Mort et al., 2007), and thus, it is necessary to utilize cpDNA regions that exhibit relatively high rates of substitution. At the beginning of this study, the *rpl16* intron (*rpl16*) had the highest sequence divergence among a number of examined cpDNA regions (Downie et al., 1996; Small et al., 1998), and remains potentially useful in species-level phylogenetic studies (Shaw et al., 2005).

We extracted genomic DNA from herbarium specimens, field collections, and cultivated accessions using the DNeasy Plant Kit (Qiagen, Valencia, CA) or FastDNA Kit (MP Biomedicals, Solon, OH). Voucher specimens are listed in Supplementary Table S1. For amplification and sequencing of ITS, we used the ITS forward (Goodwillie and Stiller, 2001) and 26S 25 reverse (Nickrent et al., 1994) primers. For amplification of *rpl16*, we used primers F71 and F1661 (Jordan et al., 1996), and primers F71 and R1516 for sequencing (Kelchner and Clark, 1997). Polymerase chain reaction conditions were 95 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 53 °C for 1 min, and 72 °C for 1 min, with a final extension step at 72 °C for 5 min. Amplified DNA was purified using the Concert Matrix Gel Extraction System (Invitrogen Corporation, Carlsbad, CA) or ExoSAP-IT (USB Corporation, Cleveland, OH) and directly sequenced.

We cloned ITS sequences that could not be directly sequenced, due to allelic differences in insertions and deletions, using the Dual Promoter TA Cloning Kit (Invitrogen Corporation, Carlsbad, CA), for a total of four to eight clones per accession; and purified clones using the FastPlasmid Mini Kit (5 Prime Inc., Gaithersburg, MD). Sequencing was performed by the Center for Genome Research and Biocomputing at Oregon State University (OSU), the University of Wisconsin Biotechnology Center, and the University of Washington Biochemistry DNA Sequencing Facility.

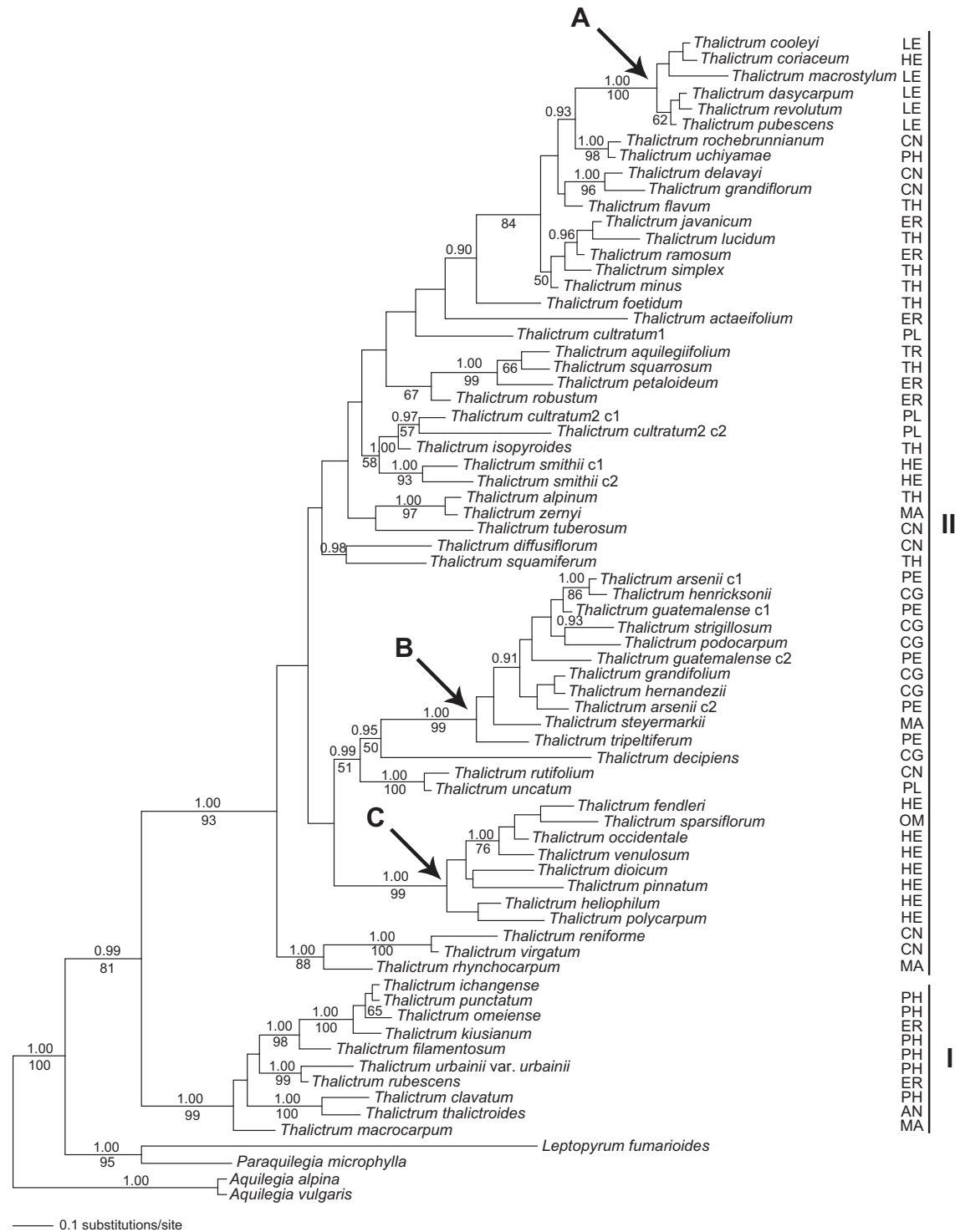
## 2.3. Phylogenetic methods

Sequences were edited in Sequencher version 4.9 (Gene Codes Corporation, Ann Arbor, Michigan, USA) and aligned manually using MacClade version 4.08 (Maddison and Maddison, 2005). Unambiguously aligned gaps from *rpl16* that were phylogenetically informative for ingroup were coded as presence/absence characters (Graham et al., 2000; Simmons and Ochoterena, 2000). The *rpl16* and ITS data sets are available through TreeBASE (<http://purl.org/phylo/treebase/phyloWS/study/TB2:S11363>).

