

HIGH MALE REPRODUCTIVE SUCCESS OF HERMAPHRODITES IN THE ANDRODIOECIOUS *PHILLYREA ANGUSTIFOLIA*

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Abstract.—Androdioecy, the coexistence of males and hermaphrodites within a population, is a rare breeding system, often considered as unlikely to evolve because of restrictive conditions for its maintenance. *Phillyrea angustifolia*, a wind-pollinated shrub, is one of the handful species reported to be androdioecious. Our previous studies have shown that natural populations of this species in southern France exhibit higher male frequencies (~50%) than predicted on theoretical grounds. Thus, the male functionality of hermaphrodites is still debated. To assess the functional breeding system of this species in the wild, a paternity analysis was performed with two highly polymorphic microsatellite loci on 729 seeds collected on 10 maternal shrubs in a natural population of 24 mature individuals of *P. angustifolia*. A large proportion of seeds were found to have been sired by pollen from outside the population. Analysis of seeds sired by individuals within the study population revealed a high male fertility of hermaphrodites resulting in a low male advantage in fertility for male plants. Intermate distances were found to have a strong impact on male reproductive success, whereas sexual morph had no effect, with males and hermaphrodites performing equivalently. This study is the first to unequivocally document the occurrence of a male function of hermaphrodites in a natural population of an androdioecious species.

Key words.—Androdioecy, male reproductive success, microsatellite, paternity analysis.

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Androdioecy (coexistence of males and hermaphrodites within a population) and gynodioecy (i.e., coexistence of females and hermaphrodites within a population) have been proposed to be possible evolutionary pathways from hermaphroditism to dioecy. They have both been addressed through numerous theoretical studies (Lewis 1942; Lloyd 1975; Charlesworth and Charlesworth 1978; Ross 1978, 1981; Bawa 1980; Charlesworth 1984; Frank 1989; Maurice et al. 1994; Schultz 1994; Ronfort 1995; Pannell 1997a; Vassiliadis et al. 2000b). Converse to gynodioecy, androdioecy is one of the rarest sexual polymorphisms observed in the wild in Angiosperms (Darwin 1877; Yampolsky and Yampolsky 1922; Charlesworth 1984; Richards 1997). Some species described as morphologically androdioecious have been shown on further study to exhibit functional dioecy in which hermaphrodites do not display male function (e.g., Anderson and Symon 1989). Only seven cases of functional androdioecy have been reported, as far as we know, in which the male fertility of hermaphrodites was checked by controlled crosses, namely *Oxalis suksdorfii* (Ornduff 1972), *Datisca glomerata* (Liston et al. 1990), *Phillyrea angustifolia* (Lepart and Dommée 1992), *Mercurialis annua* (Pannell 1997b), *Fraxinus lanuginosa* (Ishida and Hiura 1998), *Schizopepon bryoniaefolius* (Akimoto et al. 1999), and *Fraxinus ornus* (Dommée et al. 1999).

The extreme rarity of androdioecy has been explained by highly restrictive conditions for its maintenance. Theoretical

studies have shown that for males to invade a large outcrossing population of hermaphrodites and be maintained by selection, their male fertility should be at least twice as high as that of hermaphrodites (Lloyd 1975; Charlesworth and Charlesworth 1978), leading to an expected frequency of males in a population of less than 50%, with even lower male frequency in a metapopulation model (Pannell 1997a). The 2:1 male fertility ratio is called "male advantage." Under this threshold value, males cannot be maintained by selection. When male advantage reaches high values (≥ 2), male frequency increases asymptotically to 50% and hermaphrodites are expected to lose male function, at which point morphological androdioecy corresponds to functional dioecy. If hermaphrodites can self, the male advantage threshold should be even higher and the expected frequency of males lower. Indeed, selfing automatically reduces the reproductive success of males because selfed ovules are unavailable for male outcross pollination (Lloyd 1975; Charlesworth and Charlesworth 1978; Charlesworth 1984; Pannell 1997a). The expected conditions for the maintenance of functional androdioecy—large male advantage, low male frequencies, and self-incompatibility or low selfing rate in hermaphrodites—have been found in only two androdioecious species: *Datisca glomerata* (Liston et al. 1990; Fritsch and Rieseberg 1992; Rieseberg et al. 1993; Philbrick and Rieseberg 1994) and *Mercurialis annua* (Pannell 1997b,c).

In *Phillyrea angustifolia*, a wind-pollinated shrub, high male frequencies (~50%) documented in natural populations in Southern France (Vassiliadis 1999) suggest the occurrence of functional dioecy (Charlesworth 1984). Studies on the male functionality of hermaphrodites in *P. angustifolia* are

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thus of paramount importance to ascertain the reproductive status of that species. Controlled crosses show male functionality of hermaphrodites with a male advantage greater than one (1.93; Vassiliadis et al. 2000a) but less than the minimum expectation of two to explain the maintenance of males and much lower than expected values to explain high frequencies of males according to the selection model. However, these experimental results did not account for conditions occurring in natural populations (Pannell and Ojeda 2000). The effective male reproductive success of hermaphrodites may be greatly reduced by the presence of surrounding competing males in the wild. Thus, "the most unequivocal evidence for a male function in hermaphrodites under field conditions still awaits male paternity analysis in open-pollinated plants" (Pannell and Ojeda 2000, p. 500).

Paternity analyses allow direct estimation of effective male reproductive success (Snow and Lewis 1993). This approach has been stimulated by the development of highly polymorphic markers allowing unambiguous assignment of a unique father per progeny (Burke 1989, 1994; Jarne and Lagoda 1996; Schnabel 1998). Maximum-likelihood (ML) inference methods provide statistical tools to assign relative paternity or probability of paternity when markers do not allow assignment of a unique father (Meagher 1986; Thompson 1986; Devlin et al. 1988; Roeder et al. 1989; Adams et al. 1992; Smouse and Meagher 1994; Smouse et al. 1999). The male reproductive success of individuals has been estimated in plants displaying a wide range of reproductive systems, including hermaphroditic, insect-pollinated species (Broyles and Wyatt 1990; Devlin and Ellstrand 1990; Stanton et al. 1991; Devlin et al. 1992; Conner et al. 1996; Morgan 1998); monoecious, wind-pollinated trees (Schoen and Stewart 1986; Erickson and Adams 1989; Burczyk and Prat 1997; Dow and Ashley 1998; Kaufman et al. 1998; Streiff 1998); andromonoecious species (Elle and Meagher 2000); and dioecious species (Meagher 1986, 1991; Engel et al. 1999; Smouse et al. 1999).

