doi:10.1111/j.1558-5646.2008.00394.x

DISASSORTATIVE MATING, SEXUAL SPECIALIZATION, AND THE EVOLUTION OF GENDER DIMORPHISM IN HETERODICHOGAMOUS *ACER OPALUS*

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Received December 18, 2007
Accepted March 12, 2008

In sexually polymorphic species, the morphs are maintained by frequency-dependent selection through disassortative mating. In heterodichogamous populations in which disassortative mating occurs between the protandrous and protogynous morphs, a decrease in female fitness in one morph is hypothesized to drive sexual specialization in the other morph, resulting in dimorphic populations. We test these ideas in a population of the heterodichogamous species, *Acer opalus*. We assessed both prospective gender of individuals in terms of their allocations and actual parentage using microsatellites; we found that most matings in *A. opalus* occur disassortatively. We demonstrate that the protogynous morph is maintained by frequency-dependent selection, but that maintenance of males versus protandrous individuals depends on their relative siring success, which changes yearly. Seeds produced later in the reproductive season were smaller than those produced earlier; this should compromise reproduction through ovules in protandrous individuals, rendering them male biased in gender. Time-dependent gender and paternity analyses indicate that the sexual morphs are specialized in their earlier sexual functions, mediated by the seasonal decrease in seed size. Our results confirm that mating patterns are context-dependent and change seasonally, suggesting that sexual specialization can be driven by seasonal effects on fitness gained through one of the two sexual functions.

KEY WORDS: ***Acer*, gender specialization, heterodichogamy, intermorph mating, paternity analysis, sexual inconstancy.**

Most Angiosperm populations include a single floral morph that mates randomly. Less frequently, plant populations may include two or more floral morphs that differ in reproductive traits that determine the proportion of nonrandom matings. In these polymorphic populations, disassortative (intermorph) mating is predicted to be prevalent, and the evolutionary maintenance of the sexual morphs is assured by negative frequency-dependent selection (Eckert et al. 1996; Thompson et al. 2003; Barrett and Harder 2005; Pannell et al. 2005). In most cases, when matings are completely disassortative and yield progeny of equal fitnesses, the morph frequencies are expected to be equal at equilibrium. In contrast, the occurrence of assortative mating in one morph should increase its equilibrium frequency, with the possible fixation of that morph and the subsequent loss of the polymorphism when sufficiently high levels of assortative mating occur (Baker et al. 2000; Barrett and Hodgins 2006).

Disassortative mating may be driven by (1) morphological differences among the sexual morphs, as in the case of enantiostylous species, in which the two floral morphs each display reciprocal bilateral asymmetry in the placement of their stigmas and anthers (Ornduff and Dulberger 1978; Webb and Lloyd 1986; Fenster 1995; Barrett et al. 2000; Jesson and Barrett 2002); (2) by physiological differences, as in homomorphic self-incompatible species in which individuals differ from one another at a self-incompatibility locus (Clarke and Newbigin 1993; Lawrence 2000; Silva and Goring 2001; Castric and Vekemans 2004); (3) by both morphological and physiological differences, as in most heterostylous species in which self-incompatibility is linked to reciprocal placement of the stigma and anthers along the vertical axis of the flower (Barrett 1992; Barrett et al. 2000); and (4) by phenological differences, as in heterodichogamous species in which hermaphrodites are either male- or female-first in the sequence of their two sexual functions (Gleeson 1982; Renner 2001). Of these polymorphisms, most attention to date has focused on those that are governed by morphological and physiological traits, and studies of the mating patterns and gene flow in temporally polymorphic species, such as heterodichogamy, are less common.

Heterodichogamous populations usually contain two sexual morphs, protandrous and protogynous, that are expected to mate disassortatively as a consequence of both the segregation of the sexual phases within an individual (e.g., dichogamy), and the fact that the sexual morphs express the male and female phases at different times (reviewed in Renner 2001). In some heterodichogamous species, the switch between the male and female phases occurs daily (so that both phases are expressed within 24 h; Li et al. 2001); in others, the transition between the male and female phases within an individual occurs only once during the flowering season (de Jong 1976; Gleeson 1982; Pendleton et al. 1988; Dommée et al. 1990). A third type of heterodichogamy occurs in species in which all the individuals are either protandrous or protogynous, but which differ in the time at which they start flowering each day (Galil and Zeroni 1967; Gleeson 1982; Kubitzki and Kurz 1984). Although the genetic and developmental mechanisms involved in the control of the expression of the sexual phases are still poorly understood, early studies performed in a couple of species suggest that heterodichogamy is regulated by a single Mendelian locus with two alleles (Gleeson 1982; Thompson and Romberg 1985).

The above polymorphisms all involve hermaphrodites that differ in their floral strategies but that may nevertheless be equivalent in their gender, that is, in the proportion of genes transmitted through male versus female functions. Under particular circumstances, however, disassortative mating in such species may also promote the evolution of a dimorphism in gender. For example, in distylous species, a change in the pollinator fauna may drive the evolution of gender dimorphism through a process of sexual specialization (Lloyd 1979; Bawa and Beach 1981; Muenchow and Grebus 1989). Similarly, a fall-off of the quality of the seeds produced by the individuals in the population has been proposed to trigger the sexual specialization and the evolution of gender dimorphism in heterodichogamous species. This scenario was recently modeled by Pannell and Verdú (2006).

In their model, Pannell and Verdú (2006) sought the conditions under which male- or female-sterility mutations could invade a population comprising protandrous and protogynous morphs. They noted that the fall-off in seed quality might correspond to deterioration later in the season of conditions for seed development, such as increasing summer drought, and they argued that the resulting reduction in seed size would correspondingly reduce the female component of fitness of the seed producers. Implicitly, they thus assumed that protandrous individuals that produce seed later in the season than protogynous individuals, become specialists in their male function by virtue of their compromised female function. They predicted that such a population should become susceptible to the invasion of male specialists that flower at the beginning of the reproductive season, that is, male-specialist protandrous individuals. These ideas, which pertain specifically to heterodichogamous species but which have implications for the evolution of gender in populations with floral dimorphisms generally, remain largely untested.

In the present article, we present an analysis of gender and mating patterns of sexual morphs of the heterodichogamous species, *Acer opalus*, in which specialist males co-occur with protandrous and protogynous hermaphrodites. Our article has two main aims. First, we use molecular markers to assign paternity in a population of *A. opalus* to test the prediction that mating should be predominantly disassortative. Second, we directly assess the validity of the assumptions and predictions of the model developed by Pannell and Verdú (2006). *Acer opalus* presents ideal material to test this model, given recent comparative evidence for *Acer* that gender dimorphism has evolved from ancestral heterodichogamy in the genus (Gleiser and Verdú 2005).

