Euphytica
DOI 10.1007/s10681-014-1177-2

Effect of the inbreeding depression in progeny fitness of runner bean (*Phaseolus coccineus* L.) and it is implications for breeding

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Received: 11 July 2013 / Accepted: 1 June 2014

Abstract Moderate levels of selfing despite high inbreeding depression (ID) make runner bean an excellent model for mixed-mating reproductive biology studies in legumes. This work assesses the extent of the ID variation and consistency at different plant growth stages through selfing generations in seven runner bean populations. Field-collected populations after previous isolated multiplication were hand-pollinated in an isolated greenhouse during five generations to produce progeny. Generations were compared for inbreeding effects (δ) on seed germination, survival to flower, and seed weight and yield. The outcrossing rates of the founder populations and the genetic variation and Wright's ID at the population and generation level were estimated by using 35 polymorphic microsatellite loci. Neutral microsatellite loci were analyzed through generations and populations using different outlier tests to identify loci directly associated with adaptation to inbreeding. Our study revealed patterns of genetic diversity ($H_e = 0.36$) and outcrossing rates (ranged from 24 to 44 %) that are consistent with a mixed-mating system. Selfing-pollination procedure significantly affected germination and survival rates, yield, and to a lesser extent seed weight. Three loci had significant hits to genes related to embryonic development when performing BLAST searches to Phytozome database. Results showed a general inconsistency in δ across plant growth stages and populations, suggesting that different deleterious loci are acting at different stages. Inbreeding tended to purge individuals of deleterious recessive alleles to reduce ID. Variation among individuals within populations may lead to the development of inbreeding lineages with lower levels of ID. Several lines that have been self-pollinated for many generations became homozygous at almost all gene loci and produced a uniform population of true breeding progeny and acceptable performance.

Keywords Inbreeding depression · Mixed-mating system · *Phaseolus coccineus* · Microsatellites

Introduction

Cultivated species and relative wild forms have a wide range of mating-systems from selfing to cross-pollination (Fryxell 1957). Mixed mating systems, characterized by outcrossing and selfing, exist in a substantial proportion of legume species such as pigeon pea (*Cajanus cajan*), runner bean (*Phaseolus*

Electronic supplementary material The online version of this article (doi:10.1007/s10681-014-1177-2) contains supplementary material, which is available to authorized users.

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Published online: 18 June 2014

coccineus L.), yellow bush lupine (*Lupinus arboreus*) and faba bean (*Vicia faba*) (Coello and Escalante 1989; Escalante et al. 1994; Kittelson and Maronet 2000). *Phaseolus* genus offer an interesting opportunity to evaluate the interaction among self-compatibility, outcrossing rate and inbreeding depression (ID), as many annual members of the genus reproduce either by selfing or by outcrossing (Kittelson and Maronet 2000; Real et al. 2004). Runner bean is characterized by a high outcrossing rate variation (Blackwall 1971; Escalante et al. 1994; Ibarra-Perez et al. 1997), which could depend on genetic and environmental factors as well as the procedures used for its estimation (Suso et al. 2001). The genetic structure of the population regulates the mating system, thus driving the evolution of the genetic diversity by affecting the intensities of drift and selection, and determining germplasm conservation and breeding methods (Brown and Allard 1970).

Adaptive benefits of self-pollination compared to outcrossing have been indicated (Schoen and Brown 1991). Individuals with alleles promoting self-fertilization ability have an advantage in transmission over individuals which have alleles for outcrossing (Fisher 1941), because they can act as pollen parents in two ways: by fertilizing ovules on other plants (that is, outcrossing) and their own ovules (that is, selfing) (Holsinger 2000). Plants can tolerate selfing because many disadvantageous or unfit recessive alleles that could increase ID have been erased by selection (Allard et al. 1968; Ritland 1984; Goodwillie et al. 2005). Effective purging of ID depends not only on the mating system that generates inbred individuals but also on the genetic make-up of ID (Byers and Waller 1999). When ID is caused by a small number of recessive genes that have major deleterious effects on fitness is expected to vary among individuals and to respond rapidly to selection. However, ID is not expected to vary drastically among individuals and is less easily purged in the population when it is caused for many genes with small individual effects. ID delays seed germination (Sorensen 2001), depresses plant growth (Frusciante and Monti 1980), causes morphological abnormalities (Haq and Smartt 1978), and decreases seed production and fertility (Kittelson and Maronet 2000; Gilmore and Myers 2004). However, the introduction of inbreeding in runner bean breeding programs offers several advantages, such as exploitation of recessive traits, increase of the additive genetic variance, and decrease of genetic load and production of better-designed hybrids. Selfing is commonly used for population development in plant breeding, and it is well established that selfing increases genetic variance between lines, thus increasing response to genomic and phenotypic selection. Numerous studies have explored how selfing can be deployed to maximal benefit in the context of traditional plant breeding programs (Cornish 1990a, b; Liu et al. 2004). Thus, runner bean germplasm having tolerance to inbreeding is needed in order to generate high yielding inbred lines.