We reconstructed phylogenies for each molecular data set separately and for the combined molecular data set using Bayesian and likelihood analyses. For taxa with various clonal ITS sequences, we constructed a consensus sequence (including single nucleotide polymorphisms) from each monophyletic group of sequences representing a given taxon. All other clonal sequences, not forming monophyletic groups with other clones from the same accession, were included in analyses. For accessions with more than one included ITS clone, the corresponding cpDNA sequences were duplicated for use in the combined data set.

For Bayesian and likelihood analyses, models of evolution for the *rpl16* and ITS data sets were determined separately by Modeltest version 3.7 (Posada and Crandall, 1998). The models selected under the Akaike Information Criterion (Akaike, 1974) were K81uf +  $\Gamma$  and GTR + I +  $\Gamma$ , respectively. For gap data, we used the binary model, with ascertainment coding bias set to variable, in MrBayes version 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003), and the Mk model in GARLI-PART version 0.97 (Zwickl, 2006).

Bayesian analyses were conducted separately in MrBayes via the CIPRES Science Gateway version 3.1 (Miller et al., 2011) for



**Fig. 3.** Phylogenetic relationships in the genus *Thalictum* (Ranunculaceae). Bayesian all-compatible consensus tree based on the chloroplast *rpl16* intron and nuclear internal transcribed spacer region, showing two major clades, I and II, two dioecious subclades, A and C, and one andromonoecious clade, B – posterior probabilities  $\geq 0.90$  displayed above branches. Likelihood bootstrap values  $\geq 50\%$  displayed below branches. (c = clonal sequence; Sectional affiliations: AN = Anemonella, CG = Camptogastrum, CN = Camptonotum, ER = Erythrandra, HE = Heterogamia, LE = Leucocoma, MA = Macrogynea, OM = Omalophysa, PE = Pelteria, PH = Physocarpum, PL = Platycarpa, TH = Thalictum, TR = Tripterium).

each data set, and with data partitioned under the selected models for the *rpl16* and combined data sets. We used default priors of no prior knowledge for the parameters of these models. Parameters for nucleotide frequencies, substitution rates, and gamma shape were unlinked across data partitions. All partitions were allowed to evolve under different rates, and site-specific rates were allowed to vary under a flat Dirichlet prior across partitions.

Bayesian analyses were conducted with three independent Markov Chain Monte Carlo (MCMC; Yang and Rannala, 1997) analyses of five million generations for *rpl16*, 13 million generations for ITS, and 36 million generations for the combined data set. Metropolis coupling for each analysis was conducted under the default settings. Convergence was determined when the average standard deviation of split frequencies remained less than 0.01.

For the *rpl16*, ITS, and combined data sets, the first 43%, 63%, and 50% of trees, respectively, were discarded before convergence. For each analysis, the remaining trees from each run were pooled to construct a 50% majority rule consensus tree (*rpl16* and ITS) or a consensus tree with all compatible groups (combined) to obtain posterior probabilities (pp).

Likelihood analyses were conducted in GARLI version 1.0 (Zwickl, 2006) via the CIPRES Science Gateway for ITS, and in GARLI-PART with data partitioned under the selected models for the *rpl16* and combined data sets. All analyses were run under the default settings, but included five search replicates; and for the *rpl16* and combined data sets, included random starting trees, 100 attachment points evaluated for each taxon, model parameters unlinked across subsets, different subset rates allowed, a prior weight of 0.01 to topology mutations, a prior weight of 0.0015 (*rpl16*) or 0.002 (combined) to model mutations, a prior weight of 0.002 to branch-length mutations, and 10,000 generations as the first part of the termination condition.

To assess the reliability of clades in the resulting likelihood trees for each data set, we conducted 1000 nonparametric bootstrap (bs) replicates (Felsenstein, 1985) in GARLI. Bootstrap replicates were conducted under the above settings, but included one search replicate; and for ITS, included 10,000 generations as the first part of the termination condition.

#### 2.4. Character reconstruction methods

To evaluate potential selective forces involved in the evolution of dioecy or wind pollination, character states of sexual system, pollination mode, and geographic distribution were reconstructed on tree topologies using parsimony and likelihood in Mesquite version 2.74 (Maddison and Maddison, 2010). Only fully resolved phylogenies are allowed in likelihood reconstructions in Mesquite. Therefore, ancestral states were reconstructed on the fully resolved, combined Bayesian consensus tree using equally weighted parsimony (unordered states; Fitch, 1971) and likelihood, under the Markov k-state one-parameter (MK1) model, where all changes are equally probable.

The character states used for sexual system were dioecy, hermaphroditism, and monoecy (Supplementary Table S3). Monoecy has been reported from Mexican species (Boivin, 1944), but our field and herbarium observations suggest that Boivin was misled by strongly dichogamous, andromonoecious plants. No true monoecy exists in the group, but this category was used to group the andro- and gynomoecious species. The sexual system was obtained from published literature or from personal observations. We encountered two species of *Thalictrum* in the literature that appear androdioecious but are functionally dioecious, i.e. hermaphroditic plants have inaperturate pollen, *T. macrostylum* (Penny and Steven, 2009) and *T. pubescens* (Davis, 1997), and coded them as dioecious.

The character states for pollination mode were wind or insect, and were obtained from the literature or from pollination indices by Kaplan and Mulcahy (1971). For taxa in our phylogeny not included in Kaplan and Mulcahy (1971), we estimated pollination indices following their methodology when possible (Supplementary Table S4). Based on field observations and experimental confirmations of pollination indices (Guzmán, 2005; Kaplan and Mulcahy, 1971; Melampy and Hayworth, 1980; Steven, 2003; Steven and Waller, 2004), taxa were coded as followed for pollination indices (PI): wind for PI of one to two or insect for PI > two (Supplementary Table S3). We did not include mixed pollination as introduced by Kaplan and Mulcahy (1971) because little empirical evidence appeared to support its existence (Guzmán, 2005; Melampy and Hayworth, 1980; Steven, 2003; Steven and Waller, 2004).

The character states for geographic distribution were obtained from published literature (Supplementary Table S3). The categories were North America; Meso- and South America, which included Mexico and Central America; and the Old World, which included Asia, Europe, and a few African species. Polymorphic taxa are not allowed under likelihood reconstructions. Therefore, polymorphic taxa and taxa with unknown states were coded as missing.

#### 2.5. Tests for character correlations and evolutionary sequences

We tested correlated evolution using a likelihood approach in BayesDiscrete (Pagel, 1994) and a Bayesian approach in SIMMAP version 1.5 (Bollback, 2006). BayesDiscrete only allows for two-state characters and utilizes one tree; whereas, SIMMAP allows for multi-state characters, incorporates phylogenetic uncertainty by allowing multiple trees, and determines the amount of time along a branch each state occurred.