We begin by asking whether seed quality, measured as seed size, decreases over the course of a reproductive season of *A. opalus*. Because reduced seed size is likely to imply reduced survivorship under competition (Leishman et al. 2000; Leishman 2001), this question thus tests the important assumption in Pannell and Verdú's (2006) model that the protandrous morph sets up the evolution of gender dimorphism by necessarily specializing in its male function. We then estimate the gender of the sexual morphs by characterizing the prospective gender of individuals of *A. opalus* during the course of two separate reproductive seasons on the basis of patterns of investment in male and female reproductive functions (Lloyd 1980; Lloyd and Bawa 1984), as well as by estimating the siring success of the three sexual morphs through paternity analyses using microsatellite markers. We also predict the equilibrium frequencies of the sexual morphs, given the sex allocation and the mating patterns observed during a two-year phenological survey, and we use simulations to evaluate whether the sexual polymorphism observed in this species is expected to be maintained by negative frequency-dependent selection.

Table 1. **Sexual transitions matrix obtained from a six-year survey in an *A. opalus* population in the Font Roja Natural Park. Each cell (x_{ij}) represents the fraction of the possible cases in which a tree of the *i*th morph in a year (*n*) flowered as the *j*th morph the following year ($n + 1$). M: with male flowers only; PG: with protogynous flowering; PA: with protandrous flowering; N/F: nonflowering state.**

	Sex in year ($n + 1$)			
Sex in year (n)	M	PA	PG	N/F
M	0.81	0.09	0.00	0.1
PA	0.30	0.48	0.00	0.22
PG	0.00	0.00	0.74	0.26
N/F	0.27	0.07	0.42	0.24

Methods

STUDY SPECIES

Acer opalus is a deciduous small multistemmed tree with populations composed of males, protandrous, and protogynous individuals. Reproduction is characterized by a strong interannual variation in the intensity of flowering (mast flowering). Whereas protogynous trees always express both sexual phases, protandrous trees may fail to express the female phase in some years (Verdú et al. 2004). Each of the two sexual phases may last for up to three weeks, and the temporal separation of the phases within a tree is often complete. Flowers are functionally unisexual due to abortion of the pistil or the possession of anthers that fail to open. The ovaries have two locules with an ovule in each, but usually only one ovule develops after fertilization. The indehiscent fruits are schizocarps, composed of two one-seeded samaroid mericarps. The unfertilized flowers develop into parthenocarpic fruits (de Jong 1976). The developing fruits remain in the mother plant until the dispersal period. Almost all the fruits are dispersed by wind in autumn, whereas a small portion may persist in the mother tree until the following reproductive season (Gómez-Aparicio 2004).

STUDY POPULATION

The study was carried out in a wild population of *A. opalus*, located in the Font Roja Natural Park, in Eastern Spain (38° 40′ N, 0° 30′ W). This area is characterized by a Mediterranean climate, with hot and dry summers and cold winters. The mean annual precipitation in this area is 825 mm, and the mean annual temperature is 11.6°C (Laguna and García 1988). The population is located in a north-facing slope, at about 1300 m above sea level. *Acer opalus* shows a pattern of active recruitment in this population (Gómez-Aparicio et al. 2005).

SEX INCONSTANCY AND MORPH RATIOS

As sexual inconstancies have been reported in some *Acer* species (de Jong 1976; Barker et al. 1982; Primack and McCall 1986; Sakai 1990; Matsui 1995; Sato 2002), we conducted a preliminary survey on sex expression in the study population. The sexual expression of a sample of trees was followed over a six-year period from 1999 until 2004, from an initial sample of 44 trees that was enlarged to 100 trees in 2003. Sexual transitions were only ever observed between male and protandrous flowering types (Table 1), whereas the protogynous flowering type never switched to male or protandrous flowering types. From these observations, three sexual morphs were clearly distinguished: protogynous, protandrous (including individuals that may fail to produce female flowers in any one season), and male (individuals that only produced male flowers during the six-year survey). In the study population, protogynous trees comprised 48% of the population, males 39%, and protandrous trees 13%, with the latter including constant and inconstant protandrous trees. The sex ratios observed in six other Spanish populations are similar to these, with protogynous trees accounting for approximately half of each population and the males and protandrous trees accounting for the remaining half (Gleiser et al. in press).

SAMPLING REGIME FOR TIME-DEPENDENT GENDER ESTIMATES

Counts of total female and male flowers were used as estimates of the maternal and paternal investments, respectively. Fruit set and seed viability do not provide significant information, and were thus not included in the estimates of gender (the fruit set is almost 100% due to the parthenocarpic development of unfertilized ovules, and the percentage of seed viability does not differ between the protandrous and the protogynous morphs; $z = 0.22$, $P = 0.83$, $N = 850$ seeds). We sampled 10 males, 10 protogynous, and 5 protandrous trees in the flowering season of 2002, and 26 males, 19 protogynous, and 13 protandrous trees in 2003. The number of male and female flowers produced by each tree was estimated by counting the number of flowers of each type in three to five inflorescences per tree, and by then estimating the total number of inflorescences at the whole tree level. Within each inflorescence, we only considered the male flowers that were releasing pollen and the female flowers with receptive stigmas (the loss of receptivity can be noticed by a change in the color of the stigma and by the loss of turgidity of the stigmatic lobes). The total number of inflorescences per tree was estimated by counting the number of inflorescences in branches situated in different orientations (to avoid spatial effects on flowering), and by finally multiplying the mean inflorescence number per branch by the number of branches on the tree. Sampling was repeated every two to 11 days, depending on the rate of progress of flowering, until the flowering season ended. This procedure allowed us to estimate the number of male flowers releasing pollen and the number of receptive female flowers at discrete intervals across the flowering season.

TIME-DEPENDENT SEED SIZE

To test the hypothesis that seed weight might decrease with the time of its production through the reproductive season, we recorded the date of the beginning of the female phase (as a proxy to the initiation of fruit development) in 21 protogynous and six protandrous trees in 2004, and we measured the weight of their seeds at the end of the season, prior to dispersal. For each tree sampled, we collected and weighed 20 fruits per tree using a Mettler-Toledo AX205 analytical balance (Laboratory and Weighing Technologies, Greifensee, Switzerland). We analyzed the data using a Generalized Linear Mixed Model via Penalized Quasi-Likelihood, with a Gaussian distribution of errors, and with weight being the dependent variable, sexual morph and date (beginning of female phase) treated as fixed factors (with the interaction between these factors included in the model), and individual trees treated as the random factor. This and all other statistical models were run using the R statistical package (R Development Core Team 2007).