Crop domestication has occasioned changes in the reproductive system, most of the cases towards increased selfing (e.g. rice, tomato or peppers; Rick et al. 1977; Xiong et al. 1999; Gao et al. 2007). Domestication process has been unevenly distributed within the genus *Phaseolus* when one considers the distribution of traits and the level of expression compared to its wild progenitor (Gepts 2004). Runner bean species is a model where fertile individuals do not depend on pollinating insects and sterile individuals are fertilized by a "tripping" mechanism (Link 1990). Runner bean is a useful source of variability for the improvement of the primary gene pool of *P. vulgaris* (Gepts 1981; Wilkinson 1983; Santalla et al. 2004) and other members of the genus (Gilmore and Myers 2000; Gilmore et al. 2002; Schwartz et al. 2006). A previous study (Santalla et al. 2004) based on phenotypic traits revealed a high level of genetic diversity in founder populations. Microsatellites or simple sequence repeats (SSRs) have been widely developed for *Lens culinaris* (Bede 2007), *P. vulgaris* (Yu et al. 2000; Blair et al. 2003; Buso et al. 2006; Hanai et al. 2007), *Vigna unguiculata* (Gillaspie et al. 2005), *Cicer arietinum* (Radhika et al. 2007) and *Glycine max* (Powell et al. 1996; Kraic et al. 2002), and to a lesser extent for runner bean (Sicard et al. 2005; Spataro et al. 2011). A 50 % SSR transferability rate was observed between *P. coccineus*, *P. polyanthus*, *P. acutifolius* and *P. lunatus* (Yu et al. 1999).

The purpose of this paper was to evaluate the use of selfing to produce stable true-inbred lines. The development of experimental inbred lines before advanced breeding strategy implementation can elucidate a number of important considerations such as the ability to self through successive generations and the effects of ID on line extinction and measurable traits of economic importance. The ability to rapidly turn over

selfed generations in runner bean in conjunction with early trait selection can be used to adapt breeding strategies to other plant species. We examined the relationship between the levels of inbreeding, measured in terms of fitness or inbreeding effects, during five selfing generations in seven runner bean populations. The objectives of this work were (1) to assess the genetic variation, outcrossing rates and Wright's ID coefficient or fixation index (F) at the population and generation level, (2) to quantify δ (delta; Inbreeding effect) in different populations by comparing selfed and outcrossed individuals within populations, (3) to test the existence of purging during the first generations and after the establishment of inbred lines, and (4) to associate the variation in genotypic and allelic frequencies at marker loci trough the selfing generations in order to identify possible quantitative trait loci (QTLs) involved in different functional processes. Our study showed that genetic variation and outcrossing rates may partly explain the differences in ID among runner bean populations and that its effects varied considerably across plant growth stages, suggesting that different deleterious loci are acting at different stages. A positive association was observed between a selfing enhancer and low ID, which would permit selection of outstanding inbred lines as a potential source for breeding.

Materials and methods

Genetic material: greenhouse and field experiments

The plant material used in this study consisted of seven runner bean accessions, most of white-seeded populations, which represent typical accessions traditionally grown in the Centre and West of the Iberian Peninsula (Online Resource 1) where traditional farming methods have encouraged the presence of old varieties. The selection of these populations based on a previous study (Santalla et al. 2004). Spanish farmers traditionally leave the runner bean roots in the field during the winter to produce new plants in the spring although nowadays farmers grow their crop each year from seeds saved by themselves. This genetic material has been maintained by manual flower pollination in net-houses to maintain the integrity and genetic variability of each accession and to produce seed for further studies.

In 2002, one hundred plants *per* population were bulk pollinated under isolated conditions (Generation O_1). Fifty O_1 plants of each population were self-pollinated in a net house to produce S_1 generation. From 2003 to 2007, the process was repeated to obtain the next cycles of selfing. Remnant seeds from each population and generation were stored at 5 °C and 50 % humidity. Progeny performance from all generations was measured simultaneously in the field. In May 2008, eighty individuals per generation and population (80 individuals × 5 generations × 7 populations) were evaluated under field conditions (Northwest Spain, Salcedo, 42°24′N, 8°38′W, 40 masl, 14 °C mean temperature, average annual rainfall 1,600 mm), according to a random complete block experimental design with two replications. The experimental unit (40 plants) included two rows of five meters, with rows separation of 0.80 and 0.25 m between plants. The germination rate was computed after 7 days by counting the number of germinated plants. Plants showing flowering and growth abnormalities were recorded in order to assess the survival rate. Seed yield and weight per individual plant were recorded at harvest.

Microsatellite analysis

DNA was individually extracted from forty random individuals per population and generation (40 individuals × 5 generations × 7 populations) using a modified high-throughput cetyltrimethylammonium bromide (CTAB) method using stainless steel beads (Chen and Ronald 1999). A set of 35 polymorphic SSR loci (Table 1) were selected from common bean SSR markers to genotype O_1–S_4 generations per population. S_5 generation was not analyzed because after four selfing generations a high level of homozygosity would be expected. PCR reaction mixtures containing 50 ng of total genomic DNA, 1 U of Taq polymerase (BioTaq^{TM} DNA polymerase, Promega), 0.2 mM of each dNTP, 5–7.5 pmol (depending on the primer pair) of each primer, 1× PCR buffer and 1.5–25 mM $MgCl_2$ were performed in a 25 µl total reaction volume. PCR cycles, performed on a Gene-Amp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA, USA), consisted of 5 min at 94 °C, 30 cycles of 1 min at 94 °C, 1 min at 47–68 °C

Table 1 Number of alleles (na) and Nei's genetic diversity (H_e) per primer pair, allele size and PIC detected by using 35 polymorphic SSR markers for the seven runner bean populations in the outbred-founded generations (O_1)

