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Mating system of wild *Phaseolus lunatus* L. and its relationship to population size

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Using isozyme variation in a naturally pollinated seed family for 10 wild *Phaseolus lunatus* L. (Lima bean) populations, ranging in sizes from 10 to 60 reproductive individuals, we estimated levels of outcrossing (*t*) and parental inbreeding coefficient (*F*). We also examined the relationship between outcrossing rate and population size. Average estimates of the single-locus outcrossing rate (t_s) ranged from 0.024 to 0.246 (mean $= 0.091 \pm 0.065$). Estimates of the multilocus outcrossing rate (t_m) ranged from 0.027 to 0.268, and averaged 0.096 ± 0.071. Inbreeding coefficients based on genotypic frequencies of maternal plants were positive and significantly greater than zero ($F = 0.504$), suggesting an excess of homozygotes in all the populations studied. There was indirect evidence of nonrandom mating for outcrosses and this was mainly attributed to self-fertilisation since the averaged difference between t_m and t_s, which provides a measure of biparental inbreeding, represents only 1% of the autogamy rate. No significant correlation was observed between outcrossing rate and population size. Estimates of *t* showed significant heterogeneity among populations and factors explaining this tendency are suggested.
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Introduction

To be successful in the *in situ* conservation of intraspecific genetic diversity, populations managers must rely upon descriptive information about genetic structure and reproductive biology of considered species, as well as upon a good understanding of the reproductive mechanisms (Wright, 1965; Brown, 1989; Hedrick, 1990).

The present work on wild Lima bean (*Phaseolus lunatus* L.) populations located in the Central Valley of Costa Rica is a part of a wider programme aimed at describing population genetic structure, gene flow, genetic variability at microgeographical level, and at understanding mechanisms controlling the genetic structure, the reproductive biology, and the population dynamics. Isozymes electrophoresis data on these wild populations (Zoro Bi, 1999) indicated a genetic variability occurring mainly at the interpopulation level, with low allelic richness, low observed and expected heterozygosity, and low interpopulation gene flow (Zoro Bi, 1999). It was argued that the high number of significantly positive fixation indices observed for 29 populations was related to the occurrence of inbreeding resulting from a high selfing rate and/or the bottleneck effects that characterized the target populations, and/or sampling populations or plant patches that differ in gene frequencies (Wahlund effect). Since it is difficult to distinguish between the three alternatives without a thorough examination of the mating system, the present study aimed to clarify this topic. Thus, we estimated the mating system parameters for 10 populations of wild Lima bean and described patterns of outcrossing using isozyme markers. The relationship between the outcrossing rate and population size was also examined, given that the pattern of this relationship could have an important application for *in situ* management strategies.

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Materials and methods

Plant material

A total of 10 wild Lima bean populations with at least 10 plants bearing mature pods and expressing at least three polymorphic enzyme loci were sampled during the 1995, 1996, 1997, or 1998 first trimestrial period, corresponding to the time of their physiological maturity. Each population was followed for a complete season so that all individuals bearing pods during that season were sampled, resulting in 10–60 plants, sampled per population and four to six racemes per plant. Reproductive individuals can produce several racemes (about 400) with one to 20 pods per raceme, each pod containing one to five seeds. One seed was randomly chosen per raceme for electrophoretic analysis, resulting in sample sizes ranging from 58 to 334 seeds per population. These criteria were those suggested by Ritland and Jain (1981) and Shaw and Brown (1982). According to these authors, for the predominantly autogamous species, the best estimates of outcrossing rate can be obtained from few loci (three to six) and a large number of plants per

population. The selected populations were identified by several alpha-numeric codes (Rocha *et al*, 1997).