For analyses in SIMMAP, morphological models for each character were configured using the combined Bayesian consensus tree and rescaling the tree length to one, using an approach by Schultz and Churchill (1999). For the bias parameter, we used beta distribution priors with 31 categories and empirical priors, for two-state and four-state characters, respectively. For all characters, we used a gamma distribution prior for the overall evolutionary rate parameter with 90 categories. To determine values for  $\alpha$  for the beta distribution prior, and  $\alpha$  and  $\beta$  for the gamma distribution prior, we obtained values for each character by running an MCMC analysis under the default settings. Samples from the posterior distribution of these parameters were used to obtain best-fitting distributions and parameter values for  $\alpha$  and  $\beta$  using the R version 2.12.2 (R Development Core Team, 2011) script available with SIMMAP. All character states were unordered.

To account for phylogenetic uncertainty and computational time, character correlations were conducted in SIMMAP with 100 post-burnin trees from each MrBayes run, for a total of 300 trees, using the character states and priors determined above, with five samples for each tree and from the prior distribution for each character, and five posterior predictive samples simulated to determine *P*-values for the correlation *D* statistic (Huelsenbeck et al., 2003).

Finally, we tested for the evolutionary order of change in two characters using a likelihood approach in BayesTraits (Pagel and Meade, 2011), which analyzes transition rates in BayesDiscrete. Because BayesDiscrete only allows binary data, we examined flower sexuality, plant sexuality, pollination mode, and geographic distribution as two states: hermaphroditic or unisexual, cosexual (including hermaphroditic and monoecious) or dioecious, insect or wind, and Old World or New World, respectively. First, we tested for correlations between two characters with the omnibus test (Pagel, 1994). Then, we used the temporal order test, to determine whether changes in one character preceded changes in another character (Pagel, 1994), and the contingent change test, to determine whether one state of one character was dependent on the state of another character (Friedman and Barrett, 2008; Pagel, 1994; Vamosi et al., 2003).

### 3. Results

#### 3.1. Sampling and molecular results

The unaligned and aligned lengths, total base pairs analyzed, uncorrected pairwise distances, gaps scored, and number of accessions completed for each DNA region are summarized in Supplementary Table S5. Although the ITS region was shorter than the *rpl16* intron, it contained more variable sites (Supplementary Table S5).

### 3.2. Phylogenetic results

We examined the majority rule consensus trees from the *rpl16* and ITS data sets (Supplementary Figs. S1–S2) and found many of the relationships among taxa were unresolved or weakly supported with the individual *rpl16* data set. Therefore, only analyses from the combined *rpl16* and ITS data sets were used to estimate the phylogeny of *Thalictrum* and for subsequent character reconstruction and correlation analyses.

In the Bayesian and likelihood analyses of the combined data sets, *Thalictrum* is strongly supported as a monophyletic group (pp = 0.99, bs = 81%), and contains two major clades that are strongly supported and sister groups: clades I (pp = 1.00, bs = 99%) and II (pp = 1.00, bs = 93%; Fig. 3). However, relationships along the backbone of each of these clades are not well supported. We did identify a number of strongly supported groups within each major clade (Fig. 3). In particular, within clade II, we found two strongly supported dioecious, North American clades (A and C, Fig. 3) and one strongly supported andromonoecious, Meso- and South American clade (B, Fig. 3). None of the sections traditionally circumscribed for the genus (Tamura, 1995) are monophyletic (Fig. 3).

### 3.3. Parsimony and likelihood character reconstructions

The following parsimony and likelihood estimates for character reconstructions in Mesquite may be considered the minimum number of transformations required for states at the tips of our phylogeny. Parsimony analysis of sexual system evolution required seven transformations in strongly supported terminal clades (Fig. 4). Both parsimony and likelihood analyses reconstructed a hermaphroditic origin of the genus *Thalictrum* (proportional likelihood [PL] 0.995, Fig. 4). According to both analyses, the ancestral conditions for clades I and II were hermaphroditism (PL 0.975 and 0.993, respectively, Fig. 4). Dioecy has arisen twice from hermaphroditism in clade II, with one reversal to hermaphroditism in *T. sparsiflorum* (Fig. 4). Monoecy (andro- and gyno-) has arisen four times from hermaphroditism in the genus (Fig. 4). A proportional likelihood (PL) of 0.875 was previously determined as the significance threshold for likelihood reconstructions, i.e. the minimum threshold for certainty in these reconstructions (McDill et al., 2009; Schulter et al., 1997). Therefore, the likelihood reconstructions of sexual systems above are all significant.

Parsimony analysis of pollination mode evolution required 12 transformations. However, because pollination mode was very labile and the backbone of our phylogeny was resolved but not well supported, we used proportional likelihoods (PL) > 0.875 to indicate likely transitions in terminal clades, which indicated that wind pollination has arisen at least four times in clade II, with at least one reversal to insect pollination (Fig. 4).

Both parsimony and likelihood analyses reconstructed an origin of *Thalictrum* as more likely insect-pollinated (PL 0.582, Fig. 4). According to both analyses, the ancestral condition for clade I was more likely insect pollination (PL 0.658, Fig. 4). However, both analyses showed a different ancestral condition for clade II, either insect (parsimony) or wind pollination (PL 0.627, Fig. 4). Based on the PL threshold of 0.875 (McDill et al., 2009; Schulter et al., 1997), the likelihood reconstructions of pollination mode above are not significant.

Parsimony analysis of geographic distribution required five steps in strongly supported terminal clades (Fig. 5). Both parsimony and likelihood analyses reconstructed an Old World origin of the genus (PL 1.00, Fig. 5). According to both analyses, the ancestral distributions for clades I and II were most likely Old World (PL 1.00, Fig. 5). Both analyses show one and two dispersal events into North America from the Old World in clades I and II, respectively

(Fig. 5). One dispersal event into Meso- and South America from North America and from the Old World have occurred in clade II (Fig. 5). Based on the PL threshold of 0.875 (McDill et al., 2009; Schulter et al., 1997), the likelihood reconstructions of geographic distribution above are all significant.

### 3.4. Character correlations and evolutionary sequences

Significant correlations, based on character correlation *d*-statistics in SIMMAP, are summarized in Table 1 and visually represented in Figs. 4–6. Wind pollination was significantly associated with dioecy, moderately associated with monoecy (Table 1, Fig. 4), and significantly associated with North and Meso- and South America (Table 1, Fig. 5). Dioecy was significantly associated with North America (Table 1, Fig. 6), whereas monoecy was significantly associated with Meso- and South America (Table 1, Fig. 6).