We applied paternity analysis to a natural population of *P. angustifolia*, using microsatellite markers and three inference methods (exclusion-based categorical analysis, ML-based categorical analysis, and ML-based fertility estimation) to analyze the mating structure within the population, estimate the male reproductive success of males and hermaphrodites, and assess the role played by sexual phenotype, size, flowering intensity, and intermate distance on male reproductive success.

MATERIALS AND METHODS

Plant Material and Study Population

Phillyrea angustifolia is a wind-pollinated, Mediterranean shrub, with characters allowing its reestablishment after disturbance (James 1984). In the French Mediterranean region, following the 19th century rural depopulation (Lepart and Debussche 1992), it inhabits more-or-less stable environments. This species is widespread in Camargue (southern France), where it has undergone extensive population growth over the past five decades; most of the populations are aged less than 30 years, few populations of more than 50 years

(Vassiliadis 1999), and just one of more than 70 years (Tallon 1931; Molinier and Tallon 1965).

We studied one spatially restricted population in Southern Camargue (4°37'0"E, 43°22'42"N), located on a sandy bar between two shallow lagoons of the salinas of Salins de Giraud. Six populations of *P. angustifolia* were detected in the vicinity of our study population: four small (10–50 individuals) at relatively short distances (50–300 m) and two large (several hundred individuals) at further distances (780–1400 m; see Fig. 1). The density of the study population was very low (~50 individuals/ha) when compared with some populations located around in the dunes (more than 1000 individuals/ha, and an almost continuous *Phillyrea* cover; J. Lepart, pers. obs.).

In 1997, the study population (24 mature individuals; 13 hermaphrodites, and 11 males) was mapped and surveyed (Fig. 1). Individuals were assigned identification according to the population (B), the number of the individual, and the sexual phenotype of the individual (H, hermaphrodite; M, male). The percentage of males in the study population (45.8%) was near of the mean frequency of males observed in *P. angustifolia* populations in the Camargue region (~50%; Vassiliadis 1999). The limited number of mature individuals (and thus, of potential pollen donors) within the population made the paternity analysis feasible and reliable. In 1998, 60 to 250 fruits (each containing one seed) were randomly collected on each of 10 hermaphrodites (no seeds were analyzed from the individuals B1H, B15H, and B16H). We collected a large number of offspring per maternal plant to achieve an accurate representation of each male's contribution to each female's progeny and thus to obtain an accurate estimate of male reproductive success for each sexually mature individual of the population. All the mature individuals were flowering, so that height, size (i.e., length, or first diameter, and width, or second diameter of these ovoid shrubs) and flowering intensity during the 1998 flowering season (three levels: poor, medium, or high flowering intensity) were recorded for each individual.

Molecular Typing

For DNA extraction of progenies, embryos were separated from the remainder of the seed with a scalpel after soaking for 24–48 h in distilled water. DNA was extracted from embryos and from leaves of adult males and hermaphrodites according to the protocol described in Saumitou-Laprade et al. (1999).

The 24 putative parent individuals, together with 742 offspring from the 10 maternal plants analyzed, were typed for two microsatellite loci, GA6 and ATT1, following the polymerase chain reaction protocol and size typing procedure described in Saumitou-Laprade et al. (2000). These loci were chosen for their high variability: 14 and 20 alleles were identified in GA6 and ATT1, respectively, in a survey of 43 individuals from two populations, including the present study population (Saumitou-Laprade et al. 2000). Null alleles can be a source of mistyping in paternity analysis (Pemberton et al. 1995). The GA6 locus revealed a rare null allele that was easily detected in the progeny of one hermaphrodite (B18H). This null allele was also detected in two potential fathers

