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Estimation of mating system parameters in *Hedysarum coronarium* L. (Leguminosae, Fabaceae)

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Abstract – Appropriate knowledge of genetic variation and mating systems is necessary to optimise conservation and utilisation of genetic resources. The present study provides estimates of mating system parameters in three natural populations of *Hedysarum coronarium*, using isozyme markers. There was no detectable variation among populations of single- and multilocus outcrossing rates (t_s , t_m), which did not differ significantly from complete outcrossing. However, in pooled populations, estimates of t_s (0.820 ± 0.073) and t_m (0.830 ± 0.069) were both significantly different from the unity. The fixation index (F) and the biparental inbreeding rate, given by the difference between t_m and t_s , were not significantly different from zero. t_m and t_s , estimated at the seedling stage, were both smaller than those determined at the adult phase, indicating some inbreeding depression acting between these two stages.

Hedysarum coronarium / isozyme / mating system / outcrossing rate

Résumé – Estimation des paramètres du régime de reproduction chez *Hedysarum coronarium* L. (Légumineuse, Fabacée). La connaissance de la variation génétique et des modes de reproduction est nécessaire à l'optimisation de la conservation et l'utilisation des ressources génétiques. Cette étude fournit des estimations du régime de reproduction dans trois populations naturelles d'*Hedysarum coronarium* L., au moyen de marqueurs isoenzymatiques. L'analyse montre que les taux d'allogamie uni- et multilocus (t_s , t_m) présentent de faibles variations entre populations sans qu'ils ne s'écartent significativement de l'allogamie complète. Toutefois, pour l'ensemble des populations, t_s (0.820 ± 0.073) et t_m (0.830 ± 0.069) sont tous deux significativement différents de l'allogamie totale ($t = 1.0$). L'indice de fixation (F) et le taux de croisements consanguins, donné par la différence entre t_m et t_s , ne sont pas significativement différents de zéro. t_m et t_s , estimés sur des plantes au stade juvénile, sont inférieurs à ceux obtenus au stade adulte, suggérant l'existence d'une dépression de consanguinité agissant entre la germination des graines et la maturité des plantes.

Hedysarum coronarium / isozyme / régime de reproduction / taux d'allogamie

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1. Introduction

Hedysarum (Leguminosae, Fabaceae), a genus that occurs in arid, semi-arid and sub-humid regions of the Mediterranean basin, consists of several species distinguished by their morphology and mating systems. Among the Mediterranean group, the diploid ($2n = 16$) species (*H. coronarium*, *H. carnosum* Desf, *H. flexuosum* L. and *H. spinosissimum* L.) are the most widely distributed in the western basin, where they grow spontaneously [3]. These species have a good feeding value and are highly palatable to sheep. In addition, the Spanish sainfoin, *H. coronarium*, is grown as a fodder crop in Spain, Italy and in the north of Africa. In Tunisia, the ecological domain of this species has been well described [12].

There is an increasing interest in the conservation and utilisation of *Hedysarum* genetic resources. However, knowledge is limited concerning genetic variation and mating systems in the genus. Previous studies of floral biology and hand pollination have shown that *H. coronarium* was an obligate outcrosser [10]. The information gained from such studies is limited because it does not provide quantitative measures of the genetic structure of natural populations and the extent of inbreeding occurring, either through selfing or biparental inbreeding, within natural populations. Inter- and intraspecific variation in isozymes have been reported for some species of *Hedysarum* [4], but estimates of mating system parameters in *Hedysarum* using isozyme data have never been reported.

An outcrossing estimate, obtained in *H. coronarium* using qualitative markers (flower coloration), was close to 90% [6]. Competitive disadvantage of self pollen appears to be responsible for low levels of selfing [6]. However, this estimate of outcrossing rate, obtained in only one population, suffers from errors due to the use of phenotypic traits (flower color) not neutral to pollinators.

This paper presents estimates of mating system parameters derived from isozyme analysis of three natural populations of *H. coronarium* in Tunisia.

Low levels of selfing were detected in open-pollinated families in these populations, which were chosen because they are geographically isolated from other natural populations and particularly from cultivated populations of *H. coronarium*, recently introduced to Tunisia [10].