CALCULATION OF TIME-DEPENDENT PROSPECTIVE GENDER

We computed the prospective gender of individuals and morphs in our sample by accounting for their sex allocation, their time-dependent mating opportunities, and the possible time-dependent reduction in female fitness over the course of the reproductive season. To do this, we divided the flowering season into T discrete time increments corresponding to our sampling times, and we estimated the number of male and female flowers produced by individual j of morph i at time period t as p_{ijt} and c_{ijt}, respectively. We defined the prospective female and male fitness of individual j in morph i and contributed at time t as

$$w_{f,ij} = (S_i + 1)\sum_t c_{ijt}\varphi_t,$$
$$w_{m,ijt} = \frac{p_{ijt}}{P_t} C_t \varphi_t,$$

respectively, where φ_t is the relative viability of seeds produced at time t, S_i is the (constant) selfing rate of morph i, and C_t and P_t are estimates of the total number of female and male flowers, respectively, in the population during time increment t, with C_t discounted by the relevant selfing rates:

$$C_t = (1 - S_i)\sum_{i,j} c_{ijt}.$$

Selfing rates were estimated from progeny arrays from five protandrous and 22 protogynous mothers, which were genotyped with seven microsatellite markers (see paternity analyses below). Estimates of t_{m}, the multilocus outcrossing rate, were obtained with the program MLTR version 3.1 (Ritland 2002), and selfing rates were calculated as $(1 - t_{\mathrm{m}})$. We estimated the total fitness of an individual, gained at time t, as $w_{T,t} = w_{f,ijt} + w_{m,ijt}$, and its time-dependent prospective functional gender as $G_{ijt} = w_{f,ijt}/w_{T,t}$. Finally, we computed an individual's fitness and gender over the full reproductive season using appropriate summations over all time increments measured.

Because sex expression may be size-dependent in many species (Policansky 1981; Lloyd and Bawa 1984; Klinkhamer et al. 1997; Wright and Barrett 1999; Sarkissian et al. 2001), we also explored the relationship between prospective gender and size. Tree sizes were estimated by measuring the diameters of the stems at 30 cm above ground level; data were then analyzed first by considering only the largest stem, and second by considering the sum of the three largest stems to account for multistemmed trees. The statistical model used was a Generalized Linear Model with a Poisson distribution of errors, in which prospective gender was considered the dependent variable and size a continuous factor.

PREDICTION OF MORPH FREQUENCIES AT EVOLUTIONARY EQUILIBRIUM

To determine whether the sex allocation and mating opportunities estimated in each of the two years of sampling reflect the maintenance of each morph at the observed frequencies, we ran the following recursion equation until phenotype frequencies remained unchanged between generations to order 10^{-3}:

$$f'_i = f_i w_i \div \sum_j f_j w_j,$$

where f_i and $f_i{}'$ are the frequency of the ith phenotype in generations t and $t + 1$, respectively, and division by the summation term ensures that frequencies sum to one (for a similar procedure, see Pannell and Verdú 2006). Here, we assumed the observed frequencies in generation zero and the fitnesses estimated in terms of observed allocations and mating opportunities, summed over the reproductive season.

DETERMINATION OF THE ABILITY OF MORPHS TO INVADE FROM RARITY

To determine whether each of the three morphs could invade a population comprising only the other two morphs at given frequencies, we ran the recursion equation of the previous section, assuming the same allocation strategies for each of the morphs, but with fitnesses calculated on the assumption that the target morph had a frequency of zero and the other two morphs occurred at frequencies that summed to one (see Results). On this basis we then compared the fitnesses of the three morphs; we concluded that a given morph is expected to invade a population of the other two morphs from rarity if its fitness, when rare, was greater than the fitness of the other two morphs.

ESTIMATION OF MALE FUNCTIONAL GENDER BY PATERNITY ANALYSIS

We assessed functional gender by comparing the male siring success of the three sexual morphs through paternity analyses using microsatellite markers. In 2004, we selected 129 flowering trees within an area of approximately of $1000 \times 550\,\mathrm{m}^2$, and we mapped each individual using a submetric precision Global Positioning System (GPS). The sample comprised 50 males, 17 protandrous, and 62 protogynous individuals, all of which were considered as potential fathers in the paternity analyses. Leaves from these trees were collected, dried in silica gel, and then stored at −20°C until DNA extraction. In addition, we selected 22 protogynous and five protandrous mothers situated in the center of the study plot, and 24 fruits from each of these mothers were collected; this resulted in a total sample of 646 offspring available for estimating male siring success (two seeds were discarded because of amplification problems).

Dried plant tissue from each candidate parent was ground to a fine powder in liquid nitrogen, homogenized with a mortar and a pestle and stored at −20°C until DNA extraction. Genomic DNA was isolated from approximately 40 mg of dried homogenized plant material following the Cetyl-Trimethyl-Ammonium Bromide (CTAB) procedure of Doyle and Doyle (1990), with slight modifications. Embryos were carefully isolated from maternal tissues, and their genomic DNA was extracted using a DNeasy Plant Mini Kit (Qiagen). Candidate parents and embryos were genotyped using seven microsatellite loci: Aop122, Aop132, Aop450, Aop820, Aop918, Aop943 (Segarra-Moragues et al., in press), and Map9 (Pandey et al. 2004). The polymerase chain reactions were carried out on a PTC-100 (MJ Research, Waltham, MA) thermalcycler following the protocols described elsewhere (Pandey et al. 2004; Segarra-Moragues et al., in press). The amplified fragments were separated using an ABI 3700 capillary electrophoresis system (Applied Biosystems), and fragments sizes were determined with GENESCAN version 2.1 and GENOTYPER version1.1r8 software (Applied Biosystems, Madrid, Spain), using ROX-400HD as an internal size standard. The microsatellite loci used proved to be highly polymorphic in the study population, yielding a total of 73 alleles in the sample of candidate fathers, and a cumulative paternity exclusion probability (EP) of 0.999. As a consequence, each individual could be identified by a unique genotype.

