ORIGINAL ARTICLE

COMPARATIVE ANALYSES OF SEX-RATIO VARIATION IN DIOECIOUS FLOWERING PLANTS

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Dioecious plant species commonly exhibit deviations from the equilibrium expectation of 1:1 sex ratio, but the mechanisms governing this variation are poorly understood. Here, we use comparative analyses of 243 species, representing 123 genera and 61 families to investigate ecological and genetic correlates of variation in the operational (flowering) sex ratio. After controlling for phylogenetic nonindependence, we examined the influence of growth form, clonality, fleshy fruits, pollen and seed dispersal vector, and the possession of sex chromosomes on sex-ratio variation. Male-biased flowering sex ratios were twice as common as female-biased ratios. Male bias was associated with long-lived growth forms (e.g., trees) and biotic seed dispersal and fleshy fruits, whereas female bias was associated with clonality, especially for herbaceous species, and abiotic pollen dispersal. Female bias occurred in species with sex chromosomes and there was some evidence for a greater degree of bias in those with heteromorphic sex chromosomes. Although the role of interactions among these correlates require further study, our results indicate that sex-based differences in costs of reproduction, pollen and seed dispersal mechanisms and sex chromosomes can each play important roles in affecting flowering sex ratios in dioecious plants.

KEY WORDS: Biased sex ratios, clonality, costs of reproduction, dioecy, life-history traits, sex chromosomes.

The causes and consequences of sex-ratio variation in organisms with separate sexes remain a central theme in evolutionary biology. The seminal work of C. Düsing (see Edwards 2000) and R. A. Fisher (Fisher 1930) established the theoretical basis for the expected 1:1 offspring sex ratio and its maintenance by negative frequency-dependent selection. Despite this expectation, deviations from equality are commonly observed in animal and plant species (reviewed in Hardy 2002). This raises questions regarding the ecological and evolutionary mechanisms underlying biased sex ratios, at what stage(s) of the life-cycle bias is established, and how this variation is maintained.

Dioecy (separate female and male individuals) is a relatively uncommon sexual system in angiosperms (~6% of species), but has evolved independently from hermaphroditism at least 100 times (Charlesworth 2002). It has been suggested that because of the sessile nature of plants and the potential for variation in pollen and seed dispersal distances we might expect different theoretical predictions on sex-ratio bias compared to animals (Bulmer and Taylor 1980; Lloyd 1982). Among dioecious species, there is empirical evidence of frequent departures from equality in natural populations (Delph 1999; Barrett et al. 2010). Biased sex ratios have been attributed to diverse causes and may be established in seed due to factors such as local resource competition (de Jong et al. 2002), sex-ratio distorters (Taylor 1999), or as a result of the system of sex-determination (Smith 1969; Lloyd 1974). Sex-based differences in mortality and reproductive expenditure can also occur during the life cycle, resulting in biased flowering sex ratios (Lloyd and Webb 1977; Delph 1999). Despite some

advances, our current understanding of the relative importance of interactions involving life-history traits and genetic factors in affecting sex-ratio variation in plants remains limited.

Although departures from equality are frequently reported in plant populations, a number of challenges exist for investigating the causes of sex-ratio bias. First, in most plants sex cannot be determined until reproductive maturity, so most estimates of sex ratio are from samples of flowering or fruiting individuals and ignore vegetative plants. Second, many dioecious species are long-lived perennials with both sexual and clonal reproduction (Klimeš et al. 1997) and in such cases, sex ratio estimates are usually based on flowering shoots (ramets) rather than genets. Where this occurs, sex-specific differences in rates of clonal propagation (e.g., Sakai and Burris 1985; Popp and Reinartz 1988) and flowering frequency (e.g., Barrett and Thomson 1982; Bawa et al. 1982) can potentially contribute to biased ramet sex ratios. Despite these problems, flowering sex ratios remain the "sampling unit" for most studies and a considerable amount of data on the operational sex ratio (Emlen and Oring 1977; Kvarnemo and Ahnesjö 2002) of populations exists providing an opportunity to investigate general patterns and correlates of sex-ratio bias. Comparative analysis should provide insights into how life history characteristics might influence sex-based differences in survival, growth and reproduction. Moreover, explicit comparisons of flowering sex ratios between multiple independent clades with contrasting life-history traits enable hypotheses to be evaluated on the relative importance of different mechanisms causing sex-ratio bias.

Differential reproductive expenditure between the sexes is considered one of the primary drivers of biased sex ratios in plant populations. Greater reproductive investment in females (Charnov 1982) may be associated with male-biased sex ratios because of the earlier onset and more frequent flowering of males and also because of greater female mortality (Lloyd 1973; Lloyd and Webb 1977; Delph 1999; Obeso 2002). This sex-specific variation in the costs of reproduction may reflect underlying differences in the steepness of male and female gain curves (Charnov 1982). We might therefore expect that long-lived dioecious species with large maternal investment in biotically dispersed fruits would be more likely to develop male-biased ratios (e.g., Cipollini and Whigham 1994; Wheelwright and Logan 2004). Similarly, for clonal species, greater female reproductive costs may enable higher rates of clonal propagation by males, resulting in male-biased ratios (Darwin 1877; Lloyd and Webb 1977). For dioecious species in which male reproductive costs are particularly high (i.e., wind-pollinated species; Harris and Pannell 2008 and see Delph 1999), greater female bias may occur. These effects could interact with other aspects of life history such as longevity, with the amplification of sex-ratio bias following repeated episodes of reproduction.

