

OVULE DISCOUNTING IN AN OUTCROSSING, CRYPTICALLY DIOECIOUS TREE

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Abstract.—Ovule discounting denotes the reduction in the number of ovules available for cross-fertilization due to the interference of inferior pollen. Traditionally, ovule discounting has been discussed solely from the perspective of compromised outcrossing opportunities as a result of selfing, but the principle is more general. Here, we extend its applicability beyond the simple contrast between selfing and outcrossing by showing that, in the cryptically dioecious tree species *Fraxinus ornus*, ovule discounting through frequent outcrossing with inferior fathers also constitutes a substantial cost of mating. In *F. ornus*, hermaphrodites produce pollen capable of siring offspring, but these offspring are less viable than those sired by males and are inferred to produce few, if any, surviving progeny. In this paper, we used microsatellite markers to analyze the mating system and paternity in a wild population of *F. ornus*. We found that the effective number of sires per mother was low ($N_{ep} = 2.93$ to 4.95), and that paternity was correlated among progeny sampled from the same mother, but not among progeny sampled from neighboring mothers. Despite the existence of a local spatial genetic structure (up to 30 m), we found no evidence of biparental inbreeding. There was negligible selfing by hermaphrodites, but they sired approximately one fourth of the seeds produced by other hermaphrodites. Given that these progeny are not inferred to reach reproductive maturity, this constitutes a substantial cost of ovule discounting in the broad sense. We discuss the possible reasons for why hermaphrodites invest resources into inferior pollen.

Key words.—Androdioecy, cryptic dioecy, mating system, paternity, pollination, spatial genetic structure.

Received April 20, 2006. Accepted July 16, 2006.

Many hermaphroditic plants produce progeny by both self-fertilization and outcrossing (Schemske and Lande 1985; Vogler and Kalisz 2001). These mixed mating systems pose a challenge to evolutionary theory that predicts that populations should evolve toward either a complete outcrossing or a complete selfing strategy, and that intermediate selfing rates should be unstable (Lande and Schemske 1985; Charlesworth et al. 1990; Lande et al. 1994). Several hypotheses have been raised to explain the apparent mismatch between theory and observation (Porcher and Lande 2005, and references therein). One idea is that intermediate selfing rates are the inevitable outcome of trade-offs that occur during mating. For example, plants with large floral displays are known to be more attractive to pollinators, but the benefit of increased visitation can be compromised by the movement of pollinators between flowers on the same individual (Lloyd 1992; Eckert 2000). Self-pollination caused by this behavior (geitonogamy) will reduce an individual's fitness through its male function due to pollen discounting, because pollen that might have contributed to outcrossing fails to leave the plant that produced it (Nagyaki 1976; Lloyd 1992; Harder and Wilson 1998). Geitonogamy can also be costly to the female component of fitness if selfed progeny suffer inbreeding depression because of ovule discounting (i.e., ovules otherwise available for outcrossing are discounted through inferior mating; Lloyd 1992; Herlihy and Eckert 2002).

Ovule discounting has recently been implicated as a significant force shaping the evolution of the mating system in the self-compatible hermaphrodite *Aquilegia canadensis* (Herlihy and Eckert 2002). In this species, the benefits that plants might stand to gain by assuring reproduction through selfing when pollinators are scarce are compromised by the loss of ovules to self-fertilization and subsequent inbreeding depression when self and outcross pollen grains compete (Herlihy and Eckert 2002). In general, ovule discounting should constitute an important cost whenever inferior pollen preempts or competes with superior pollen during fertilization. However, it has previously been considered only in the context of competition between self and outcross pollen, and only for hermaphroditic populations in which all individuals have the same sex allocation (Lloyd 1992; Schoen et al. 1996; Herlihy and Eckert 2002). In this article, we show that ovule discounting through frequent outcrossing with inferior fathers also constitutes a substantial cost of mating in the cryptically dioecious species, *Fraxinus ornus*. Our observations thus extend the applicability of the concept of ovule discounting beyond the simple contrast between selfing and outcrossing.