2. Materials and methods

2.1. Floral biology

H. coronarium usually flowers from late March until June. Flowers are pollinated by native bee species (especially *Apis mellifera*). This species is predominantly allogamous. The flowers are self-compatible but protandrous. They can produce inbred seeds in several different ways: pollen from one flower may fertilize ovules from the same flower (within flower selfing, limited by protandry) or ovules of another flower on the same inflorescence or on another inflorescence on the same individual (geitonogamous selfing). Flowers will also produce inbred seeds when pollen and ovule parents are related (biparental inbreeding). Each mature plant produces 15–30 inflorescences, each inflorescence containing 20–40 flowers. In an inflorescence, the flowering is acropetous; each flower remaining receptive for about two days after anthesis.

The pods usually contain 2–4 seeds and are covered with very small thorns. Pods are retained on the plants till maturation in late July. Seed dispersal, via pod transport, is ensured by animals, especially sheep.

2.2. Site and electrophoresis descriptions

The study was carried out at “Menzel Abderrahman” located 5.5 km southwest of Bizerte, North Tunisia (37° 29 N, 7° 45 E). This site, where *H. coronarium* is the predominant herb, is completely isolated from other populations of this species by the “Canal of Bizerte”.

Three “populations” were sampled. A population, in this case, consisted of a group of plants separated from other groups by at least 0.5 km. In all cases, there was a fairly continuous distribution of plants in the areas separating the populations. These populations were designated “Pop m1”, “Pop m2” and “Pop m3”. They were similarly uniform and dense: the average distance between flowering plants was 10 cm. Annual rainfall at the site ranged from 300 to 400 mm.

Twenty maternal plants were sampled in each population. Plants were selected on the basis of the presence of an adequate seed production, with the additional criterion that they were separated from each other by at least 1 m. Pods were collected in July 1998, from about five inflorescences per maternal plant. Seeds were extracted from twenty randomly selected pods per plant, and kept separate by plant and pod. One seed was randomly selected from each pod, scarified and sown in a Petri dish on moistened filter paper at 20–25 °C in the dark in the laboratory. After seven days, ten seedlings were randomly chosen per family, that is the progeny of one plant.

At least ten families per population were used for isozyme study at the seedling stage. About ten

families per population were placed for growth in a greenhouse at the Faculty of Bizerte. They were cultivated until maturity under uniform environmental conditions, and used for electrophoretic study at the adult phase. The number of families per population and the mean number of progeny per family are indicated in Table I.

For electrophoretic analysis, the overall sample size (56 families providing 394 progenies) was sufficient for indirect estimation of maternal genotypes from the progeny arrays using the method of Brown and Allard [5].

Extracts were made by grinding 25 mg of excised seedlings, or leaflets (in the case of the adult stage) in 0.10 ml buffer solution consisting of 0.1 M Tris-HCl, pH 7.5, containing 7% sucrose, 5% PVP-40 (polyvinylpyrrolidone), 1% mercaptoethanol and 20 mM bisulfite sodium. The samples were centrifuged in a SIGMA microcentrifuge (10 000 rpm $\approx$ 10 448 g; for 15 minutes), and the supernatant used as the enzyme extract. Electrophoresis was performed in 12% starch gels. All samples were run in morpholine-citrate buffer, pH 6.1 [7] at 4 °C using 160 volts for about four hours. Gels were stained for phosphoglucose isomerase (PGI, EC 5.3.1.9), phosphoglucose mutase

Table I. Sampling parameters for mating system analyses in three populations of *H. coronarium*.

Population	Life stage	Number of families	Total number of progenies	Mean number of progeny per family
Pop m1	Seedling	10	80	8.00
	Adult	9	56	6.22
	Total	19	136	7.16
Pop m2	Seedling	10	80	8.00
	Adult	9	55	6.11
	Total	19	135	7.10
Pop m3	Seedling	11	88	8.00
	Adult	7	35	5.00
	Total	18	123	6.83
Overall	Seedling	31	248	8.00
	Adult	25	146	5.84
	Total	56	394	7.04

(PGM, EC 2.7.5.1), 6-phosphogluconic dehydrogenase (6 PGD, EC 1.1.1.44) and shikimate dehydrogenase (SKDH, EC 1.1.1.25), according to the procedures of Wendel and Weeden [30]. Only two loci, Pgi-2 and Skd were polymorphic. There is no information available on the inheritance of isozymes in *H. coronarium*, so genetic interpretation of the isozyme data was based on (i) banding patterns of isozymes (i.e. one or two bands for codominant, monomeric enzymes, or either one or three bands for codominant, dimeric enzymes); and (ii) the possibility of a maternal parent genotype that could generate the progeny array. Loci were numbered consecutively, with the most anodal being designated locus 1 (e.g. Pgi-1, Pgi-2, etc.). For a given locus, alleles were designated in the same way (the most anodal being number one).