Marker	LG	na	H_e	Allele size in bp (range)	PIC
BM98	3	5	0.614	226–244	0.746
BM141	9	4	0.519	180–188	0.651
BM143	2	4	0.158	114–130	0.265
BM149	4	5	0.471	225–243	0.622
BM154	9	6	0.571	184–266	0.543
BM156	2	6	0.547	188–204	0.553
BM159	3	2	0.107	193–199	0.153
BM164	2	4	0.351	148–162	0.451
BM167	2	8	0.512	200–296	0.579
BM181	3	6	0.354	172–190	0.448
BM187	6	3	0.363	160–166	0.346
BM202	9	3	0.364	146–150	0.374
BM212	10	4	0.386	184–198	0.504
BMd02	2	4	0.122	100–109	0.126
BMd05	Unliked	2	0.380	117–120	0.333
BMd12	6	4	0.334	158–167	0.426
BMd14	1	4	0.552	178–186	0.520
BMd15	4	7	0.360	174–204	0.340
BMd17	2	2	0.160	94–106	0.180
BMd21	9	4	0.329	100–158	0.227
BMd23	Unliked	3	0.222	128–132	0.424
BMd25	8	2	0.169	112–118	0.173
BMd36	3	7	0.615	164–208	0.757
BMd46	9	4	0.307	320–350	0.564
BMd50	5	3	0.211	109–121	0.343
BMd51	Unliked	2	0.150	107–109	0.360
BMd56	Unliked	4	0.169	180–198	0.146
IAC85	Unliked	6	0.465	190–200	0.466
AG1	3	5	0.273	134–150	0.446
GATs11	10	5	0.521	160–224	0.446
GATs91	2	7	0.399	200–234	0.531
J04555	4	6	0.421	153–177	0.561
M75856	11	2	0.369	137–153	0.375
Pvtttc001	Unliked	4	0.370	166–178	0.496
X04660	4	5	0.439	216–236	0.673
Average		4.3	0.361		0.432

LG common bean linkage group

(depending on the set of primers used) and 1 min at 72 °C, plus a final extension of 30 min at 72 °C. The amplified fragments were multiplexed depending on their size, and analyzed in an ABI PRISM 3130xl genetic analyzer with a 600LIZ size standard internal weight marker (35 fragments from 20 to 600 bp) (Applied Biosystems Inc.) and a Performance Optimised Polymer 7 (Applied Biosystems, Inc.). Genotypes for each marker were determined using the Genescan 3.7 and GeneMapper softwares (Applied Biosystems Inc.).

Assessment of genetic parameters and ID effects

The 35 SSR markers were characterized for polymorphic information content (PIC), number of alleles (na) and gene diversity (H_e, Nei 1987) using the PowerMarker v. 3.0 software (Liu and Muse 2005). A locus was considered polymorphic if the frequency of the most common allele did not exceed of 0.95. Each allele detected was coded in base pairs (bp). Total number of alleles (n_T), the mean number of alleles per locus (A), observed heterozygosity (H_o), Nei's unbiased estimate of gene diversity or heterozygosity expected (H_e, Nei 1987) and the percentage of polymorphic loci P (%) (El Mousadik and Petit 1996; Petit et al. 1998) were estimated for the polymorphic markers in each generation by using the GeneAlex 6 software (Peakall and Smouse 2006). Wright's ID coefficient or fixation index (F), which informs of the intrapopulation inbreeding by its deviation from expected heterozygosity, was estimated as $F = 1 - (H_o/H_e)$, where H_o is the observed frequency of heterozygotes and H_e is the heterozygosity expected for each marker. F measures the excess of homozigosity due to self-fertilization. The expected heterozygosity was calculated as $H_e = 1 - \sum p_i^2$, where p_i is the observed frequency of the ith allele. The expected F coefficients would be 0.5, 0.75, 0.875, 0.938 and 0.969 for S_1–S_5 generations of selfing, respectively (Crow 1986). Estimates of multilocus outcrossing rate (t) were computed according to $t = (1 - F)/(1 + F)$. This procedure assumes equilibrium conditions (Crow and Kimura 1970) and it is an indirect measure of mating events, since Wright's coefficients may decrease through the lifetime as ID reduces the frequency of inbred progeny (Ritland 1996). The advantage of calculating outcrossing based on the Wright's coefficient is that it is a cumulative measure across years and, therefore, less sensitive to yearly variation in selfing rates.

The effects of generation and population on the germination and survival rates, and seed yield and weight traits were calculated using a two-way mixed-model ANOVA, where population and generation were random and fixed factors, respectively. The germination and survival rates values were arcsine-transformed prior to analyses to meet the assumption of normality of residuals in ANOVA. In those variables for which the ANOVA showed significant differences between generations, separation of means was carried out with Tukey's procedure for multiple comparisons ($P \leq 0.05$). Standard errors and coefficients of variation were determined for the quantitative traits studied.

Differences between selfed and outcrossed progeny were computed by the parameter δ (delta; Inbreeding effect), which measures the effects of inbreeding on the viability and fitness of the population under conditions of selfing, and it was calculated for each generation as $\delta = 1 - w_s/w_o$, where w_o is the measured fitness of the outcrossed progeny and w_s is the measured fitness of the selfed progeny. Fitness measurements were estimated using germination and survival to flowering rates, seed weight and yield values. Negative δ values indicate that selfed progeny had higher fitness than the O_1 generation (Hedrick and Kalinowski 2000).