Fraxinus ornus (Oleaceae) is an insect-pollinated tree in which males cooccur with individuals that produce both seeds and pollen (Dommée et al. 1999; Verdú 2004; Verdú et al. 2004). The species has previously been described as androdioecious (Dommée et al. 1999), but the fact that males typically occur at a frequency of 0.5 indicates that *F. ornus* is functionally dioecious and that hermaphrodites are thus func-

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tionally female (Verdú et al. 2004, and see Discussion). If hermaphrodites do sire progeny in nature, either through selfing or outcrossing, or both, and if these progeny do not themselves contribute their genes to subsequent generations (as the sex ratios suggest), then any siring performed by hermaphrodites would represent a cost of outcrossing that is exactly analogous to the cost of ovule discounting through self-fertilization and inbreeding depression. Here, we address whether hermaphrodites do in fact sire any progeny in natural populations.

Our primary aim in this article was to use molecular markers to estimate the extent to which hermaphrodites of *F. ornus* sire progeny in nature, relative to the siring success of males, and thus to gauge the cost of ovule discounting. *Fraxinus ornus* is self-compatible, and selfing occurs in bagged inflorescences under experimental conditions (Verdú et al. 2004). However, the selfing rate in natural populations is not yet known. We thus also estimated the selfing and outcrossing rates in the wild to assess the contribution made to ovule discounting by self versus outcross fertilizations. Of course, inbreeding depression can arise via other forms of inbreeding than selfing. To determine the extent of local mating and biparental inbreeding, we also analyzed the spatial pattern of pollen dispersal by computing the maximum-likelihood (ML) regression coefficient for the effect of distance on male reproductive success. In addition, we assessed fine-scale spatial genetic structure by computing pairwise kinship coefficients by distance classes. Finally, we estimated the extent to which progeny produced by the same mother, or by mothers separated by different distances in the population, were sired by the same or different fathers. If paternity is correlated among progeny of the same mother as well as among progeny of different but neighboring mothers, then this would point to the importance of spatial proximity in determining mating patterns; if hermaphrodites also act as fathers in some of these matings, then this would imply a spatial dimension to the cost of ovule discounting, with mothers surrounded by hermaphrodites bearing the highest ovule discounting. By contrast, if paternity of progeny of the same or neighboring mothers is uncorrelated, then we would expect that all hermaphrodites in the population should bear a similar cost of ovule discounting, regardless of their position.

METHODS

Study Species and Population

Fraxinus ornus (Oleaceae) is a small deciduous, wind-dispersed tree that inhabits in mixed woods, thickets, and rocky places in the Mediterranean region and Central Europe. Its main pollinators are bees although many insects visit the flowers (for a list of flower visitors, see Wallander 2001). On average, male trees have more inflorescences than hermaphrodites (25.29 ± 3.5 vs. 15.7 ± 2.9 , mean \pm SE), but the inflorescences from both genders have a similar number of flowers (890 ± 150 vs. 860 ± 600 for males and hermaphrodites, respectively; Verdú 2004). The mean flowering period of a single tree ranges from nine to 22 days; a difference of 18 days may separate the start of the flowering period of early and late flowering trees within the population. Hermaphrodite flowers are protogynous, with stigmas recep-

TABLE 1. Allelic richness (A), gene diversity (H_e) and paternity exclusion probability (EP , Jamieson and Taylor 1997) in adult trees, grouped by sexual morph, for the five microsatellite loci used in this study.

Locus	N	A	H_e	EP
Hermaphrodites				
FEMSATL4	87	18	0.8605	0.7253
FEMSATL16	87	10	0.7960	0.5973
FR16	87	10	0.6336	0.4437
FR39	87	5	0.5360	0.3064
FR41	87	4	0.4271	0.2386
Overall	87	9.4	0.6506	0.9675
Males				
FEMSATL4	78	17	0.8776	0.7500
FEMSATL16	78	9	0.8085	0.6151
FR16	78	9	0.7212	0.5325
FR39	78	5	0.6199	0.3710
FR41	78	4	0.3534	0.1916
Overall	78	8.8	0.6761	0.9771

tive when flowers open and pollen released only one week later (Wallander 2001). However, self-pollination by geitonogamy is not totally prevented by protogyny because of the high asynchrony between flowers within a tree (i.e., pollen from early flowers may pollinate the stigmas of late flowers). The study was carried out in La Font Roja Natural Park, a well-preserved population in eastern Spain (for further details, see Laguna and García 1988; Verdú 2004).

