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Mating system and multiple paternity in *Hedysarum coronarium* L. (Fabaceae)

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Abstract – The mating system was studied in four populations of *Hedysarum coronarium* under the mixed-mating model (MMM) and the effective selfing model (ESM) by analysis of progeny arrays. Multilocus population outcrossing rates ranged from 0.822 to 1.013. Pollination experiments indicated that *H. coronarium* is self-compatible, and therefore the high outcrossing rates were attributed to inbreeding depression. This view was supported by high levels of seed abortion in self pollination when compared to open pollination. The effective selfing rate ranged from $E = 0.026$ to $E = 0.183$. Positive covariance of selfing with gene fixation ($D = 0.085$) was observed in one population and could be attributed to subpopulation structure and mating between relatives.

Estimates of the level of multiple paternity under the correlated mating model (CMM) was assessed at the progeny array level. The correlation of the outcrossed paternity (r_p) ranged from 0.029 to 0.342 and according to the outcrossing rates, up to 80% of the total seeds in a progeny array may have different male parents.

***Hedysarum coronarium* / outcrossing rate / progeny array / effective selfing / multiple paternity**

Résumé – Régime de reproduction et paternité multiple chez *Hedysarum coronarium* L. (Fabaceae). L'étude du régime de reproduction a été réalisée chez quatre populations de *Hedysarum coronarium* en utilisant le modèle de reproduction mixte et le modèle d'autofécondation par l'analyse des tableaux de descendance. Les taux d'allogamie multilocus des populations varient de 0,822 à 1,013. Des expériences de pollinisation ont montré que *H. coronarium* est autocompatible et les taux d'allogamie élevés peuvent être attribués à la dépression de consanguinité. Ce point de vue est soutenu par l'augmentation du taux d'avortement des graines en autopolinisation manuelle.

Le taux d'autofécondation (E) varie de 0,026 à 0,183. Une covariance positive ($D = 0.085$) entre E et l'indice de fixation des parents a été observée chez une des quatre populations. Elle peut être attribuée à la présence de subdivision au sein de cette population et à des croisements entre apparentés.

Le taux de paternité multiple a été évalué sous le modèle de reproduction corrélée. La corrélation de paternité chez les descendants issus d'allocroisement (r_p) varie de 0,029 à 0,342. En se basant sur les taux d'allogamie obtenus, la proportion des graines issues d'une plante mère et ayant des parents mâles différents peut s'élever à 80 %.

***Hedysarum coronarium* / autofécondation / paternité multiple / tableau de descendants / taux d'allogamie**

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1. Introduction

Mating systems have a profound influence on the extent and structure of genetic variation in plant populations. Outbreeding populations are expected to have greater genetic diversity, higher levels of heterozygosity and less differentiation among populations than self-fertilized populations [5, 9]. Plants exhibit a variety of mating systems which include self-fertilization, outcrossing, or a mixture of both. The consequences of these different mating systems will markedly affect the structure and the evolution of populations [9].

The mixed mating model [4, 18, 33] gives a robust procedure for estimating mating system parameters. However, other approaches have been developed permitting further investigations of demographic and genetic causes of mating patterns. The effective selfing model [25, 26] calculates mating system parameters in spatially structured populations by considering mating among relatives and selfing in the estimate of effective selfing.

In multiseeded fruit, outcrossed progeny may share the same male parent if siring pollen derives from one source [35] or one pollination event [23]. Correlation of outcrossed paternal parentage has been reported for several species [15, 22, 23].

The genus *Hedysarum* (Fabaceae) contains a number of species distinguishable by their different morphology, reproductive systems, karyotype features and geographical origins [1]. In Tunisia, the Dorsal mountains separate the semi-temperate ecological domain of *H. coronarium* and *H. capitatum* to the north and the arid ecological domain of *H. carnosum* and *H. eu-spinosissimum* to the south [2].

Hedysarum coronarium is distributed in the western mediterranean basin where it grows wild. It is grown for fodder in a number of countries including Spain, Italy and North Africa. In Tunisia, the species has been studied by numerous authors and exhibits considerable variability [11, 17, 39, 40] and a predominantly allogamous mating system [7, 14].

In this study, mating system parameters of *H. coronarium* were estimated. The information obtained should help to explain the distribution of genetic diversity in this species.