2.3. Data analysis

Outcrossing rates of seedling and adult stages and population-level inbreeding coefficients were estimated for each population and for the three combined populations using the MLT and ML2T programs of Ritland [25].

MLT, based on the procedure of Ritland and Jain [27], uses the Expectation-Maximization method for determining maternal and pollen allele frequencies and the Newton-Raphson method for joint maximum likelihood estimation of t_m (the multilocus outcrossing rate), t_s (the single-locus outcrossing rate averaged over loci), and F (the fixation index of the maternal parents). Standard errors for these parameters were generated using 100 bootstrap estimates, with resampling among families. Family size is sufficient to provide minimum variance estimates of F [24]. MLT uses the method of Brown and Allard [5] to infer maternal genotypes for each family. Multilocus estimates of outcrossing improve the chance of detecting outcrosses when parents are related, so that the difference between t_m and t_s provides a measure of the amount of biparental inbreeding [23].

ML2T estimates the outcrossing rates of two groups within a population assuming that the outcross pollen pools are the same for both groups

(e.g. life stages). As the ML2T model uses the entire population data set to calculate pollen allele frequencies, it is expected to give more reliable estimates of the stage-specific outcrossing rate than those produced by MLT using each group as a separate population. Standard errors of outcrossing rates were estimated from 250 bootstrap analyses, using families as the unit of observation. However, the population level estimate of F produced by MLT may be more reliable as fewer parameters are estimated than the ML2T [16]. The significance of departures from expectations for panmixia were assessed by t-test [1, 15].

An assumption of the multilocus models for the estimation of mating system parameters is that the loci are unlinked [27]. To test for non-random associations among alleles at two loci, the digenic composite disequilibrium coefficient Δ_{AB} [29] was calculated for all pairwise combinations of polymorphic loci, using the Weir's [29] LD86 program. Δ_{AB} is equal to zero if the allelic state at one locus is not correlated with that at another. Deviations from zero were detected using χ^2 with one degree of freedom. Estimates of disequilibrium were calculated in each population from progeny genotypes. However, this estimation does not fulfil the assumption of independence in the χ^2 test due to common maternal, and often paternal, parents. A significant result needs to be interpreted with caution and could be the result of violation of the assumption of independence [17]. The maternal genotypes could not be used to test for gametic disequilibrium because of small sample sizes.

Wright's F -statistics (F_{IS} , F_{IT} and F_{ST}) were estimated on the maternal populations according to Nei [20], using the POPGENE computer program (version 1.32) of Francis Yeh (distributed by the author at <http://www.ulberta.ca>). F_{IS} is the fixation index arising from non-random mating within populations. F_{ST} is the fixation index arising from population differentiation. F_{IT} is the overall fixation index arising from differentiation between individuals in the whole populations. The significance of F_{IS} and F_{ST} values was tested by the χ^2 tests of Li and Horvitz [18] and of Workman and Niswander [31], respectively. The resulting F_{ST} values were then used to generate indirect estimates of gene

flow among populations. The number of migrants exchanged per generation, Nm , was estimated as $(1/F_{ST} - 1)/4$ [32].

Expected selfing rate (s_e) at inbreeding equilibrium was calculated from the observed fixation index (F_{IS}) by the equation $F_{IS} = s_e/(2-s_e)$ [21]. Expected selfing rate (s_e) and observed multilocus selfing rate (s_m) should be equal if the populations are at inbreeding equilibrium in absence of selection [11].

3. Results

3.1. Determination of informative isozyme loci

The banding patterns for PGI indicated the presence of two zones of activity: Pgi-1 and Pgi-2, which migrated anodally. Pgi-1 was monomorphic in our samples. Pgi-2 showed three bands, present either as a single band or in a triple-band, corresponding to the dimeric expression of an heterozygous locus. The slowest band was found only in the pollen pools; while the fastest one was highly frequent in both ovule and pollen pools.