All flowering individuals (179 trees) within a belt transect of dimensions 450×40 m were tagged and mapped using a submetric precision global positioning system, and their gender was recorded. All of these individuals were considered potential fathers of the seed progeny collected. Leaflets from all 179 putative fathers (92 males and 87 hermaphrodites) and 20 seeds from each of 20 hermaphrodites (mothers) were collected, dried in silica gel, and stored at -20°C prior to DNA extraction. These mothers were all selected from the middle of the transect to maximize the potential paternity from within the plot. Genomic DNA was isolated using cetyltrimethylammonium bromide (CTAB), following a procedure modified after Doyle and Doyle (1987). We used between 15 and 30 mg of leaf material and eluted the extract in a final volume of 100 μl TE buffer. The DNA extracts were diluted tenfold for polymerase chain reaction (PCR).

Five nuclear microsatellite loci were used in the analysis: FEMSATL4, FEMSATL16, FR39, FR41 (Lefort et al. 1999; Verdú et al. 2004), and FR16. FR16 is a new marker developed by the company Bionostra S.L. (Madrid, Spain), consisting of a (GA) repeat motif amplified by FR16.f 5'-CCT CTA GAT AGT CGA CTG G-3' and FR16.r 5'-CAC TAC ACA CTG CTT GAA AC-3' (GenBank accession number for the cloned sequence is DQ857293). Together, these microsatellites yielded a high overall exclusion probability (see Table 1). Polymerase chain reactions (25 μl) contained: autoclaved deionized water, $1 \times$ buffer (containing 10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM MgCl_2 ; Netzyme), 100 μM of each dNTP (Roche Diagnostics, Burgess Hill, U.K.), 400 nM of each primer (Applied Biosystems, Foster City, CA), 1 U Taq polymerase (Netzyme), and 10 μl of the diluted DNA extract. Polymerase chain reactions were performed on

a PTC-100 programable thermal cycler using a modification of a published protocol for *F. excelsior* (Heuertz et al. 2001), with 4 min at 95°C; 30 cycles of 30 sec at 94°C; 45 sec at the primer-specific annealing temperature; 1 min at 72°C; and a final extension of 5 min at 72°C. Fluorescent labeling of the forward primers allowed detection of amplification products on an automated DNA sequencer (ABI PRISM 3700 DNA sequencer). Sizing of fragments was performed using Genescan 2.1 (Applied Biosystems) by comparison with an internal size standard (Genescan-400HD Rox).

Mating System Estimates

Inbreeding in the hermaphrodite adult trees sampled, F , and the multilocus (t_m) and single-locus (t_s) population outcrossing rates were estimated from the progeny arrays using MLTR software (Ritland 2002). Correlated mating was estimated by two different methods: (1) Ritland's multilocus estimator of the correlation of outcrossed paternity within progeny arrays, r_p (implemented in MLTR); and (2) the kinship approach of Hardy et al. (2004), which is based on pairwise kinship coefficients between paternal gametes of offspring pairs within mothers (F_s). In the absence of selfing and biparental inbreeding, r_p and $2F_s$, are both estimators of the proportion of full-siblings within progeny arrays (Ritland 1988; Hardy et al. 2004). However, the approach of Hardy et al. (2004) can also provide estimates of among-mother correlated paternity (F_{ij}) at different spatial (or temporal) scales and may thus yield insights into a population's pollination biology. For example, limited mate availability, that is, a low number of pollen donors, would result in higher pairwise kinship coefficients between pollen gametes sampled from mothers nearby one another (i.e., among-mother correlated paternity) than those computed among mothers further away from one another (because mothers close together will be mated by the same, scarce, fathers that are locally available).

The importance of ovule discounting depends on the strength of pollen competition. If pollen is limiting (weak pollen competition), fertilizing ovules with less fit pollen might not have any impact on the number of ovules fertilized with more fit pollen. In this case, there might be little or no ovule discounting even when low fitness pollen sources are used. To determine the extent of pollen competition, we compared seed set obtained in open-pollinated inflorescences with that obtained in inflorescences in which pollen from males was experimentally supplied. Pollinations were performed on 10 hermaphrodites, with four replicates per treatment (for experimental details, see Verdú et al. 2004). The proportions of seeds sired as a result of supplementary pollination and open-pollination were compared using a generalized linear mixed model via penalized quasi-likelihood (glmmPQL; the MASS library on the R statistical package; Ihaka and Gentleman 1996), with a Poisson distribution of errors and a canonical link.