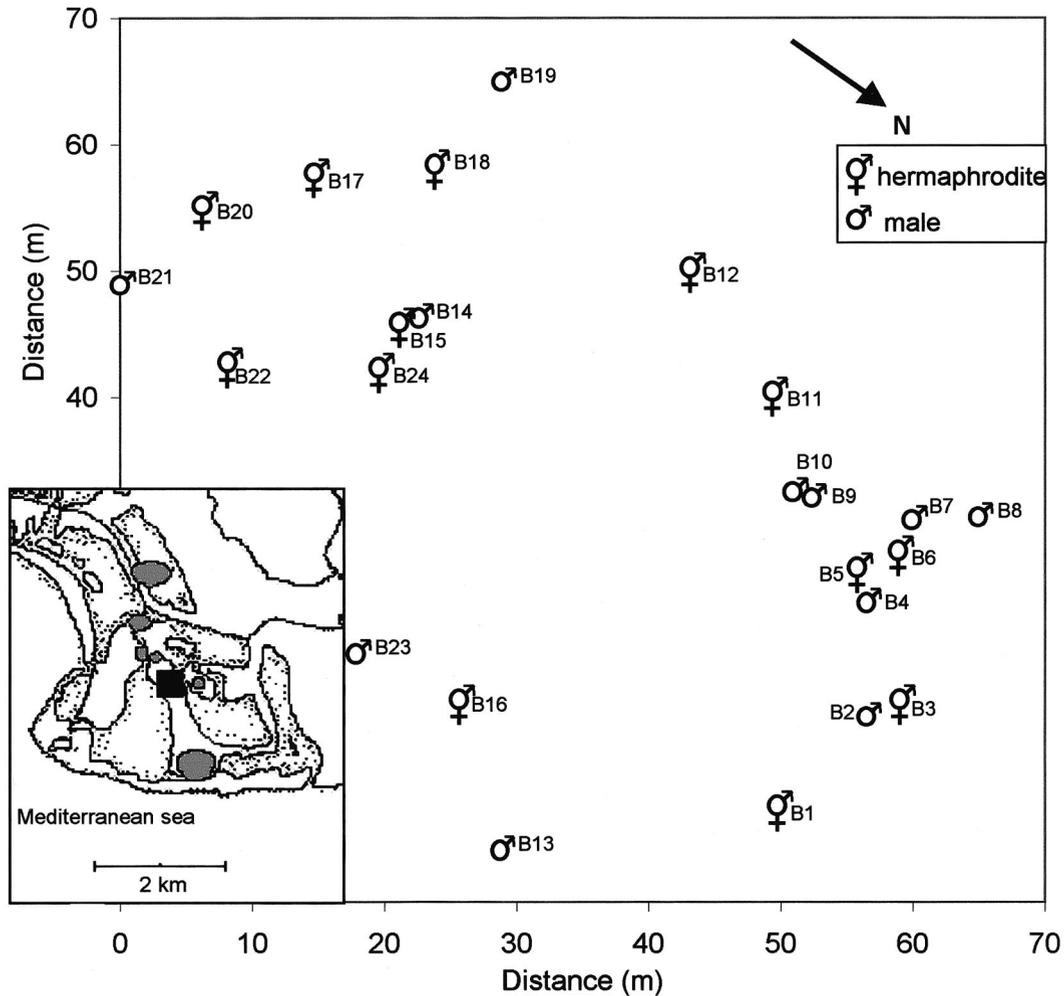


FIG. 1. Map of the Salins population, with male and hermaphrodite individuals indicated. Inset: localization of the study population (black square) and of surrounding populations (gray ellipses).

(B1H and B21M) by checking the Mendelian inheritance of the other allele present in their presumed progeny. We encoded all the offspring of the hermaphrodite B18H bearing this allele, as well as nine other offspring for which paternity was unambiguously assigned (with B1H or B21M as unique father) on the basis of the ATT1 locus. We discarded from the analysis the 13 seeds that had several possible fathers, among which there was at least one possible father bearing a null allele (B1H, B18H, or B21M). The estimated frequency of any null allele was then only 3%. Finally, a total of 729 seeds, unambiguously typed at the two loci, were included in the paternity analysis (see Table 1).

Statistical Analyses

Genotypic and paternity analyses

For each microsatellite locus, the number of alleles (N_{all}), the observed heterozygosity (H_o), the gene diversity (H_e), and the level of inbreeding (F_{IS} ; Weir and Cockerham 1984) were estimated by using Genetix version 4.0 (Belkhir et al. 2000). Tests for deviations from Hardy-Weinberg expecta-

tions at each microsatellite locus and for genotypic linkage disequilibria among loci were computed with Genepop version 3.2 (Raymond and Rousset 1995). The exclusion probability for each locus and for both loci combined was calculated by using the paternity analysis software Cervus (ver. 1.0, Marshall et al. 1998).

A paternity analysis was carried out to: (1) estimate the number of offspring sired by individuals outside the population (i.e., offspring for which no pollen within the population matches) and thus estimate the external pollen gene flow; and (2) assign a single father to a certain proportion of the remaining progeny sired within the population. The paternity analysis was first performed by using an exclusion-based categorical method. For each mother-offspring pair, we excluded as many as possible of the potential pollen donors (i.e., males or hermaphrodites), and unequivocally assign paternity to the unique, if any, genetically compatible father. However, exclusion-based categorical analysis may be insufficient to definitively resolve paternity (Chakraborty et al. 1988; Marshall et al. 1998). Therefore, we performed a ML-based categorical analysis following Meagher (1986)

TABLE 1. Pollen-donor origin: exclusion-based categorical and maximum-likelihood-based (ML-based) categorical analyses. For each maternal plant (mother) analyzed, the number of offspring analyzed (N_{off}) is given together with the result of the exclusion-based categorical analysis: (1) the minimum estimate of the number and percentage of offspring for which the pollen donor is outside the population; (2) the number of offspring: (a) for which more than one father is possible within the population, (b) for which one father is assigned unambiguously, and (c) that are derived from selfing (of the number of offspring with one father only). The number of offspring for which one father is assigned at the 95% and 80% levels of confidence according to the ML-based categorical method (Cervus software; Marshall et al. 1998) is also given.

Mother	N_{off}	Exclusion-based categorical analysis						ML-based categorical analysis	
		(1)		(2)			One father (95%) N	One father (80%) N	
		Pollen donor outside		Pollen donor inside					
N	%	More than one father N	One father N	Selfing N					
B3H	77	35	45.5	23	19	0	19	30	
B5H	75	35	46.7	29	11	1	12	25	
B6H	65	24	36.9	20	21	0	21	30	
B11H	77	46	59.7	10	21	0	21	26	
B12H	80	46	57.5	16	18	0	23	32	
B17H	68	31	45.6	18	19	0	19	25	
B18H	76	39	51.3	8	29	0	29	33	
B20H	77	40	51.9	19	18	1	19	25	
B22H	67	30	44.8	9	28	1	28	34	
B24H	67	19	28.4	7	41	0	41	41	
Total	729	345	47.3	159	225	3	232	301	
(inside)					(384)		(232)		

using the software Cervus (ver. 1.0; Marshall et al. 1998). This analysis is based on the calculations of Mendelian segregation probabilities. Given the genotypes of offspring, their known mothers and the potential fathers within the population, the probability that a given offspring with a known mother was sired by a given male is calculated. Then, the paternity is assigned to the male with the highest log-likelihood ratio (LOD score, or log of the likelihood ratio, as defined in Meagher 1986), that is, to the most-likely father. The method implemented in the software Cervus uses computer simulations of paternity inference to evaluate the statistical significance of LOD scores. It thus allows assignment of paternity to the most-likely father, given that the difference between the LOD score of the most-likely father and that of the second most-likely father is statistically significant (Marshall et al. 1998).