Evidence for deviation from neutral expectations

Neutral loci were identified in all populations in O_1 generations by using the F_{ST}-outlier detection method as implemented in the LOSITAN software (Beaumont and Nichols 1996; Antao et al. 2008). This method uses the available data to derive a distribution of F_{ST} and expected heterozygosity. Loci that deviate from the expected distribution of neutrality were identified on the basis of excessively high or low F_{ST} at founder populations and were discarded from the analysis. After outlier loci were discarded, LOSITAN was run for each population (seven single analyses). Identifying loci that show divergent patterns of differentiation among samples collected over time from a single population is conceptually similar to searching for outliers in samples collected at a single time point from different populations. We adapted the original method to fit our scenario by generating the expected neutral distribution through simulations of drift within a single population. We based simulations on 15,000 iterations and a 99 % confidence interval, options for neutral and forced mean F_{ST} for the mutation model option, according to an infinite allele model, were used. We therefore supplemented the LOSITAN analysis with three statistical methods (simulation or ST, Waples and significance of F_T tests), which take into account the effect of genetic drift, were applied by using TAFT2.3 software (temporal allele frequencies test; Sandoval-Castellanos 2010) in order to identify the allele frequency changes through selfing generations. ST test is based on computer simulations under a Bayesian background, incorporating binomial sampling for change of generation and hypergeometric sampling for getting effective population (Sandoval-Castellanos 2010). Waples test (1989) uses a χ^2 adjusted. ST and Waples tests include the implementation of two sampling schemes (Nei and Tajima 1981) named Plan I and Plan II, which consist of taking individuals before or after selfing, respectively. Plan I was used in this work because DNA was extracted from each individual plant regardless if survive or not to the next generation. Both statistical tests include the use of values of N (population size) and N_e (effective size or number of reproductive organisms) changing over generations. The statistic named F_T accounts for the differences among individuals taken at different times after subtracting the mean gene drift. This average-gene-drift term is named F^S. F_T is calculated as $F_T = F_{ST} - F^S$ and F_{ST} according to Nei (1973) as $F_{ST} = (H_T - H_S)/H_T$, where H_S is the average Hardy–Weinberg heterozygosity and $H_T = 1 - \sum p_i^2$ for any number of alleles. This F_T test considers Empirical Bayes and Full Bayesian algorithms. Empirical Bayes algorithm assigns the allele frequencies obtained from an estimation made with the observed allele frequencies from all the individuals to the founder generation. It is faster but theoretically less accurate than the Full Bayesian algorithm. Full Bayesian algorithm was selected for this work, which simulates a vector of allele frequencies for the population in the founder generations and then simulates the taking of individuals and change of generation with a random number and binomial or hypergeometrical distributions. For each combination of parameters, 100,000 simulations were run, and the results of significant tests (with $\alpha = 0.05$) were recorded.

Results

SSR variation through populations and selfing generations

The level of variation at SSR loci was expressed in terms of the number of alleles and the genetic diversity at each locus (Table 1). A total of 152 alleles were detected, and the number of alleles observed at each locus varied considerably, ranged from 2 (BM159, BMd05, BMd17, BMd25, BMd51 and M75856) to 8 (BM167) alleles, with a mean value of 4.3 and an average genetic diversity of 0.36 over the 35 loci. Marker BM159 showed the lowest genetic diversity ($H_e = 0.11$), while BM98 and BMd36 markers exhibited the highest genetic diversity ($H_e = 0.61$) values. Rodríguez et al. (2013) detected a similar diversity value ($H_e = 0.31$) in runner bean populations from the Iberian Peninsula and Italy. Similar results were also obtained by Spataro et al. (2011) and Santalla et al. (2010) after the analysis of 148 Portuguese populations using 12 SSR markers ($H_e = 0.36$) and 22 Spanish populations using 42 SSR markers ($H_e = 0.35$), respectively.

The total number of alleles, the observed and expected heterozygosity, the estimated Wright's ID or fixation index F and the percentage of polymorphic loci for each population and generation are shown in Table 2. The average percentage of polymorphic loci ranged from 63 to 83 %, while the genetic diversity ranged from 0.26 to 0.40 in the founder or O_1 generation. The average observed heterozygosity decreased from the O_1 to S_4 generations at the rate expected (over 90 %). The crop was not completely allogamous because outcrossing rates (t) in the founder populations ranged from 24 to 44 %. Only a few studies have examined the variation in the outcrossing rates of runner bean populations (Blackwall 1971; Escalante et al. 1994). The amount of outcrossing in runner bean usually varies from 20 to 50 % (Blackwall 1971), although Escalante et al. (1994) revealed higher outcrossing rates in two Mexican populations (60 and 70 %). Homozygosity was high as expected from a partially selfing species and values of F varied from 0.40 to 0.61 based on genotypic frequencies in the founder populations. Levels of homozygosity and selfing rates estimates indicated relatively frequent outcrossing events and suggested that a moderate level of inbreeding occurs within these populations. The estimated F coefficients were not significantly different from the expected values after several generations of selfing ($1 - (1/2)^n$) in most of the populations studied, except for PHC-0012, where the decrease in the heterozigosity was lower compared to the other populations. This population showed a more severe ID, which counter-selected inbred genotypes and favored the maintenance of heterozygotes, being the population with the highest outcrossing rate and genetic diversity. These results proved that the population level F varies with the population's history of inbreeding and highlight the importance of genetic associations between selfing-modifier traits and viability in mating system evolution (Uyenoyama and Waller 1991).

