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Siring Success and Paternal Effects in Heterodichogamous *Acer opalus*

GABRIELA GLEISER[1,*], JOSÉ GABRIEL SEGARRA-MORAGUES[1],
JOHN RICHARD PANNELL[2] and MIGUEL VERDÚ[1]

[1]*Centro de Investigaciones sobre Desertificación (CSIC-UV-GV), Camí de la Marjal s/n,*
Apartado Oficial, 46470 Albal (Valencia), Spain and [2]*Department of Plant Sciences, University of Oxford,*
South Parks Road, Oxford OX1 3RB, UK

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- *Background and Aims* Heterodichogamy (a dimorphic breeding system comprising protandrous and protogynous individuals) is a potential starting point in the evolution of dioecy from hermaphroditism. In the genus *Acer*, previous work suggests that dioecy evolved from heterodichogamy through an initial spread of unisexual males. Here, the question is asked as to whether the different morphs in *Acer opalus*, a species in which males co-exist with heterodichogamous hermaphrodites, differ in various components of male in fitness.
- *Methods* Several components of male fertility were analysed. Pollination rates in the male phase were recorded across one flowering period. Pollen viability was compared among morphs through hand pollinations both with pollen from a single sexual morph and also simulating a situation of pollen competition; in the latter experiment, paternity was assessed with microsatellite markers. It was also determined whether effects of genetic relatedness between pollen donors and recipients could influence the siring success. Finally, paternal effects occurring beyond the fertilization process were tested for by measuring the height reached by seedlings with different sires over three consecutive growing seasons.
- *Key Results* The males and protandrous morphs had higher pollination rates than the protogynous morph, and the seedlings they sired grew taller. No differences in male fertility were found between males and protandrous individuals. Departures from random mating due to effects of genetic relatedness among sires and pollen recipients were also ruled out.
- *Conclusions* Males and protandrous individuals are probably better sires than protogynous individuals, as shown by the higher pollination rates and the differential growth of the seedlings sired by these morphs. In contrast, the fertility of males was not higher than the male fertility of the protandrous morph. While the appearance of males in sexually specialized heterodichogamous populations is possible, even in the absence of a fitness advantage, it is not clear that males can be maintained at an evolutionary equilibrium with two classes of heterodichogamous hermaphrodites.

Key words: *Acer opalus*, heterodichogamy, male fertility, microsatellites, paternal effects, pollen competition, pollination rates, genetic relatedness.

INTRODUCTION

The repeated evolution of dioecy in flowering plants has attracted the attention of biologists ever since Darwin (1877) first wondered about the advantages that might accrue to plants that gave up one or other of their sexual functions. One hypothesis is that dioecy may evolve from hermaphroditism as a result of selection for inbreeding avoidance (B. Charlesworth and D. Charlesworth, 1978; Thomson and Barrett, 1981; Webb, 1999). An alternative, though not mutually exclusive, idea (Charnov *et al.*, 1976; B. Charlesworth and D. Charlesworth, 1978; Freeman *et al.*, 1997), is that dioecy may evolve through a gradual process of gender specialization and frequency-dependent selection (Darwin, 1877; Lloyd, 1979; Willson, 1979; Givnish, 1980). This second process might occur, for example, in monoecious populations in which one class of individual produces increasingly more male flowers, with the other class becoming increasingly female (Lloyd, 1972; D. Charlesworth and B. Charlesworth, 1978; Lloyd, 1980; Renner and Ricklefs, 1995; Renner and Won, 2001).

Gender specialization might be particularly likely as a path to dioecy in hermaphroditic populations that already possess floral dimorphisms, with each of the two morphs specializing increasingly in one or other of the two genders. For example, heterostyly might evolve towards dioecy if long- and short-styled plants increasingly accentuate their female and male functions, respectively; indeed, there is good evidence that this path has been followed in a number of species (Ornduff, 1966; Lloyd, 1979; Muenchow and Grebus, 1989; Barrett, 2002). Dioecy might also evolve from heterodichogamy, where the protandrous and protogynous morphs become increasingly male and female, respectively (Pendleton *et al.*, 1988, 2000; Sato, 2002; but see Dommée *et al.*, 1990, for the opposite transition). In both cases, the evolution of dioecy may be triggered if there is an asymmetry between the sexual morphs in the amount of fitness gains through each sexual function (Lloyd, 1979; Pannell and Verdú, 2006), or if the incompatibility system typical of heterostylous species is lost, with selection then favouring alternative means of avoiding self-fertilization (Ornduff, 1966; Lloyd, 1979).

The heterodichogamous pathway to dioecy has received less attention than the heterostylous path (Webb, 1999;

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* For correspondence. E-mail gabriela.gleiser@uv.es

but see Renner, 2001). However, heterodichogamy is phylogenetically widespread (Renner, 2001), and dioecy has been noted in several families with heterodichogamous members (de Jong, 1976; Kubitzki and Kurz, 1984; Pendleton *et al.*, 1988; reviewed in Renner, 2001). Recently, Pannell and Verdú (2006) modelled the conditions for the spread of unisexuals in heterodichogamous populations in which a sexual asymmetry was the result of decreasing fitness returns on female function as the flowering season approached its end. This model showed that males or females could invade a heterodichogamous population even if their fitness was only slightly greater than that of the male or female fitness of hermaphrodites, respectively; whereas it is well known that, for unisexuals to invade monomorphic outcrossing populations, they need to be at least twice as fit as the corresponding sexual function of the hermaphrodites (Lloyd, 1975; B. Charlesworth and D. Charlesworth, 1978; Charlesworth, 1984; Pannell, 2002). Pannell and Verdú's (2006) model also predicted morph frequencies at equilibrium following the initial invasion of males or females. Two possibilities exist: either an equilibrium is established in which unisexuals are maintained with both protandrous and protogynous individuals, or the unisexuals completely displace one of the hermaphrodite morphs.