Male reproductive success estimation

The male reproductive success of the individuals located within the population was studied in two ways. First, we estimated the male reproductive success based on the 232 progenies for which a single father was assigned within the population by the ML-based categorical analysis at the 95% confidence level (Cervus ver. 1.0; see above), as the proportion of offspring sired per each individual. Second, male reproductive success of males and hermaphrodites was estimated by using a ML-based fertility analysis program (PatQuest, kindly provided and implemented to analyze microsatellite loci by T. R. Meagher) based on the likelihood methodologies described in Meagher (1986), Smouse and Meagher (1994), and Smouse et al. (1999). This second analysis was carried out to consider all the offspring for which at least one father ($N = 384$; i.e., 52.7% of the total number of progeny analyzed) was assigned within the population. As

for the ML-based categorical analysis performed with the Cervus software, Patquest generates the Mendelian segregation probabilities. Then it computes the likelihood of the male parentage spectrum (a vector of the relative male reproductive successes $\{\lambda_k\}$ for each candidate father within the population, $\sum \lambda_k = 1$) and maximizes this likelihood by an iterative algorithm, readjusting progressively the λ_k estimates. A log-likelihood ratio (LLR) test statistic is then used to determine if the males vary significantly in their reproductive contributions.

Conversely to the exclusion-based and the ML-based categorical analyses described earlier, the ML-based fertility analysis uses all the available data but it has been shown to underestimate the variance in male reproductive success (Marshall et al. 1998). We tested for the congruence between estimates obtained by the ML-based categorical method and the ML-based fertility analysis by calculating a Pearson correlation coefficient. The mean and variance of male reproductive success were also compared for both methods with that of hermaphrodites by using an *F*-test on arcsine-transformed data (Sokal and Rohlf 1995).

Phenotypic traits and distance effects analyses

Fitness through the male function is related to traits such as pollen production, flower size, flower display, as well as to the distances between potential mates (see references in Elle and Meagher 2000; Meagher and Vassiliadis 2002). In the study population, the distances between recipient hermaphrodites (10 individuals) and each potential father (23 individuals possible for each hermaphrodite, excluding selfing) were calculated using the map coordinates (Fig. 1). In addition to sex, the size and the flowering intensity were also recorded.

Pollen-mediated gene flow empirically follows a log-linear

model with distance, first developed by Adams and Birkes (1991; e.g., Burczyk et al. 1996; Burczyk and Prat 1997; Kaufman et al. 1998; Streiff 1998; Engel et al. 1999; Elle and Meagher 2000). To disentangle between a sex effect and phenotypic traits or distance effects on the male reproductive success of the individuals within the population, we followed the model developed by Smouse et al. (1999) and implemented in the PatQuest software. The model and analyses are fully detailed in Smouse et al. (1999). Basically, male fertilities are estimated using likelihood criteria to obtain ML estimates of regression coefficients of the following log-linear model:

$$\log(\lambda_{jk}) = \gamma \cdot \delta_{jk} + \beta_1 \cdot z_{1k} + \beta_2 \cdot z_{2k} + \beta_3 \cdot z_{3k}, \quad (1)$$

where λ_{jk} represents the fertility of the k th male over the j th female; δ_{jk} the ln distance between these two individuals; z_{1k} , z_{2k} , z_{3k} the measurements of the sexual phenotype, flower intensity, and individual size of the k th male; and γ and β_i are estimated regression coefficients that relate the distance and the i th feature to male fertility. Following Smouse et al. (1999) and Elle and Meagher (2000), a LLR test and a bootstrap analysis (1000 permutations) were carried out to test both for the significance of the model incorporating all traits and for individual features.

No male features were significant and only a distance effect was observed. A numerical representation of the distance effect was thus obtained by using the following equation:

$$\log(\lambda_{jk}) = \gamma \cdot \delta_{jk} \quad (2)$$

over all the possible intermate distances. As for the male reproductive success estimation (see above), these results were compared to those obtained through the ML-based categorical analysis carried out with the software Cervus ($N = 232$ offspring). The frequency distribution of the effective fertilizations grouped as an average by intermate class-distance (13 5-m distance classes) was determined, and we fitted an equation to these points analogous to (2). As for the above analyses, the curves do not take into account the seeds sired by individuals from other populations (47.3% of the seeds). Finally, we estimated the frequency of potential mates for each distance.

RESULTS

Microsatellites Polymorphism

The two loci used, ATT1 and GA6, did not exhibit any linkage disequilibrium (exact test, $P = 1$) and were highly polymorphic as expected (see Appendix 1), with 17 and 13 alleles, respectively, over the 24 individuals analyzed. Gene diversity values reached 0.918 and 0.893 for ATT1 and GA6, respectively. With such a large allelic diversity, each parental plant was characterized by a unique genotype when taking into account both loci (Appendix 1). The expected exclusion probability estimated for the 24 individuals of the population reached 95.2% over the two loci. Of the 53 possible haploid (pollen) genotypes produced by the 24 individuals, six, five, and one pollen genotypes corresponded to two, three, and four possible fathers, respectively, and the large majority (41,

or 77%) were produced by a unique possible father within the circumscribed population.

Mating Structure

The number of offspring for which no pollen donor could be assigned within the population represented a minimum estimation for the external gene flow or apparent gene flow. The results for each hermaphrodite recipient are presented in Table 1. Over all progenies, 345 (47.3%) of the offspring were assigned to a pollen donor not present within the population, and thus whose sexual phenotype was not known. In the further analysis, we will focus on the remaining 384 (52.7%) progenies sired within the population. We could not account directly for cryptic gene flow because we do not have estimates of allele frequencies from outside the population. Incorporating cryptic gene flow would probably increase our estimate of external gene flow, resulting in a higher variance among male fertility estimates (Sork et al. 1999).