A single anodal zone of activity, containing three bands, was found for SKD, either as a single band or in a pair. The locus Skd is typical of a codominantly monomeric locus with three alleles. These three alleles were present in both gamete pools (ovule and pollen) and expressed in coleoptile (seedling) and leaflet tissues.

3.2. Ovule and pollen allelic frequencies

Estimates of allelic frequencies and their standard errors for ovule (maternal plants) and pollen pools for two polymorphic loci (Pgi-2 and Skd) are listed in Table II. Differences between any pairs (ovules/pollen) of allelic frequencies were checked for significance ($P < 0.05$) by the Workman and Niswander [31] homogeneity test. Ovule and pollen pool allelic frequencies did not differ significantly at the 5% level (Tab. II), indicating that maternal plants contribute equally to the pollen pool [17] and are representative of the local populations [11]. Furthermore, ovule allelic frequencies did not differ significantly among maternal populations for each polymorphic locus surveyed (Tab. III). The summed χ^2 value indicated no significant spatial differentiation over loci ($\chi^2 = 2.195$; $P > 0.05$).

Table II. Estimates of pollen and ovule allele frequencies in three populations of *Hedysarum coronarium* (N is the number of alleles in each pool). The χ^2 test of heterogeneity was performed after grouping the lowest frequencies at each locus.

Population (N)	Locus Pgi-2				Locus Skd			
	1	2	3	χ^2 (1)	1	2	3	χ^2 (1)
Pop m1:								
Ovule (38)	0.842	0.158	0		0.395	0.553	0.053	
Pollen (136)	0.698	0.293	0.009	3.121 NS	0.436	0.518	0.046	0.146 NS
Pop m2:								
Ovule (38)	0.868	0.132	0		0.342	0.579	0.079	
Pollen (135)	0.858	0.142	0.001	0.025 NS	0.439	0.495	0.066	0.741 NS
Pop m3:								
Ovule (36)	0.750	0.250	0		0.389	0.611	0	
Pollen (123)	0.845	0.155	0.001	1.729 NS	0.294	0.706	0.001	1.164 NS
Overall:								
Ovule (112)	0.813	0.188	0		0.357	0.598	0.045	
Pollen (394)	0.814	0.183	0.003	0.002 NS	0.415	0.548	0.038	0.884 NS

NS: $P > 0.05$.

Table III. Comparison between allelic frequencies in the maternal gene pool of three populations studied in *H. coronarium*. The χ^2 test of heterogeneity was performed after grouping the lowest frequencies for Skd.

Locus	Allele	Population			χ^2 (df=1)
		Pop m1	Pop m2	Pop m3	
Pgi-2	1	0.842	0.868	0.750	1.935 NS
	2	0.158	0.132	0.250	
Skd	1	0.395	0.342	0.389	0.260 NS
	2	0.553	0.579	0.611	
	3	0.053	0.079	0.000	
Overall					2.195 NS

NS: $P > 0.05$.

Table IV. F-statistics and gene flow (Nm) based on inferred maternal genotypes of three populations of *H. coronarium* (n = number of maternal plants).

Locus	n	F_{IS}	F_{IT}	F_{ST}	Nm
Pgi-2	56	-0.120 NS	-0.100	0.018 NS	14.1
Skd	56	0.172 NS	0.175	0.004 NS	58.7
Mean	56	0.067 NS	0.076	0.009 NS	27.5

NS: $P > 0.05$.

Table V. Single locus (t_s) estimates of the outcrossing rate for three populations of *H. coronarium* and χ^2 values for goodness-of-fit of two isozyme loci to the mixed mating model. Degrees of freedom (9, for triallelic loci) are approximate; we used $\alpha = 0.01$, as suggested by Ritland [22]. Standard errors (SE) of t_s are based on 100 bootstraps.

Population	t_s (SE)		Locus Pgi-2	Locus Skd
	Pgi-2	Skd		
Pop m1	0.738* (0.139)	0.860 (0.154)	2.75	5.66
Pop m2	0.437* (0.104)	1.093 (0.108)	1.11	2.83
Pop m3	0.773* (0.120)	0.860 (0.134)	2.58	1.90
Overall	0.657* (0.078)	0.910 (0.086)	9.53	15.17

^a Single-locus estimates.