Electrophoretic analysis

To estimate the gene frequencies and mating system parameters, we selected six polymorphic loci resolved from six enzymatic systems: alcohol dehydrogenase (ADH, E.C. 1.1.1.1.), diaphorase (DIA, E.C. 1.8.1.4.), fluorimetric esterase (fEST, E.C. 3.1.1.−), malate dehydrogenase (MDH, E.C. 1.1.1.37), phosphoglucomutase (PGM, E.C. 5.4.2.2), and shikimate dehydrogenase (SKDH, E.C. 1.1.1.25). Enzyme extraction was carried out by grinding 5-day-old cotyledon tissues in a potassium phosphate buffer, pH 7.0, containing 20% sucrose (Sigma # S-8501), 5% PVP-40, 0.05% triton X-100 (Sigma #T-8532), 14 mM 2-mercaptoethanol (Sigma #M-6250), and 0.1 M KH_2PO_4. The pH value was adjusted to 7.0 with a solution of 5 N NaOH. Electrophoresis was performed using a horizontal 10% starch-gel (Sigma #S-4501) containing 3% sucrose. Two buffer systems were employed: continuous histidine-citrate, pH 6.1 for ADH and MDH, and discontinuous lithium-borate, pH 8.1/Tris-citrate, pH 8.4 for DIA, fEST, PGM, and SKDH. The techniques for gel electrophoresis and histochemical staining procedures are those reported elsewhere (Zoro Bi *et al*, 1999).

Loci were labelled sequentially, with those migrating closest to the anodal end designated as number 1. The *Centro Internacional de Agricultura Tropical* (CIAT, Cali, Colombia) accession G25221, a Mexican wild form, was used as the control for our analyses. The allozyme from this genotype is designated 100 and all other allozymes are assessed according to their relative migration rate. The genetic control and the quaternary structure of the analysed enzyme systems were discussed previously (Zoro Bi *et al*, 1999).

Data analysis

We used the multilocus mixed-mating model and the estimation procedure of Ritland and Jain (1981), implemented by Ritland (1990), to estimate the following parameters: average single-locus (t_s) and multilocus (t_m) outcrossing rate, ovule and pollen allele frequencies, and the inbreeding coefficient of maternal parents (F). The model uses the Newton–Raphson method for joint maximum-likelihood estimation of outcrossing (t_s and t_m) and F, the expectation-maximisation method for determining maternal and pollen allele frequencies, and the method of Brown and Allard (1970) for inferring maternal plant genotypes. The calculation of outcrossing rate using the multilocus procedure improves the chance of detecting outcrosses when parents are related, so that the difference between t_m and t_s represents an estimate of the biparental inbreeding (Shaw *et al*, 1981; Ritland, 1984). Assumptions of this model are: (1) no genetic changes due to mutation or selection following fertilisation; (2) no assortative mating; (3) no heterogeneity in the incorporated pollen pool; and (4) outcrossing is independent of maternal genotypes. Since assumption 3 is often violated in plant species (Godt and Hamrick, 1991), we tested for each population the homogeneity between pollen and ovule allele frequencies using Fischer exact tests (Sokal and Rohlf, 1995). The goodness-of-fit of the observed to the expected frequencies of progeny genotypes under the mixed-mating model assumptions was tested for each locus using a χ^2 test generated by MLT (Ritland, 1990). Only data for loci showing a good fit to this model were used to estimate the mating system parameters. This method provides not only greater statistical power, but is also free of the inbreeding equilibrium assumption (Ritland, 1983). Standard errors of mating system estimates, including $t_m - t_s$, were calculated by bootstraping, where the unit of resampling was the maternal family arrays. The mean maternal plant inbreeding coefficient based on progeny genotypes (F) was compared to the expected inbreeding coefficient at equilibrium $F_e = (1-t)/(1+t)$ (Fyfe and Bailey, 1951). If populations are at a genetic equilibrium and genotypic frequencies are determined solely by the mating system, F and F_e are equal (Brown, 1979). Discrepancies between F and F_e reflect the amount by which a population deviates from inbreeding equilibrium. For each population, we tested the significance level of t_s and t_m by a one-tailed Student's t-test based on the null hypothesis that t_s or $t_m = 1$ (Sokal and Rohlf, 1995). To verify the importance of the biparental inbreeding in autogamy for wild Lima bean, we also compared $t_m - t_s$ to zero using a Student's test. The significance level of F was tested using a χ^2 test (Li and Horvitz, 1953), whereas the mean $F_e - F$ was tested for significant difference from zero using a paired-t-test. Variability of t_m among the analysed populations was tested using a χ^2 test suggested by Godt and Hamrick (1991). The test was carried out by subtracting each population estimate from the global mean, dividing these differences by the standard error associated with each population outcrossing rate, squaring these quantities, and summing over populations. This statistics was tested as χ^2-value, with degree of freedom being one less than the number of populations.