Female-biased sex ratios are less frequent than male-biased sex ratios in flowering plants (Delph 1999; Barrett et al. 2010) and several mechanisms have been proposed to account for this pattern. Sex-specific differences in mortality may contribute to female bias (Lloyd 1974), and these can intensify during the life cycle to produce skewed sex ratios (e.g., Shelton 2010a,b). For species with sex chromosomes, female bias may result from the differential performance of female- and male-determining microgametophytes (the "certation" hypothesis; Correns 1922) and/or sex-based differences in mortality (Stehlik and Barrett 2005). Degeneration of nonrecombining Y-chromosomes in species where males are the heterogametic (XY) sex may account for the establishment of female bias in seed (Smith 1963) and its amplification across the life cycle due to sex-specific differences in growth and/or survival (Stehlik et al. 2007). Based on the assumption that female bias is associated with the degree of genetic differentiation between the X and Y chromosomes, Lloyd (1974) predicted a relation between the magnitude of female bias and degree of sex chromosome differentiation (heteromorphism). According to this hypothesis, stronger female bias might be expected in species with heteromorphic compared to homomorphic sex chromosomes. Although species with sex chromosomes are relatively infrequent in angiosperms, their wide taxonomic dispersion offers opportunities for comparative analyses to examine these currently untested ideas.

The wide taxonomic distribution of dioecy and the diversity of life-history traits in angiosperms provide opportunities for comparative analysis to evaluate the relative importance of mechanisms that may cause biased sex ratios. Although dioecious species are characterized by a suite of particular life-history traits (Renner and Ricklefs 1995; Vamosi et al. 2003), they often possess different pollen and seed dispersal mechanisms (abiotic vs. biotic), substantial variation in fruit size (0.001 mm–20 kg) and fleshiness, contrasting modes of reproduction (sexual and clonal) and a wide range of longevities, from herbaceous annuals to long-lived perennials including herbs, shrubs, and trees. This variation provides considerable scope for sex-based differences in survival and reproduction to influence sex ratios.

Despite the large literature on sex ratios in flowering plants, only one cross-species study has examined the correlates of sex-ratio variation (Sinclair et al. 2012). This study examined predictions made by de Jong et al. (2002) that relative pollen and seed dispersal distances, through their effects on sib mating and local resource competition, should influence sex-ratio variation. They examined the extent to which life form and pollen and seed dispersal mechanisms were associated with biased flowering sex ratios. However, testing this hypothesis requires data on seed sex ratios (see de Jong et al. 2002), which as discussed above are not available for most dioecious species, including those used in their survey. In addition, the authors included species that are not dioecious

Table 1. Summary of the questions and predictions regarding the influence of life-history traits and sex chromosomes on sex-ratio variation addressed in this study.

	Question	Predictions
(1) Costs of reproduction		
(i) Longevity and growth form	Does sex-ratio bias vary among growth forms and increase with longevity?	Increased sex-ratio bias in longer lived trees. Less sex-ratio bias in annual and short-lived species.
(ii) Clonal reproduction	Does clonality increase sex-ratio bias?	Increased male bias in clonal species. Greatest bias in long-lived clonal species.
(iii) Pollen vector	Is there a difference in sex-ratio bias among species with biotic versus abiotic pollination?	Increased female bias in species with abiotic pollination.
(iv) Seed dispersal vector	Is there a difference in sex-ratio bias among species with biotic versus abiotic seed dispersal?	Greater male bias in species with biotic seed dispersal.
	Do species with fleshy fruits show greater male-biased sex ratios?	Greater male bias in species with fleshy compared to nonfleshy fruits.
(2) Sex chromosomes	Do species with sex chromosomes have female-biased sex ratios?	Female bias in species with sex chromosomes.
	Does the magnitude of female bias relate to sex chromosome differentiation?	Greater female bias in species with heteromorphic sex chromosomes.

but are sequential hermaphrodites, with environmental sex change (e.g., *Arisaema triphyllum*, see Table 4 in Sinclair et al. 2012) and also they failed to take into account for the non-independence of species traits owing to shared ancestry. Comparative analyses of sex-ratio variation are best undertaken using phylogenetic methods due to the non-independence of species (Felsenstein 1985; Harvey and Pagel 1991; Freckleton et al. 2002). Such approaches have provided important insights into the causes of sex-ratio variation in diverse animal groups (reviewed in Mayhew and Pen 2002). Accordingly, when considering sex-ratio variation in plants, incorporating phylogenetic information enables examination of the importance of life-history traits while controlling for the shared evolutionary history of species.

Here, we investigate the correlates of sex-ratio variation among 243 species of flowering plants using cross-species and phylogenetic comparative analysis. We first consider the frequency of departures from equality and the phylogenetic distribution of biased sex ratios. We then investigate sex-ratio variation in relation to life-history traits, particularly those associated with sex-based differences in reproductive costs. Despite their long history (Darwin 1877), hypotheses regarding the importance of life history and sex-based differences in reproductive costs (reviewed in Lloyd & Webb 1977, Delph 1999) remain largely untested by comparative analysis. If higher female expenditure contributes toward sex-ratio variation, we predict greater male bias in species that: (i) are longer lived with repeated episodes of reproduction, (ii) reproduce clonally, (iii) have biotic seed dispersal and/or fleshy fruits. Conversely, for species in which

male reproductive costs may often be higher (i.e., wind-pollinated species), we predict female-biased sex ratios. We also examine whether sex chromosomes are associated with female-biased sex ratios and whether the degree of bias differs between species with homomorphic versus heteromorphic sex chromosomes. By examining these factors in the context of sex-specific differential reproductive costs and the possession of sex chromosomes, our study provides a novel contribution to current understanding of the mechanisms underlying sex-ratio variation in flowering plants. For ease of presentation, we summarize the specific questions motivating individual analyses and their predictions in Table 1.

Methods
DATA COLLECTION
To compile a database of sex ratios we searched for published studies using the ISI Web of Science (1899–2010) and key word combinations: "sex ratio(s)" "female bias" "male bias" "dioecy" "dioecious" "plant(s)." In addition, we surveyed the lists of articles obtained by these searches and reviews on sex ratios and sexual dimorphism (e.g., Rottenberg 1998; Geber et al. 1999; Obeso 2002). Only studies meeting the following four criteria were included in our comparative analysis: (i) the species was dioecious, (ii) the study reported the number of individuals sampled, (iii) sex ratio samples were obtained by direct counts and were not based on plant density, and (iv) the sex ratio of a population was based on a minimum sample of 10 individuals. We excluded species with environmental sex determination including gender diphasy

(sequential hermaphroditism). For seven species (*Sagittaria latifolia*, *Wurmbea dioica*, *Schiedea globosa*, *Corema album*, *Astilbe biternata*, *Thymelaea hirsuta*, *Pachycereus pringlei*) known to have sub-dioecious populations (males, females, and hermaphrodites), sex ratios were only obtained from dioecious populations where hermaphrodite frequency was < 5%. From these criteria our survey resulted in sex ratios for 243 species from 144 publications between 1942 and 2010 (see Appendices S1 and S2). For all species, we recorded the number of females and males and total sample size for each population, and where available, we also recorded information on population size. Throughout this article the sex ratio of a population = males/(females + males).