Fitness variation through populations and selfing generations

The germination and survival rates means were similar among O_1, S_4 and S_5 generations in all populations, but significant differences were found among S_1, S_2 and S_3 generations (Fig. 1). The variation on the germination (52–80 %) and survival (52–86 %) rates was significantly affected by selfing in S_1 and S_2 generations for most of the populations studied. Thus, PHC-0035 and PHC-0012 populations had a significant negative selfing effect in S_1 generation, with over 50 % of individuals failed to germinate and flower. ANOVA analyses revealed significant differences among generations and population-by-generation interactions for all traits, and significant differences among populations for seed weight (data not shown). These results indicated a significant difference between the overall mean for the outcrossed and selfed plants.

Table 3 shows the means comparison for seed weight and yield for each population and generation. Lower values were found in the S_1 than O_1 generations for both traits, except for PHC-0016, PHC-0022, PHC-0035 and PHC-0038 for seed weight, and PHC-0016, PHC-0022 and PHC-0038 for yield. From S_3 generation, most of the selfed individuals followed a development similar to that of the O_1 individuals and even with an significant increase in seed size and yield, which could suggest an association between selfing and seed physiology and/or plant performance.

Inbreeding effects (δ) for the germination and survival to flowering rates (Fig. 2) ranged from 0.354

Table 2 The total number of alleles (n_T), mean number of alleles per locus (A), observed heterozygosity (H_o), expected heterozygosity with random mating (H_e), Wright's ID coefficient or fixation index (F), and the percentage of polymorphic loci P (%) detected by using 35 polymorphic SSR markers for the seven runner bean populations at the O_1–S_4 generations

Generation	N	n_T	A	H_o	H_e	F	P (%)
PHC-0008 ($t = 0.43 \pm 0.08$)							
O_1	37	80	2.28	0.217	0.362	0.400 ± 0.059	71.4
S_1	34	75	2.14	0.104	0.314	0.668 ± 0.063	62.9
S_2	30	73	2.09	0.069	0.284	0.757 ± 0.052	54.3
S_3	29	69	1.97	0.023	0.229	0.899 ± 0.026	51.4
S_4	40	67	1.91	0.021	0.216	0.903 ± 0.023	51.4
PHC-0012 ($t = 0.44 \pm 0.04$)							
O_1	34	84	2.40	0.243	0.403	0.397 ± 0.041	82.9
S_1	30	77	2.20	0.190	0.348	0.454 ± 0.058	80.0
S_2	26	65	1.86	0.100	0.254	0.606 ± 0.052	68.6
S_3	40	61	1.74	0.069	0.221	0.687 ± 0.038	54.3
S_4	40	50	1.43	0.041	0.175	0.765 ± 0.049	37.1
PHC-0016 ($t = 0.33 \pm 0.04$)							
O_1	40	62	1.77	0.100	0.262	0.503 ± 0.074	62.9
S_1	36	61	1.74	0.098	0.245	0.600 ± 0.042	60.0
S_2	26	58	1.66	0.030	0.181	0.834 ± 0.027	58.8
S_3	34	55	1.57	0.010	0.136	0.926 ± 0.046	45.7
S_4	40	53	1.54	0.008	0.119	0.933 ± 0.020	42.9
PHC-0019 ($t = 0.33 \pm 0.03$)							
O_1	40	67	1.91	0.143	0.288	0.503 ± 0.031	65.7
S_1	26	63	1.80	0.082	0.250	0.672 ± 0.043	61.9
S_2	34	60	1.71	0.044	0.210	0.790 ± 0.045	57.1
S_3	27	56	1.60	0.028	0.178	0.842 ± 0.046	48.6
S_4	40	49	1.40	0.009	0.110	0.918 ± 0.022	34.3
PHC-0022 ($t = 0.30 \pm 0.02$)							
O_1	32	71	2.03	0.151	0.326	0.536 ± 0.039	74.3
S_1	25	69	1.97	0.131	0.318	0.588 ± 0.042	71.4
S_2	24	66	1.89	0.059	0.287	0.794 ± 0.020	62.9
S_3	27	65	1.86	0.040	0.269	0.851 ± 0.018	61.9
S_4	39	64	1.83	0.022	0.243	0.909 ± 0.015	60.0
PHC-0035 ($t = 0.24 \pm 0.02$)							
O_1	31	67	1.91	0.122	0.313	0.610 ± 0.027	65.7
S_1	21	62	1.77	0.100	0.266	0.624 ± 0.025	60.0
S_2	24	61	1.74	0.046	0.242	0.809 ± 0.083	57.1
S_3	24	57	1.63	0.036	0.190	0.810 ± 0.029	48.6
S_4	40	51	1.46	0.017	0.169	0.900 ± 0.026	45.7
PHC-0038 ($t = 0.32 \pm 0.03$)							
O_1	38	69	1.97	0.150	0.311	0.517 ± 0.031	68.6
S_1	31	67	1.91	0.092	0.276	0.667 ± 0.038	68.6
S_2	26	64	1.83	0.069	0.255	0.729 ± 0.044	62.9
S_3	34	59	1.69	0.039	0.221	0.823 ± 0.028	54.3
S_4	40	53	1.51	0.009	0.147	0.938 ± 0.018	40.0

The multilocus outcrossing rates (t) were calculated in the founder populations (O_1)