The high exclusion probability values also allowed unambiguous assignment by exclusion-based categorical analysis a unique father to 225 of 384 offspring for which at least one father (including selfing) within the population was compatible (Table 1). Only three of 225 offspring with a unique father within the population could have been produced by selfing (Table 1). Over the 384 progenies with at least one father in the study population, this corresponds to a maximum selfing rate of 0.8%. When using the ML-based categorical analysis (as implemented in Cervus), 232 and 301 offspring were assigned at the 95% and 80% confidence levels, respectively (Table 1). Given that only two loci were used, these results were not much different from those obtained by a simple exclusion (only seven additional seeds were assigned at the 95% confidence level). The results of the ML-based categorical analysis at the 95% confidence level are detailed in Appendix 2.

Male and Hermaphrodite Reproductive Success

The percentage of seeds sired by each pollen donor located in the study population is given in Figure 2. The results obtained through the two male fertility estimation methods (i.e., ML-based categorical and ML-based fertility) are indicated. Both estimates of relative male reproductive success have the same general mean (0.042, $N = 24$) and are highly correlated (Pearson moment correlation, 0.92 $P < 0.001$), which indicates they give overall congruent results. However, the variance was lower with the ML-based categorical analysis (CV = 87.0%) than with the ML-based fertility analysis (111.2%). Two individuals, B24H and B4M, have a null reproductive success with the ML-based categorical analysis but a success that is not null with the ML-based fertility analysis. Only one individual, B3H, had a null reproductive success according to both methods (Fig. 2).

In the following text, the results obtained by the ML-based categorical and ML-based fertility analyses are given with subscripts 1 and 2, respectively. Local hermaphrodites were shown to sire 119 (51.3%) and 271.7 (48.7%) seeds of the 232 and 384 seeds analyzed through the ML-based categorical and ML-based fertility analyses, respectively. They had

Male reproductive success

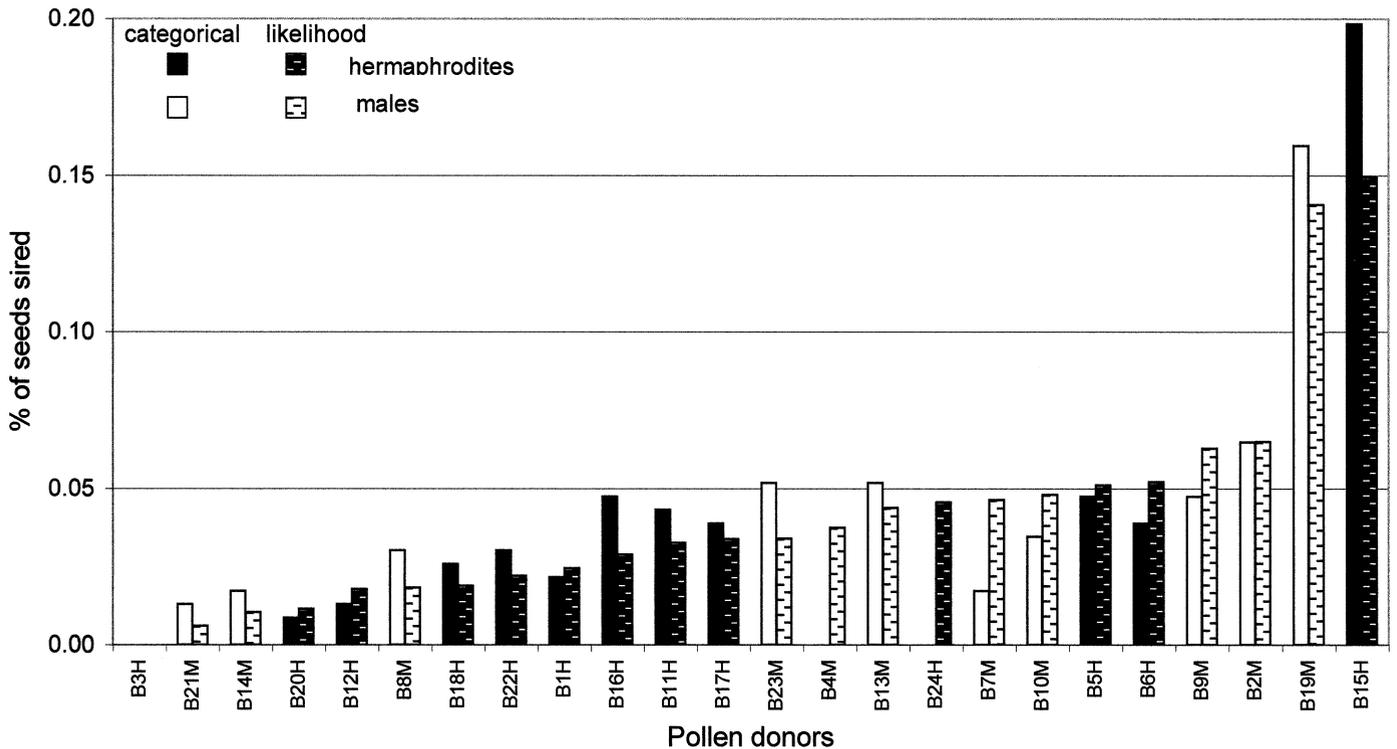


FIG. 2. Male reproductive success of each individual of the population (male, white; hermaphrodite, black), estimated by two methods: (1) plain: the frequency of progeny assigned to a unique father within the population by using a maximum-likelihood-based categorical analysis ($N = 232$ seeds; Cervus software); and (2) dashed: a global maximum-likelihood-based fertility estimate over all progeny with at least one putative father in the population ($N = 384$ progeny; PatQuest software). Individuals are ranked according to estimate (2).

a substantial male reproductive success (mean₁ = 0.039, mean₂ = 0.038, $N = 13$), and according to both estimates, the most fertile individual is a hermaphrodite (B15H, Fig. 2). High variance for both the male reproductive success of males ($CV_1 = 97\%$, $CV_2 = 79\%$, $N = 11$) and hermaphrodites ($CV_1 = 128\%$, $CV_2 = 98\%$, $N = 13$) were observed. Males had a slightly better male reproductive success than hermaphrodites (mean₁ = 0.044, mean₂ = 0.047, $N = 11$). However, this male advantage ($MA_1 = 1.12$, $MA_2 = 1.24$) was not significantly different from one (F -test, $P_1 = 0.65$, $P_2 =$

0.45; and see sex effect in Table 3) and thus was much lower than the minimum expected of two according to the selection model.