We used three alternative methods to assess male mating success, including both direct and indirect approaches. First, we used a categorical likelihood-based paternity approach, as implemented in the Famoz program (Gerber et al. 2000, 2003), in which the most-likely father was assigned to each offspring on the basis of LOD scores (log-likelihood ratios, Meagher 1986; Marshall et al. 1998; Gerber et al. 2000). The significance threshold for the LOD scores was determined by simulations, which build an empirical statistical test minimizing both type I and II errors (Gerber et al. 2000). In these simulation runs, we considered a departure from HW equilibrium of 0.1 (as estimated from our data with GENEPOP version 3.4; Raymond and Rousset 1995) and a mistyping error of 0.0001 to be significant. We considered as true fathers only those candidates for which the highest LOD score fell above the threshold value (i.e., no "ties" were considered), and for which father–offspring genotype mismatches could be ruled out. The male reproductive success of each sexual morph was then estimated directly for the subset of progeny for which a father could be assigned, as the proportion of seeds sired by each morph.

In addition, the siring success of the three sexual morphs was estimated using two indirect methods. The first one was a non-spatial Bayesian method of fractional paternity inference implemented in the PATRI program (Nielsen et al. 2001; Signorovitch and Nielsen 2002). This method performs maximum-likelihood analyses of hypotheses regarding the relative male reproductive success of different ecological or behavioral groups (e.g., sexual morphs), and may be more appropriate for open populations because it accommodates incomplete sampling of fathers (i.e., gene flow from outside the population is possible). PATRI provides a maximum-likelihood estimate of α, the relative siring success of the groups defined a priori (in this case, protogynous, protandrous, or male individuals). It also allows different models of male fertility to be compared by likelihood-ratio tests against a null model of equal fertilities ($\alpha = 1$). For non-nested models, we used the minimization of the Akaike information criterion (AIC) to select the best model (Posada and Buckley 2004). Because this method does not allow missing data, we discarded from the analysis two loci suspected of containing null alleles (Aop918 and Aop132). Finally, as the distance between mates can also be an important determinant of male fertility, a second indirect method based on a mating model that includes distance between candidate fathers and mothers was applied using the PatQuest version 4.0 program (Thomas R. Meagher and collaborators, University of St Andrews, U.K). In this method, male fertility is estimated from a log-linear

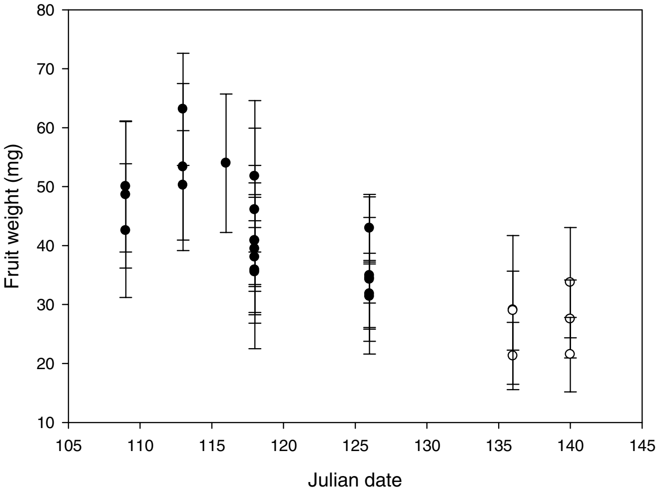

Figure 1. Early flowering of the female phase produces heavier seeds, as shown by the decreasing relationship between fruit weight and time. Each point indicates the mean fruit weight and standard deviation for each sampled mother, with protogynous mothers represented by solid symbols and protandrous mothers represented by open ones.

regression model as follows (Smouse et al. 1999; Elle and Meagher 2000; Vassiliadis et al. 2002; Verdú et al. 2006):

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

where λ_{jk} is the male fertility of the kth father over the jth mother, δ_{jk} is the distance between them, z_k is an indicator variable that represents the sexual group to which the kth father belongs, and γ_d and β are the estimated regression coefficients. The siring success of one sexual morph over any other morph is obtained by calculating exp(β), and the significance of the estimated parameters is assessed by pedigree-based bootstrap permutations using 1000 iterations (Morgan and Conner 2001).

Results

TIME-DEPENDENT SEED SIZE

Seed size (estimated as fruit weight) depended significantly on the time of the initiation of fruit development ($t = -4.61, P = 0.0001$, Fig. 1), and was independent of the sex of the fruit-bearing tree ($t = -1.05, P = 0.30$); the interaction between the time effects and the sexual morph was statistically nonsignificant. In our calculations of gender (below), we thus began by assuming a diminishing value of ϕ that reached 0 at the end of the season. We assessed the sensitivity of our fitness estimates to this assumption by also estimating male and female fitness for the three morphs, for both years, with $\varphi = 1$, that is, by assuming that seed viability remains constant across the season.

PROSPECTIVE FUNCTIONAL GENDER

Selfing rates were low and similar for both heterodichogamous morphs ($S_{PG} = 0.10$ for the protogynous morph and $S_{PA} = 0.13$ for the protandrous morph). Fitness gains accrued to individuals in a highly time-dependent manner. Prospective fitness values were highest through the earliest sexual phases of the heterodichogamous morphs, as can be observed by the highest male fitness values obtained in 2002 for the protandrous morph, and the highest female fitness values obtained both years for the protogynous morph (Fig. 2). Although almost all the population's female function was accomplished by the protogynous morph in both years, male function was mostly achieved by the protandrous morph in the first year of study and mostly by males in the second year (Fig. 2).

Prospective fitness calculations were significantly influenced by mast flowering, particularly with regard to the male function (Table 2). In fact, the differences in fitness between males and protandrous trees were due to an interannual variation in the intensity of male flowering in these two morphs. During the flowering season of 2002, the majority of the male individuals produced few flowers, so that almost all the pollen in the population was dispersed by the protandrous morph (the mean total male flowers ± 1 SE produced by males was 2700 ± 600 and by protandrous trees 19,100 ± 7300; $z = 283$, $P < 0.01$). In contrast, in 2003 males significantly increased their flower production (the mean total male flowers ± 1 SE produced by males and protandrous trees, respectively, were 31,300 ± 1600 and 12,900 ± 2100; $z = -329$, $P < 0.01$), thus achieving more than twice the prospective male fitness of the protandrous morph (Table 2A). The highest female fitness estimates were obtained both years by the protogynous morph, whereas the protandrous morph was prospectively less than 10% as fit as the protogynous morph (Table 2A).