To examine correlates of sex-ratio variation we obtained information on seven life-history traits for all species: (i) growth form (annual, herbaceous perennial, shrub, tree, vine, or hemi-parasite = mistletoe), (ii) herbaceous or woody, (iii) pollination system (abiotic = wind or water; biotic = animal), (iv) seed dispersal (abiotic = wind, water, or gravity; biotic = animal), (v) clonal propagation (1 = yes, 0 = no), (vi) fleshy fruits (1, 0), and (vii) sex chromosomes (1, 0), which were further classified according to the degree of sex chromosome differentiation into heteromorphic (cytologically distinct) or homomorphic (cytologically similar, recombination suppressed and male-specific regions present) according to Ming et al. (2011). Information on these traits was obtained from articles containing the sex ratio data, ISI Web of Science (1899–2010) and Google Scholar using species and trait as key word combinations, and the LEDA database (Kleyer et al. 2008). Information on clonal propagation was also obtained from the clonal database CLO-PLA (Klimešová and Klimeš 2010). For the sex chromosome analysis, we obtained sex ratio data for 54% (21 of 39) of angiosperm species with well-described sex chromosome systems (see Ming et al. 2011).

Statistical Analysis

DEVIATIONS FROM EQUALITY

We used a weighted average (Sokal and Rohlf 1995), based on population sample size, to obtain an average sex ratio for the 126 species (51.9%) for which sex ratios were sampled from more than one population. For the remaining 117 species (48.1%) with only one population sample this value was used (see Fig. 1; Appendix S1). For the 52 species where surveys were undertaken over multiple years we used the average sex ratio. Given the large variation in population size and sampling intensity resulting from differences in life history (e.g., annual vs. long-lived trees), we truncated sample size to a maximum of 2000 individuals for 22 species (original sample size ranged from 2019 to 126 444). We used a goodness-of-fit test (*G*-test) in R (R Core Development Team 2008) to examine if the sex ratio for each species was significantly different from 0.5. Sex ratios were classified as equal
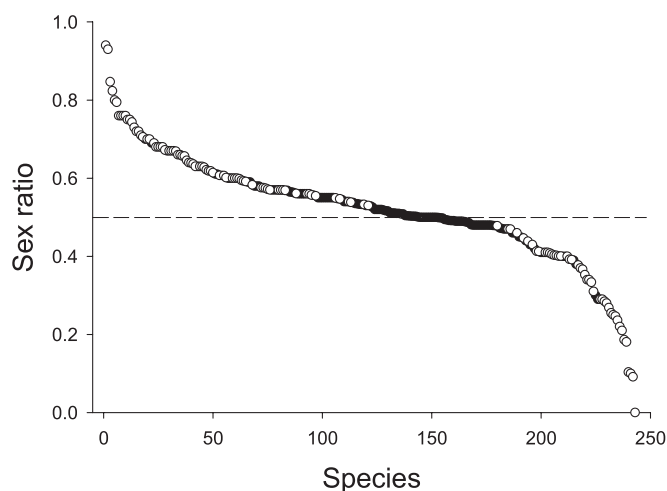
Figure 1. Variation in sex ratios in 243 dioecious species from 123 genera and 61 families of flowering plants. Deviations from equality were assessed using *G*-tests. Closed circles represent species with sex ratios at equality (no significant deviation from 0.5), whereas open circles above and below 0.5 represent species with significant male and female bias, respectively. There is considerable overlap of points representing species with sex ratios that are not significantly different from equality.

when they did not differ significantly from 0.5, male-biased when they were significantly > 0.5, and female-biased when they were significantly < 0.5.

CORRELATES OF SEX-RATIO VARIATION

We employed both a phylogenetic controlled comparative approach and cross-species (non-phylogenetically controlled) analysis to examine whether sex-ratio variation among the 243 species was related to: (i) growth form, (ii) growth form and clonality, (iii) pollination system and seed dispersal, (iv) fleshy fruits, and (v) presence of sex chromosomes. We present both phylogenetic controlled and cross-species analysis to provide an assessment of the robustness of our results to the assumptions that underlie both types of analysis. We examined the importance of these traits (and their interactions) according to specific hypotheses (Table 1). We initially included growth form in the models examining pollination system and seed dispersal (model iii), and fleshy fruits (model iv). However, as a nonsignificant term in both models, growth form was removed so that the simplest model is presented. Our phylogenetically controlled analysis used a generalized estimating equations (GEE) method implemented in the APE package in R (Paradis and Claude 2002; Paradis et al. 2004). This procedure uses phylogenetic information to account for shared evolutionary history and the non-independence of species (Felsenstein 1985; Harvey and Pagel 1991) and uses a binomial data structure appropriate for the analysis of sex ratio data (Wilson and Hardy 2002). Accordingly, we used a logit link function (binomial family) to

implement the GEE function in APE (Paradis et al. 2004). We used *Phylomatic* (Webb and Donoghue 2005) to generate phylogenetic trees for the 243 species based on the angiosperm phylogeny APG3 supertree (Stevens 2001). Due to incomplete phylogenetic information we fixed all branch lengths within genera to one. To account for phylogenetic uncertainty, we generated 200 trees by introducing random variation to branch lengths (normal distribution, variance multiplier of 0.1) in Mesquite (Maddison and Maddison 2011). We then ran all GEE analyses for each tree independently, to assess the sensitivity of each analysis to a particular phylogeny. Analyses where *P* values for the majority of trees were < 0.05 (or 0.1) indicate that the effects were robust to the use of a particular phylogeny. This criterion was used to provide a guide for assessing the sensitivity of the analysis outcomes to phylogenetic uncertainty. For comparison, we also examined these relations using a cross-species approach that treats each species as independent and does not account for their shared evolutionary history. For this analysis we used a GLM (logistic regression) with a binomial distribution and a logit link function.