2. Materials and methods

Hedysarum coronarium is an entomophilous species, and bees (*Apis mellifera* L.) visit the flowers. Both self- and cross-fertilization may occur and both depend fully on insects. A plant can self-fertilize after mechanical stimulation of the stigma by visiting insects, so-called “tripping”.

A pollination program was conducted in the greenhouse of the Faculté des Sciences de Bizerte (Tunisia) to determine the levels of self-compatibility of *H. coronarium*. Thirty plants per population, raised from seeds (collected from wild populations), were grown to flowering and a mean of fourteen plants from each population were allocated to hand self-pollination and open-pollination. Percentage of pod set, mean seed numbers per pod and mean number of aborted seeds per pod were calculated for each of the two pollination treatments.

The populations used in the study of the mating system are listed in Table I. At each sampling site, seeds were harvested in early June. From each adult, several multipoded infrutescences (up to ten) were collected. Seeds from the same maternal parent were bulked. Twenty seeds per maternal parent were germinated and only eight were used in electrophoresis. From each seedling, the coleoptile was crushed with a glass rod in 50 µl of extraction buffer [41]. Extraction was performed directly in Eppendorf tubes over an ice bath. Extracts were absorbed onto 3 × 10 mm whatman paper wicks, and inserted into a 13% (w/v) starch gel for horizontal electrophoresis. The buffer used was Histidine pH 6.2 [41]. The enzyme systems studied were: isocitric dehydrogenase (IDH), shikimic dehydrogenase (SKDH), 6-phosphogluconate dehydrogenase (6-PGD), phosphogluco-isomerase (PGI), Malate dehydrogenase (MDH) and phosphoglucomutase (PGM). Only two loci were

Table I. Populations and progeny array characteristics.

Population	Number of families	Total progeny size	Density	Morph	Flowers
El Alia	24	192	Low	Prostrate	Red violet corolla
Maktar	28	224	Medium	Prostrate	Violet corolla
Tunis	24	192	Medium	Prostrate	Red violet corolla
Amairia	25	200	High	Erect	Red violet corolla

polymorphic enough to be used in mating system analysis: Pgi-2 and Skdh-1.

We used the computer program MLTR version 1.1 [30] to estimate multilocus t_m and average single-locus outcrossing rates t_s at population level under the mixed-mating model (MMM). Standard errors of t_m , t_s and their difference $t_m - t_s$ were calculated using 300 bootstrap replicates.

In contrast to the mixed mating model, the effective selfing model (ESM) assumes equality of gene frequencies among pollen and ovules. At least 24 maternal parents are needed to obtain reliable estimates. The program ESR developed by Ritland [29] estimates the total amount of “apparent selfing”, also termed “effective selfing”, at a single locus caused by either self-fertilization or mating to relatives. Joint maximum likelihood estimates of gene frequency, the inbreeding coefficient of parents (F), the selfing rate of inbred (S_i) and outbred parents (S_o) were calculated [26, 32]. Three further estimates were subsequently obtained based on ESM [31, 32]. Firstly, the effective selfing rate $E = F S_i + (1 - F) S_o$ which equals the self-fertilization rate when there are no consanguineous matings, although population substructure may cause it to be greater than the self-fertilization rate [31]. Secondly, the inbreeding assortative selfing rate (D), also called the covariance of effective selfing with parental gene fixation, $D = F(1 - F)(S_i - S_o)$. Positive D maintains the variation of F among neighbourhoods and is an indicator of nonself inbreeding. The third parameter is the change in the fixation index from parents to progeny (ΔF). Changes in F -values between generations will give an indication of whether any selection against homozygotes or heterozygotes occurs during later stages of the life cycle.

Correlated mating is another facet of the mating system, and the correlated mating model (CMM) is a parametric approach to mating system estimation that accounts for covariances among mating events [27, 28]. Two parameters of correlated matings were estimated with the program MLTR: the correlation of outcrossing rate within progeny arrays (r_l) and the correlation of outcrossed paternity within progeny arrays (r_p) which is the probability that a randomly chosen-pair of progeny from the same array are full sibs.

For the four populations studied, MLTR was used to estimate the single-plant multilocus outcrossing rate jointly with gene frequencies (p) of the outcrossing pollen pool.

To measure the effective selfing caused by consanguineous matings, p estimates were regressed upon additive values of maternal genotypes [31, 33]. Additive values of 0, 0.5 and 1 were assigned to maternal genotypes aa , Aa and AA , respectively, where “ A ” is the most common allele and “ a ” the class of other alleles at that locus.