A pattern of sexual specialization was found in the hermaphrodite morphs (Fig. 3). Prospective gender estimates suggest that the protandrous morph was specialized in male function (mean prospective gender of 0.01 for 2002 and 0.1 for 2003), and the protogynous morph specialized in female function (mean prospective gender of 0.81 for 2002 and 0.95 for 2003). Furthermore, we found that sex expression was independent of the size of the trees, as shown by a lack of any correlation between gender estimates and size ($t = -2.61 \times 10^{-14}$, $P = 0.99$ for the case in which only the largest stem was considered; $t = -8.85 \times 10^{-16}$, $P = 0.99$ for the case in which size was estimated as the sum of the largest three stems).

Fitness estimates depended little on whether seed viability was assumed to remain constant or declined over the course of the reproductive season; although female fitness values for the protandrous morph were slightly higher in the case of constant progeny viability (Table 2B), they were still significantly lower than female fitness values of the protogynous morph.

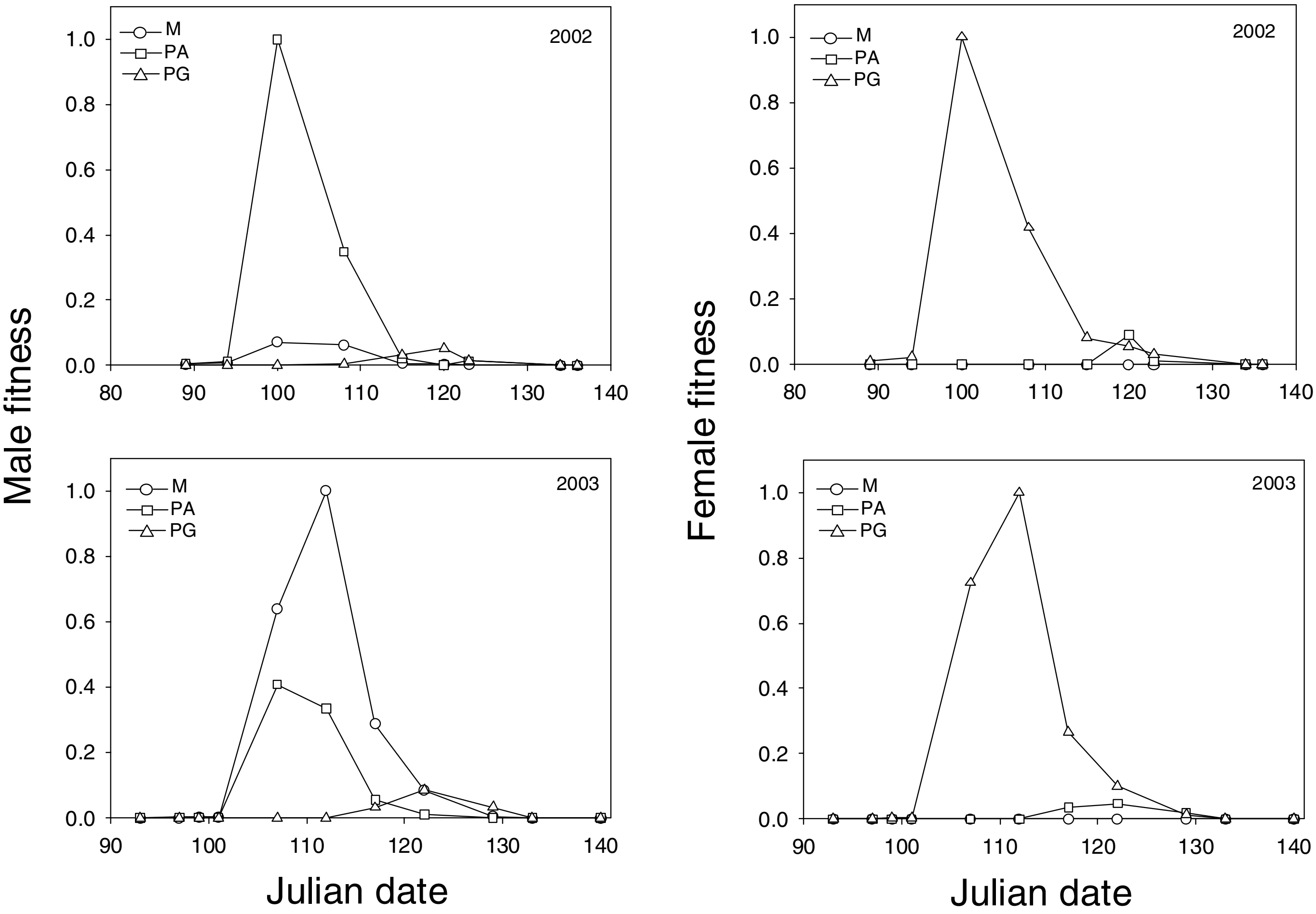

Figure 2. Variation in male and female prospective fitness over the course of the flowering season. Fitness functions were calculated from the sex allocations and flowering frequencies observed in the 2002 and 2003 flowering seasons, under the assumption that seed viability decreases with time. Values were obtained for each point of time and for the three morphs: protogynous (triangles), protandrous (squares), and male (circles). Time is expressed as Julian dates.

MORPH FREQUENCIES AT EVOLUTIONARY EQUILIBRIUM

Our estimates of the predicted morph frequencies at equilibrium reflected the between-year variation in male flower production by male and protandrous individuals. Thus, in 2002 the observed sex allocations and the context of mating implied the maintenance of a purely heterodichogamous population at equilibrium (i.e., they implied the loss of the male morph), whereas in 2003 the gender distribution was consistent with the selection of a gender dimorphism (i.e., the maintenance of males with female-specialized protogynous trees; Table 3). Although selection on the male and protandrous strategies was found to fluctuate from one year to the next, in both years it favored the establishment of the protogynous morph at frequencies of around 0.5. The estimates of the equilibrium frequencies depended very little on whether φ remained constant or decreased over time, and so we present only estimates obtained for decreasing φ, in accord with our empirical estimates (Table 3).

ABILITY OF MORPHS TO INVADE FROM RARITY

Fitness estimates calculated for hypothetical dimorphic populations showed that the protogynous morph should be expected to increase in frequency when invading a population composed of males and protandrous trees, as both years it obtained the greatest total fitness when the initial frequency was set to zero (Table 4A). Frequency-dependent selection favored the invasion and maintenance of males in populations composed of protandrous and protogynous trees only under the sex allocation and mating context observed in 2003 (Table 4B), in which males obtained more than twice the male fitness of the protandrous morph (Table 2A). Alternatively, the invasion and maintenance of the protandrous morph could only be explained under the allocation pattern and phenology of 2002 (Table 4C), in which this morph represented almost all the male function in the population (Table 2A). Again, results depended little on whether φ was constant or decreasing, and are thus only presented for decreasing φ (Table 4).