Results

VARIATION IN SEX RATIOS

We found significant variation in flowering sex ratios among the 243 dioecious species (Fig. 1), representing 123 genera and 61 families. The average number of populations sampled for each species was 4.5 (range 1–82). In about half (49.8%, *n* = 121) of all species in our sample sex ratios did not deviate significantly from equality. For species with significant deviation from 0.5, male bias was almost twice as common as female bias, with 76 species (31.3%) showing significant male-biased sex ratios compared to 46 species (18.9%) with significant female bias (see Appendix S1). Species with biased sex ratios had a wide taxonomic distribution (Fig. 2). Female bias occurred in 20 (33%) of the 61 families, whereas male bias was found in 37 (61%).

CORRELATES OF SEX-RATIO VARIATION

(i) Do sex ratios vary with growth form and clonality?

Growth form had a significant effect on sex ratio in the cross-species analysis (Fig. 3a, Table 2a). Dioecious vines and trees had significantly male-biased sex ratios, whereas the mean sex ratios of annuals, herbaceous perennials, and shrubs were close to equality and not significantly different from one another (Fig. 3a, Appendix S3). In contrast, mistletoes had more female-biased sex ratios (Fig. 3a, Appendix S3). In the analysis controlling for phylogeny, the effect of growth form on sex ratio was less apparent and sensitive to phylogenetic relationships among species, with a significant difference (*P* < 0.05) among growth forms in only 10.5% of phylogenetic trees (Fig. 4a).

Table 2. Generalized linear model (GLM, logistic regression) analyses of the effect of growth form, clonality, pollen and seed dispersal vectors, fleshy fruits and sex chromosomes on average sex ratio in 243 dioecious angiosperm species

	df	F	P
(a) Growth form (a, hp, s, t, v, m)[1]	5,235	2.78	**0.019**
(b) Clonality and growth form			
Clonality[2]	1,223	11.76	**<0.001**
Growth form	2,219	3.55	**0.031**
Clonality[3]	1,219	10.89	**0.001**
Growth form × clonality	2,219	2.42	0.091
(c) Pollen and seed dispersal			
Pollen vector	1,239	35.44	**<0.001**
Seed vector	1,239	5.24	**0.023**
Pollen vector x seed vector	1,239	1.51	0.220
(d) Fleshy fruits	1,241	11.45	**<0.001**
(e) Sex chromosomes	1,241	36.47	**<0.001**
(f) Sex chromosomes (hom., het.)[4]	1,20	7.35	**0.013**

Significant *P* values (*P* < 0.05) are highlighted in bold.
[1] Six categories of growth form [annual (a), herbaceous perennial (hp), shrub (s), tree (t), vine (v), mistletoe (m)].
[2] Model 1 (only clonality).
[3] Model 2 (growth form and clonality).
[4] hom = homomorphic sex chromosomes and het = heteromorphic sex chromosomes.

Clonal reproduction had a significant effect on sex ratio in the cross-species analysis, with clonality associated with a higher proportion of females, whereas nonclonal species had a higher proportion of males (Table 2b, model 1, clonal mean ± SE = 0.48 ± 0.01, nonclonal mean = 0.53 ± 0.01). This result was also apparent in the phylogenetically controlled analysis (Fig. 4b) with clonality having a significant effect (*P* < 0.05) on sex ratio in 87% of phylogenetic trees. When growth form was included in the analysis, there was some evidence for variation in the influence of clonality among growth forms in the cross-species analysis (growth form × clonal: *P* = 0.091; Table 2b, model 2, Fig. 3b), and this was also evident after controlling for phylogeny (GEE growth form × clonal 72% of phylogenetic trees *P* < 0.05). For herbaceous plants, we found female-biased sex ratios in species capable of clonal reproduction compared to male bias in species without clonal reproduction. In comparison, for shrubs or trees there was no difference in sex ratio among clonal and nonclonal species (Fig. 3b, Appendix S3).

(ii) Is sex-ratio variation correlated with pollen and seed dispersal vectors?