Evaluation of the relative fitness of different morphs requires an assessment of their average reproduction contribution through both male and female functions. Female fitness is relatively easily estimated in terms of the numbers and quality of seeds produced. In the case of male function, reproductive success can be affected both by pollination success and post-pollination processes (Bertin, 1988; Marshall and Folsom, 1991; Stanton, 1994). In insect-pollinated species, for example, pollen flow is determined by pollinator behaviour (Beach and Bawa, 1980; Stanton *et al.*, 1986; Stanton, 1994), but also by variation in the ability of pollen grains to fertilize ovules following particular pollination events (Bertin, 1988; Willson, 1994; Morand-Prieur *et al.*, 2003; Verdú *et al.*, 2004*a*). There is some evidence for differential fertilization success between different sires, with selection amongst pollen grains favouring those sires that produce the most vigorous pollen tubes (Mulcahy, 1979; Marshall, 1998; Pasonen *et al.*, 1999; Aronen *et al.*, 2002; Morand-Prieur *et al.*, 2003). Alternatively, non-random mating may be mediated by genetic relatedness between the pollen and the seed parent, either through pollen discrimination by stigmas (e.g. 'female mate choice', Waser and Price, 1993; Souto *et al.*, 2002), self-incompatibility systems (Matton *et al.*, 1994; Silva and Goring, 2001), or as a result of the effects of inbreeding and outbreeding depression (Willson and Burley, 1983; D. Charlesworth and B. Charlesworth, 1987; Waser and Price, 1994). Finally, differences in siring success may extend beyond the reproductive stages, where post-zygotic selection may act on offspring genotypes sired by different male parents (Marshall and Whittaker, 1989; Marshall and Folsom, 1991; Willson, 1994; Verdú *et al.*, 2004*a*). Paternal effects on progeny quality are almost unknown, but they have seldom been sought. Recently, they were reported in cryptically dioecious populations of the long-lived woody angiosperm *Fraxinus ornus*, where progeny with male fathers grew faster than those sired by hermaphrodites (Verdú *et al.*, 2004*a*), and it is thus possible that they may be more common.

In this paper, we conduct a functional analysis of the sexual system of the heterodichogamous species *Acer opalus*, focusing on pre- and post-pollination effects on male fitness (i.e. pollination rates, pollen viability, pollen performance in competition, genetic incompatibility and post-zygotic effects). Within the genus *Acer*, the evolution of unisexuality started with an initial spread of unisexual males into heterodichogamous populations (de Jong, 1976; Sato, 2002; Gleiser and Verdú, 2005). In *A. opalus*, males already co-occur with protandrous and protogynous morphs. In this study, an estimation was made of the siring success of the three sexual morphs, aiming to determine: (1) whether the siring success of males is greater than in the other two (hermaphroditic) morphs; and (2) whether there is evidence of sexual specialization in the hermaphrodites, manifested as differences in their siring success. It was found that males and protandrous individuals enjoy increased pollinator visitation and, remarkably, we document a further example of morph-specific paternal influences on offspring quality. The results are discussed in terms of observed sex ratios from a number of populations and theoretical predictions for the maintenance of males with protandrous and protogynous hermaphrodites (Pannell and Verdú, 2006).

MATERIALS AND METHODS

Study species, study population and sex ratios

Acer opalus is a deciduous tree with populations composed of males and protandrous and protogynous individuals. Flowering occurs in spring and is characterized by a strong inter-annual variation in intensity (masting). Sexual inconstancy occurs in the protandrous morph, which may fail to express the female phase some years (termed inconstant protandrous trees hereafter; Verdú *et al.*, 2004*b*), but never in the protogynous trees (which always express both sexual phases when they flower). The flowers are functionally unisexual because of abortion of the pistil or incapacity of the anthers to open, and are arranged in inflorescences that are mixed (with male and female flowers) in the case of the hermaphrodite morphs. Both types of flowers are actinomorphic, with a yellowish perianth that encloses a nectary. The ovaries have two locules with two ovules each, although fertilization rarely occurs in more than one of the ovules. The indehiscent fruits are schizocarps with two one-seeded samaroid mericarps. Non-fertilized female flowers normally develop into sterile parthenocarpic fruits (de Jong, 1976). Fruit development is completed by summer and almost all the fruits are dispersed by wind in autumn.

The study was conducted in a wild population of *A. opalus* in the Font Roja Natural Park, in eastern Spain (UTM 30SYH1482); the population is located on a north-facing slope at about 1250 m a.s.l.. The area experiences a Mediterranean climate, with cold winters and a dry, hot summer that lasts for over 3 months. The study population is composed of 48 % of protogynous, 39 % of male and 13 % of protandrous trees, with the latter including constant and inconstant protandrous trees (Verdú *et al.*, 2004*b*; Fig. 1). Sex ratios were also checked in six other populations situated in south-eastern Spain (see co-ordinates in Fig. 1).

Pollination effects

Hesse (1979) reported *A. opalus* to be both insect- and wind-pollinated on the basis of pollen morphology. To test this hypothesis (i.e. to determine the relative roles of wind and insect pollinations in this species), pollination was assessed under experimental conditions in the field. Twelve protogynous trees were selected prior to anthesis; protandrous trees were not used in this experiment because the low number of female flowers that they produce does not allow to perform all the treatments within a single mother (and thus to control the maternal effects). In each tree, one to three inflorescences were enclosed in bags that allowed the passage of air-borne pollen but excluded pollinators. Unbagged branches were also tagged as controls for open pollination (wind plus insect pollination). Soon after the beginning of the female phase, the unopened male flowers were removed and the number of female flowers were counted in both the bagged inflorescences and the control branches. The following autumn, the fruits obtained from these treatments were harvested and the number of viable seeds obtained were scored in each treatment. The percentage of viable seeds was compared between treatments using a generalized linear model (GLM) with a binomial distribution of errors, in which treatment was considered as a fixed effect. Generalized linear models allow dealing with non-