Effects of Distance and Gender on Pollination Success

To determine whether pollination was restricted by distance (i.e., males preferentially pollinate mothers located nearby), we computed regression coefficients for the effect

of distance on log male reproductive success, as implemented in PatQuest version 4.0 software (Thomas R. Meagher and collaborators, University of St. Andrews, U.K.). Transition probabilities based on genetic data were used to estimate the male contributions (λ_k , for each of the $k = 1, 2, \dots, 179$ putative fathers, males and hermaphrodites, with $\sum \lambda_k = 1$) to the overall progeny array using maximum-likelihood (Roeder et al. 1989; Smouse and Meagher 1994). Analyses were limited to those offspring with at least one genetically compatible father within the population ($N = 328$) (Vassiliadis et al. 2002). The effect of distance, γ_d , on male reproductive success was estimated using a log-linear model (Smouse et al. 1999; Burczyk et al. 2002; Vassiliadis et al. 2002; Wright and Meagher 2004):

$$\log(\lambda_{jk}) = \gamma_d \delta_{jk}, \quad (1)$$

where λ_{jk} represents the fertility of the k th father over the j th mother and δ_{jk} is the log distance between these individuals. Statistical significance of γ_d was computed using standard log-likelihood ratios (LLR) and pedigree-based bootstraps analyses, the latter following Morgan and Conner (2001). This model was further extended to account simultaneously for the effects of intermate distance and paternal gender (male or hermaphrodite) on male reproductive success, as follows:

$$\log(\lambda_{jk}) = \gamma_d \delta_{jk} + \beta z_k, \quad (2)$$

where $\exp(\beta)$ corresponds to the relative numbers of ovules sired by a male compared to those sired by a hermaphrodite, and z_k is an indicator variable for the k th candidate father that takes the value of 0 for hermaphrodites and 1 for males.

Differences in siring success between males and hermaphrodites were also analyzed using (1) a Bayesian method that takes into account incomplete sampling of potential fathers but not pollination distances (Nielsen et al. 2001; Signorovitch and Nielsen 2002) and (2) a method that accounts for both pollen immigration and intermating distance effects, the 'neighborhood' model (Burczyk et al. 1996; Bacles et al. 2005). These analyses produced very similar results to those obtained with the likelihood method described above and are thus not shown.

Fine-Scale Spatial Genetic Structure

Pairwise kinship coefficients between adult trees at distance class r , $F(r)$, were used to construct spatial autocorrelograms. Kinship coefficients were computed as a correlation coefficient between allelic states following Loiselle et al. (1995), as implemented in SPAGeDi version 1.1 software (Hardy and Vekemans 2002). Incautious interpretation of correlograms can be misleading because they may depend strongly on the sampling scheme. Thus, we also computed the S_p statistic, which has been shown to be robust to different common biases, at least when the pairwise kinship coefficients vary linearly with r or $\ln(r)$ (Vekemans and Hardy 2004). When properly computed, and when the spatial genetic structure is truly representative of isolation-by-distance processes in a two-dimensional space, the S_p statistic is related to the inverse of Wright's neighborhood size, N_b .

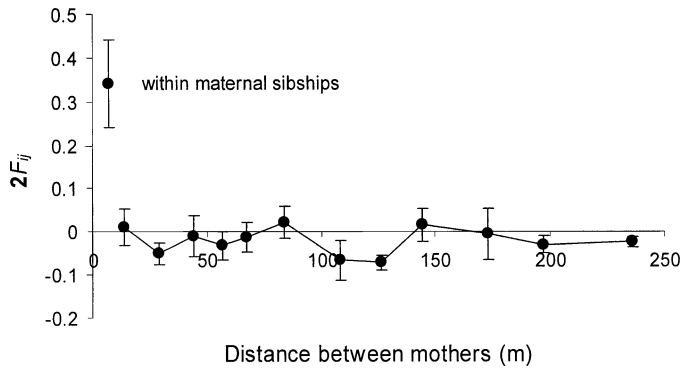


FIG. 1. Correlated paternity, estimated as twice the average kinship coefficient between the paternal genes of offspring pairs within maternal sibships (i.e., correlated mating) and among sibships according to the distance separating the mothers. Error bars indicate 95% confidence intervals.