We found that pollen and seed dispersal vector (abiotic or biotic) had a significant effect on sex ratio in both the cross-species analysis (Fig. 3c, Table 2c) and after controlling for phylogeny (GEE analysis, Fig. 4c). Pollen dispersal vector had the strongest
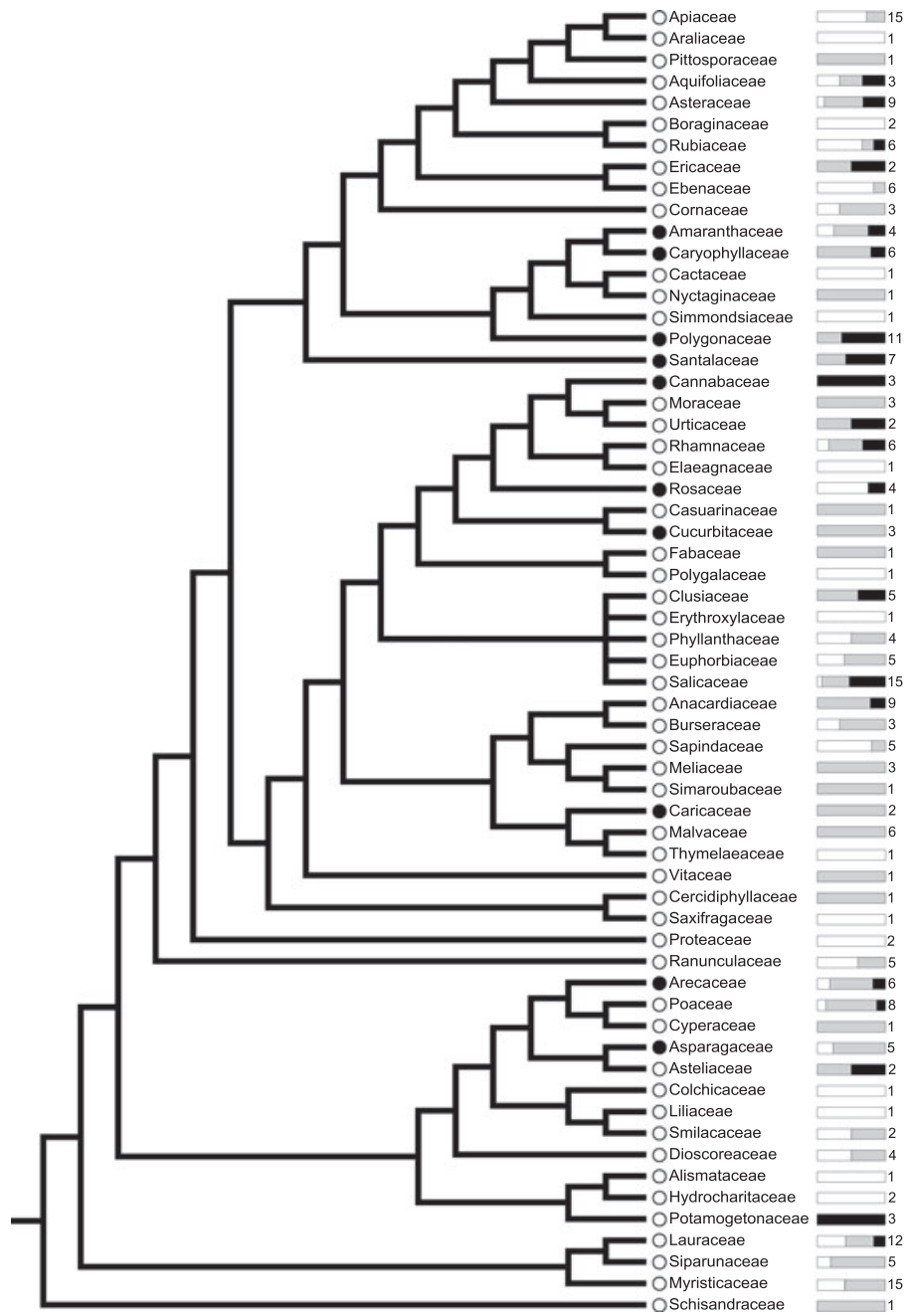
Figure 2. The phylogenetic distribution of sex-ratio variation and sex chromosomes among families of flowering plants. The phylogenetic analysis of angiosperm families follows (Stevens 2001). Filled circles indicate the presence of sex chromosomes within a family. Bar plots represent the proportion of species with no significant deviation from equality (grey) and significant male-biased (white) and female-biased (black) sex ratios. The number adjacent to each bar plot is the sample size of species in each family.

effect on sex ratio (pollen vector: GLM $P < 0.001$, GEE 87% of phylogenetic trees $P < 0.05$), with female-biased sex ratios more evident in species with abiotic pollination. In contrast, species with biotic pollen dispersal were more likely to exhibit male-biased sex ratios (Fig. 3c). The effect of biotic seed dispersal on sex ratio (seed dispersal vector: GLM $P = 0.023$, GEE 81% of phylogenetic trees $P < 0.05$) was similar for species with both abiotic and biotic pollen dispersal for both the cross-species

analysis (pollen vector × seed vector: $P > 0.05$, GLM Table 2c, Fig. 3c) and the GEE analysis (GEE Fig. 4c). For both types of pollen dispersal, an increase in the proportion of males was found in species with biotic compared to abiotic seed dispersal. However, the effect of seed dispersal vector on sex ratio was most prominent for species with abiotic pollen dispersal. Here, there was a significant difference in sex ratio among abiotically pollinated species with abiotic and biotic seed dispersal, but not
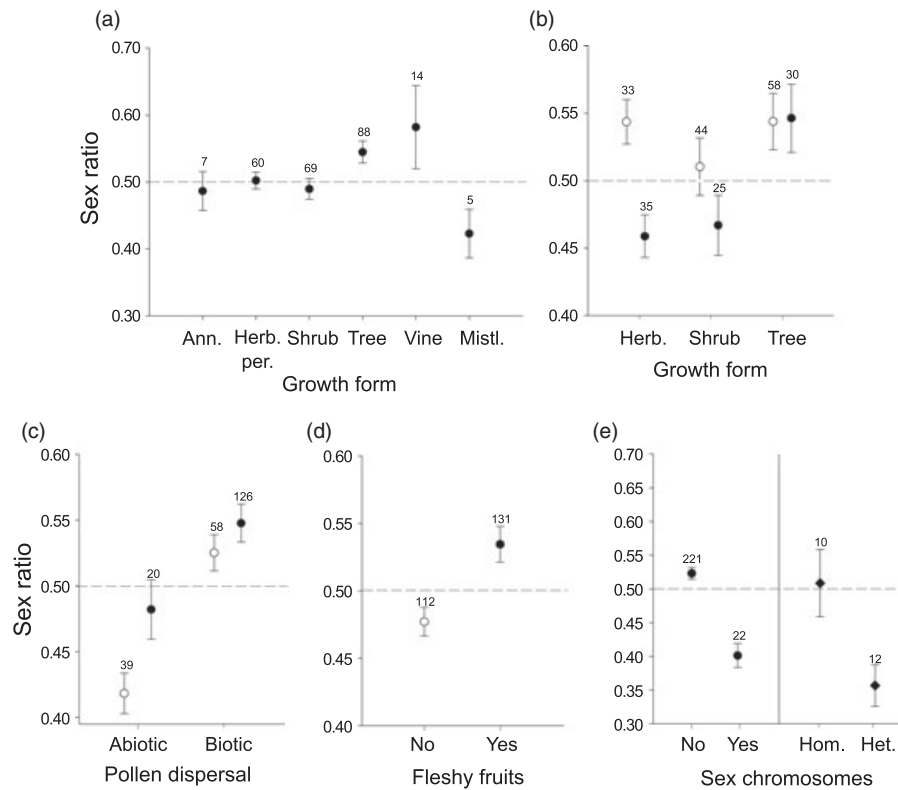
Figure 3. Generalized linear model (GLM, logistic regression) analyses to examine the effect of: (a) growth form, (b) clonality and growth form, (c) pollen and seed dispersal vectors (abiotic, biotic), (d) fruit type (fleshy, nonfleshy), and (e) sex chromosomes (presence, absence, and homomorphic or heteromorphic sex chromosomes) on sex ratios (proportion male) in 243 dioecious species. The number of species in each category is indicated above the mean sex ratio. For (a) Ann. = annual, Herb. per. = herbaceous perennial, Mistl. = mistletoe. For (b) Herb. = herbaceous and open symbols indicate nonclonal species and closed symbols clonal species. For (c) open symbols indicate abiotic seed dispersal and closed symbols indicate nonclonal species and clonal species. For (d) open symbols indicate nonfleshy fruits and closed fleshy fruits. Vertical bars are ± 1 standard error. For significant differences among groups for all analyses see Appendix S3.