N number of individuals analyzed

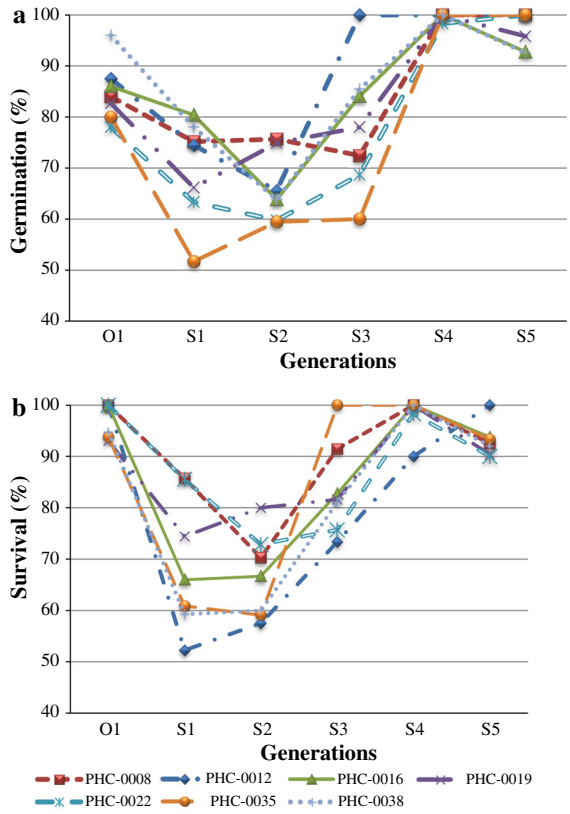

Fig. 1 Germination (**a**) and survival to flowering rates (**b**) for the seven runner bean populations through O_1 and S_1–S_5 generations

(PHC-0035) to −0.282 (PHC-0038) and from 0.478 (PHC-0012) to −0.075 (PHC-0022) through S_1–S_5 generations, respectively, with a recovery after the S_3 generation, which could indicate the rapid purging of lethal, sub-lethal, or highly deleterious alleles by selfing. S_1 generation could be considered an unbiased sample of all progenies that can be obtained by self-pollination of their respective progenitors. Ranges from 52 % (PHC-0035) to 80 % (PHC-0016) for the germination rate, and from 52 % (PHC-0012) to 85 % (PHC-0008 and PHC-0022) of survival to flower rate at the S_1 generation were observed, which could cause an underestimation of inbreeding effects (δ). The inbreeding effects (δ) for seed size and yield (Fig. 2) ranged from 0.132 (PHC-0008) to −0.188 (PHC-0022) and from 0.776 (PHC-0008) to −3.09 (PHC-0016) through S_1–S_5 generations, respectively. The δ values for seed yield were higher at S_1 and S_2 generations and with a continuous relative decrease up to the S_3 generation. The PHC-0008 was the population with the highest positive δ values for both seed traits, although negative values were observed at the S_5 generation that indicated a mean fitness slightly superior to the founder population.

Detection of outlier loci through selfing generations

The identification of loci that exhibited divergent patterns of variation when compared the founder (O_1) with selfing (S_1–S_4) generations, may offer opportunities to identify markers associated with adaptation to inbreeding. In order to avoid false positives caused by population structure, a global outlier test using all founder seven populations with LOSITAN software was applied to the 35 SSR markers, and initially detected five outlier loci outside the desired confidence interval (0.99 % CI). BMd51 marker showed a high F_{ST} value, while BM156, BMd05, BMd14 and GATs11 markers presented low F_{ST} values, compared to neutral expectations, which implies that these loci could be subjected to positive and balancing selection, respectively. After removing the five outlier loci, we used different tests (simulation, Waples, significance of F_T tests and simulated LOSITAN F_{ST} value; Table 4), which revealed from one to three outlier loci per population in at least two of the statistic tests used.

We performed multiple database searches against proteome and genome files downloaded from the Phytozome database (http://www.phytozome.net/) (Goodstein et al. 2012). The sequences of these outlier markers were compared with sequences from other species that codify for known genes. Gene and site annotations for the strongest hits (lowest e-value) for each sequence are reported (Table 5). BM154 and BM187 markers were co-located with QTLs for seed yield (Blair et al. 2006; Rodríguez-Suárez et al. 2007; Checa and Blair 2008) and GATs91 with a seed weight QTL (Blair et al. 2006). An alignment with high identity (89 %) was observed between BM154 and a patched family protein of *Arabidopsis*, which is needed for embryo development (Bürglin 2008). BM154 was detected as outlier in most of the populations except for PHC-0035, and 75 % of the individuals in S_4 generation had the private allele of 188 bp (data not shown). BM187, outlier detected in PHC-0012 and PHC-0038, showed a 79 % of identity with a metal-

Table 3 Mean values and standard deviation for 100-seed weight and yield traits for the seven runner bean populations through O_1–S_5 generations