3. Results

Pod set following open-pollination ranged from 45.7% in the Tunis population to 57.3% in the Amairia population with a mean of 53.3%. Pod set following hand self-pollination varied from 24% in the El Alia population to 28.2% in the Amairia population with a mean of 26.2%. Mean seed numbers per pod over the four populations were 2.12 and 2.62 for hand self-pollination and open-pollination respectively. Mean number of aborted seeds per pod for hand self-pollination varied from 0.57

in the Tunis population to 0.92 in the El Alia population. Lower seed abortion was observed after open-pollination, values ranged from 0.04 (in El Alia) to 0.15 (in Tunis). These results of pollination experiments indicate that *Hedysarum coronarium* is self-compatible and self-fertile.

Multilocus and average single-locus estimates of the outcrossing rate and their differences are given for each population in Table II. Gene frequencies for the ovule and pollen pools are presented in Table III. t_m values ranged from 0.822 to 1.013, suggesting a predominance of outcrossing in the set of populations studied. It was found that t_s values were slightly lower than t_m in two populations (El Alia and Tunis) hence positive ($t_m - t_s$) differences.

The analysis of data under the ESM (Tab. IV) gave mean effective selfing rates (E) which ranged from 0.026 in Amaïria to 0.183 in El Alia. Considering inbred and outbred parents, strong divergence was found for the apparent selfing rate between them in the El Alia population ($S_i > S_o$) (Tab. IV). Similarly, this population differed from the three others in terms of covariation of E with F: the populations of Amaïria, Tunis and Maktar did

Table II. Population estimates of the outcrossing rate for *Hedysarum coronarium* under the mixed mating model.

Population	t_m (SD)	t_s (SD)	$t_m - t_s$ (SD)
El Alia	0.822 (0.126)	0.819 (0.110)	0.003 (0.039)
Maktar	0.925 (0.090)	0.937 (0.099)	-0.012 (0.020)
Tunis	0.833 (0.066)	0.828 (0.064)	0.005 (0.020)
Amaïria	1.013 (0.055)	1.030 (0.051)	-0.017 (0.026)

t_m , multilocus outcrossing rate, t_s , single-locus outcrossing rate averaged over loci, and their difference. Variance estimates based upon 300 bootstraps (unit of resampling Family within population).

not show any covariation as a consequence of nearly zero F-values (Tab. IV). The positive covariation ($D > 0$) observed in the El Alia population is known to maintain variation of F among population subdivisions [31, 32] and may reflect the occurrence of mating between relatives. Maternal parents appeared inbred in the El Alia population ($F = 0.159$) (Tab. IV).

There is great variation among estimates of single-plant outcrossing rates for the populations studied (Fig. 1). These estimates can be strongly

Table III. Allele frequencies for two variable loci in ovule and pollen pools for each population of *Hedysarum coronarium*.

	Skdh-1					Pgi-2			
	1	2	3	4	5	1	2	3	4
EL Alia									
Ovule	-	0.646	-	0.354	-	-	0.083	0.917	-
Pollen	-	0.490	-	0.510	-	-	0.061	0.939	-
Maktar									
Ovule	0.018	0.411	-	0.571	-	0.024	0.246	0.702	0.018
Pollen	0.07	0.493	-	0.437	-	0.035	0.170	0.786	0.019
Tunis									
Ovule	-	0.490	0.020	0.449	0.041	-	0.250	0.688	0.063
Pollen	-	0.343	0.013	0.590	0.055	-	0.189	0.618	0.193
Amaïria									
Ovule	0.019	0.692	-	0.269	0.019	-	0.137	0.843	0.020
Pollen	0.015	0.676	-	0.304	0.005	-	0.168	0.807	0.025

Figure 1. Distribution of outcrossing rates in El Alia (a), Amaïria (b), Tunis (c), and Maktar (d) populations of *Hedysarum coronarium*.

affected by the influence of maternal genotype and population marker diversity on the probability of detecting outcrosses [43].

Although higher in Amaïria, El Alia and Maktar, r_t was close to zero ($r_t = 0.053$) in Tunis (Tab. V), indicating more homogeneity of the outcrossing rate among maternal parents in this population.