To examine the relationships between population sizes and outcrossing rates (t_m), Spearman rank correlation coefficients (r) and their probabilities (P) were calculated using the SAS statistical package version 8.2 (SAS Institute Inc., 1990).

Results

The homogeneity test (two-tailed test with $\alpha/2 = 0.025$) indicated that pollen and ovule allele frequencies in the 10 populations of wild *P. lunatus* were not significantly different for all enzyme loci analysed (Table 1). Such results suggested that maternal individuals contributed equally to the pollen pool. Results of goodness-of-fit tests showed that of the 10 sampled populations, t_m and t_s could be estimated simultaneously for nine populations (Table 1): E25 with data from four loci, E50 with data from three loci, E76 with data from three loci, E84 with data from two loci, E88 with data from one locus, E100 with data from three loci, G1 with data from two loci, J48 with data from three loci, and KM30 with data from two loci. Significant departure from the expected frequencies of progeny genotypes was detected for the three loci analysed in population TR54.

The multilocus and mean single-locus outcrossing estimates for wild Lima bean from the Central Valley of Costa Rica were $t_m \pm SE = 0.096 \pm 0.071$ and $t_s \pm SE = 0.091 \pm 0.065$, respectively (Table 2). Significant self-fertilization (ie t_m and $t_s < 1$) was detected in the

Table 1 Estimated pollen and ovule allele frequencies at six enzyme loci in 10 populations of wild *Phaseolus lunatus*, significance levels of exact tests of homogeneity for allele frequencies between pollen and ovule pools (*P*), and chi-square goodness-of-fit tests for mixed-mating model[a]

Population	*Locus*	*Allele frequencies (SE)*		P	χ^2
		Pollen	*Ovule*		
E25	*Adh-2*	0.745 (0.177)	0.987 (0.012)	0.361	<0.01
	Mdh-2	0.294 (0.189)	0.355 (0.072)	0.107	1.14
	Pgm-2	0.531 (0.289)	0.079 (0.037)	0.188	0.08
	Skdh	0.001 (<0.001)	0.050 (0.052)	0.398	4.00
E50	*Adh-2*	0.999 (<0.001)	0.818 (0.031)	0.173	0.41
	Mdh-2	0.001 (<0.001)	0.295 (0.096)	0.147	0.82
	Pgm-2	0.001 (<0.001)	0.227 (0.076)	0.159	4.05
E76	*Adh-2*	0.002 (0.100)	0.850 (0.099)	0.240	2.77
	Dia-1	0.488 (0.325)	0.700 (0.106)	0.199	31.75***
	Mdh-2	0.999 (0.100)	0.100 (0.087)	0.307	0.01
	Pgm-2	0.678 (0.276)	0.700 (0.100)	0.163	31.32***
	Skdh	0.001 (<0.001)	0.050 (0.052)	0.398	4.00
E84	*Adh-2*	0.132 (0.120)	0.472 (<0.001)	0.125	4.08
	Dia-1	0.660 (0.151)	0.917 (<0.001)	0.241	27.72***
	Mdh-2	0.868 (0.111)	0.750 (<0.001)	0.149	0.92
	Pgm-2	0.610 (0.183)	0.194 (<0.001)	0.176	40.96***
	Skdh	0.053 (0.034)	0.083 (<0.001)	0.162	26.20***
E88	*Adh-2*	0.323 (0.069)	0.447 (0.088)	0.153	8.63*
	f. Est-2	0.111 (0.062)	0.158 (0.069)	0.189	9.67**
	Mdh-2	0.500 (0.124)	0.474 (0.104)	0.129	11.50**
	Pgm-2	0.696 (0.092)	0.605 (0.085)	0.141	3.65
E100	*Adh-2*	0.863 (0.112)	0.800 (<0.001)	0.063	5.38
	Dia-1	0.999 (0.004)	0.983 (<0.001)	0.280	13.03**
	Mdh-2	0.510 (0.208)	0.458 (<0.001)	0.078	2.66
	Pgm-2	0.185 (0.158)	0.133 (<0.001)	0.037	5.05
G1	*Adh-2*	0.888 (0.105)	0.865 (0.051)	0.140	1.11
	Mdh-2	0.360 (0.166)	0.392 (0.070)	0.105	1.30
	Skdh	0.104 (0.111)	0.041 (0.025)	0.257	10.44**
J48	*Adh-2*	0.932 (0.084)	0.980 (0.014)	0.299	0.26
	Mdh-2	0.271 (0.151)	0.265 (0.050)	0.100	0.51
	Pgm-2	0.999 (<0.001)	0.990 (0.010)	0.414	<0.01
	Skdh	0.001 (<0.001)	0.059 (0.027)	0.183	10.32**
KM30	*Adh-2*	0.825 (0.119)	0.789 (0.066)	0.035	28.77***
	Pgm-2	0.255 (0.221)	0.013 (0.008)	0.192	0.80
	Skdh	0.001 (<0.001)	0.026 (0.022)	0.294	5.50
TR54	*Adh-2*	0.778 (0.252)	0.476 (0.093)	0.123	35.38***
	Mdh-2	0.997 (0.001)	0.286 (0.073)	0.133	38.48***
	Pgm-2	0.636 (0.225)	0.357 (0.093)	0.108	23.60***