Generation	N	100-seed weight (g)			Seed yield (g/plant)		
PHC-0008							
O_1	67	152.9	(7.38)	AB	59.3	(6.84)	B
S_1	58	**132.8**	(20.49)	C	**13.4**	(9.32)	D
S_2	43	**139.2**	(35.72)	BC	**21.8**	(18.21)	CD
S_3	53	143.7	(11.77)	B	**32.4**	(21.72)	C
S_4	80	152.0	(18.57)	B	**81.1**	(32.56)	AB
S_5	74	**162.**0	(9.60)	A	**86.8**	(25.51)	A
PHC-0012							
O_1	62	161.90	(16.02)	AB	30.7	(25.12)	B
S_1	31	**150.8**	(25.21)	B	**7.9**	(6.26)	C
S_2	30	160.2	(43.32)	AB	**13.6**	(4.93)	BC
S_3	59	166.5	(20.37)	AB	35.5	(3.43)	BC
S_4	80	**172.3**	(13.28)	AB	**56.7**	(15.78)	A
S_5	72	**173.**1	(9.64)	A	**62.4**	(12.35)	A
PHC-0016							
O_1	61	155.9	(11.87)	B	26.1	(6.42)	BC
S_1	48	154.9	(18.23)	B	18.1	(19.90)	C
S_2	34	**168.2**	(28.99)	A	23.7	(6.33)	C
S_3	56	156.0	(19.44)	B	46.3	(12.74)	BC
S_4	80	159.6	(17.39)	A	**59.3**	(12.58)	B
S_5	70	161.5	(6.65)	AB	**106.8**	(19.95)	A
PHC-0019							
O_1	62	189.9	(24.85)	B	48.5	(8.52)	B
S_1	39	**172.9**	(33.91)	C	**21.1**	(13.55)	C
S_2	54	**193.1**	(43.61)	A	**25.3**	(8.22)	C
S_3	44	191.2	(16.86)	B	59.1	(12.05)	B
S_4	80	**196.2**	(13.17)	A	**68.4**	(30.46)	B
S_5	68	**194.8**	(4.39)	A	**94.1**	(22.97)	A
PHC-0022							
O_1	62	155.7	(21.02)	DE	28.8	(11.46)	CD
S_1	43	145.3	(25.06)	E	17.0	(18.50)	D
S_2	35	**160.3**	(40.07)	DE	20.3	(17.67)	D
S_3	42	168.1	(13.25)	CD	40.5	(18.55)	C
S_4	77	**170.5**	(12.93)	BC	**66.5**	(16.25)	B
S_5	72	**185.0**	(8.27)	A	**83.0**	(14.19)	A
PHC-0035							
O_1	60	154.7	(12.88)	AB	23.9	(7.87)	CD
S_1	25	146.9	(27.44)	B	**11.9**	(9.48)	D
S_2	28	166.5	(28.24)	A	17.4	(7.10)	CD
S_3	48	163.0	(6.35)	AB	**39.1**	(10.08)	BC
S_4	80	165.2	(10.33)	AB	**56.7**	(5.92)	B
S_5	75	**169.8**	(3.42)	A	**74.4**	(3.98)	A

Table 3 continued

Generation	N	100-seed weight (g)			Seed yield (g/plant)		
PHC-0038							
O_1	73	145.8	(15.44)	B	25.9	(12.33)	CD
S_1	43	145.3	(14.06)	B	17.5	(13.76)	CD
S_2	31	**157.4**	(16.92)	A	18.8	(18.26)	CD
S_3	35	153.1	(16.21)	AB	34.1	(11.03)	BC
S_4	80	**160.2**	(17.42)	A	**50.1**	(16.82)	AB
S_5	68	**163.0**	(11.92)	A	**70.1**	(12.13)	A

The bold values differ significantly at $P \leq 0.05$ from generation O_1

Means followed by the same letter in columns do not differ significantly ($P < 0.05$) by Tukey's test