among biotically pollinated species with abiotic and biotic seed dispersal (Fig. 3c, Appendix S3).

There was a significant difference in sex ratio among species with fleshy compared to nonfleshy fruits for both the cross-species analysis (Fig. 3d, Table 2d) and after controlling for phylogeny (Fig. 4d, $P < 0.05$ in 100% of phylogenetic trees). More male-biased sex ratios were found in species with fleshy fruits compared to those with nonfleshy fruits.

(iii) Are sex chromosomes associated with female bias?

The presence of sex chromosomes had a significant effect on sex ratio in the cross-species analysis (Table 2e, Fig. 3e) and this also remained significant for the GEE analysis (Fig. 4e, $P < 0.05$ in 100% of phylogenetic trees). Female-biased sex ratios were found in species with sex chromosomes, compared to more male-biased ratios in species without sex chromosomes (Fig. 3e). This relation between sex chromosomes and female bias was also evident from inspection of the phylogenetic distribution of sex ratios among dioecious families (Fig. 2). Families

with sex chromosomes were associated with a higher frequency of female-biased sex ratios. For species with sex chromosomes, we found a significant difference in sex ratio between species with homomorphic versus heteromorphic sex chromosomes in the cross-species analysis (Fig. 3e, Table 2f), with greater female bias in species with heteromorphic sex chromosomes. However, controlling for phylogeny, sex chromosome type had no consistent effect on sex ratio, with a marginally significant difference ($P < 0.1$) in sex ratio between species with homomorphic versus heteromorphic sex chromosomes in only 1% of phylogenetic trees (Fig. 4f).

Discussion

Our survey of flowering sex ratios among 243 dioecious angiosperm species revealed near continuous variation (Fig. 1) from strongly male- to female-biased sex ratios (1.0–0.06). Nearly half of the species exhibited significant deviations from equality, with male-biased ratios almost twice as common as female-biased
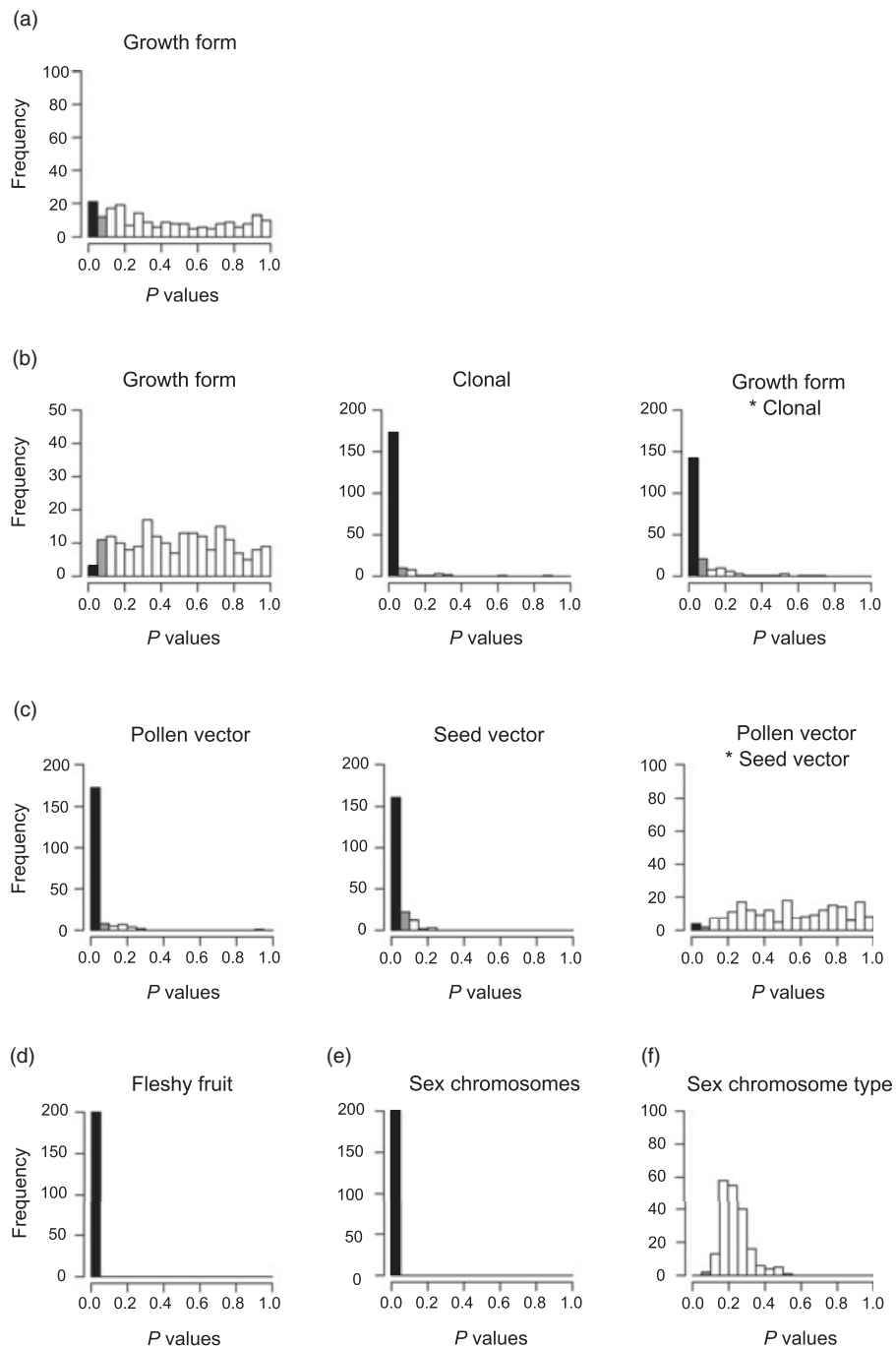
Figure 4. Frequency histograms of *P* values from the generalized estimating equation (GEE) phylogenetically-controlled comparative analysis to examine the effect of: (a) growth form, (b) clonality and growth form, (c) pollen and seed dispersal vectors (abiotic, biotic), (d) fruit type (fleshy, nonfleshy), (e) sex chromosomes (presence, absence), and (f) homomorphic or heteromorphic sex chromosomes on sex ratios in 243 dioecious plant species. Black and grey-shaded bars represent the number of phylogenetic trees where *P* values were <0.05 and 0.05 to 0.1, respectively. Analyses were repeated for 200 bootstrap trees to assess the sensitivity of each factor to a particular phylogeny.