Correlation of outcrossed paternity was very low ($r_p = 0.029$) in Tunis (Tab. V) suggesting frequent and successful multiple matings in this population. Although r_p varied among populations, a value of 1 was never reached, therefore excluding the monopaternal origin of outcrossed progeny. The average mating pool (neighbourhood size) is calculated as the inverse of r_p and is the number of males that gives rise to this correlation, assuming all males have equal mating probabilities and consecutive matings are independent [28]. This neighbourhood size varied between 2–3 plants in El Alia and 34–35 plants in Tunis (Tab. V).

The regression of outcrossing-pollen allele frequency on additive maternal genotype value varied widely among loci and populations (Tab. VI). Significant regression was observed only in the El Alia population at the Pgi-2 locus. A negative value of the coefficient is unlikely to be related to consanguineous matings.

4. Discussion

Variation in outcrossing rates between populations appears to be a common phenomenon in mixed mating species [5, 10, 37]. Our study of the mating system in *H. coronarium* indicates that outcrossing varies both within and between populations. The multilocus estimate is less affected by selection and non-random outcrossing than are single-locus estimates [5]. Differences between multilocus and mean single-locus outcrossing rates for individual populations provide a measure of biparental inbreeding [29, 33]. These differences were negligible for all the populations studied, thus it seems that biparental inbreeding is negligible. Localized seed dispersal, coupled with local pollen transfer, can lead to the development of population

Table IV. Estimates of *Hedysarum coronarium* mating system parameters under the ESM.

Parameters	Population			
	EL Alia Estimate (SD) 95% CI	Maktar Estimate (SD) 95% CI	Tunis Estimate (SD) 95% CI	Amaïria Estimate (SD) 95% CI
E	0.183 (0.087) (0.018 ; 0.382)	0.110 (0.084) (0.001 ; 0.227)	0.140 (0.064) (0.004 ; 0.254)	0.026 (0.23) (0.001 ; 0.082)
S_i	0.716 (0.298) (0.001 ; 0.999)	0.001 (0.417) (0.001 ; 0.999)	0.094 (0.427) (0.001 ; 0.999)	0.010 (0.473) (0.001 ; 0.999)
S_o	0.082 (0.101) (0.001 ; 0.362)	0.110 (0.089) (0.001 ; 0.297)	0.140 (0.067) (0.001 ; 0.252)	0.026 (0.008) (0.001 ; 0.026)
F	0.159 (0.124) (0.001 ; 0.444)	0.001 (0.046) (0.001 ; 0.157)	0.001 (0.040) (0.001 ; 0.125)	0.007 (0.066) (0.001 ; 0.242)
D	0.085 (0.048) (0.000 ; 0.150)	0.000 (0.015) (-0.023 ; 0.052)	0.000 (0.018) (-0.009 ; 0.062)	0.000 (0.019) (0.000 ; 0.072)

E, average effective selfing rate; S_i and S_o , effective selfing rates of inbred and outbred parents; F, parental inbreeding coefficient; D, covariation of parental F with E: $D = F(1-F)(S_i - S_o)$. Standard deviations (in parentheses) and 95% confidence intervals based upon 1000 bootstraps.

subdivision and biparental inbreeding [19]. Nevertheless, t_m was not strictly equal to unity except for population Amaïria.

The value of parental inbreeding was zero in three of the four populations (Maktar, Tunis and Amaïria). This suggests that parental inbreeding depression may occur before the adult stage [37]. This may be due to the presence of selection against biparentally inbred progeny, which can occur if genetic load is high [37]. Several lines of evidence indicate that selection operates against inbred progeny in self-compatible species [20]. This view is supported by high levels of seed abortion in hand self-pollination when compared to open-pollination. Thus, higher levels of embryo abortion may be responsible for maintaining elevated levels of outcrossing rates in populations [21]. The protandrous nature of flower development in *H. coronarium*, sequential flowering on the same inflorescence during anthesis period and competitive inferiority of self pollen reported for this species [8] are additional causes of a high outcrossing rate.

Analysis of the mating system under ESM indicates the occurrence of biparental inbreeding in the El Alia population since (i) the covariation of E

Table V. Correlated mating parameters of *Hedysarum coronarium* under the sibling-pair model.

Population	r_i (SD)	r_p (SD)	Neighbourhood size ($1/r_p$)
El Alia	0.990 (0.558)	0.342 (0.202)	2–3
Maktar	0.717 (0.586)	0.129 (0.139)	7–8
Tunis	0.053 (0.153)	0.029 (0.077)	34–35
Amaïria	0.990 (0.891)	0.184 (0.204)	5–6

r_i , correlation of outcrossing rate within progeny arrays; r_p , correlation of outcrossed paternity within progeny arrays. Variance estimates based upon 300 bootstraps (unit of resampling = family within population).