RESULTS

Mating System

The coefficient of inbreeding (based on progeny arrays) for mother trees was low and not significantly different from zero (0.077 ± 0.124). A similar low albeit significant value (0.068 $P < 0.05$) was obtained from the estimate of kinship between genes within individuals using all sampled trees. Both multilocus (1.095 ± 0.051) and single-locus outcrossing rates (0.979 ± 0.029) were high and not significantly different from unity, indicating low levels of both selfing and biparental inbreeding. A simple estimate of minimum incoming gene flow is provided by the percentage of offspring that are not compatible with any sampled father (9.14%). However, the true levels of immigrant pollen in *Fraxinus ornus* were probably much higher than this minimum estimate, as reliable estimates are usually two to fourfold greater than estimates based on exclusion analysis alone (Adams and Burczyk 2000, and references therein).

Correlated mating among progeny with the same mother was high in *F. ornus*, being 0.20 ± 0.07 as estimated by Ritland's MLTR and 0.34 ± 0.10 using the pairwise kinship approach of Hardy et al. (2004). The lower value of the estimate based on Ritland's approach is consistent with its recognized tendency to underestimate correlated mating (Hardy et al. 2004). The high level of correlated paternity among progeny of the same mother meant that the effective number of sires per mother was low ($N_{ep} = 2.93$ to 4.95). Correlated paternity among progeny sampled from mothers in close proximity was extremely low and similar to that from mothers separated by hundreds of meters (Fig. 1). Thus, there was no evidence for pollen limitation, which was confirmed by our fruit (seed) set experiment: seed set per inflorescence under supplementary pollination (173 ± 23) (mean \pm SE, here and below) was not significantly greater than natural levels of seed set (132 ± 20 ; $t = -1.29$; $df = 52$; $P = 0.20$).

Effects of Distance and Gender on Male Reproductive Success

The most likely estimate of γ_d , the effect of intermate distance on male reproductive success, was -0.1922 and sig-

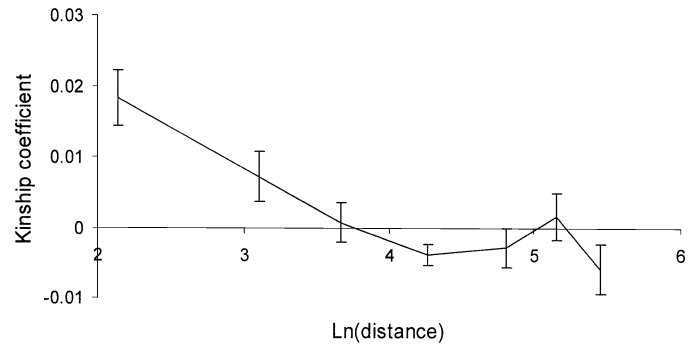


FIG. 2. Spatial autocorrelogram based on kinship coefficients for pairs of adult trees and the logarithm of the distance (m). Bars indicate standard errors estimated by jackknifing over loci.

nificant, as shown by both a standard LLR test, which compares this model with a simpler model that excludes intermate distance ($P = 0.052$) and Morgan and Conner's (2001) bootstrap test ($P = 0.049$). A significant negative value of this coefficient indicates an excess of nearby matings and shorter pollen dispersal distances than expected by random mating. The most likely estimate of intermate distance effect changed little ($\gamma_d = -0.2172$) when the gender effect was added to the likelihood function. The LLR tests showed that models that include gender effects (i.e., male vs. hermaphrodites) were better than simpler models in which both γ_d and β were set to zero ($P = 0.001$), or in those in which intermate distance, γ_d , was the only effect included ($P = 0.005$). The maximum-likelihood estimate of male siring success was 2.21 times higher in males than in hermaphrodites ($\beta = 0.7930$). Similar results were obtained by a Bayesian non-spatial method, with relative mating success of males to hermaphrodites of 1.49 (assuming 200 potential fathers) to 2.94 (assuming 500 potential fathers) (see methods in Nielsen et al. 2001; Signorovitch and Nielsen 2002), and a 'neighborhood' model, with relative mating success of males to hermaphrodites of 1.73 (see methods in Burczyk et al. 1996; Bacles et al. 2005). Recognizing that the inverse of $\exp(\beta)$ corresponds to the relative numbers of ovules sired by a hermaphrodite compared with those sired by a male, and given that the sex ratio in the population is 1:1, the inverse values of the ML estimate obtained here would suggest that hermaphrodites sire $\sim 45\%$ as many ovules as are sired by males.