ratios. Using comparative analyses we found that several life-history traits (growth form, clonality, fleshy fruits, pollen and seed dispersal mechanisms) were significantly associated with the observed variation in sex ratios (Fig. 3). We also demonstrated that female-biased ratios were associated with the presence of sex chromosomes, suggesting that genetic factors can also play a role in modifying sex ratios. The associations identified through our comparative analyses highlight the complex interactions that influence sex-ratio variation and we focus our discussion on interpreting the causes of departures from equality.

LIFE-HISTORY TRAITS AND SEX RATIOS

If differences in the cost of reproduction between sexes cause deviations from equality, we expect bias in the direction of the sex with less reproductive investment. Given that males usually invest less in reproduction they are likely to have an earlier onset of first reproduction and flower more frequently leading to male-biased flowering sex ratios (Lloyd and Webb 1977; Delph 1999). In some species it has also been suggested that greater mortality in females may contribute to male-biased sex ratios (Allen and Antos 1993; Delph 1999), particularly under stressful conditions (Espirito-Santo et al. 2003). We may also expect the degree of bias to differ among growth forms (life-history groups) that vary in longevity due to repeated episodes of reproduction (Lloyd 1973). In our cross-species analysis, we found greater male-biased ratios in populations of trees compared to shrubs and herbs, a result consistent with Lloyd's hypothesis (Fig. 3a). Similarly, Sinclair et al. (2012) report a tendency for male-biased sex ratios in populations in trees. In our study, this association was weaker after controlling for phylogeny (Fig. 4a), indicating that phylogenetic relationships also contribute to differences in sex ratio among groups. This highlights the importance of controlling for phylogeny in comparative studies, as differences among groups may be largely driven by shared evolutionary history, rather than life-history variation. We found no difference in sex-ratio bias among shrubs, herbaceous perennials and annual species, despite differences in longevity among these groups. Longevity and the number of reproductive episodes may not necessarily scale increasingly from herbaceous perennials to shrubs to trees, and therefore there is likely some overlap among groups in these traits. Sex-based differences in allocation to sexual versus asexual reproduction, and in the costs associated with the type of pollen and seed vectors, may also contribute to variation within life-history groups. Indeed, 64% ($n = 14$) of vines and 74% of trees ($n = 88$) included in our study had fleshy fruits, which may have contributed to the observed male bias. However, a similarly high percent of shrubs had fleshy fruits (68%, $n = 69$), but the mean sex ratio of these species was close to equality. This indicates that several factors may interact to determine patterns of sex-ratio variation among life-history groups.

Differences in investment in sexual reproduction may result in variation in rates of clonal propagation between the sexes. If males have lower reproductive costs they should have more resources for clonal growth leading to a greater number of flowering shoots and male-biased ramet sex ratios. In contrast to these expectations, we found that clonal reproduction was associated with more female-biased sex ratios in both the cross-species analysis (Fig. 3b) and after taking account of phylogenetic relationships (Fig. 4b). The difference in sex ratio between clonal and non-clonal species was particularly evident for herbaceous species (Fig. 3b). This novel result is puzzling and could reflect differ-

ences among life-history groups in the allocation strategies of the sexes. Sexual size dimorphism in herbaceous plants is often opposite to woody plants (shrubs and trees), with females larger in size than males (Lloyd and Webb 1977; Obeso 2002) and if larger size is associated with more flowering ramets this could contribute to female-biased sex ratios. Correlations among traits such as pollination vector and clonality may also contribute to the observed pattern of female bias in clonal herbs. We found that abiotic pollination was associated with female-biased sex ratios (Fig. 3c) so that the more frequent occurrence of abiotic pollination in clonal herbs (20 of 35 species) compared to nonclonal herbs (9 of 33 species) may contribute to the observed female bias. Also, due to the limited information on clonal characteristics for many species in our survey we used a simple dichotomous classification for clonality. However, plants possess a wide range of clonal growth strategies with consequences for resource sharing among ramets. In contrast to strategies where ramets become physiologically independent, connected ramets are able to access and share resources in heterogeneous environments (Klimeš et al. 1997). This difference may influence the resources available for sexual reproduction and flowering sex ratios.