[a]Since all analysed loci were diallelic, only the frequency of the most anodally migrating allele is presented. *$P<0.05$; **$P<0.01$; ***$P<0.001$. SE: standard error.

nine populations in which t_m and t_s were calculated simultaneously. Accordingly, the means t_m and t_s were significantly lower than 1 (t_m: Student's $t=38.05$, $P<0.001$; t_s: Student's $t=41.96$, $P<0.001$). For the nine selected populations, the differences t_m-t_s were not significantly different from zero, indicating the absence of biparental inbreeding. Consequently, the mean t_m-t_s was not different from zero (Student's $t=1.15$; $P=0.280$).

Inbreeding coefficients based on genotypic frequencies of maternal plants were positive and significantly greater than zero, suggesting an excess of homozygotes in all the populations (Table 2). In all cases, the equilibrium inbreeding coefficients estimated from the multilocus outcrossing rates were greater than the observed maternal inbreeding coefficients (Table 2), a trend that was statistically significant (paired-$t=8.71$, $P<0.001$). The mean equilibrium inbreeding coefficient for the nine populations was 0.830. This result indicated the occurrence of inbreeding disequilibrium in the studied populations.

Significant heterogeneity in multilocus outcrossing rates was observed among the nine analysed populations ($\chi^2=23.60$, $P=0.003$).

A nonsignificant correlation was found between the multilocus outcrossing rate and population size expressed as both the number of individuals in population

Table 2 Sample sizes (m is the number of plants and n is the number of seeds), estimates ($\pm$SE) of multilocus (t_m) and single-locus outcrossing (t_s) rates, biparental inbreeding ($t_m - t_s$), inbreeding coefficients of maternal parents (F), expected inbreeding coefficients at equilibrium (F_e), and difference between F_e and F for nine wild Lima bean populations