Fine-Scale Spatial Genetic Structure

We found significant spatial structure in the two shorter distance classes, up to 30 m, with pairwise kinship coefficients varying approximately linearly with the logarithm of the distance between adult trees (Fig. 2). The S_p statistic was 0.028, which translates to a neighborhood size of about 193. Unfortunately, few fathers were identified by unambiguous exclusion, and thus we were not able to directly assess whether fathers sired offspring of related hermaphrodites. No differences in spatial genetic structure were found for males and hermaphrodites; both sex morphs showed similar autocorrelograms and S_p values (data not shown).

DISCUSSION

It is clear from our results that in populations of *Fraxinus ornus* pollen is widely dispersed, and that both males and hermaphrodites sire seeds under natural conditions. While a considerable proportion of the seeds sampled in the study were sired by individuals outside the demarcated population, the significant negative regression coefficient for the effect of distance on male reproductive success indicated that fathers were more likely to be positioned near to the mothers than further away. Moreover, there was a significant fine-scale spatial genetic structure that was not different between males and hermaphrodites. Finally, we found significant correlations in paternity among progeny sampled from the same mother, but not among progeny sampled from neighboring mothers. These results have several implications for our understanding of the pollination biology and sexual system of *F. ornus*. In particular, they raise important questions concerning the function of pollen dispersed by hermaphrodites and its evolutionary implications. We discuss these issues below.

The Mating System of Fraxinus ornus

Although *F. ornus* is self-compatible and fully capable of producing viable self-fertilized seeds in experimental pollinations (Dommée et al. 1999; Verdú et al. 2004), we found very little evidence for self-fertilization in a population under natural conditions. Thus, *F. ornus* can be characterized as a self-compatible outcrossing tree. Dommée et al. (1999) speculated that hermaphrodites might be maintained in *F. ornus* by selection during colonization phases, when an ability to produce progeny by self-fertilization would confer the advantage of reproductive assurance. This explanation has been suggested for several plant and animal species in which males coexist with self-fertile hermaphrodites (reviewed in Pannell 2002a). In all these functionally androdioecious species (Charlesworth 1984; Pannell 2002b), the high variability in male frequencies among populations represents a telltale signature of colonization, but this signature is absent in *F. ornus*, where equal numbers of males and hermaphrodites have always been observed. It is plausible that single hermaphrodites would produce progeny via selfing, but this would seem to be an unusual situation for the species. Indeed, hermaphrodite-sired progeny appear unlikely to survive to reproductive maturity (Verdú et al. 2004, and see below), so that we might expect populations colonized by single self-fertilizing hermaphrodites not to persist beyond the first generation.

Several features of the pattern of gene flow within populations of *F. ornus* deserve comment. First, we found no evidence for pollen limitation on seed set. In conjunction with the fact that almost all ovules were outcrossed rather than self-fertilized, this finding argues against the role that pollen limitation might play in selecting for combined sexes and thus androdioecy in *F. ornus* (Wolf and Takebayashi 2004).

Second, whereas pollen-producing individuals positioned close to prospective mothers were more likely to sire their progeny than those further away, many seeds were sired by individuals at greater distances; indeed, many seeds were sired by fathers growing outside our sampled area. This pat-

tern of within-population gene flow is unremarkable and typical of gene flow documented for many plant populations, both of animal-pollinated and wind-pollinated species (Epperson 1993; Ennos 2001; and see review by Smouse and Sork 2004; Vekemans and Hardy 2004). The low to moderate fine-scale spatial genetic structure found in *F. ornus* is consistent with restricted seed and pollen dispersal, with the S_p values being typical of small outcrossing trees (see table 3 in Vekemans and Hardy 2004).

Third, there was substantial correlated mating within sibships, that is, progeny with both the same mother and the same father. On its own, the fact that nearby fathers were more likely to sire progeny on a given mother is unsurprising. However, we found correlated paternity only within single mothers but not among seeds sired across neighboring mothers. This suggests that the receptivity of ovule-bearing individuals to pollen is probably limited to a brief period of time, so that a different pool of pollen-producing individuals competes to sire seeds on different mothers. If gene flow is regulated by subtle but important differences in the temporal window of opportunity for siring opportunities on particular mothers, then it is also plausible that dichogamy may be the main mechanism by which self-fertilization is prevented, although Verdú et al. (2004) provided some evidence for some mechanism of weak autoincompatibility, too. Alternatively, the high correlated paternity found within single mothers but not across neighboring mothers could be explained by pollinator type and behavior, because it has been shown for other mating system features (reviewed in Harder and Barrett 1995; Brunet and Sweet 2006).