Table VI. Regression of the gene frequencies of the pollen outcrossing pool upon maternal genotypes for *Hedysarum coronarium*.

Locus	Population			
	El Alia	Maktar	Tunis	Amaïria
Skdh-1	0.03 (NS)	0.102 (NS)	0.055 (NS)	-0.102 (NS)
Pgi-2	-0.441** ($p=0.014$)	-0.234 (NS)	-0.159 (NS)	-0.059 (NS)

Significance tested with ANOVA on the regression coefficient.

with F is positive ($D = 0.085$) and (ii) $S_i > S_o$ indicating that inbred parents effectively self more than outbred parents. Similar results were reported in subdivided populations [12, 25, 31]. A positive value of F ($F = 0.159$) in El Alia indicates that selfed progeny survived to maturity. This may be an example of the “heterozygosity paradox” of Brown, who found that homozygosity showed a puzzling excess in outbreeders [3]. In addition, covariance of selfing with gene fixation may have consequences on fitness in El Alia. In fact, for inbred or homozygous neighbourhoods where effective selfing rates are higher, deleterious recessives become less frequent and alleles advantageous in the homozygous condition may increase in frequency [32].

Variation of the mating system within populations is assessed by among-plant variation of the outcrossing rate. The distribution of family t_m estimates (Fig. 1) is nearly bimodal in the El Alia population with more than 50% of plants virtually completely selfed or outcrossed which seems in accord with the high correlation of outcrossing ($r_t = 0.990$). Within progeny arrays in the Amaïria and Maktar populations, classes of intermediate outcrossing are observed and high r_t values may be attributed to the presence of important classes of highly selfing (Amaïria) or highly outcrossing (Maktar) plants. In the Tunis population, t_m for maternal plants were more dispersed and hence may explain a low correlation of outcrossing within progeny arrays ($r_t = 0.053$). Variation in estimates of single-plant outcrossing rates may arise from variation in the distance to nearest neighbours [36] (as in the El Alia population), genetic differences in levels of self-fertility [6] and asynchrony in flowering times [13, 21, 24].

Correlation of outcrossed paternity within progeny arrays (r_p) was recently estimated for the herbaceous annual *Centaurea solstitialis* [37] and for two woodland shrub *Daviesia suaveolens* and *D. mimosoides* [43]. In these three pollinator-dependent outbreeders, r_p estimated with MLTR ranged from 0.047 to 0.636. In our study, r_p varied from 0.029 in Tunis to 0.342 in El Alia. The last value indicates that 34% of outcrossed seeds share

the same male parent and 66% do not. As the outcrossing rate is 0.82 in the El Alia population, 54% of the total seeds in the progeny array share different paternal parents. The chance of producing outcrossed full-sibs is increased in a small sparse population like the El Alia. A similar result was reported for two populations of *Daviesia mimosoides* [43]. In the three other populations of *H. coronarium*, despite the differences observed in their outcrossing rates, the proportion of multipaternal seeds was approximately the same (80%), reflecting a high diversity of pollen donors in these populations. A decrease in r_p increases the neighbourhood size as in the Tunis population where the average “paternal mating pool” was 34–35 which is larger than the progeny array sample size. A similar result was obtained by Sun and Ritland [37] in one population of *Centaurea solstitialis*.

H. coronarium is becoming an important forage species and breeding programs for its improvement should include determination of its mating system and quantitative estimation of its genetic parameters. The half-sib relationship among progeny within open-pollinated families often assumed [42] when estimating additive genetic variance and the heritability of traits, is not usually met as shown by our results. With a half-sib relationship, the genetic correlation among progeny is 0.25, indicating that the progeny have 1/4 of their genes in common. Any degree of correlated mating, mating among relatives, or self-fertilization will cause an increase in the coefficient of relationship [38].

The violation of the half-sib assumption will result in overestimations of additive genetic variance, heritability and, subsequently, genetic gains [16]. In an entomophilous species, like *H. coronarium*, the combination of pollinator behaviour and genetic substructuring may create a hierarchy of correlated matings within inflorescences and individual plants [5]. Thus, according to our study, 20% to 46% of the total seeds in the progeny array are full-sibs. Therefore, the coefficient of relationship is expected to be higher than 0.25.

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