Population	*Sample sizes*		$t_s \pm SE$	$t_m \pm SE$	$t_m - t_s \pm SE$	F	F_e	$F_e - F$
	m	n						
E25	39	144	0.024 ± 0.019***	0.027 ± 0.020***	0.003 ± 0.004	0.717***	0.947	0.230
E50	18	86	0.082 ± 0.050***	0.056 ± 0.039**	−0.027 ± 0.013	0.617***	0.894	0.277
E76	10	58	0.057 ± 0.023***	0.074 ± 0.029***	0.017 ± 0.013	0.288*	0.862	0.574
E84	19	109	0.134 ± 0.033*	0.141 ± 0.033*	0.007 ± 0.007	0.424***	0.753	0.329
E88	21	151	0.246 ± 0.060*	0.268 ± 0.064*	0.022 ± 0.015	0.261**	0.577	0.316
E100	60	334	0.065 ± 0.019***	0.082 ± 0.023***	0.018 ± 0.006	0.613***	0.848	0.235
G1	38	137	0.073 ± 0.047*	0.081 ± 0.051*	0.008 ± 0.006	0.442***	0.850	0.408
J48	51	198	0.078 ± 0.037***	0.082 ± 0.038***	0.004 ± 0.004	0.500***	0.848	0.348
KM30	37	204	0.059 ± 0.082*	0.057 ± 0.082*	−0.002 ± 0.003	0.676***	0.892	0.216
Mean	32	157	0.091 ± 0.065***	0.096 ± 0.071***	0.006 ± 0.015	0.504 ± 0.164	0.830 ± 0.108	0.326***

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

($r = 0.092$, $P = 0.814$) and the number of seeds collected ($r = 0.234$, $P = 0.544$). Thus, for wild Lima bean, it appeared that population size was not significantly associated with the level of multilocus outcrossing rate.

Discussion

In this study, outcrossing rates ranged from 0. 027 to 0.268 across nine populations. These data were close to those reported from previous study (Harding and Tucker, 1969). Indeed, using cultivated forms of Lima bean and morphological markers, these authors observed that the outcrossing rates ranged from 0.032 to 0.242. *P. lunatus* could therefore be classified as mixed-mating and predominantly selfing, according to the criterion of Schemske and Lande (1985). The high rates of apparent selfing (up 90%) in populations of wild Lima bean were consistent with the flower morphology (Webster *et al*, 1979) and the main pollinator foraging behaviour (Hardy *et al*, 1997). Indeed, using a cultivated form of Lima bean to study the morphogenesis of the reproductive structures, Webster *et al* (1979) showed that this plant was primarily self-pollinated. Morphological characteristics of the reproductive structures that facilitate self-pollination included the stage of floral development at the time of anther dehiscence and the relative positions of anthers and stigma within the keel at the time of pollen shedding. Coincidental maturation of pollen and receptivity of the stigmatic surface reported for this plant material also enhanced the capacity of self-pollination. In addition, from direct observations made in the valley, Hardy *et al* (1997) noted that the common bee (*Apis mellifera*) was the main pollinator of wild Lima. It is well known that individuals of this species tend to forage in restricted areas (Free, 1993). According to Godt and Hamrick (1991), the genetic effect of such pollinator behaviour is to reduce the single-locus outcrossing estimates. In our case, by analysing seeds from different pods per plant, we may have reduced the effect of correlated mating on the estimates of outcrossing.

We used three approaches to get some insight into the nature of outcrossed matings: (i) the estimation of pollen and ovule pool allele frequencies and the test of homogeneity between them, (ii) the magnitude and significance of the difference between multilocus and monolocus outcrossing estimates, and (iii) the comparison of the maternal inbreeding coefficient (F) to the expected inbreeding coefficient at equilibrium (F_e). No difference was found between pollen and maternal allele frequencies at any locus analysed, suggesting that plants had equal pollination success. Comparison between F and F_e was a second way to obtain information about patterns of outcrossing. Indeed, the assumptions that make the F_e estimation model different from that of F are that: (1) the population has reached its equilibrium genotypic composition, and (2) selfing is the only factor that influences the inbreeding coefficient. Consequently, the observed significant difference between the two estimates could arise from (1) the fact that the populations were not yet at equilibrium, and/or (2) the existence of selection in favour and/or against certain genotypes (Ennos, 1981) or additional sources structuring the genetic variation (eg biparental inbreeding). A study to compare the genetic variation at different stage of wild Lima bean life cycle, namely soil seeds bank, seedlings and adults (Cabin, 1996; Cabin *et al*, 1998) is required to identify which of inbreeding disequilibrium or selection are responsible. Nevertheless, it is worth noting that the founder effects resulting from extinction/recolonisation episodes that characterised the study populations (Rocha *et al*, 1997) could enhance the effects of subsequent selection by facilitating the establishment and expansion of favoured genotypes. Indeed, in the target area, many wild Lima bean populations are found in coffee plantations, fallow land or along hedges, so that weeding practices contribute to the destruction of plants. Recolonisation of the cleared sites could be due to any nearby plants, to new individuals emerging from the soil seed bank, or to human activities (such as seed transportation over longer distances on shoes or tools). We found that the mean multilocus estimate of selfing (90%) was similar to the mean monolocus estimate (91%). On average, only 1% of the apparent selfing in the populations was due to biparental inbreeding. Thus, in the wild Lima bean, the autogamy could be attributed mainly to the true selfing. The breeding system could explain the high level of homozygosity observed in the studied populations.