Siring Success of Hermaphrodites in Fraxinus ornus

We have shown that hermaphrodites of *F. ornus* not only disperse viable pollen, but also sire seeds in the wild. Indeed, our best estimates suggest that about 30% of the seeds produced in the *F. ornus* study population were sired by hermaphrodites (i.e., $N_h/[N_h + 2.21N_m]$, where $N_h = 87$ hermaphrodites and $N_m = 92$ males, and assuming that males sire 2.21 as many seeds as do hermaphrodites). This observation might be explained by the larger numbers of flowers produced by males. On face value, the relatively high siring success of hermaphrodites would suggest that *F. ornus* should be described as androdioecious (e.g., Dommée et al. 1999). Similar evidence has been cited in arguments for the functionality of androdioecy in *Phillyrea angustifolia*, a shrub in the same family that occupies similar habitats in the Mediterranean Basin (Lepart and Dommée 1992; Pannell and Ojeda 2000; Vassiliadis et al. 2002). However, the 1:1 sex ratios observed in populations of *F. ornus* strongly suggest that the species is cryptically dioecious, and that hermaphrodites are thus cryptically female.

Although the relative production of seeds and pollen by hermaphrodites in gender dimorphic populations provides a useful basis for hypothesizing their potential prospects as male versus female parents (e.g., Lloyd 1980), we emphasize that the sex ratio of populations at equilibrium potentially provides the most robust indicator of hermaphroditic functional gender. To see this, let m and $(1 - m)$ be the proportions of males and putative hermaphrodites in a population, where

the hermaphrodites are outcrossing and produce both seeds and pollen and the males produce only pollen. Further, let r be the number of reproductively successful progeny sired by hermaphrodites relative to those sired by males ($r < 1$ may be due to a greater amount of pollen produced by males than hermaphrodites, or a greater quality, or both). At equilibrium, the fitness of males and hermaphrodites must be equal. With this assumption, it is easy to show (e.g., Lloyd 1975) that the expected proportion of males at equilibrium is then $m = (1 - 2r)/(2 - 2r)$, or that $r = (1 - 2m)/(2 - 2m)$. It is thus clear that $r = 0$ when $m = 0.5$, that is, that when the sex ratio is 1:1, hermaphrodites sire little to no successful progeny and are therefore functionally female.

It is important to emphasize that the sex ratio model here is the primary sex ratio, that is, the proportion of males to females or hermaphrodites prior to any gender-dependent mortality. The sex ratios observed in populations of *F. ornus* are secondary, and thus might reflect deviations from the primary sex ratio due to mortality. We cannot discount the possibility of differences in mortality between males and hermaphrodites, particularly as differences in ecophysiology have been observed between the genders (Verdú 2004), and more data are clearly needed. However, it appears unlikely that viability effects would bring the sex ratio, by coincidence, to 50:50 in all of the populations studied. In a previous paper, we showed that hermaphrodite-sired progeny grow more slowly than those sired by males and, in a competitive environment, it may be suspected that they fail to reach reproductive maturity (Verdú et al. 2004). Therefore, hermaphrodites of *F. ornus* appear to be functionally female, and the species is probably cryptically dioecious, not androdioecious.

Cryptic dioecy is known in several other plant species in which apparent hermaphrodites produce pollen but do not sire progeny (Mayer and Charlesworth 1991). Typically, the low siring success of hermaphrodites in these species is due to pollen inviability or to anthers that do not open properly (Anderson and Symon 1989; Mayer and Charlesworth 1991; Cane 1993; Dunthorn 2004). In other words, any genetic contributions made by potential hermaphrodites through pollen are prevented before any progeny can be sired, that is, a generation earlier than appears to be the case in *F. ornus*. Thus, *F. ornus* differs from other known cryptically dioecious species in that its hermaphrodites do actually sire seeds. This raises two interesting questions.