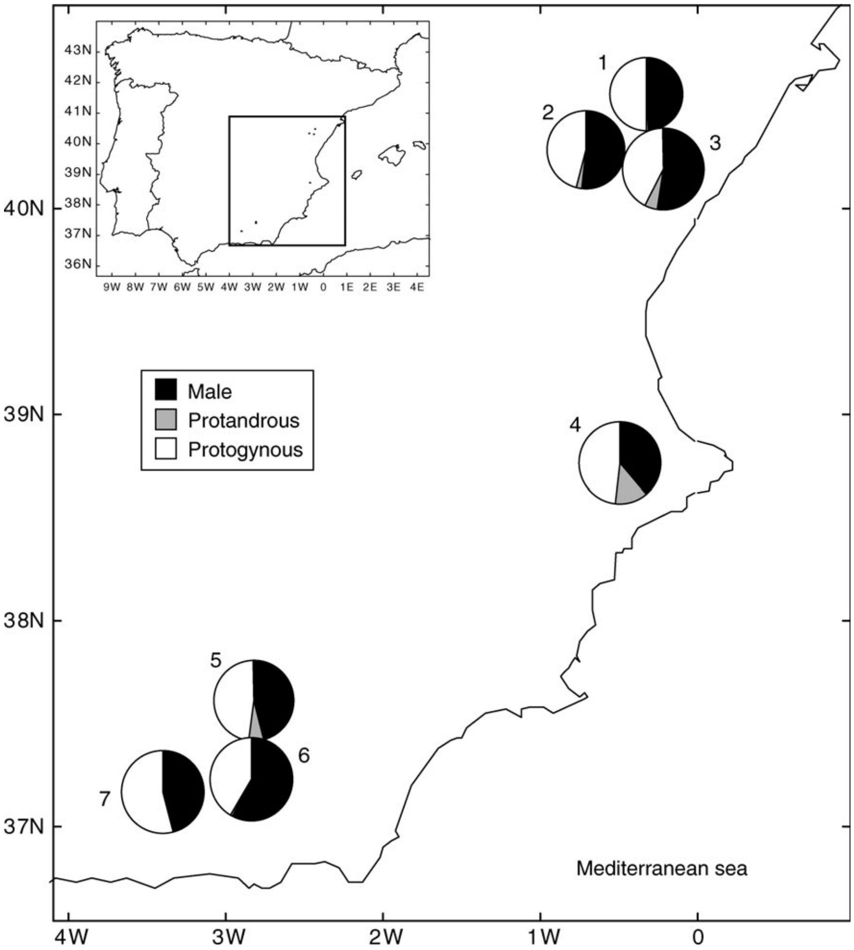

FIG. 1. Sex ratios and the location of seven populations of *A. opalus* surveyed in south-eastern Spain. The pie charts show the proportion of the male, protandrous and protogynous morphs. Proportions were obtained on the basis of trees in flower. The populations sampled were (from north to south): 1, Villafranca del Cid (UTM 30TYK2778), with 76 flowering trees and 20 non-flowering trees; 2, Linares de Mora (UTM 30TYK0662), with 44 flowering trees and 81 non-flowering trees; 3, Peñagolosa (UTM 30TYK2359), with 23 flowering trees and 20 non-flowering trees; 4, Font Roja (UTM 30SYH1482), with 129 trees; 5, Barranco de la Canaleja, Sierra de Baza (UTM 30SWG1438), with 63 flowering trees and 15 non-flowering trees; 6, Calar de San Sebastián, Sierra de Baza (UTM 30SWG1433), with 70 flowering trees and 15 non-flowering trees; and 7, Sierra Nevada (UTM 30SVG5904), with 50 flowering trees and 20 non-flowering trees. Sex ratios from the Font Roja population were obtained from 129 trees whose sex expression was followed over the course of 6 years; thus, all the trees showing sexual inconsistencies were identified in this population, so that they were not mistaken as males. Note that the ratio M + PA : PG did not depart significantly from 1 : 1 in any population ($\chi^2_{pop1} = 0$, $P = 1$; $\chi^2_{pop2} = 0{\cdot}36$, $P = 0{\cdot}55$; $\chi^2_{pop3} = 0{\cdot}39$, $P = 0{\cdot}53$; $\chi^2_{pop4} = 0{\cdot}19$, $P = 0{\cdot}66$; $\chi^2_{pop5} = 0{\cdot}14$, $P = 0{\cdot}70$; $\chi^2_{pop6} = 2{\cdot}06$, $P = 0{\cdot}15$; $\chi^2_{pop7} = 0{\cdot}21$, $P = 0{\cdot}65$).

normal error distributions, different variance-to-mean relationships and correlated data by implementing a link function describing the relationship between the linear predictor and the mean of the distribution function (McCullagh and Nelder, 1989). The model described above as well as the others reported below were run with the help of the MASS (Venables and Ripley, 2002) and the nlme (Pinheiro *et al.*, 2006) packages of R (R Development Core Team, 2006).

Pollination rates among sexual morphs were compared in 2004; these were estimated as the number of pollinator visits to a tree within a period of 2 min. We selected 22 adult trees of similar sizes (seven males, five protandrous and ten protogynous trees) along a transect in a scattered way in order to avoid positional and temporal effects (location of the tree and date of recording). Pollination rates were recorded between 1000 h and 1400 h on sunny days when the highest pollinator activity was expected to occur. Censuses were made every 4 to 5 days during the course of the entire flowering season. The number of pollinators on focal trees was counted from a point sufficiently distant to avoid disturbing the foraging behaviour of the pollinators, and also in order to be able to monitor the entire canopy of the sampled tree. As the leaves of this deciduous species begin to emerge at the early end of the flowering season, it was easy to count the number of pollinators visiting the flowers without the visual interference of the leaves. In addition, the number of male flowers releasing pollen was estimated in each focal tree every time a pollinator census was made. The pollination rates in the male stage were compared among sexual morphs across the flowering season by means of a generalized linear mixed model via penalized quasi-likelihood (glmmPQL) with a Poisson distribution of errors, with sexual morph, number of flowers and time treated as fixed factors, and tree as a random grouping factor. An autoregressive of order 1 covariance structure (corAR1) was introduced into the model to account for the temporal correlation between the measures (Pinheiro and Bates, 2000). We also tested whether the number of male flowers differed among the three sexual morph by means of a glmmPQL with a Poisson distribution of errors, where sexual morph was considered a fixed factor and tree a random factor.