This study revealed significant heterogeneity in outcrossing rates among populations of *P. lunatus*. Inter-population differences in outcrossing rates are generally attributed to variation in population size, population

density, floral morphology, floral phenology, and pollinator availability (Donnelly *et al*, 1998; Fausto *et al*, 2001; Neel, 2002). For wild *P. lunatus*, the heterogeneity observed in outcrossing rates seems unrelated to population size since among the 10 studied populations, we found a nonsignificant correlation. Similar results have been reported for several plant species (van Treuren *et al*, 1993; Raijmann *et al*, 1994; Kunin, 1997). Given that wild Lima bean populations have complex shapes in the target site, it was difficult to estimate the density with certainty. However, visual examination of the spatial distribution of plants in these populations indicated apparent differences in densities. Indeed, the study populations were characterised by linear or patchy and gregarious distribution, with different number of plants per patch and sometimes, few flowers per plant. In these conditions, the contribution of density to the observed outcrossing heterogeneity could be expected. There was no variation in floral morphology among the populations of wild Lima bean in the target site so that this hypothesis could be discarded. Presently, an in-depth study on the floral phenology of wild Lima bean populations from the target site is in progress. Results from such studies could allow us to check the relationship occurring between the interpopulation outcrossing rates variation and the possible variation in flower appearance period. Such observations should contribute to checking the origin of the heterogeneity of outcrossing rates among the studied populations.

Sampling populations through several years (1995–1998) could also influence the outcrossing rate homogeneity. This influence is important if only seedling recruitment is temporally or spatially limited (Aspinwall and Christian, 1992; Raijmann *et al*, 1994). For wild Lima bean, the mean seed germination rate within a year was 78%, and 4% of the germinating seeds reach the matured age the same year (Degreef, 1998), indicating that this hypothesis could also explain the observed tendency. For this study, our sampling efforts were mainly devoted to obtaining samples of at least 10 matured plants and expressing at least three polymorphic loci in order to satisfy the used models assumptions. Since in the target site only 16% of the 400 recorded populations displayed more than five plants, it was difficult to obtain a sufficient number of populations answering the indicated purpose within a year. Further studies should examine how temporal variation in outcrossing rates may interact with temporal and spatial variation in seedling recruitment (Schemske *et al*, 1994).

From the heterogeneity of outcrossing rates observed among the studied populations and the significant differences between F and F_e, we could also conclude that these populations were at different stages of mating system evolution. Indeed, theoretical models of mating system evolution predict various equilibrium outcomes, ranging from mixed-mating to stable equilibria at either extreme of outcrossing continuum (Lande and Schemske, 1985; Uyenoyama, 1986). The mating system evolution is a complex process which can be affected by a number of biological factors as well as abiotic conditions (Schemske and Lande, 1985; Godt and Hamrick, 1993). Accordingly, for wild Lima bean, a two-pronged study is needed to evaluate the mating system evolution. First, genetic variation in mating system must be quantified in experimental situations in which environmental effects are controlled. Such study should look for the existence of significant levels of genetic variation in relation to outcrossing rates (Kalher *et al*, 1975). Secondly, environmental effects must be considered through detailed studies of the factors that affect outcrossing rates (eg pollinator abundance and density).

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