It has been proposed that pollination and seed dispersal mechanisms have the potential to influence plant sex ratios (Bulmer and Taylor 1980). Specifically, ESS theory and inferences concerning the intensity of local resource competition indicate that restricted pollen and seed dispersal should result in female-biased seed sex ratios (de Jong et al. 2002). However, tests of these theoretical predictions using empirical data have been equivocal (de Jong and Klinkhamer 2005, Table 10.2), in part, because most data in the literature involves flowering rather than seed sex ratios. Mechanisms of pollen and seed dispersal (abiotic or biotic) are also commonly associated with differences in the relative investment that the sexes make in reproduction, and this probably also explains some of the among-species variation in sex ratios. For example, in species with abiotic pollination males may incur higher reproductive costs than females as a consequence of producing large quantities of nitrogen-rich pollen (Harris and Pannell 2008). In contrast, in species with biotic seed dispersal, females are likely to incur high reproductive costs due to investment in rewards for seed dispersers (e.g., fleshy fruits). Consistent with these predictions (Table 1), we found greater female bias in species with abiotic pollination and seed dispersal, whereas male-biased sex ratios were associated with biotic pollen and seed dispersal. The latter result likely reflects the fact that most species with animal-dispersed seed had fleshy fruits (89% of species). Indeed, species with fleshy fruits had significantly male-biased sex ratios compared to those with nonfleshy fruits (Fig. 3d). Sinclair et al. (2012) also found that male bias was apparent in insect-pollinated, biotically dispersed trees. Although our understanding of these associations remains limited (Obeso 2002), it is more likely that

any signal of the ESS seed sex ratio will be erased by repeated flowering episodes owing to ecological factors associated with differential reproductive expenditure of the sexes. Most dioecious species are perennial and this may guarantee the decoupling of primary (seed) and flowering (operational) sex ratios in plants.

SEX CHROMOSOMES AND FEMALE BIAS

We detected an association between sex chromosomes and female-biased sex ratios in both the cross-species analysis and after controlling for phylogenetic relationships (Fig. 3e). This result provides new insights into the importance of genetic factors for sex-ratio variation. Our finding is consistent with the hypothesis that female bias is associated with Y chromosome degeneration in species in which males are the heterogametic sex (XY; Smith 1963; Lloyd 1974). Although the proximate genetic mechanism(s) involved require further investigation, recent empirical evidence using sex-specific markers indicates that both preferential fertilization by female-determining microgametophytes and sex-based mortality contribute to female-biased ratios (Stehlik and Barrett 2005; Stehlik et al. 2007).

The relation between the degree of sex chromosome differentiation and female bias may provide insight into the contribution of Y chromosome degeneration to sex-ratio variation. Following Lloyd's (1974) hypothesis, we predicted that female bias would increase with the degree of sex chromosome differentiation. In accord with this prediction we found evidence of greater female bias in species with heteromorphic compared to homomorphic sex chromosomes in the cross-species analysis (Fig. 3e; but see Fig. 4f). This was not apparent in the phylogenetically controlled analysis suggesting that the difference observed in the cross-species analysis could be driven by shared evolutionary history. However, it is important to recognize that other factors may also influence the degree of sex-ratio bias. For example, for species with viable YY genotypes, recombination may reduce the accumulation of deleterious recessive alleles on the Y chromosome. Accordingly, less female bias might be expected in species with viable YY genotypes. In our study equality or male bias was found in several species with viable YY genotypes (i.e., *Asparagus officinalis*, *Spinacia oleracea*; Ming et al. 2011 for YY viability information). In contrast, more female-biased ratios were found in species in which the YY genotype is known to be inviable (i.e., *Humulus japonicus*, *Silene latifolia*). Selfish genetic elements and X-linked meiotic drive can also influence sex-ratio variation (Taylor 1999) and variation among species in effective population size and life history are also likely to influence the extent to which genetic systems influence flowering sex ratios.

CONCLUSIONS AND FUTURE DIRECTIONS

Since the discussion of sex-ratio variation initiated by Darwin in the first edition of "*The Descent of Man and Selection in Relation to Sex*" (Darwin 1871, p. 316), evolutionary biologists have been fascinated by the principles and processes governing sex ratios. Although much of this work has focused on animal species, flowering plants with their diversity of life histories provide a rich source of data for studies of sex-ratio variation. We conclude by identifying three general problems that future efforts might focus on to improve understanding of the mechanisms causing biased sex ratios in plants. First, and perhaps the easiest to implement, involves an increased attention to more extensive sampling of natural populations. Only 5.8% ($n = 14$) of the species in our survey reported data from > 15 populations, limiting opportunities to detect robust patterns of sex-ratio bias. Future efforts should be directed toward sampling more populations over a broader geographical range and encompassing wider environmental and demographic contexts. Second, sex-specific markers have only been employed to study sex ratios in natural populations of a few dioecious species (e.g., Eppley et al. 1998; Stehlik and Barrett 2005; Shelton 2010a,b). Genomic approaches should provide outstanding opportunities for the rapid identification of large numbers of sex-specific markers (e.g., Bergero and Charlesworth 2011), enabling identification of the sex of diverse life-history stages, including seeds (e.g., Stehlik & Barrett 2005). This should allow investigators to determine primary sex ratios, and to identify life-cycle stages where biases develop. Sex-specific markers will also enable comparisons of sex-specific differences in clone size and the relations between ramet and genet sex ratios. Finally, a better understanding of resource currencies (e.g., carbon, nitrogen, see Harris and Pannell 2008) and the reproductive expenditure of the sexes are needed to evaluate costs of reproduction. Novel insights into the causes of sex-ratio variation are likely to come from precise determination of the stages at which bias occurs and the factors influencing the survival, growth, and flowering of females and males in natural populations.

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Supporting Information

Additional Supporting information may be found in the online version of this article at the publisher's website:

Appendix S1. Mean sex ratio and life history characteristics of the 243 species used in the cross species and phylogenetically controlled comparative analyses.

Appendix S2. Complete reference list of studies used in the comparative analyses of sex-ratio variation.

Appendix S3. Least significant differences among groups (LSD) for the generalized linear model (GLM, logistic regression) analyses of the effect of growth form, clonality and pollen and seed dispersal vectors on sex-ratio variation.