Post-pollination effects: siring success in the absence of pollen competition

The siring success of the three sexual morphs was compared by means of controlled pollinations in the field. Twelve protogynous trees were selected before the beginning of the flowering period in 2003, and the flowering buds were enclosed using pollen-proof bags. Protandrous trees were not used as ovule donors because of the low number of female flowers that they normally produce (see above). Once the female flowers opened, the immature male flowers were removed from the bagged inflorescences to avoid interference from self pollen on the stigmas. Receptive stigmas were then brushed with pollen from males, protandrous and protogynous trees. To control for maternal effects, each mother received pollen from the three different pollen donors. Nevertheless, as a consequence of phenological constraints, pollen from the protogynous trees could only be used in five of the twelve trees. The number of pollinated flowers per treatment was counted and the number of viable seeds from each treatment was estimated after fruit harvesting in autumn (fruit set was not an informative measure as unfertilized flowers typically develop into parthenocarpic fruits). The proportion of viable seeds sired by male, protandrous and protogynous trees was compared using a glmmPQL with a Binomial distribution of errors, in which pollen donor was considered as a fixed factor and mother as a random clustering factor. A dispersion parameter was included in the model to account for over-dispersion (McCullagh and Nelder, 1989). Furthermore, to ensure that the viable seeds obtained by this experiment were sired by the pollen added to the stigmas in this experiment, two additional ‘control’ treatments were performed: a treatment in which female flowers were bagged and no pollen was eventually added, so as to ensure that the apparently non-functional anthers did not release pollen; and a second treatment in which these anthers were emasculated from the female flowers, in order to check for the possibility of apomixis.

Post-pollination effects: siring success under pollen competition

The pollen competition treatments were performed in ten protogynous mothers selected in 2004. Flower buds were enclosed in pollen-proof bags and male flowers were removed prior to anthesis. Once the female flowers became receptive, stigmas were brushed with three different pollen combinations: pollen from a male and a protandrous pollen donor (M-PA pollen competition, with 11 different combinations of pollen donors); pollen from a male and a protogynous pollen donor (M-PG pollen competition, with five different combination of pollen donors); and pollen from a protandrous and a protogynous pollen donor (PA-PG pollen competition, with two different combinations of pollen donors; see Appendix). To ensure that each stigma received pollen from the two pollen donors (and in similar quantities), we brushed each stigmatic branch with pollen from a single pollen donor until the surface of the branch was covered with pollen grains. The number of female flowers assigned to each treatment (M-PA, M-PG and PA-PG) was unbalanced, as it was difficult to pair protogynous trees releasing pollen with protogynous trees whose stigmas were still receptive. As a consequence, we obtained few seedlings from the treatment involving competition between protogynous and protandrous pollen donors, as the scarcity of protandrous trees in the population also reduces the number of possible crosses. Fruits were harvested at the end of fruit ripening in autumn and seedlings were obtained from these seeds following the protocol described in Gleiser *et al.* (2004); this involved cold stratification for 3 months at 4 °C and the subsequent excision of the embryos (removal of testa and pericarp) prior to germination. Germination was performed in a germination chamber with a constant temperature of 20 °C and a 12 h light, 12 h dark photoperiod. Once the two first true leaves of the germinated embryos emerged, seedlings were transplanted to alveolar containers, and they were then grown in a greenhouse with regular watering.

Paternity was assigned to 101 seedlings on the basis of microsatellite markers. Leaves from mothers and putative fathers were collected in the field and dried in silica gel (Chase and Hills, 1991), ground in liquid nitrogen and then stored at −20 °C before DNA extraction. Plant tissue from seedlings was homogenized with a pestle and mortar immediately prior to DNA extraction. Genomic DNA was isolated following the cetyl-trimethyl-ammonium bromide (CTAB) procedure of Doyle and Doyle (1987) with slight modifications, from approximately 40 mg of dried homogenized plant material (from mothers and putative fathers) and from around 50 mg of fresh plant tissue (from seedlings). The purified DNA recovered by ethanol precipitation was re-suspended in 50 μL of autoclaved deionized water. All the trees involved in the pollen competition experiment were genotyped using seven microsatellite loci (Pandey *et al.*, 2004; Segarra-Moragues *et al.*, 2008). In order to assign paternity to seedlings from known mothers, several of these SSR loci were selected depending on their suitability to distinguish the genotypes from the mother and the different pollen donors. The paternity of the seedlings was unambiguously assigned using the SSR loci Aop122, Aop132, Aop450 (Segarra-Moragues *et al.*, 2008) and Map9 (Pandey *et al.*, 2004). Amplifications of microsatellites were conducted on a PTC-100 (MJ Research/Bio-Rad) thermalcycler following the protocols described elsewhere (Pandey *et al.*, 2004; Segarra-Moragues *et al.*, 2008). The products were analysed with an ABI 3700 automated sequencer (Applied Biosystems), and the amplified fragment lengths assigned to allelic sizes with GENESCAN v. 2·1 and GENOTYPER v.1·1r8 software (Applied Biosystems), using ROX-400HD as the internal lane size standard. The proportion of seedlings sired by each morph within each type of competitive pollination treatment was compared by means of a Chi-square test.

In addition, to assess whether the outcome of the competition experiment resulted from differences in pollen germination ability or genetic interactions between the pollen grain and the maternal tissue, we calculated, for each experimental cross, the genetic distances between each mother and the competing sires using Populations 1·2·28 Software (Langella, 2002). Mothers and sires were genotyped for five microsatellite loci: Aop122, Aop450, Aop820, Aop943 (Segarra-Moragues *et al.*, 2008) and Map9 (Pandey *et al.*, 2004). As we had no *a priori* information on the best-fitting model for the microsatellite markers used, we calculated the genetic distances assuming both an infinite-allele model (IAM; Kimura and Crow, 1964) and a stepwise mutation model (SMM; Kimura and Ohta, 1978).