* Rejection of the null hypothesis: $t_s = 1.0$ ($P < 0.01$).

Analysis of Wright's F-statistics provided further information on population structure and genetic differentiation among maternal populations (Tab. IV). F_{IS} values indicated no significant deviations from Hardy-Weinberg expectations within adult populations. F_{ST} values, ranging from 0.004 to 0.018 among loci with an average of 0.009 over all alleles and loci, were not significantly different from zero and indicated a high genetic similarity between the three populations studied in *H. coronarium*. These F_{ST} values produced estimates of gene flow among populations (Nm) ranging from 14.1 (Pgi-2) to 58.7 (Skd), with a weighted mean of 27.5 migrants per generation. These results suggested that the three populations of *H. coronarium* may experience a substantial gene flow between each other.

3.3. Mating system parameters

χ^2 tests for goodness-of-fit of the observed progeny frequencies to those expected under the mixed-mating model were not significant (at 1% level, as suggested by Ritland [22]) for all population $\times$ locus combinations (Tab. V). These χ^2 tests were generated by the MLT program. The two loci, Pgi-2 and Skd, were used for mating system study in *H. coronarium*.

Intra-population estimates of single-locus outcrossing rates (t_s) varied from 0.437 (Pop m2, locus Pgi-2) to 1.093 (Pop m2, locus Skd, Tab. V). Values for t_s can exceed 1.0 when using the maximum-likelihood estimation procedure of Ritland and Jain [27], but it provides statistically unbiased estimates [24]. Here, estimates derived from the locus Skd uniformly indicated complete outcrossing ($t_s = 1.0$) in all populations. But incomplete outcrossing was detected for the locus Pgi-2 ($t_s < 1.0$; Tab. V). The lowest t_s , obtained for Pop m2, was 0.437 (SE $\pm$ 0.104; 19 families), a value significantly smaller than the unity ($t = 5.413$; $P < 0.05$).

Differences among single-locus outcrossing estimates have been reported for several species [8, 11, 26]. Single-locus estimates of the outcrossing rate (t_s) often showed considerable variation, since

they are more sensitive than multilocus estimates to violation of the assumptions underlying the mixed-mating model [27, 28]. These assumptions include (1) equal survival of selfed and outcrossed zygotes from fertilization to germination, (2) homogeneity of allele frequencies in the pollen pool, (3) a uniform selfing rate among maternal parents and (4) Mendelian segregation of marker genes. Since the goodness-of-fit tests (Tab. V) revealed no significant departures from the mixed-mating model expectations, all conditions were satisfied in *H. coronarium*. It may be noted here that, for the multilocus estimates of mating system parameters, loci must be unlinked [27]. This condition was satisfied, since no significant departures from equilibrium between Pgi-2 and Skd were detected in any population studied in *H. coronarium* (Tab. VI).

Single-locus estimates are also expected to show a downwards bias caused by mating among related individuals (biparental inbreeding). A slight reduction in t_s , compared to t_m (Tab. VII) was observed in nine cases among twelve comparisons ($t_m - t_s$), including population-level and life stage estimates. However, the difference was significant in one case only: the seedling stage for Pop m1 ($t_m - t_s = 0.069 \pm \text{SE } 0.029$; 10 families; $t = 2.379$; $P < 0.05$).

At the population-level, multilocus outcrossing rates (t_m) were ranged from 0.815 (SE ± 0.123) to 0.902 (SE ± 0.081) and did not differ significantly from unity (Tab. VII). However, the t_m estimated for pooled populations was 0.830 (SE ± 0.069) statistically different from unity ($t = 2.464$; 56 families; $P < 0.05$).

Regarding life stage, the t_m estimates for the seedling stage were consistently lower than those estimated on the adult phase (except in Pop m3, Tab. VII), but these differences were not significant ($P > 0.05$). At the adult-stage, all t_m estimates showed no significant departure from complete outcrossing (Tab. VII) while for the seedling-stage, the t_m values were significantly different from unity in Pop m2 ($t_m = 0.706 \pm 0.109$; $t = 2.697$; $P < 0.05$) and in pooled populations ($t_m = 0.806 \pm 0.072$; $t = 2.694$; $P < 0.05$).

Table VI. Estimates of gametic disequilibrium Δ_{AB} between Pgi-2 and Skd for each population studied in *H. coronarium* (SD: standard deviation; n: number of plants).