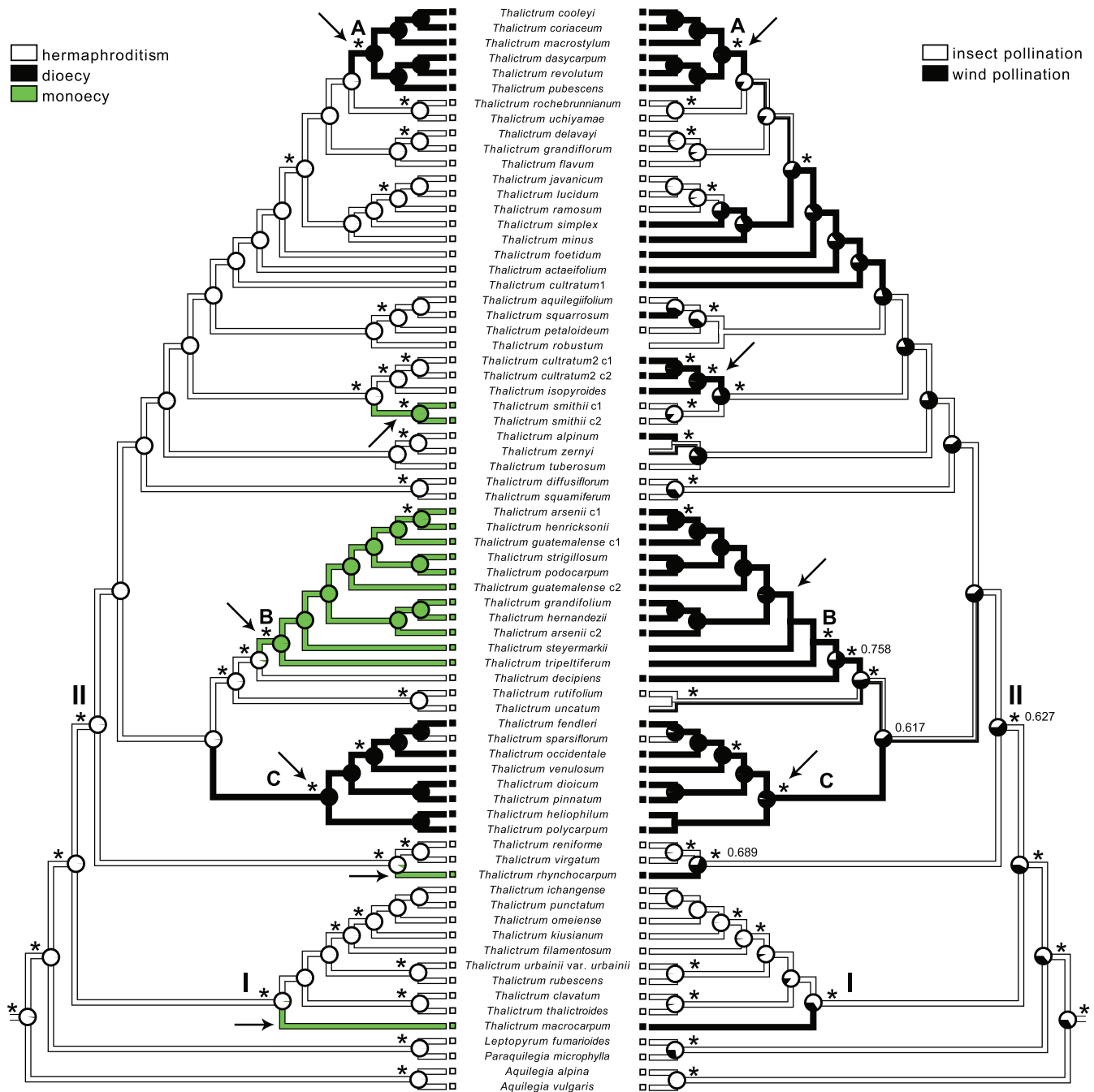
According to the omnibus test in BayesDiscrete, flower sexuality and pollination mode, plant sexuality and pollination mode, and pollination mode and geographic distribution were significantly correlated; whereas, flower sexuality and geography were moderately correlated (Table 2). Both the temporal and contingent change tests did not show strongly significant results for the origin of one character state preceding or depending on another character's state, which may be due to lack of support along the backbone of the phylogeny. However, the origin of unisexual flowers as dependent on wind pollination, i.e. wind pollination arising before unisexual flowers, was marginally significant ( $P = 0.059$ , Table 2).

Although we could not confidently detect the evolutionary sequence of events among characters with tests in BayesDiscrete, both SIMMAP and BayesDiscrete character correlation tests show that wind pollination, unisexual flowers (including dioecy), and New World distribution (including North America and Meso- and South America) are all strongly correlated (Tables 1 and 2).

If we examine the evolution of wind pollination and unisexual flowers across the combined Bayesian consensus phylogeny, likelihood values are greater than 50% in three instances, indicating that wind pollination more likely originated prior to dioecy in clade C (PL 0.617) and prior to monoecy in clade B (PL 0.758) and in *T. rhyncho carpum* (PL 0.689; Fig. 4). Yet, if we use the published PL threshold of 0.875 (McDill et al., 2009; Schulter et al., 1997), likelihood reconstructions of pollination mode are not significant. Based on this phylogeny, we could not detect the evolutionary sequence of events for clade A or *T. macro carpum* because wind pollination and unisexual flowers appear to have evolved along the same branch (Fig. 4). In any event, we found no evidence for unisexual flowers arising before wind pollination in *Thalictrum*. Similarly, wind pollination appears to have originated prior to New World distribution in clade C (PL 0.617; Fig. 5), but is unclear in clades A and B because both wind pollination and New World colonization appear to have occurred along the same branch (Fig. 5).