Post-pollination effects: paternal effects on seedling growth

Total seedling height was measured on the seedlings obtained from the pollen competition experiment, from which maternal and paternal genotypes were known. Measures were taken with a digital calliper to the nearest 0·01 mm, after the first, second and third growing seasons (the beginning of summer 2005, 2006 and 2007). Data were analysed with a linear mixed-effects model, with the sex of the seedlings' sire considered as a fixed factor, and seedlings as the random factor in which the measures were repeated. The temporal correlation of the measures of height was controlled by introducing an autoregressive of order 1 covariance structure into the model (Pinheiro and Bates, 2000).

RESULTS AND DISCUSSION

Sex ratio variation in Acer opalus

In all seven populations sampled, protogynous trees made up about half the individuals, with the other half composed of males and a small fraction of protandrous trees (Fig. 1). The frequency of the protandrous morph may have been under-estimated, because some may have been mistaken as males (see Materials and Methods). However, in the Font Roja population, where sexes have been repeatedly scored over 6 consecutive years, the frequency of inconstant protandrous individuals was significantly lower than the frequency of pure males. Any under-estimation of the frequency of protandrous trees in these populations is thus expected to be small.

The sex ratios observed in populations of *A. opalus* surveyed in this study are consistent with theoretical expectations arising from Pannell and Verdú's (2006) model, where males that invade heterodichogamous populations tend to replace the protandrous hermaphrodites while the frequency of the protogynous morphs remains unaffected. Our study therefore contributes to the growing body of evidence (de Jong, 1976; Sato, 2002; Gleiser and Verdú, 2005) that males have invaded heterodichogamous populations of species in the genus *Acer*. In *Acer*, the frequency of males varies among species from very low, as in *A. japonicum* (Sato, 2002), to frequencies around 50 %, as in *A. opalus* (this study). It would thus seem that trimorphism can persist, but also that males can invade to such an extent that the sexual system becomes subdioecious and ultimately dioecious. This pattern is anticipated in the theoretical analysis of Pannell and Verdú (2006), who found that the protandrous morph can persist in trimorphic populations even at low frequencies, but also that full dioecy can evolve in such populations. Phylogenetic comparative analysis indeed suggests that dioecy has evolved repeatedly from hermaphroditism in *Acer* (Gleiser and Verdú 2005, Renner *et al.*, 2007).

Morph-specific pollinator visitation

Nearly all flowers from the pollinator-exclusion treatment (which allowed the passage of wind-borne pollen) failed to produce viable seeds (0·3 % of viable seeds). Open-pollination treatments produced a significantly greater number of seeds (13 % of viable seeds; $z = 6{\cdot}3$, $P \ll 0{\cdot}0001$), suggesting that wind plays little or no role as a pollination vector for *A. opalus*.

Acer opalus trees were mainly visited by bumble bees (*Bombus terrestris*) and honey bees (*Apis mellifera*), as well as by low numbers of various flies and other bees. The pollination rates varied significantly across the flowering season ($t = 3{\cdot}4$, d.f. $= 46$, $P = 0{\cdot}0013$), and increased with the number of male flowers ($t = 4{\cdot}06$, d.f. $= 46$, $P = 0{\cdot}0002$). Males and protandrous trees received significantly more visits to their male phase than the protogynous trees (pollination rates of 1·0, 0·85 and 0·57 for males, protandrous and protogynous, respectively, with values standardized with respect to the highest value: $t = 0{\cdot}51$, d.f. $= 20$, $P = 0{\cdot}61$ for the M-PA contrast; $t = -1{\cdot}9$, d.f. $= 20$, $P = 0{\cdot}04$ for the M-PG contrast; and $t = -2{\cdot}2$, d.f. $= 20$, $P = 0{\cdot}04$ for the PA-PG contrast). The number of male flowers produced by each sexual morph was not significantly different ($t = -0{\cdot}53$, d.f. $= 20$, $P = 0{\cdot}6$ for the M-PA contrast; $t = -1{\cdot}9$, d.f. $= 20$, $P = 0{\cdot}07$ for the M-PG contrast; and $t = -1{\cdot}6$, d.f. $= 20$, $P = 0{\cdot}1$ for the PA-PG contrast). Thus, investment in male flowers by males and protandrous trees paid greater rewards due to increased pollination rates than by protogynous trees. These differences in the pollination rates are probably due to a decrease in the availability of pollinators at the time when most protogynous trees release their pollen, probably because the number of species that start their flowering period increases (e.g. the competition for pollinator attraction is higher). As a consequence, the male fitness of protogynous individuals, which delay the dispersal of pollen, is likely to be reduced.

Morph-specific fertilization success

The production of viable seeds as a result of apomixis or selfing with pollen from the apparently non-functional anthers of female flowers could both be ruled out, as the number of viable seeds obtained by these treatments was negligible (four viable seeds out of 814 flowers treated). The three sexual morphs produce viable pollen, although the percentage of viability of seeds obtained with supplemented pollen never exceeded 60 %. The percentage of seeds sired by each morph did not differ significantly ($48{\cdot}0 \pm 5{\cdot}6$, $46{\cdot}8 \pm 3{\cdot}6$, $46{\cdot}0 \pm 11{\cdot}2$, mean $\pm$ 1 s.e. for males, protandrous and protogynous morphs, respectively; $t = -0{\cdot}4$, $P = 0{\cdot}7$). Pollen performance in competition was also similar among sexual morphs, as the number of seedlings sired by each morph within each competition treatment was not significantly different (Fig. 2). Thus, once pollen grains arrive at a stigma, males and both heterodichogamous hermaphrodites are equally capable of fertilizing ovules.

These results thus differ from those found for other sexually polymorphic species in which males occur with hermaphrodites, such as *Fraxinus lanuginosa* (Ishida and Hiura, 1998), *Phillyrea angustifolia* (Vassiliadis *et al.*, 2000), *Fraxinus ornus* (Verdú *et al.*, 2004*a*) and *F. excelsior* (Morand-Prieur *et al.*, 2003), where pollen from males tended to be more successful at fertilizing ovules than that from hermaphrodites. The difference in the pattern of siring success between *A. opalus* and these other species, all of which belong to the Oleaceae, possibly reflects the fact that androdioecy in the Oleaceae probably evolved from monomorphic hermaphrodite populations, where the appearance of males can only be explained if males are more than twice as fertile as the male function of the hermaphrodites (Lloyd, 1975; B. Charlesworth and D. Charlesworth, 1978; Charlesworth, 1984; Pannell, 2002). By contrast, the critical threshold for male invasion is reduced from two to one in dimorphic heterodichogamous populations if each of the morphs begins to specialize in either male or female functions (Pannell and Verdú, 2006).