Population (n)	Δ_{AB}	SD	$\chi^2_{(1)}$
Pop m1 (136)	0.033	0.022	2.58
Pop m2 (135)	-0.001	0.018	0.00
Pop m3 (123)	0.000	0.017	0.00
Pooled populations (394)	0.010	0.012	0.83

Wright's fixation index (F) is a measure of inbreeding in the maternal plants relative to the entire population [32] (Tab. VII). This index can vary from -1 to 1. A negative value indicates that there is a greater proportion of heterozygous plants than expected under random mating. It could originate from selection against homozygotes at any time after fertilization. All population-level F estimates were not significantly different from zero (Tab. VII), indicating the neutrality of both loci (Pgi-2 and Skd) studied in *H. coronarium*.

4. Discussion

The results yielded by the mating system analysis of three populations of *H. coronarium* showed homogeneity, at population-level, both for single-locus outcrossing rates (t_s), which varied from 0.810 ± 0.114 to 0.852 ± 0.94 , and for multilocus outcrossing rates (t_m), that ranged from 0.815 ± 0.123 to 0.902 ± 0.081 . These single- and multilocus estimates did not differ significantly from complete outcrossing ($t = 1.0$), because their associate standard errors were large due to the small sample of families per population. When these three populations were pooled, the overall estimates of t_m (0.830 ± 0.069) and t_s (0.820 ± 0.073) differed significantly from unity.

This multilocus outcrossing estimate in *H. coronarium* ($t_m = 0.830 \pm 0.069$), based upon allozyme markers, was in accordance with a previous estimate [6] ($t = 0.90$), based upon flower colour

Table VII. Population-level and life stage multi- and single-locus outcrossing rates (t_m , t_s) and population-level estimates of inbreeding coefficient (F) in three populations of *H. coronarium*. Standard errors (SE) are in parentheses (n = number of families).

Population	t_m (SE)	t_s (SE)	$t_m - t_s$ (SE)	F (SE)
Pop m1 (n = 19)	0.837 (0.110)	0.810 (0.114)	0.027 (0.030)	0.159 (0.249)
Seedling (n = 10)	0.792 (0.134)	0.723 ^a (0.130)	0.069 ^b (0.029)	
Adult (n = 9)	0.857 (0.214)	0.869 (0.215)	-0.012 (0.074)	
Pop m2 (n = 19)	0.815 (0.123)	0.823 (0.129)	-0.008 (0.024)	0.094 (0.224)
Seedling (n = 10)	0.706 ^a (0.109)	0.722 ^a (0.134)	-0.016 (0.039)	
Adult (n = 9)	0.845 (0.259)	0.837 (0.269)	0.009 (0.038)	
Pop m3 (n = 18)	0.902 (0.081)	0.852 (0.094)	0.050 (0.032)	-0.167 (0.149)
Seedling (n = 11)	0.863 (0.113)	0.829 (0.128)	0.034 (0.032)	
Adult (n = 7)	0.694 (0.199)	0.686 (0.212)	0.008 (0.035)	
Overall (n = 56)	0.830^a (0.069)	0.820^a (0.073)	0.010 (0.016)	0.013 (0.109)
Seedling (n = 31)	0.806 ^a (0.072)	0.779 ^a (0.075)	0.027 (0.018)	
Adult (n = 25)	0.820 (0.123)	0.804 (0.134)	0.015 (0.024)	

Life stage and population-level estimates were produced by Ritland's [25] ML2T (250 bootstraps) and MLT (100 bootstraps) programs, respectively.

^a t_m , t_s significantly lower than unity ($P < 0.05$).

^b ($t_m - t_s$) statistically greater than zero ($P < 0.05$).

markers. Results from both studies indicated significant, but low levels of selfing (and/or biparental inbreeding) in this species.

Mean single-locus selfing rate, which measures the apparent selfing including biparental inbreeding, was estimated in *H. coronarium* as $(1 - t_s) = 0.180 \pm 0.073$ (Tab. IV), slightly greater than $s_m = 0.170 \pm 0.069$. So, consanguineous matings only contributed for 1% of apparent selfing.