## 4. Discussion

Here, we present the first comprehensive phylogenetic analysis of the genus *Thalictrum* and examine the evolution of sexual systems and pollination modes within the group by various character reconstruction methods and correlation tests. Based on reconstructions of ancestral character states, we identified at least two instances each of evolution to dioecy, andromonoecy, and gynomoecy from hermaphroditism (Fig. 4). Wind pollination likely evolved early in the genus, with several losses and gains, and preceded the evolution of unisexual flowers in a few cases (Fig. 4). Significant correlations were detected between wind pollination, unisexual flowers (including dioecy, andro-, and



**Fig. 4.** Parsimony and likelihood reconstructions of ancestral states of sexual system (left) and pollination mode (right) in *Thalictrium* and outgroups (Ranunculaceae), using the Bayesian consensus tree based on the chloroplast *rp16* intron and nuclear internal transcribed spacer region. Proportional likelihoods of ancestral states at nodes are represented by pie charts. Proportional likelihoods of ancestral states at strongly supported ancestral nodes (i.e., Bayesian posterior probabilities  $\geq 0.95$  or likelihood bootstrap values  $\geq 70\%$ ) indicated with asterisks below nodes. Transitions to dioecy and monoecy and wind pollination in terminal clades with proportional likelihoods  $>0.875$  indicated by arrows. Numerical values of proportional likelihoods indicated at nodes that show wind pollination as more likely to have occurred prior to unisexual flowers. Two major clades in the genus, I and II, two dioecious subclades, A and C, and one andromonoecious clade, B.

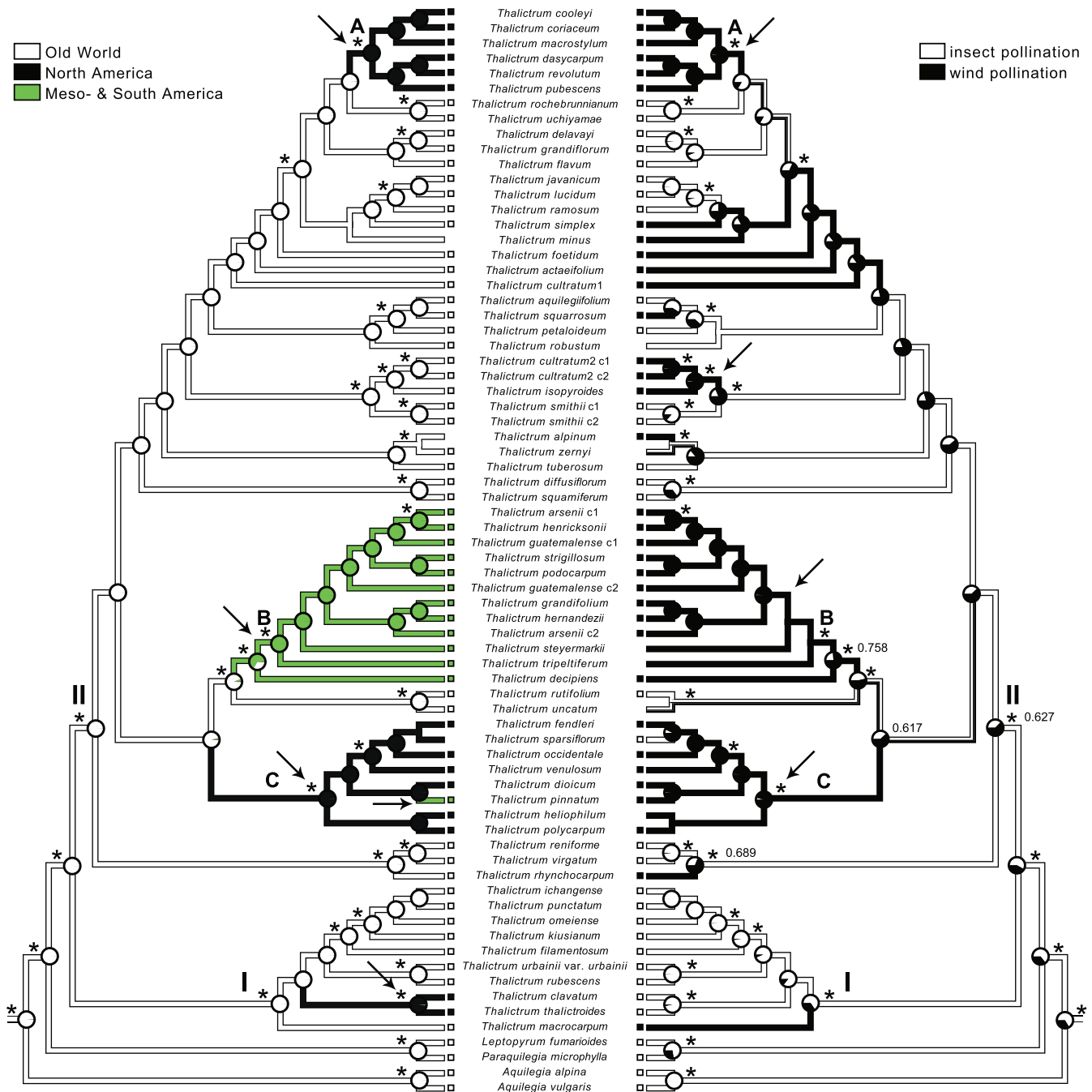
gynomonoecy), and colonization of the New World, suggesting that new habitats also played a role in the evolution of these traits.

#### 4.1. Evolution of sexual systems

Our phylogenetic character reconstructions indicate that dioecy has arisen from hermaphroditism in *Thalictrium*. No gynodioecious or fully monoecious species have been reported in *Thalictrium*; therefore, it is unlikely for these sexual systems to have played a role in the evolution of dioecy in this group. Moreover, *Thalictrium* is relatively recent, having originated approximately seven million

years ago (Bastida et al., 2010). Therefore, extinction of intermediate sexual systems is less likely in its terminal clades, supporting the claim that intermediate sexual systems did not play a role in the evolution of dioecy in this genus.

Three alternative pathways to dioecy from hermaphroditism have been suggested in the literature: distyly, andromonoecy or androdioecy, and direct evolution (Ross, 1982). In addition to this study, the evolution of dioecy from hermaphroditism without intermediate sexual systems has been shown in additional phylogenetic studies (Mitchell and Diggle, 2005; Soza and Olmstead, 2010; Torices et al., 2011; Weiblen et al., 2000). In Ranunculaceae,



**Fig. 5.** Parsimony and likelihood reconstructions of ancestral states of geographic distribution (left) and pollination mode (right) in *Thalictrium* and outgroups (Ranunculaceae), using the Bayesian consensus tree based on the chloroplast *rp16* intron and nuclear internal transcribed spacer region. Proportional likelihoods of ancestral states at nodes are represented by pie charts. Proportional likelihoods of ancestral states at strongly supported ancestral nodes (i.e., Bayesian posterior probabilities  $\geq 0.95$  or likelihood bootstrap values  $\geq 70\%$ ) indicated with asterisks below nodes. Transitions to North and Meso- and South America and wind pollination in terminal clades with proportional likelihoods  $>0.875$  indicated by arrows. Numerical values of proportional likelihoods indicated at nodes that show wind pollination as more likely to have occurred prior to unisexual flowers. Two major clades in genus, I and II, two dioecious subclades, A and C, and one andromonoecious clade, B.

dioecy has evolved at least three other times, including *Clematis*, *Hamadryas*, and *Paroxygraphis* (Tamura, 1993), and appears to have evolved from hermaphroditism in *Hamadryas* (Hoot et al., 2008).