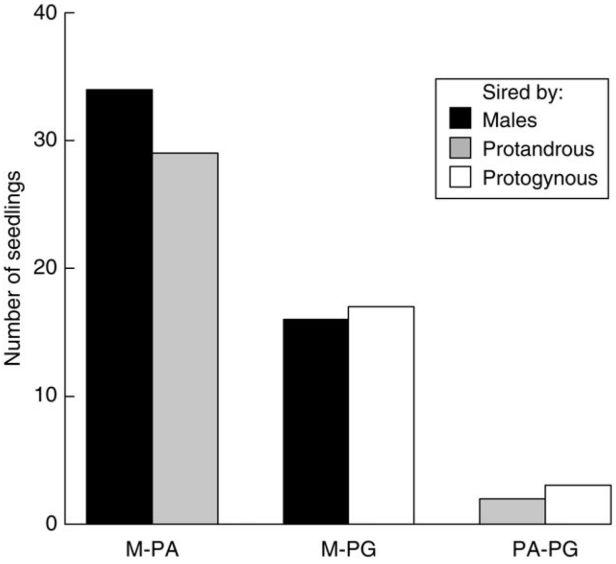

FIG. 2. The number of seedlings sired by each pollen donor in the pollen competition experiments was not significantly different ($\chi_1^2 = 0{\cdot}39$, $P = 0{\cdot}53$ for the M-PA pollen competition; $\chi_1^2 = 0{\cdot}03$, $P = 0{\cdot}86$ for the M-PG pollen competition; and $\chi_1^2 = 0{\cdot}2$, $P = 0{\cdot}65$ for the PA-PG pollen competition). Three different types of pollen competition were performed: male–protandrous pollen competition (M-PA); male–protogynous pollen competition (M-PG); and protandrous–protogynous pollen competition (PA-PG). The bars show the number of seedlings sired by male, protandrous and protogynous pollen donors as indicated.

Seedling paternity followed a random pattern in relation to the genetic relatedness between parents (Appendix). Although the inferred genetic distances differed under IAM versus SSM assumptions, the results where similar: neither pollen superiority of sires, nor mate-sorting by females (female choice) seem to have produced departures from random mating. Evidence for siring superiority of a particular pollen donor would be shown if its pollen performed consistently better across all the pollen recipients (Marshall, 1998) and against all pollen competitors. However, this did not apply to *A. opalus*: in almost all the competition treatments, both competing sires were able to sire seeds, a pattern consistent across mothers (see, for example, the seedlings from mother no. 3 with sire 1 = 1 and sire 2 = 6, or seedlings from mother no. 10 with sire 1 = 1 and sire 2 = 6; Appendix). Female choice due to genetic relatedness could be ruled out, because the most successful fathers of seeds produced by a given mother were both the closest or the most distantly related to her (see, for example, the seedlings from mother no. 9 with sire 1 = 5 and sire 2 = 11; Appendix).

Morph-specific paternal effects on progeny performance

It was found that paternal effects occurring at a post-pollination stage caused differences in the male fitness of the three sexual morphs of *A. opalus*, as the protogynous-sired seedlings grew more slowly than those sired by male and protandrous fathers (Fig. 3). Seedling growth may constitute a competitive advantage if, for example, there is strong resource competition among emerging seedlings. In fact, competition among seedlings is likely to occur at the time of emergence in *A. opalus*, as seed dispersal is highly limited in space (Gómez-Aparicio *et al.*, 2007), and emerging seedlings are highly aggregated around adult trees (Gómez-Aparicio, 2004). In addition, if seedling height is associated with the first age of reproduction, taller seedlings will benefit from earlier reproduction. Additional studies are needed to support these hypotheses.

Although the ecological and evolutionary consequences of the differences in seedling growth are clear, the mechanisms involved in the superiority of male and protandrous-sired seedlings are still not understood. Maternal effects acting in early seedlings stages have been largely studied, and are associated with the quality of the cytoplasm, the endosperm and the tissues surrounding the embryo (Roach and Wulff, 1987). In contrast, little is known on the existence of paternal effects acting beyond the siring of seeds. Paternal effects have been reported in species with different life histories. In the annual weed *Raphanus sativus*, pollen-donor identity was shown to affect offspring traits such as height, number of leaves and three other traits closely related to fitness: total weight, date of first flowering and number of flowers (Marshall and Whittaker, 1989). Paternal effects were also reported in the morphologically androdioecious but cryptically dioecious tree *Fraxinus ornus*, where seedlings sired by males also grew taller than ‘hermaphrodite’-sired ones (Verdú *et al.*, 2004*a*). To date, the genetic mechanisms underlying these effects still remain unclear.

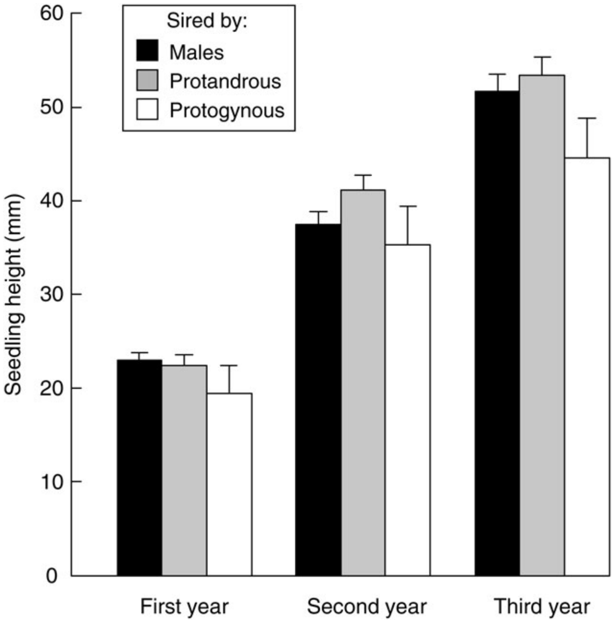

FIG. 3. Seedling height (±1 s.e.) measured after the first, second and third growing periods (2005, 2006 and 2007) of seedlings sired by male, protandrous and protogynous fathers. The protogynous-sired seedlings grew more slowly than those sired by male and protandrous fathers (M vs. PG: $t = -3{\cdot}17$, $P = 0{\cdot}0018$; PA vs. PG: $t = -3{\cdot}47$, $P = 0{\cdot}0006$; M vs. PA: $t = -0{\cdot}67$, $P = 0{\cdot}5$).