It is well known that significant gametic disequilibrium coefficients (Δ_{AB}) affect multilocus outcrossing rate (t_m) estimates [14]. Positive Δ_{AB} causes a downward bias of t_m and negative Δ_{AB} causes a slight upward bias of t_m . This general downward bias rule is relevant for studies that aim at detecting biparental inbreeding by comparing between single-locus (t_s) and multilocus (t_m) outcrossing rate estimates. If t_m is downward biased by gametic disequilibrium (while t_s is unaffected), biparental inbreeding will be underestimated or perhaps not even detected.

Since, in *H. coronarium*, all gametic disequilibrium coefficients (Δ_{AB}) were not significantly

different from zero, t_m was not underestimated. Consequently, the difference between single- and multilocus outcrossing rates defines without any bias the selfing rate due to biparental inbreeding.

Biparental inbreeding may be caused by the combined effects of limited seed dispersal and near-neighbour pollination. Non-significant biparental inbreeding in *H. coronarium* (1% in pooled populations) indicated a large seed dispersal, leading to the absence of population subdivision when associated with a high level of outcrossing. Bees mediate most pollen dispersal in *H. coronarium*, and travel predominantly to near-neighbours [9]. These neighbours may not be related, if seed dispersal is unrestricted and populations undifferentiated. Genetic similarity between maternal populations has been established on the basis of non-significant F_{ST} estimates for each polymorphic locus and for both loci. Seeds of *H. coronarium* are animal-dispersed, but mean seed dispersal distance is unknown.

Non-significance of biparental inbreeding in *H. coronarium* could also be related to inbreeding depression, acting before the adult stage.

Reduction of selfing and biparental inbreeding rates at adult-stage, in comparison with seedling stage (Tab. VII), confirmed this hypothesis. However, inbreeding depression is particularly severe during seed maturation and germination [8, 15]. Our data do not provide a measure of this phenomenon at these early stages.

Previous studies based on hand pollination have shown that allogamous *Hedysarum* species are self-compatible [2, 9]. The rate of self-compatibility averaged 66% [9] among several populations of *H. coronarium*. In contrast, the current study detected, in naturally pollinated populations of *H. coronarium*, high levels of outcrossing which, per population, did not differ significantly from the unity. These results confirm that this species is an obligate outcrosser, as indicated by the low levels of seed set in bagged inflorescences [9, 10]. However, a significant amount of selfing was detected in pooled populations indicating that *H. coronarium* is predominantly outcrossing. Because this species is self-compatible but highly outcrossing, it must have a low level of self-pollination. Differentiation in timing of the receptivity of stigma and pollen shed in the same flower (e.g. protandry, as observed in *H. coronarium* [6]) may contribute to avoid self-pollination. Competitive disadvantage of self-pollen [6] is another mechanism which may be responsible for the high outcrossing rate measured in this species.

According to the classical rule that high levels of outcrossing should be favoured when heterozygosity confers advantage [19], it is expected that, in *H. coronarium*, observed inbreeding coefficients (F) in maternal plants must be significantly negative. We found no evidence of any form of selection, since all observed Wright's fixation indices (F) were not significantly different from zero (Tab. VII). However, most F -values, except for Pop m3, were positive and showed a non-significant heterozygosity deficit relative to panmictic expectations. F -statistics analyses on maternal genotypes (Tab. IV) confirmed these results, providing an overall populations and loci F_{IS} estimate close to 0.067.

When populations are at inbreeding equilibrium, the fixation index (F_{IS}) predicts the expected multi-locus outcrossing rate (t_e) by the equation $t_e = (1 - F_{IS}) / (1 + F_{IS})$ [21]. Substituting the observed inbreeding (F_{IS}) of 0.067 (Tab. IV) into the equation yields an expected outcrossing rate of 0.874, which is nearly the observed value for t_m (0.830 ± 0.069) in pooled populations of *H. coronarium* (Tab. VII). Similar situations were reported for other species [11, 13].

It is evident from the results of the present study that natural populations of *H. coronarium* maintain high levels of outcrossing, reducing the genetic diversity among populations. The low levels of selfing detected in this species are most likely due to the combination of the protandrous nature of flowers and the competitive disadvantage of self pollen. Low levels of selfing can also result from poor survivorship of selfed embryos, but seed abortion is low (5% or less, unpublished data) and thus unlikely to significantly reduce selfing. Further studies into the factors regulating selfing in *H. coronarium* are needed.

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