One scenario for the evolution of dioecy from hermaphroditism in *Thalictrium* has been proposed upon examination of the floral ontogeny of the dioecious species *T. dioicum* (Di Stilio et al., 2005; Diggle et al., 2011). *Thalictrium dioicum* flowers are unisexual from inception (type II), rather than the more widespread condition of unisexual by abortion (type I), and expression of organ identity genes indicates that unisexuality may have arisen by homeosis (Di Stilio et al., 2005). The two mutations needed for the evolution

of dioecy (Charlesworth and Charlesworth, 1978a) could have involved stamen identity function, under the B class genes of the ABC model of flower development (Coen and Meyerowitz, 1991). For example, a loss-of-function mutation in the B class genes would result in female flowers by homeotic conversion of stamens to carpels, while a gain-of-function mutation in these genes would result in male flowers by homeotic conversion of carpels to stamens (Di Stilio et al., 2005).

Both types of unisexual flowers are found among dioecious species of *Thalictrium*. Type II flowers (unisexual from inception; Fig. 2f–g) are the predominant form in unisexual species in clades



**Table 1**  
Character correlation *d*-statistic among sexual systems, pollination modes, and geographic distribution in *Thalictrum* (Ranunculaceae), as measured in SIMMAP (Bollback, 2006).

Sexual systems	Pollination modes		Geographic distribution		
	Insect	Wind	North America	Meso- and South America	Old World
Hermaphroditism	<b>+0.076 (0.004)<sup>c,d</sup></b>	<b>−0.076 (0.004)</b>	<b>−0.067 (0.015)</b>	<b>−0.065 (0.021)</b>	<b>+0.132 (0.001)</b>
Dioecy	<b>−0.045 (0.020)</b>	<b>+0.045 (0.020)</b>	<b>+0.087 (0.003)</b>	−0.010 (0.137)	<b>−0.076 (0.006)</b>
Monoecy	−0.031 (0.055)	+0.031 (0.055)	−0.020 (0.074)	<b>+0.075 (0.007)</b>	<b>−0.055 (0.033)</b>
<i>Geographic distribution</i>					
North America	<b>−0.032 (0.045)</b>	<b>+0.032 (0.045)</b>			
Meso- and South America	<b>−0.043 (0.023)</b>	<b>+0.043 (0.023)</b>			
Old World	<b>+0.075 (0.003)</b>	<b>−0.075 (0.003)</b>			

<sup>a</sup> “+” Indicates positive association.

<sup>b</sup> “−” Indicates negative association.

<sup>c</sup> *P*-values shown in parentheses.

<sup>d</sup> Bold values indicate *P* < 0.05.

A and C (Fig. 3); whereas a few species with type I flowers (unisexual by abortion) are found in clade A (Fig. 3), as evidenced by *T. macrostylum* (Penny and Steven, 2009) and *T. pubescens* (Davis, 1997; Kaplan and Mulcahy, 1971). Potential mutations in B class genes may be responsible for the origin of dioecy in clade C (Fig. 3), as hypothesized for *T. dioicum*; however, dioecy may have arisen differently in clade A (Fig. 3), containing both type I and type II flowers. The presence of morphologically androdioecious, but functionally dioecious, taxa in clade A (*T. macrostylum*, *T. pubescens*, Fig. 3; Davis, 1997; Penny and Steven, 2009) indicates that female-sterile mutations could have arisen first in this clade (these taxa contain both male and hermaphrodite plants), followed by male-sterile mutations that do not involve B class genes (stamens are retained on hermaphrodites, but pollen is sterile). The presence of both morphologically androdioecious and dioecious taxa in this clade points to androdioecy as a potential pathway in the evolution of dioecy in this more recent dioecious clade.

At least two origins of andro- and gynomonoeocism from hermaphroditism have persisted in *Thalictrum* (Fig. 4). Andromonoecy occurs in one large clade in Meso- and South America (clade B, Fig. 6) and in *T. rhynchocarpum* from Africa. The only two gynomonoeocious species are *T. macrocarpum* from Spain (Guzmán, 2005) and *T. smithii* from Asia (Zheng-yi et al., 2001). Gynomonoeocism has been hypothesized as a potential pathway to the evolution of full monoecy in angiosperms (Charlesworth and Charlesworth, 1978b), but has also been shown as a terminal condition in Asteraceae (Torices et al., 2011). Andromonoecy has also been shown to evolve from hermaphroditism multiple times as a terminal condition (Torices et al., 2011; Weiblen et al., 2000). Since no fully monoecious species are known in *Thalictrum*, andromonoecious and gynomonoeocious species represent terminal conditions that evolved from hermaphroditism, rather than being a step towards the evolution of other sexual systems.

#### 4.2. Evolution of pollination mode

According to our reconstructions and outgroup comparisons, insect pollination is the most likely ancestral state for the origin of *Thalictrum*. The rest of the genera in Ranunculaceae are reported to be exclusively insect-pollinated (Leppik, 1964; Kaplan and Mulcahy, 1971), supporting insect pollination as the ancestral mode for *Thalictrum*. Wind pollination arose later on in the genus, as early as in the ancestor of clade II (Fig. 4), although the probability is lower than 0.875.