Phenotypic Traits and Distance Effects

The mean values of the phenotypic and intermate distances are presented in Table 2; none of the phenotypic traits values were significantly different in males and hermaphrodites. The results of the LLR and bootstrap analyses (carried out with

TABLE 2. Phenotypic traits and intermate distances over all individuals and for each sexual type. Mean values and standard errors (in parentheses) are given for the measured phenotypic features and distances to recipient for males, hermaphrodites, and all individuals. P , t -test values for each parameter, comparing males and hermaphrodites.

	Total $N = 24$	Males $N = 11$	Hermaphrodites $N = 13$	P
Flowering intensity	3.26 (0.18) ¹	3.27 (0.26)	3.25 (0.24) ¹	0.48
Individual height (H)	1.64 (0.10)	1.59 (0.13)	1.68 (0.14)	0.33
First diameter (D1)	3.15 (0.29)	3.15 (0.45)	3.16 (0.36)	0.48
Second diameter (D2)	3.00 (0.24)	2.92 (0.39)	3.08 (0.30)	0.37
Individual size (H × D1 × D2)	30.1 (5.98)	29.6 (10.1)	30.5 (6.97)	0.47
Distance to recipients	32.8 (1.14)	32.85 (1.70)	32.75 (1.52)	0.48
	($N = 230$)	($N = 110$)	($N = 120$)	

¹ Data missing for B24H.

TABLE 3. Effects of sex, distance, and phenotypic traits on the relative male fertility. Sex, sexual phenotype of the pollen donor (male or hermaphrodite); flower, flowering intensity (low, medium, high); individual size, first diameter \times second diameter \times height; coefficient, the gamma and beta values that indicate the direction of the relationship either positive or negative. Log-likelihood ratios (LLR) for each fitted model, as well as chi-square and bootstrap tests of significance, are also given (see Materials and Methods for details).

	Coefficient	LLR	<i>P</i> (chi-square)	<i>P</i> (bootstrap)
Distance	-0.3391	43.869	<0.0001	0.0280
Sex	-0.1094	0.8649	0.8339	0.7705
Flower	0.19688	6.1229	0.1059	0.4545
Size	0.00576	6.7872	0.0790	0.4685
Full model		69.03	<0.0001	

Patquest) testing for the phenotypic traits and distance effects are shown in Table 3. None of the phenotypic features were shown to have a significant effect on the male reproductive success.

The distances between potential mates ranged from 3.2 m to 67.9 m (Fig. 1). The ML-based estimate for the distance parameter γ is -0.34 , which is significantly negative (Table 3). Thus, intermate distance had a significant effect on male reproductive success. The distance effect is mainly due to an excess of successful fertilization at short distances when compared to random-mating expectations (Fig. 3). These high values of male reproductive success at short distances were observed for pairs including either one hermaphrodite pollen donor (pair B15H-B24H) or one male (pair B19M-B18H). When removing B15H and B19M from the analysis, the distance effect was still significant.

DISCUSSION

The reproductive status of the androdioecious species *P. angustifolia* has previously been debated because of several features not in agreement with theoretical expectations (Lepart and Dommée 1992; Vassiliadis 1999). Among those features, the high male frequencies ($\sim 50\%$) have been interpreted as a clue for a possible sexual specialization of hermaphrodites as females (Lepart and Dommée 1992). To definitely ascertain the occurrence of a male function of hermaphrodites in the wild, we used a paternity analysis on 729 offspring collected on open-pollinated shrubs.

The microsatellite markers employed in this study exhibited high variability, allowing an accurate estimation of mating success and patterns in this natural population. The high value of the exclusion probability, greater than 95%, was obtained with only two loci and corresponds to values generally obtained with such markers in similar studies in plants (e.g., more than 99% with nine loci in *Pithecebollium elegans* a tropical tree [Chase et al. 1996]; 99.99% with six loci in *Quercus petraea* and *Q. robur* [Streiff et al. 1999]). This high polymorphism allowed us to identify the seeds sired by outside and unknown fathers (47.3%) and to examine the sexual status of male parent for 384 seeds (52.7%) sired by individuals located in the study population. From the ML-based fertility analysis car-