CONCLUSIONS

The sex ratios of *Acer opalus* populations conform to theoretical predictions on heterodichogamous ancestral populations where males tend to replace the protandrous morph. The fact that the fitness of males was not found to be superior to the male fitness of the protandrous morph explains the occurrence of a trimorphism in the populations, composed of mainly males and protogynous trees, and a small proportion of protandrous individuals. However, in spite of the occurrence of three sexual morphs, there is strong evidence of a functional dimorphism in the species. In a forthcoming study we will show that female fitness declines during the course of the flowering season, resulting in a female-biased gender in the protogynous morph that induces a frequency-dependent bias towards maleness in the protandrous morph. Here, we have shown that the protandrous morph enjoys a male-fitness advantage over the protogynous hermaphrodites due to a higher attraction of pollinators to the male phase, and also due to the differential growth of protandrous-sired seedlings in comparison with the protogynous-sired ones. The genetic mechanisms involved in these paternal effects remain to be investigated.

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APPENDIX

Genetic distances between the protogynous ovule donor (mothers) and the two competing pollen donors (sires 1 and 2) in the pollen competition experiment. Each row explains the experimental cross for each seedling; different sires are described by different identification numbers (ID); the sexual morph of each sire is also indicated between brackets (M: male, PA: protandrous, PG: protogynous). Genetic distances were calculated both by assuming a stepwise mutation model (SMM; *ASD*, Goldstein *et al.*, 1995) and an infinite allele mutation model (IAM; *Da*, Nei *et al.*, 1983). Numbers in bold type show the genetic distances of the pollen donors that sired the seedling in the experiment. The 'winner' column indicates whether the successful sire had the highest genetic distance from the mother (indicated by ' + ') or whether the successful sire had the lowest (blank space) or equal (' = ') genetic distance from the mother.

			Genetic distances (SMM)			Genetic distances (IAM)		
Mother	Sire 1 ID (morph)	Sire 2 ID (morph)	Sire 1	Sire 2	Winner	Sire 1	Sire 2	Winner
1	5 (M)	10 (PG)	**38·4**	52·8		**0·66**	0·52	+
1	5 (M)	10 (PG)	**38·4**	52·8		**0·66**	0·52	+
1	6 (PA)	10 (PG)	28·8	**52·8**	+	0·62	**0·52**	
1	6 (PA)	10 (PG)	**28·8**	52·8		**0·62**	0·52	+
1	6 (PA)	10 (PG)	28·8	**52·8**	+	0·62	**0·52**	
1	6 (PA)	10 (PG)	28·8	**52·8**	+	0·62	**0·52**	
1	9 (M)	11 (PG)	**34**	107·6		**0·56**	0·32	+
2	3 (M)	6 (PA)	**94**	37·2	+	**0·86**	0·56	+
2	8 (M)	12 (PA)	72	**54·8**		0·50	**0·60**	+
3	1 (M)	6 (PA)	**76**	73·6	+	**0·52**	0·62	
3	1 (M)	6 (PA)	76	**73·6**		0·52	**0·62**	+
3	1 (M)	6 (PA)	**76**	73·6	+	**0·52**	0·62	
3	1 (M)	6 (PA)	76	**73·6**		0·52	**0·62**	+
3	5 (M)	12 (PA)	**90·4**	85·2	+	**0·66**	0·56	+
3	5 (M)	12 (PA)	**90·4**	85·2	+	**0·66**	0·56	+
3	5 (M)	12 (PA)	90·4	**85·2**		0·66	**0·56**	
4	8 (M)	13 (PA)	**32·4**	34·8		**0·66**	0·60	+
4	8 (M)	13 (PA)	32·4	**34·8**	+	0·66	**0·60**	
4	8 (M)	13 (PA)	32·4	**34·8**	+	0·66	**0·60**	
4	8 (M)	15 (PA)	32·4	**98·4**	+	0·66	**0·66**	=
4	8 (M)	15 (PA)	**32·4**	98·4		**0·66**	0·66	=
4	8 (M)	15 (PA)	32·4	**98·4**	+	0·66	**0·66**	=
4	8 (M)	15 (PA)	**32·4**	98·4		**0·66**	0·66	=
5	8 (M)	12 (PA)	114·4	**77·6**		0·72	**0·52**	
5	8 (M)	4 (PA)	114·4	**90**		0·72	**0·52**	
5	8 (M)	4 (PA)	114·4	**90**		0·72	**0·52**	
5	8 (M)	4 (PA)	114·4	**90**		0·72	**0·52**	
5	8 (M)	4 (PA)	**114·4**	90	+	**0·72**	0·52	+
5	8 (M)	4 (PA)	**114·4**	90	+	**0·72**	0·52	+
5	8 (M)	4 (PA)	114·4	**90**		0·72	**0·52**	
6	5 (M)	13 (PA)	**34·8**	36·4		**0·46**	0·36	+
6	5 (M)	13 (PA)	34·8	**36·4**	+	0·46	**0·36**	
6	5 (M)	13 (PA)	**34·8**	36·4		**0·46**	0·36	+
6	5 (M)	13 (PA)	**34·8**	36·4		**0·46**	0·36	+
6	5 (M)	14 (PA)	**34·8**	30	+	**0·46**	0·36	+
6	5 (M)	14 (PA)	34·8	**30**		0·46	**0·36**	+
6	5 (M)	14 (PA)	**34·8**	30	+	**0·46**	0·36	+
6	5 (M)	14 (PA)	**34··8**	30	+	**0·46**	0·36	+
6	5 (M)	14 (PA)	**34·8**	30	+	**0·46**	0·36	+
6	5 (M)	14 (PA)	**34·8**	30	+	**0·46**	0·36	+
6	5 (M)	14 (PA)	**34·8**	30	+	**0·46**	0·36	+