*Thalictrum* is unusual compared to other members of Ranunculaceae because it exhibits floral characteristics that are linked to wind pollination, yet these characteristics are plesiomorphic (ancestral) for the genus and occur in insect-pollinated species as well. For example, the lack of petals and nectar, characteristic traits of the genus, and presence of uniovulate carpels, a plesiomorphic

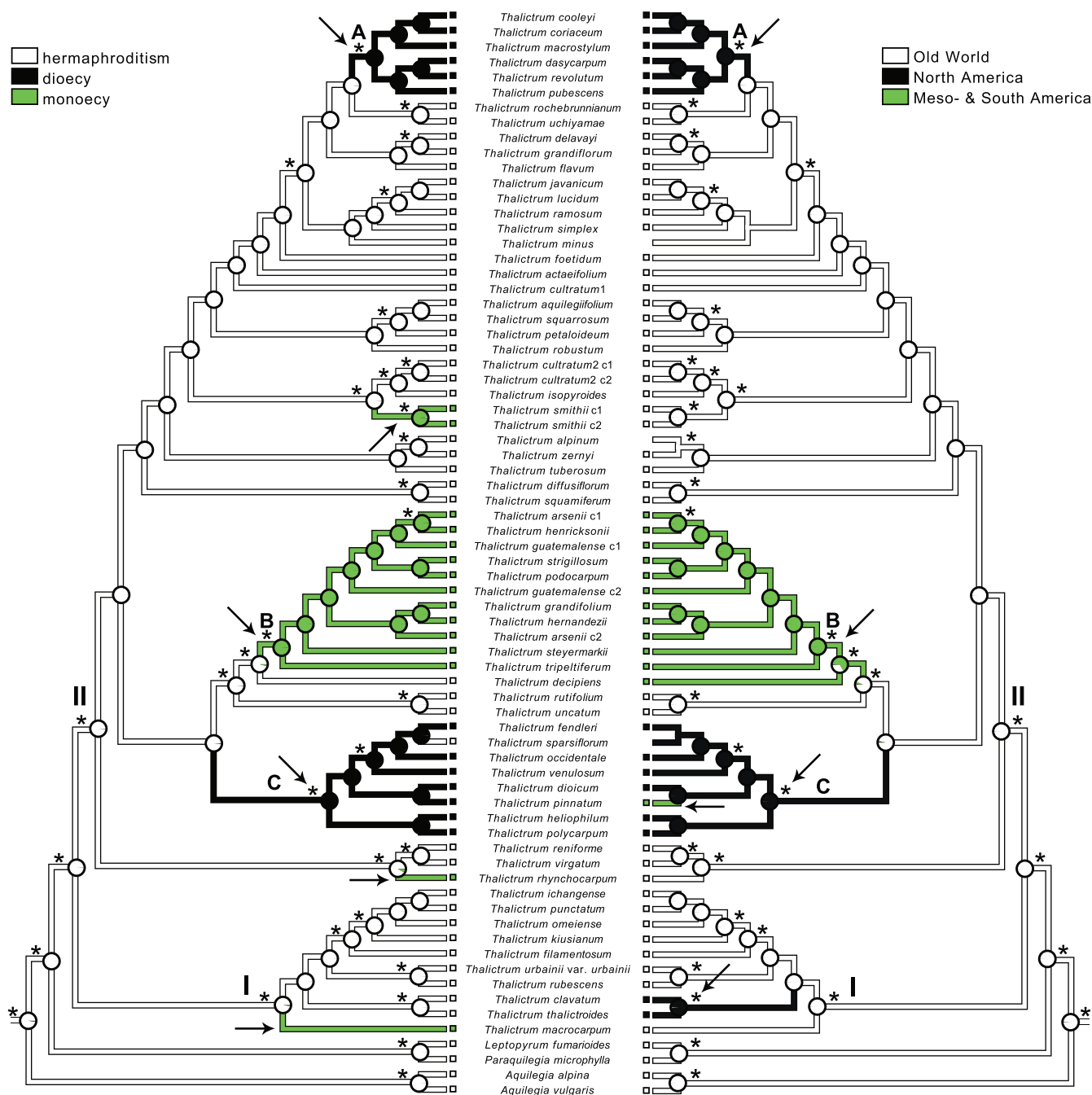
trait of the family (Endress, 1987), are typically associated with wind pollination (Faegri and Van der Pijl, 1979; Friedman and Barrett, 2008); yet occur in all species (including insect-pollinated species). Wind pollination has been suggested to arise more often in species pollinated by generalist insects, such as flies and pollen-collecting bees (reviewed in Friedman and Barrett, 2008); and many of the insect-pollinated species in *Thalictrum* have small, white, inconspicuous flowers, floral traits typically associated with generalist pollinators. All of these floral characteristics support a scenario for the early evolution of wind pollination from insect pollination in *Thalictrum*, possibly due to pollinator limitation in new habitats.

Pollination mode is a labile character in *Thalictrum*. If we consider wind pollination to have evolved early in the genus, we see transitions from insect to wind, back to insect, and finally back to wind pollination over the whole phylogeny (Fig. 4). At least four transitions to wind pollination are strongly supported and have persisted in the genus in clades A–C and in the ancestor of *T. cult-ratum* and *T. isopyroides* (Fig. 4), with at least one reversal to insect pollination (*T. sparsiflorum*). A few examples of other potential transitions from wind back to insect pollination can be seen for *T. rochebrunnianum* and *T. uchiyamae*; *T. delavayi* and *T. grandiflorum*; *T. javanicum*, *T. lucidum* and *T. ramosum*; *T. aquilegiifolium* and *T. petaloideum*; and *T. smithii* (Fig. 4). The frequent reversion from wind back to insect pollination is supported by the fact that diverse traits can act as pollinator attractants in the genus. Some species have developed purple, showy sepals (e.g. *T. diffusiflorum*, Fig. 2b), while others have developed showy stamens with purple filaments (e.g. *T. aquilegiifolium*, Fig. 2d) or white to pink petaloid stamens with conical cells (e.g. *T. filamentosum*, *T. kiusianum* and *T. omeiense*, Fig. 2c; Di Stilio et al., 2009).

Low pollinator abundance may select for the evolution of wind pollination to increase reproductive assurance, while low wind intensity or high pollinator abundance may select for the evolution of insect pollination (assuming higher efficiency of pollen transfer by insects; Culley et al., 2002). The genus *Thalictrum* allows us to study transitions between wind and insect pollination among closely related species. Field pollination studies would increase understanding of the selective factors involved in these transitions, while helping to determine whether additional species have a mixed pollination system, involving both wind and insects (Kaplan and Mulcahy, 1971).