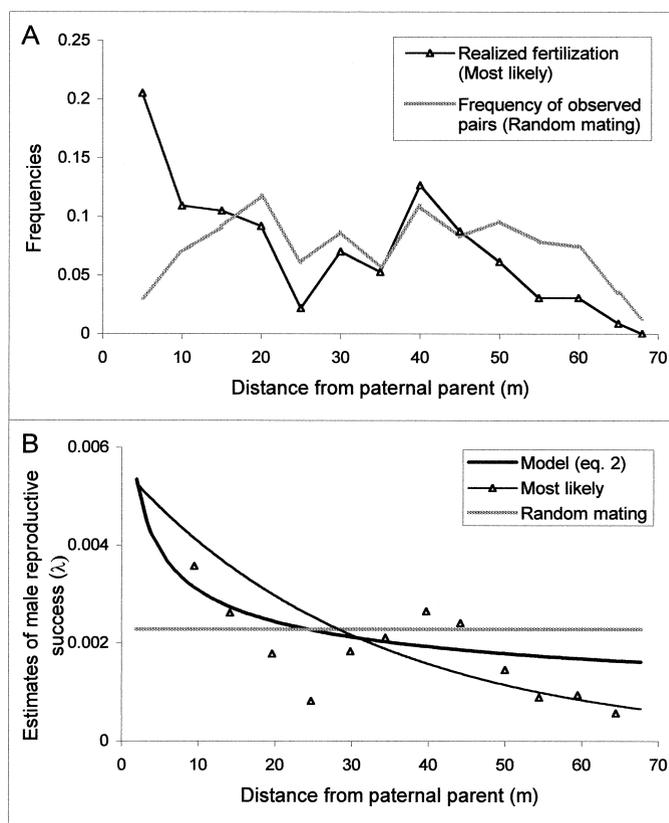


FIG. 3. Effect of the distance on male reproductive success. (A) Frequency of fertilization success based on the maximum-likelihood-based categorical method averaged by intermate class-distance ($N = 232$ progeny, 13 5-m distance classes; for 0–5 m, the value 0.015 is not on the graph) and frequency distribution of possible couples (random-mating), as a function of intermate class-distance (13 5-m distance classes). (B) Male reproductive success as a function of intermate distances. Bold line: regression estimate of male reproductive performance (based on eq. 2 with maximum-likelihood estimate of $\gamma = -0.34$) as a function of intermate distance. Open triangles: male reproductive success estimates calculated from the maximum-likelihood-based categorical method (see A). Thin line: fitted equation to these observed data ($\log[\lambda_{jk}] = -0.85(\delta_{jk} - 3.48)$, $R^2 = 0.69$). Gray line: expected male reproductive success under random mating hypothesis, with no distance effect.

ried out on these 384 seeds, the cumulative male reproductive success of hermaphrodites ($N = 13$) was shown to reach 48.7% (51.3% following the ML-based categorical analysis; $N = 232$ seeds). One of the hermaphrodites (B15H) sired nearly 15% of the seeds (20% with the ML-based categorical analysis). From the analysis of the sexual status of plants siring seeds within the study population, the male function of hermaphrodites of the androdioecious *P. angustifolia* was thus ascertained unequivocally.

In previous single-donor controlled crosses, the male advantage estimated was 1.93 (Vassiliadis et al. 2000a). In the present study, when considering only the siring success within the population, the pollen produced by the hermaphrodites appears as efficient as the pollen of the males; the male advantage measured was not significantly different from one (Table 3; sexual phenotype). Thus, the result suggested by

controlled-crosses is only reflected as a trend in the study population.

To our knowledge this is the first study on androdioecious species in which male reproductive success has been directly measured by a paternity analysis. In two other species studied, *Datisca glomerata* and *Mercurialis annua*, both of them wind-pollinated, the male reproductive success was indirectly estimated by quantifying flower and/or pollen production, resulting in an estimated male advantage of 2.93 in *D. glomerata* (Philbrick and Rieseberg 1994; Spencer and Rieseberg 1995) and four to 10 in *M. annua* (Pannell 1997b). In these species, the male advantage estimates are well above the threshold of two and in a range expected in selection models. However, given the present analysis, it would be of interest to confirm these high male advantages by paternity analyses.

Given the high male frequency in the studied population (45.8%) and the high reproductive success of hermaphrodites shown in the present study, the question of the maintenance of androdioecy in *P. angustifolia* still awaits explanation. More specifically, one can wonder what may explain the large male function observed in hermaphrodites and the concomitant maintenance of males at such a high level.

The male advantage estimation and the sexual effect analysis were carried out only on a part (52.7%) of the total seeds analyzed. A large number of seeds (47.3%) were found to have been sired by individuals located outside of the study population and for which the sexual status of the paternal parent was unknown. Such high estimates of extrapopulation gene flow have already been commonly reported in paternity studies of wind-pollinated trees (30–69% in conifers, Adams and Birkes 1991; 56% in *Pinus attenuata*, Burczyk et al. 1996; 54% in *Pseudotsuga menziesii*, Burczyk and Prat 1997; 57% in *Quercus macrocarpa*, Dow and Ashley 1998; 65% in *Q. robur* and 69% in *Q. petraea*, Streiff et al. 1999). For *P. angustifolia*, this high value of the minimum estimate of the external gene flow is not surprising, given that it is a wind-pollinated species and the study population was located in an open, windy landscape; other populations were found in the vicinity (see Materials and Methods) even if the largest populations were more than 500 m away; and the density of the study population was very low, so that in absence of synchronicity of flowering in the population, the pollen from individuals outside the population may compete with local pollen. The high external gene flow observed might bias downward the male fertility and male advantage estimation in two ways: (1) the progeny sired by external pollen may have been sired by more males than hermaphrodites; and (2) conversely, pollen from males in the study population may have sired more seeds outside the population than pollen from hermaphrodites. In both cases, the real male advantage could reach higher values, similar to those observed in previous controlled crosses (Vassiliadis et al. 2000a). Additional paternity analyses would need to encompass the surrounding populations to ascertain this point. However, our main conclusions still hold: in the wild, the pollen of hermaphrodites is functional; and her-

maphrodites may sire a large number of seeds as pollen donors, at least at a local scale.

Our results may be related to specific features of the study population. Indeed, to make the paternity analysis feasible we sampled a population characterized by a low density. In androdioecious species, the evolution toward dioecy and complete separation of sexes can be prevented by a selective advantage for male function in hermaphrodites, as already suggested for the weeds *D. glomerata* and *M. annua* (Liston et al 1990; Pannell 1997a). In *P. angustifolia*, Lepart and Dommée (1992) suggested that androdioecy might be a case of leaky dioecy in which females have a limited male function to ensure reproductive success during the establishment of new low-density populations. Although the study population is of low density (50 individuals/ha) and seems to be a newly established one (populations in southern Camargue are usually aged less than 30 years; Vassiliadis 1999), we cannot ascertain, without comparing with old and dense populations, that the hypothesis proposed by Lepart and Dommée (1992) holds.