Continued

Appendix *Continued*

			Genetic distances (SMM)			Genetic distances (IAM)		
Mother	Sire 1 ID (morph)	Sire 2 ID (morph)	Sire 1	Sire 2	Winner	Sire 1	Sire 2	Winner
6	5 (M)	14 (PA)	**34·8**	30	+	**0·46**	0·36	+
6	5 (M)	14 (PA)	**34·8**	30	+	**0·46**	0·36	+
6	5 (M)	14 (PA)	**34·8**	30	+	**0·46**	0·36	+
6	5 (M)	14 (PA)	34·8	**30**		0·46	**0·36**	
6	7 (M)	13 (PA)	56·8	**36·4**		0·66	**0·36**	
6	7 (M)	13 (PA)	**56·8**	36·4	+	**0·66**	0·36	+
6	7 (M)	13 (PA)	**56·8**	36·4	+	**0·66**	0·36	+
6	7 (M)	13 (PA)	**56·8**	36·4	+	**0·66**	0·36	+
6	7 (M)	13 (PA)	56·8	**36·4**		0·66	**0·36**	
6	7 (M)	13 (PA)	**56·8**	36·4	+	**0·66**	0·36	+
6	7 (M)	13 (PA)	56·8	**36·4**		0·66	**0·36**	
6	7 (M)	13 (PA)	56·8	**36·4**		0·66	**0·36**	
6	7 (M)	13 (PA)	**56·8**	36·4	+	**0·66**	0·36	+
6	7 (M)	14 (PA)	56·8	**30**		0·66	**0·36**	
6	7 (M)	14 (PA)	56·8	**30**		0·66	**0·36**	
6	7 (M)	14 (PA)	56·8	**30**		0·66	**0·36**	
7	8 (M)	4 (PA)	20·8	**13·2**		0·62	**0·62**	=
7	8 (M)	4 (PA)	20·8	**13·2**		0·62	**0·62**	=
7	8 (M)	4 (PA)	**20·8**	13·2	+	**0·62**	0·62	=
8	2 (M)	10 (PG)	59·6	**67·2**	+	0·62	**0·62**	=
8	2 (M)	10 (PG)	**59·6**	67·2		**0·62**	0·62	=
8	2 (M)	10 (PG)	59·6	**67·2**	+	0·62	**0·62**	=
8	2 (M)	11 (PG)	59·6	**113·6**	+	0·62	**0·62**	=
8	2 (M)	11 (PG)	59·6	**113·6**	+	0·62	**0·62**	=
8	5 (M)	10 (PG)	44·4	**67·2**	+	0·48	**0·62**	+
8	5 (M)	11 (PG)	44·4	**113·6**	+	0·48	**0·62**	+
8	5 (M)	11 (PG)	**44·4**	113·6		**0·48**	0·62	
8	5 (M)	11 (PG)	44·4	**113·6**	+	0·48	**0·62**	+
8	5 (M)	11 (PG)	44·4	**113·6**	+	0·48	**0·62**	+
8	5 (M)	11 (PG)	44·4	**113·6**	+	0·48	**0·62**	+
8	5 (M)	11 (PG)	**44·4**	113·6		**0·48**	0·62	
8	5 (M)	11 (PG)	**44·4**	113·6		**0·48**	0·62	
8	5 (M)	11 (PG)	**44·4**	113·6		**0·48**	0·62	
8	5 (M)	11 (PG)	**44·4**	113·6		**0·48**	0·62	
8	5 (M)	11 (PG)	44·4	**113·6**	+	0·48	**0·62**	+
8	5 (M)	11 (PG)	**44·4**	113·6		**0·48**	0·62	
8	5 (M)	11 (PG)	**44·4**	113·6		**0·48**	0·62	
8	5 (M)	11 (PG)	**44·4**	113·6		**0·48**	0·62	
9	4 (PA)	11 (PG)	**26·4**	136		**0·52**	0·46	+
9	5 (M)	11 (PG)	**33·2**	136		**0·52**	0·46	+
9	5 (M)	11 (PG)	33·2	**136**	+	0·52	**0·46**	
9	5 (M)	11 (PG)	**33·2**	136		**0·52**	0·46	+
9	5 (M)	11 (PG)	33·2	**136**	+	0·52	**0·46**	
9	5 (M)	11 (PG)	33·2	**136**	+	0·52	**0·46**	
9	5 (M)	11 (PG)	33·2	**136**	+	0·52	**0·46**	
9	5 (M)	11 (PG)	33·2	**136**	+	0·52	**0·46**	
9	5 (M)	11 (PG)	**33·2**	136		**0·52**	0·46	+
9	5 (M)	11 (PG)	33·2	**136**	+	0·52	**0·46**	
9	5 (M)	11 (PG)	**33·2**	136		**0·52**	0·46	+
9	5 (M)	11 (PG)	33·2	**136**	+	0·52	**0·46**	
10	1 (M)	6 (PA)	**90·4**	39·6	+	**0·48**	0·52	
10	1 (M)	6 (PA)	**90·4**	39·6	+	**0·48**	0·52	
10	1 (M)	6 (PA)	90·4	**39·6**		0·48	**0·52**	+
10	1 (M)	6 (PA)	**90·4**	39·6	+	**0·48**	0·52	
10	1 (M)	6 (PA)	90·4	**39·6**		0·48	**0·52**	+
10	3 (M)	6 (PA)	**79·6**	39·6	+	**0·80**	0·52	+
10	5 (M)	12 (PA)	46·8	**91·6**	+	0·66	**0·56**	
10	5 (M)	12 (PA)	**46·8**	91·6		**0·66**	0·56	+
10	8 (M)	12 (PA)	46	**91·6**	+	0·76	**0·56**	
10	8 (M)	12 (PA)	**46**	91·6		**0·76**	0·56	+