Table 2. Male, female, and total prospective fitness for the three morphs, calculated for 2002 and 2003. Prospective fitness estimates were obtained under the assumption that (A) seed viability falls linearly with progression of the flowering season, and that (B) seed viability remains constant throughout the season. Values were standardized according to the highest value within each category.

	M	PA	PG
(A) *Linear decrease in seed viability*			
2002			
Male fitness	0.109	1	0.070
Female fitness	0	0.062	1
Total fitness	0.104	1	0.836
2003			
Male fitness	1	0.404	0.075
Female fitness	0	0.047	1
Total fitness	0.474	0.237	1
(B) *Constant seed viability*			
2002			
Male fitness	0.115	1	0.137
Female fitness	0	0.121	1
Total fitness	0.104	1	0.911
2003			
Male fitness	1	0.379	0.125
Female fitness	0	0.076	1
Total fitness	0.461	0.246	1

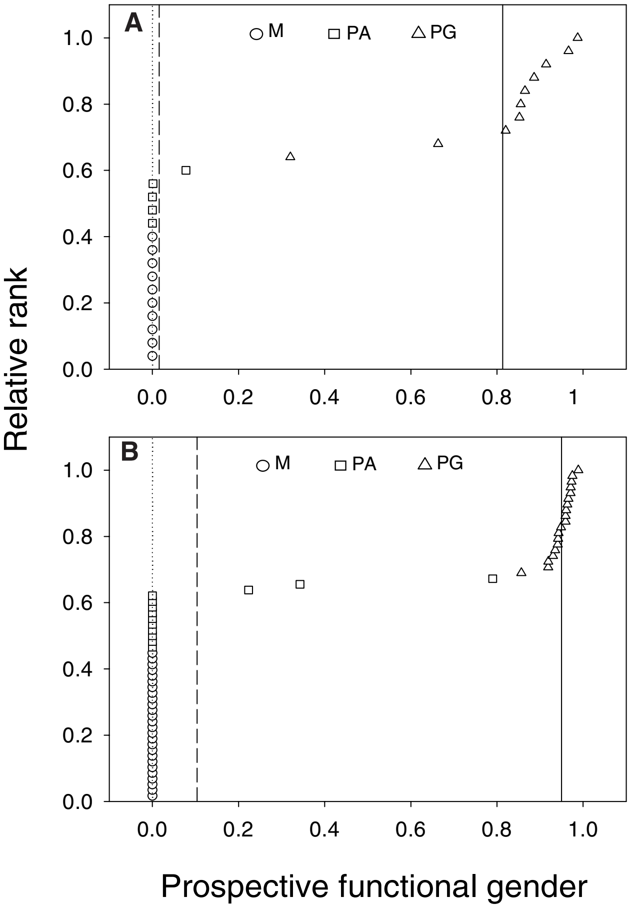

Figure 3. Prospective functional gender (functional femaleness) of males (circles), protandrous (squares), and protogynous (triangles) trees in 2002 (A) and 2003 (B). Vertical lines show the mean values of prospective functional gender for the protogynous (solid line), protandrous (dashed line), and male (dotted line) morphs.

PATERNITY ANALYSES: MATING PATTERNS AND SIRING SUCCESS

A single most-likely father could be assigned to 240 seeds (37%). Among these, 105 seeds were sired by males, 46 by protandrous, and 89 by protogynous fathers. Because the number of candidate fathers from each sexual morph was unbalanced, these values had to be recalculated taking into account the number of genotyped sires of each sexual morph. The relative siring success, after controlling for an unbalanced number of sires, was 0.53 for protogynous, 0.78 for males, and 1.0 for protandrous fathers. Mating unions (excluding selfing events) were predominantly disassortative: there were no intramorph matings observed within the protandrous morph, whereas 21.2% of matings occurred between protogynous individuals.

Using Nielsen's Bayesian approach (Nielsen et al. 2001; Signorovitch and Nielsen 2002), we could reject the null model that morphs do not differ in their siring ability (Table 5). Rather, the best-fitting model indicated that the siring success of protogynous fathers was 0.57 that of males and protandrous trees, which did not differ from one another (i.e., $\alpha_{PG} = 0.57$, $\alpha_M = \alpha_{PA} = 1$; Table 5). Similarly, we can reject a model that assumes that mating success was independent of the interplant distance; the spatially explicit log-linear regression model was significantly better than the null model (LR test = 476.6, df = 2, $P < 0.0001$), with the significantly negative estimate of the distance parameter indicating increased mating among neighbors than among more spatially separated individuals s ($\gamma_d = -1.005$, $P < 0.0001$). This analysis also showed that protogynous individuals enjoyed significantly lower siring success than males and protandrous individuals ($\beta = -1.34$, $P = 0.02$). Given that $\exp(\beta) = 0.26$, this analysis suggests that the relative siring success of the protogynous sex morph was approximately half that estimated on the basis of the other two methods above.

Discussion

Our study shows that mating in *A. opalus* is nonrandom, being determined both by phenology and by the distance to mates. Matings were predominantly disassortative, although intramorph crosses were also detected within the protogynous morph. Our simulations indicate that the protogynous morph is being maintained in the populations by frequency-dependent selection; however,

Table 3. Equilibrium frequencies and prospective fitness for the three morphs, estimated from the sex allocations and phenology observed in 2002 and 2003, under the assumption that seed viability decreases linearly with time (see text for details).

	M	PA	PG
2002			
Male fitness	0.121	1	0.095
Female fitness	0	0.062	1
Total fitness	0.114	1	1
Equilibrium frequency	0	0.421	0.579
2003			
Male fitness	1	0.429	0.039
Female fitness	0	0.047	1
Total fitness	1	0.475	1
Equilibrium frequency	0.428	0	0.572

males and protandrous trees seemed to be competing as male parents, and the outcome of this competition depended on the mast flowering observed in these morphs. Our results also indicate that seed size in *A. opalus* (a proxy to female fitness) decreases with the progression of the flowering season, and that the heterodichogamous morphs are sexually specialized in the sexual phase they first express. Overall, our findings are consistent with the theoretical scenario modeled by Pannell and Verdú (2006), where sexual specialization, driven by a decrease in female fitness with time, eases the conditions for the appearance of male specialists.