More interestingly, of the four features tested for their effects on male reproductive success (namely sexual phenotype, individual size, flowering intensity, and intermate distance), only intermate distance played a significant role. Distance effect depends on pollination mode. An excess of near-neighbor matings as compared to random mating expectation was reported in insect-pollinated plant studies (Meagher 1986; Devlin and Ellstrand 1990; Godt and Hamrick 1993; Schnabel and Hamrick 1995; Smouse et al. 1999). Results for wind-pollinated plants are more varied; such distance effects were found in some wind-pollinated trees (Burczyk and Prat 1997; Kaufman et al. 1998; Streiff et al. 1999) but not in some conifers (Adams and Birkes 1991; Latta et al. 1998; Schuster and Mitton 2000). Here, we found an isolation-by-distance effect (male reproductive success declining with intermate distance) together with a substantial amount of successful pollen from outside the population (47.3%). The significant distance effect at short distance is mainly due to a few highly successful short-distance crosses, which may involve phenological synchronization or other pairwise factors. Indeed, other potential close pairs did not show this high fertilization success (e.g., pair B2M-B3H is closer but less successful than pair B2M-B12H). Moreover, in the study population, only one or two fathers sired most of the progeny of any given recipient. Although we cannot exclude the hypothesis of pollen competition (e.g., Ellstrand 1984; Marshall and Ellstrand 1986; Broyles and Wyatt 1990; Arnold 1994; Stanton 1994; Willson 1994; but see Krauss 2000), this homogeneity within a progeny together with the low density of individuals (which decrease the intensity of pollen competition) suggest that phenological synchronization between individuals may be very important in the siring success.

In conclusion, the study population exhibited a low density and a small number of potential fathers (with slightly more hermaphrodites than males), a situation in which pollen competition should be low and male reproductive success could mainly depend on random and pairwise factors such as the spatial positions of individuals (intermate distance effect) and

their phenologies. Thus, the characteristics of the study population could partially explain our results.

The task now is to explain more generally the seeming contradiction of the maintenance of a high male fertility in hermaphrodites and of functional androdioecy in *P. angustifolia*, while the frequency of males often reaches 50%, a case for which functional dioecy is expected (Charlesworth 1984). We propose two possible and nonexclusive hypotheses, based on our field experience and on a theoretical study.

First, in denser populations, increased pollen competition and resource allocation trade-off in hermaphrodites would result in a higher male advantage. Male reproductive success would then depend on population age and density. Sexual differentiation between males and hermaphrodites would select against evolution toward hermaphroditism. To better understand why so many males are present in the natural populations, similar paternity studies are needed on other populations of *P. angustifolia*, varying in density and in their level of isolation from surrounding populations.

Second, the maintenance of high frequencies of males with a low male advantage can be explained by a new theoretical model that includes a genetic linkage between self-incompatibility and female sterility loci (Vassiliadis et al. 2000b). *Phillyrea angustifolia* is known to be self-incompatible (less than 0.8% of the seeds analyzed here derived from selfing; Vassiliadis et al. 2000a). In that model, the spread of a dominant female-sterility allele in a hermaphroditic population, and thus the maintenance of androdioecy, can be induced under complete linkage between a self-incompatibility locus and the sex locus, as long as the number of self-incompatibility alleles is small. Under such conditions, functional androdioecy with frequencies of males equal or larger than 50% can be maintained, even with a male advantage in fertility lower than two. The characterization of the genetic determinism of sexual phenotypes and of the linkage between sex and self-incompatibility would be of great importance for further study of androdioecy in *P. angustifolia*.

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APPENDIX 1

Genotypes at both microsatellite loci, ATT1 and GA6 (alleles are defined as the size of alleles in base pair, null allele is indicated by the size 50), for all mature individuals of the studied population. The sexual phenotype is indicated at the end of the name of each individual (H, hermaphrodite; M, male). For each locus, the number of alleles (N_{all}), the observed (H_o) and expected (H_e) heterozygosity, the f -value (P -value of the test for Hardy-Weinberg expectation), and the exclusion probability (EP) are given (see Materials and Methods).

Individuals	ATT1		GA6	
B1H	89	92	50	109
B3H	71	130	107	117
B5H	71	130	113	117
B6H	121	130	109	127
B11H	135	143	123	137
B12H	71	89	109	137
B15H	71	147	107	127
B16H	80	135	115	115
B17H	71	121	111	117
B18H	104	135	50	137
B20H	127	127	109	121
B22H	127	135	125	137
B24H	71	92	109	109
B2M	89	92	109	115
B4M	71	121	109	109
B7M	121	127	109	115
B8M	108	120	111	113
B9M	127	127	115	127
B10M	74	133	127	127
B13M	74	86	111	127
B14M	71	71	105	105
B19M	100	130	109	115
B21M	89	89	50	111
B23M	135	135	109	137
N_{all}	17		13	
H_o	0.792		0.792	
H_e	0.918		0.893	
f -value (P_{HW})	0.141 (0.02)		0.115 (0.24)	
EP	0.798		0.753	

APPENDIX 2

Maximum-likelihood-based categorical analysis, detailed within-population mating structure. The number of offspring obtained by the maximum-likelihood-based categorical analysis at the 95% confidence level (Cervus software; Marshall et al. 1998) for each pollen donor–hermaphrodite pair is indicated (total $N = 232$).

	B3H	B5H	B6H	B11H	B12H	B17H	B18H	B20H	B22H	B24H	Per pollen donor
B1H	5										5
B3H											0
B5H		1	8		1			1			11
B6H	1	1			1	2	1	2		1	9
B11H							1		8	1	10
B12H		1		1				1			3
B15H	2		1	3		3	2	1	3	31	46
B16H	1	1		3	3		1	1	1		11
B17H		1	2						6		9
B18H		1	1	2					2		6
B20H								1	1		2
B22H			1	1	2		1	1	1		7
B24H											0
B2M	3			2	5	2	1	1	1		15
B4M											0
B7M			4								4
B8M		2						1		4	7
B9M	3			3	2	1	1		1		11
B10M		3	1	1				1	2		8
B13M	2		2	3	1					4	12
B14M						2	1	1			4
B19M	1		1	1	2	8	17	7			37
B21M					1				2		3
B23M	1	1		1	5	1	3				12
Per maternal plant	19	12	21	21	23	19	29	19	28	41	232