First, what developmental mechanism causes the seeds sired by hermaphrodites to be of lower quality than those sired by males? Verdú et al. (2004) suggested that a linkage between the sex-determining locus and a viability locus or loci might explain the observed siring effects on fitness. There is some evidence for a similar effect in the androdioecious clam shrimp *Eulimnadia texana*, in which the dominant hermaphrodite-determining allele is linked to apparently deleterious recessive alleles (Weeks et al. 2001; Weeks 2004). Here, hermaphrodites that are heterozygous at the sex-determining locus have higher fitness than those that are homozygous (Weeks et al. 2001). On reflection, it is clear that this kind of situation would be insufficient to explain observed progeny fitnesses and adult sex ratios in *F. ornus*. The 1:1 sex ratios of *F. ornus* require that no hermaphrodite-sired

progeny reach reproductive maturity. However, under a scenario similar to that observed in *E. texana*, we should expect not all, but only one quarter, of the progeny sired by mature hermaphrodites to be homozygous recessive at the viability locus, and thus of reduced fitness. Vassiliadis et al. (2000) proposed a different model to explain high male frequencies in *Phillyrea angustifolia*, in which they considered linkage between the sex-determining locus and a self-incompatibility locus. However, this explanation would seem to be immediately inapplicable to *F. ornus*, which we have shown is self-compatible (see also Verdú et al. 2004). The proximate reasons for the reduced fitness of hermaphrodite-sired progeny thus remains unclear and awaits further work.

The second question concerns why hermaphrodites should invest valuable resources in producing and dispersing pollen that fails to transmit genes to subsequent generations. Because *F. ornus* is insect pollinated but produces no nectar, it is likely that pollen functions as a pollinator reward, as it does in other cryptically dioecious species (Anderson and Symon 1989; Mayer and Charlesworth 1991; Cane 1993). However, this explanation simply begs the further question of why viable pollen is dispersed to other stigmas in the population. In other cryptically dioecious species, pollen grains are often inviable, or anthers do not open (Mayer and Charlesworth 1991). In these species, it is possible that the siring ability of pollen grains produced by morphological hermaphrodites has been reduced by selection because of fitness benefits of avoiding inbreeding depression. As noted above, however, *F. ornus* is almost entirely outcrossed, possibly as a result of dichogamy. It is of course possible that the behavior of pollen produced by hermaphrodites reflects an ancestral state where hermaphrodite-sired seeds were more viable than they are now. However, a convincing ultimate explanation for the investment by cryptic females of resources into pollen production remains outstanding.

Conclusions

This study adds to a series of papers on the reproductive biology of *F. ornus* (Dommée et al. 1999; Verdú 2004; Verdú et al. 2004) and confirms that hermaphrodites of the species do indeed sire seeds in natural populations. The 1:1 sex ratios of *F. ornus* populations indicate that the species is cryptically dioecious (Charlesworth 1984; Mayer and Charlesworth 1991), and thus that hermaphrodite-sired progeny cannot substantially contribute genes to subsequent generations (Verdú et al. 2004). Our result therefore indicates that, ultimately, ovules fertilized by pollen from hermaphrodites must be discounted from the population of ovules available for outcrossing by males, and that this ovule discounting represents a substantial mating cost.

Our results pose more problems than they solve. In particular, the proximate (developmental) mechanisms for the reduced growth rates of hermaphrodite-sired progeny are not yet clear (Verdú et al. 2004). Nor do we know the ultimate (selective) reasons for the maintenance of expensive pollen production by cryptic females. Answers to these questions await further work. However, it is worth noting that *F. ornus* occurs within a genus and family in which putative androdioecy occurs surprisingly often (Wallander 2001). Given

that androdioecy is not easy to evolve or to maintain (Lloyd 1975; Charlesworth and Charlesworth 1978; Charlesworth 1984; Pannell 2002a), it is therefore tempting to think that understanding the basis of cryptic dioecy in *F. ornus* might point to explanations for the maintenance of males with hermaphrodites in other species of the genus and family. An important question for these other species will continue to be whether or not their hermaphrodites function as males, and to what extent.

ACKNOWLEDGMENTS

We are grateful to J.A. Bellido, P. García-Fayos, G. Gleiser, and R. M. López for assistance in the field and laboratory, the Parque Natural del Carrascal de la Font Roja for permission to conduct work on its land, R. Nielsen and J. Signorovitch for assistance with PATRI analyses, and S. Otto and two anonymous referees for helpful comments on the manuscript. S. C. González-Martínez was supported by the Ramón y Cajal Fellowship RC02-2941. The work was funded by the European Union grant FRAXIGEN (EVK2-CT-2001-00180) and the Spanish Ministerio de Educación y Ciencia (project AGL2005-07440-C02-02/FOR).

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Corresponding Editor: S. Otto