HETERODICHOGAMY, GENDER INCONSTANCY AND SEXUAL SPECIALIZATION

In an evolutionary stable heterodichogamous population, the two genetic morphs (protandrous and protogynous) are expected to be found at equal frequencies (Gleeson 1982). Accordingly, in *A. opalus*, protogynous individuals comprise half of the population, whereas the other half is comprised of males and protandrous trees. During the course of a six-year survey, we observed that the majority of males were consistent in their sex expression, but that some individuals switched their sex expression between pure male and protandrous hermaphrodite. In contrast, all individuals classed as protogynous remained in their class for the duration of the six-year study. Sexual lability thus appears to be restricted to a few individuals that alternate between male and protandrous flowering strategies. This pattern of sex expression in *A. opalus*, together with the sex ratios, suggests that, in an ancestral heterodichogamous population, the emergence of sex inconstancy in protandrous individuals may have predated and triggered the appearance of pure males. A similar sexual structure was reported by Sato (2002) for *A. japonicum*, where sexual inconstancy was only observed in some males that switched to protandrous flowering.

Table 4. Hypothetical fitness values obtained for three different scenarios: (A) populations without protogynous individuals, (B) populations without males and (C) populations without protandrous trees, under the assumption that seed viability decreases with time. Fitness values were standardized with respect to the highest value of each row.

	M	PA	PG
(A) *Populations without protogynous trees*			
Frequency	0.5	0.5	0
2002			
Male fitness	0.059	0.012	1
Female fitness	0	0.062	1
Total fitness	0.024	0.042	1
2003			
Male fitness	0.511	0.069	1
Female fitness	0	0.047	1
Total fitness	0.030	0.049	1
(B) *Populations without males*			
Frequency	0	0.5	0.5
2002			
Male fitness	0.123	1	0.140
Female fitness	0	0.062	1
Total fitness	0.084	0.743	1
2003			
Male fitness	1	0.370	0.051
Female fitness	0	0.047	1
Total fitness	1	0.394	0.544
(C) *Populations without protandrous trees*			
Frequency	0.5	0	0.5
2002			
Male fitness	0.088	1	0.01
Female fitness	0	0.062	1
Total fitness	0.088	1	0.130
2003			
Male fitness	1	0.427	0.044
Female fitness	0	0.047	1
Total fitness	0.757	0.369	1

Within flowering plants, the existence of gender variation is a common phenomenon (Lloyd and Bawa 1984). Gender may vary in response to a plant size, health, or internal resource status, or in response to environmental conditions. In *A. pensylvanicum*, for example, directional changes from male to female flowering with progressive forest crown closure were reported by Hibbs and Fischer (1979), who suggested that sex changes in this species might constitute a life-cycle strategy in which plants are first male and then female as they age. Gender inconstancy differs from such "gender diphasy" (Lloyd and Bawa 1984; Schlessman 1988), in that individuals in the former category are specialized in their gender over their lifetime, whereas those in the latter category belong

Table 5. Three alternative hypotheses of male fertility were tested against a null hypothesis of equal fertility using the PATRI software. In the first alternative model, the siring success of males and protandrous individuals were fixed to be equal, and the relative siring success of protogynous fathers was estimated (H_1); in the second alternative model (H_2), the siring success of protandrous and protogynous sires were fixed, and the siring success of males was estimated; and in the third model, the siring success of protandrous individuals was calculated, whereas for the other two sex morphs success were fixed (H_3). The three models were tested against the null one by means of log-likelihood ratio tests (LR tests). α_M, α_{PA}, and α_{PG} are the relative siring success of males, protandrous, and protogynous sex morphs, respectively. Estimates were obtained by assuming a prior of 200 potential fathers in the population; priors of 400 and 600 potential fathers were also assumed, but the estimates are not shown because they were similar to the ones presented here.

		Log-likelihood	df	LR test	*P*-value	AIC	α_M	α_{PA}	α_{PG}
H_0	$\alpha_M=\alpha_{PA}=\alpha_{PG}$	−5243.45	0	–	–	10,487	–	–	–
H_1	$\alpha_M=\alpha_{PA}$; α_{PG}	−5236.68	1	13.538	0.0002 *	10,475	–	–	0.57
H_2	$\alpha_{PA}=\alpha_{PG}$; α_M	−5241.89	1	3.116	0.07	10,486	1.30	–	–
H_3	$\alpha_M=\alpha_{PG}$; α_{PA}	−5239.31	1	8.294	0.004 *	10,481	–	1.78	–

$^*P < 0.05$

to a single gender class that may switch their sexuality in any season according to circumstances. Lability is in fact a common feature of many gender dimorphisms, such as dioecy, subdioecy, and gynodioecy (Lloyd and Bawa 1984; Wolfe and Shmida 1997; Barrett et al. 1999). In some subdioecious populations, for example, females are usually canalized in their sex expression, whereas the pollen-bearing morphs (males and hermaphrodites) may also produce a few fruits from time to time. Gender variation in the sexually dimorphic populations of *A. opalus* would appear to constitute a special case of this type of gender inconstancy, where the female specialists (protogynous individuals) show a canalized pattern of sex expression, and the males and protandrous individuals may vary in their sex expression from one season to the next, irrespective of their size. In fact, our observations in *A. opalus* are similar to those reported for *A. rubrum* (Primack and McCall 1986; Sakai 1990), and *A. japonicum* (Sato 2002), two other species in the genus whose populations are sexually dimorphic.

TIME-DEPENDENT SEED WEIGHT AS A DRIVER OF SEXUAL SPECIALIZATION

Our results suggest that reproductive success through the female phase may decrease with time over the reproductive season, as late flowering resulted in the production of smaller fruits, probably due to a decrease in resource availability as the season progresses. Flowering in this Mediterranean species starts in spring, and the filling of fruits ends in summer, when temperatures are high and soil moisture levels are low. Because fruits produced earlier enjoy a longer developmental period, it would appear that protandrous individuals, which delay the beginning of their female function, produce smaller fruits. If seeds in these fruits are less viable, as we might expect (see below), delayed fruiting should reduce the female function of protandrous plants and thus skew their gender toward increased maleness. Furthermore, given that the mean functional gender in a population must be 0.5 (Lloyd 1980; Lloyd and Bawa 1984), a necessary consequence of increased male function in the protandrous morph is an enhanced female function in the protogynous morph, driven by the low availability of ovules toward the end of the flowering period and accentuated by the greater time available for the development of their fruits. Accordingly, our calculations indicate that the prospective functional gender of the protogynous and protandrous morphs of *A. opalus* were strongly female- and male-biased, respectively.