#### 4.3. Biogeography

*Thalictrum* has originated in the Old World and colonization of the New World (North, Meso- and South America) from Old World ancestors occurred independently at least four times (Fig. 5). Moreover, these derived New World lineages are strongly supported in our phylogenetic analyses (Fig. 2). The majority of Old World taxa



**Fig. 6.** Parsimony and likelihood reconstructions of ancestral states of sexual system (left) and geographic distribution (right) in *Thalictrium* and outgroups (Ranunculaceae), using the Bayesian consensus tree based on the chloroplast *rpl16* intron and nuclear internal transcribed spacer region. Proportional likelihoods of ancestral states at nodes are represented by pie charts. Proportional likelihoods of ancestral states at strongly supported ancestral nodes (i.e., Bayesian posterior probabilities  $\geq 0.95$  or likelihood bootstrap values  $\geq 70\%$ ) indicated with asterisks below nodes. Transitions to dioecy and monoecy and North and Meso- and South America with proportional likelihoods  $> 0.875$  indicated by arrows. Two major clades in genus, I and II, two dioecious subclades, A and C, and one andromonoecious clade, B.

are Eurasian, with a few African species (i.e. *T. rhynchocarpum*, *T. zernyi*). This suggests that the origin of the genus was Eurasia and that the major dispersal events into Africa and the New World were from Eurasian ancestors. In addition, the majority of outgroups to *Thalictrium* within subfamily Thalictroideae (Wang et al., 2009) also have a Eurasian distribution (Ziman and Keener, 1989), supporting this origin.

#### 4.4. Character correlations and evolutionary sequences

We observed strong correlations between pollination mode, sexual system, and geographic distribution in *Thalictrium* (Tables

1 and 2). In particular, strong correlations existed between wind pollination and dioecy (Table 1). Dioecy was strongly associated with North America and (andro)monoecy with Meso- and South America (Table 1). Further studies of closely related species with different sexual systems in North America versus Meso- and South America may help elucidate whether conditions favoring the evolution of one or the other sexual system are more prevalent in temperate versus tropical habitats.

Upon testing evolutionary sequences of events, dioecy was not more likely to arise in wind-pollinated species or vice versa (plant sex, Table 2). However, the probability that unisexual flowers were more likely to evolve in wind-pollinated species was marginally

**Table 2**  
Likelihood ratio values for tests of correlated evolution between sexual systems, pollination modes, and geographic distribution in *Thalictrum* (Ranunculaceae) using BayesDiscrete (Pagel and Meade, 2011).

Test/character pair	Flower sex (X), pollination (Y)	Plant sex (X), pollination (Y)	Flower sex (X), geography (Y)	Pollination (X), geography (Y)
<b>Omnibus (4 d.f.):</b> Correlation between X and Y	<b>18.69<sup>a</sup> (0.001)<sup>b</sup></b>	<b>12.95 (0.012)</b>	9.22 (0.056)	<b>10.41 (0.034)</b>
<b>Temporal order (1 d.f.):</b> Change in X precedes change in Y or vice versa	1.71 (0.191)	0.261 (0.610)	1.30 (0.254)	0.42 (0.518)
<b>Contingent change (1 d.f.):</b> Origin in Y depends on state of X	2.50 (0.114)	2.12 (0.145)	1.41 (0.234)	0.94 (0.331)
<b>Contingent change (1 d.f.):</b> Origin in X depends on state of Y	3.55 (0.059)	0.90 (0.343)	0.41 (0.522)	0.99 (0.320)

<sup>a</sup> Bold values indicate  $P < 0.05$ .

<sup>b</sup>  $P$ -values shown in parentheses.

significant ( $P = 0.059$ ; flower sex, Table 2). For clade II, for example, the evolution of wind pollination seemed to have preceded the evolution of andromonoecy and dioecy in clades B–C, with proportional likelihoods  $>0.50$  (Fig. 4), and provide some indication that wind pollination may have evolved prior to dioecy and unisexual flowers in the genus *Thalictrum* (Fig. 1, Hypothesis I).

Although a recent large-scale phylogenetic study across angiosperms found that the evolution of wind pollination was more likely after the establishment of dioecy or unisexual flowers (Fig. 1, Hypothesis II; Friedman and Barrett, 2008), we do not see any evidence for this pattern of transitions in *Thalictrum*. Two additional studies have found wind pollination preceding the origin of unisexual flowers, showing support for Hypothesis I: one in the grass family, Poaceae (Givnish et al., 2010; Malcomber and Kellogg, 2006), and the other in *Fraxinus* (Wallander, 2008). It seems, therefore, reasonable to assume that different selective forces and evolutionary pathways to dioecy, unisexual flowers, and wind pollination exist within and between genera.

Using generic-level phylogenies to examine the evolution of traits has advantages, yet also inherent limitations. Not only is it difficult to find a genus with the necessary variation in the traits of interest; the number of transitions in a given trait may also be limited. For example, in *Thalictrum*, even if we increased taxonomic sampling, we do not expect to increase the number of transitions in sexual systems or geographic distributions. All dioecious species are expected to belong to the two North American clades; whereas, most andromonoecious species occur in Meso- and South America and are expected to belong to clade B (Fig. 4). Furthermore, we have sampled all known gynomonoeious species in the genus (Supplementary Table S2). A limited number of transitions lowers the statistical power of tests, as exemplified in this study.

On the other hand, if we increase taxonomic sampling or molecular regions used within the genus, we may obtain a better estimate of the number of transitions among pollination modes along the backbone of the phylogeny, as increased sampling will likely provide more support among relationships along the backbone. The lability in pollination mode may also suggest that different selective forces may be acting in different clades of *Thalictrum*. Differential speciation and/or extinction rates among the different pollination modes may also result in a higher number of estimated transitions. Methods that incorporate diversification rates in ancestral state reconstructions (FitzJohn et al., 2009) will be important to use in future research.

#### 4.5. Conclusions and future directions

Evolutionary transitions among sexual systems and biogeography were strongly supported on our phylogeny, yet transitions between pollination mode were less supported. Although the contingent change test was marginally significant, our data suggest that wind pollination may have preceded the evolution of unisexual flowers in this genus. We see no evidence for the alternate pathway on the phylogeny.

The phylogeny clearly highlights closely related species with different sexual systems and/or different pollination modes that would benefit from field research to further clarify the factors involved in the evolution of these traits. Furthermore, we now have a phylogenetic framework to continue to investigate the genetic mechanisms and developmental pathways involved in the separate transitions to unisexual flowers and pollination modes (Di Stilio et al., 2005, 2010, 2009). Our phylogeny could also be used to test other evolutionary mechanisms, such as whether dichogamy influenced the evolution of andro- and gynomonoeicy (Brunet and Charlesworth, 1995) in this group. Moreover, our study highlights that compiling results from different genera across angiosperms should uncover trends that may otherwise be obscured in larger-scale phylogenetic studies.

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#### Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympv.2012.01.009.

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