Variations in seed size may result in differences in seed performance, and thus may be an important trait upon which selection may act. Although the evidence is to some extent species-dependent, in many cases heavier seeds are more successful than smaller ones within a species (Stanton 1984; Jones et al. 1997; Baskin and Baskin 1998; and many references therein). In *A. opalus*, specifically, it is known that germination rates decrease significantly with decreasing seed weight (logistic regression with $R^2 = 0.26$, $P < 0.0001$, $n = 759$ seeds, L. Gómez-Aparicio and E. Baraza, pers. comm.). Because seedling emergence in this species begins in spring, a delay may be unfavorable because later emergents will face the lower moisture availability and high temperatures of the Mediterranean summer before they have established a sufficient root system (Verdú and Traveset 2005). Confirmation of these hypotheses awaits further experimental work.

FREQUENCY-DEPENDENT SELECTION AND THE EVOLUTIONARY MAINTENANCE OF THE SEXUAL MORPHS

When levels of disassortative mating are greater than random mating, sexual polymorphisms are expected to be evolutionarily maintained by negative frequency-dependent selection (Gleeson 1982; Baker et al. 2000; Barrett 2002). Our likelihood-based categorical paternity analysis showed that matings were mainly disassortative in *A. opalus*; whereas no intramorph crosses occurred within the protandrous morph (other than a few selfing events), only a 21%

of the seeds sired by protogynous trees were assortative crosses. *Acer opalus* was found to be mainly outcrosser, although the fact that a small number of seeds were self-fertilized suggests that the species is at least partially self-compatible. Disassortative mating would thus appear to be a consequence of the reciprocal phenology of the morphs, rather than of physiological self-incompatibility as in many other polymorphic species (reviewed in Barrett 2002).

The effect of frequency-dependent selection assessed by simulations was evident for the protogynous morph, which obtained the greatest fitness as a female parent in both years, irrespective of its initial frequency. Moreover, when the initial frequency of this morph was set to zero, it also had the greatest fitness as a male parent, indicating that selection should favor an increase in frequency of late pollen dispersers, which are the only ones able to fertilize the ovules produced by the protandrous morph. The results of the simulations were different for the male and protandrous morphs: the maintenance of these morphs as a consequence of their fitness gains as male parents depended significantly on the strong variation in the male flower production of these morphs. In other words, the interannual fluctuations in male flower production produced fluctuations in the direction of selection, favoring the maintenance of males one year (with the displacement of the protandrous morph) and the maintenance of protandrous individuals the following year (with the consequent displacement of males). These results also demonstrate that males and protandrous trees tend to compete directly with one another as male parents. It is important to note that our results are based on samples from a single population; sampling across populations is needed to generalize our results further.

SIRING SUCCESS AND THE APPEARANCE OF MALES IN HETERODICHOGAMOUS POPULATIONS

Unlike other heterodichogamous systems (e.g., Bai et al. 2007), gene flow through pollen was found to be unequal among morphs. Paternity analyses showed that the siring success of the protogynous morph was only a fourth of that enjoyed by both males and protandrous individuals once the effects of isolation by distance were accounted for. The best-fitting model in the fractional paternity analysis indicated that the siring success of males and protandrous morphs were similar. Pannell and Verdú (2006) demonstrated theoretically that when the ancestral population is dimorphic, such as in heterodichogamous populations, male mutants may appear even if they do not have a higher fertility, provided there is some degree of sexual specialization in the hermaphrodites. Our study now demonstrates the existence of sexual specialization in an heterodichogamous species, with fitness assessed with molecular markers. Our paternity analysis showed that the protandrous morph is sexually specialized toward maleness, being as successful as a pollen donor as the extreme male specialists. We therefore propose that selection against a delayed female function may have induced sexual specialization in protandrous individuals, thereby favoring the appearance of pure males.

Concluding Remarks

Our study shows that *A. opalus* is essentially dimorphic in gender, with protogynous individuals belonging to a mainly female class and males and protandrous individuals, together, belonging to a male class. Our results are consistent with predictions of Pannell and Verdú’s (2006) model and thus suggest that the gender specialization in *A. opalus* may have evolved as a result of the spread of male individuals, that is, via a potentially androdioecious path. However, gender switching in some individuals between male and protandrous hermaphroditic sex expression indicates that the sexual system of this species is more complex than those modeled by Pannell and Verdú (2006), and these complexities do not seem to be unusual in the genus.

The variation among species of *Acer* in their sexual systems prompted speculations by de Jong (1976) regarding the evolutionary paths that might link them. More recently, evolutionary hypotheses have been tested by two different comparative analyses in which ancestral states were reconstructed (Gleiser and Verdú 2005; Renner et al. 2007). These studies have reached somewhat different conclusions about sexual-system evolution in the genus, largely as a result of different interpretations of sexual-system categories for the included species. In particular, Renner et al. (2007) have questioned the existence of heterodichogamous sexual systems within the genus *Acer*. However, the sex ratios observed in *A. opalus*, together with surveys on sex expression over six years and the mating patterns inferred from our paternity analysis, provide fairly convincing evidence that this species of *Acer*, at least, is indeed heterodichogamous.

Renner et al. (2007) also doubted the existence of gender specialization in *Acer*, arguing (p. 2716) that “the ability of some fraction of trees to change sex expression set up conditions that slowed down or prevented the evolution of sex specialization (dioecy).” In contrast, our paternity analysis in a natural population would seem to confirm the existence of sexual specialization in *A. opalus*. Macroevolutionary studies such as those of Gleiser and Verdú (2005) and Renner et al. (2007) may be useful in generating and testing hypotheses, but population-level studies, such as ours on *A. opalus* and those cited above on other species of the genus, point to their limits. Recently, one of us (JRP) participated in a workshop on plant reproduction biology in China in which several experienced plant reproductive ecologists failed to agree on how to characterize the sexual system of *A. truncata* after several days of intense sampling. That experience, and our study of the sex expression of *A. opalus* over several years, urge caution against drawing conclusions on the basis of limited population-level sampling across the genus.

ACKNOWLEDGMENTS

We thank the staff of the Parque Natural Carrascal de la Font Roja for giving us permission to perform this study in the natural park. This study was funded by a MEC/CICYT (REPROFOR AGL2005-07440-C02-02/FOR) project grant to MV, a I3P/CSIC postdoctoral grant to JGS-M and a FPU/MEC Ph.D. grant to GG.

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Associate Editor: J. Shykoff