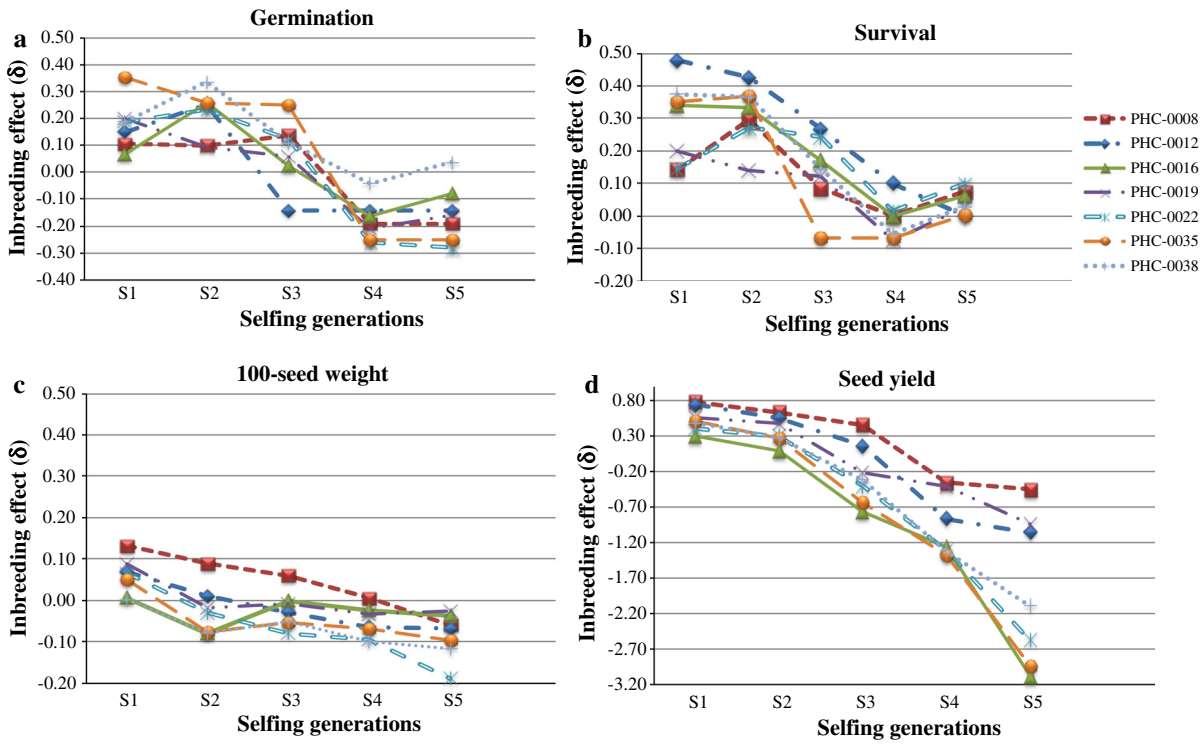

Fig. 2 Inbreeding effect (δ) for germination (**a**), survival to flowering (**b**), 100-seed weight (**c**), and yield (**d**) for the seven runner bean populations through S_1–S_5 generations

nicotiamine transporter YSL7-LIKE. The YSL family plays an important role in the transport of metals through and between vascular tissues to support gametogenesis and embryo development (Curie et al. 2009). The private 164 bp allele of the BM187 locus was fixed in both populations. X04660 was significant in PHC-0012, PHC-0019 and PHC-0022 and presented an identity of 87 % with Tesmin/TSO1-like CXC protein domain from *Arabidopsis*, which seems to play a role in the development of both male and female reproductive tissues and is required for fertility (Hauser et al. 2000; Song et al. 2000). Therefore, BM154, BM187 and X04660 markers seem to be associated to genes related to plant and embryo

Table 4 Outlier loci identified from different tests for the seven runner bean populations through O_1– S_4 generations

Population	Locus	No of alleles	Probability values of the tests[a]			H_e/F_{ST}^b
			ST	F_TT	WT	LOSITAN
PHC-0008	BM154	2	**0.0306**	0.0200	**0.0000**	0.505/0.059
PHC-0012	BM154	3	**0.0030**	0.0501	**0.0000**	**0.376/0.336**
	BM187	2	**0.0000**	0.0961	**0.0140**	**0.395/0.578**
	X04660	2	**0.0000**	0.0657	**0.0008**	**0.471/0.470**
PHC-0016	BM154	4	0.0528	**0.0074**	**0.0068**	**0.387/0.408**
PHC-0019	BM154	2	**0.0050**	**0.0378**	0.1718	**0.367/0.298**
	GATs91	2	**0.0014**	**0.0278**	**0.0000**	**0.417/0.274**
	X04660	2	**0.0104**	**0.0250**	**0.0029**	**0.333/0.271**
PHC-0022	BM154	2	0.2783	**0.0023**	0.1432	**0.669/0.457**
	X04660	2	**0.0219**	**0.0219**	**0.0056**	**0.347/0.336**
PHC-0035	BM167	3	**0.0002**	**0.0253**	**0.0000**	**0.066/0.130**
	GATs91	2	**0.0060**	**0.0264**	**0.0014**	0.303/0.196
PHC-0038	BM154	3	**0.0073**	**0.0221**	**0.0025**	**0.441/0.424**
	BM187	2	**0.0322**	**0.0170**	**0.0208**	**0.417/0.374**

[a] *ST* simulation test, F_TT test of significance of F_T, *WT* Waples test. Bold numbers indicate significant results at $\alpha = 0.05$

[b] Pairwise estimate of expected heterozygosity and F_{ST} were obtained using the Lositan software (Beaumont and Nichols 1996; Antao et al. 2008). Bold numbers indicate detection of loci at the 99 % confidence level

Table 5 Candidate loci through the generations in the runner bean populations and their alignments to known protein sequences

Locus	LG	Alignment length (bp)	Putative gene function	Species	E-value	Associated QTL
BM154	9	111	Patched family protein	*A. Thaliana*	5e−11	Pods/plant[a]
BM187	6	300	Metal-nicotiamine transporter YSL7-LIKE	*G. max*	6e−78	Grain yield[a]
GATs91	2	71	Uncharacterized protein	*G. max*	1e−18	100 seed weight (g)[a]
X04660	4	186	Tesmin/TSO1-like CXC protein	*A. Thaliana*	6e−25	
BM167			Unknown. No BLAST similarity			

LG common bean linkage group

[a] Blair et al. (2006), Rodríguez-Suárez et al. (2007), Checa and Blair (2008)

development which indicates that could play an important role in the adaptation to selfing.

Discussion

Genetic variability in founder runner bean populations

Phaseolus coccineus is native to Mexico, Guatemala and Honduras (Delgado Salinas 1988) and the wild forms are probably not all ancestral to the cultivated form. Although the gene-diversity levels are much higher in origin centers than European varieties (Vargas-Vázquez et al. 2011, 2013), similar levels were observed over all of Europe (Rodríguez et al. 2013). Alvarez et al. (1998) compared Spanish and Mexican accessions of *P. vulgaris* and *P. coccineus*, and they suggested that the runner bean maintained a high level of diversity after its introduction into Europe. Other studies have suggested changes in the structure of the genetic variation, probably due to selection, to rapid adaptation to the new growing conditions, or to demographic processes, such as bottleneck and founder effects (Sicard et al. 2005; Spataro et al. 2011). A moderate level of genetic diversity ($H_e = 0.36$) was detected in this experiment, which it is in accordance with other runner bean

studies (Spataro et al. 2011; Rodríguez et al. 2013). Wright's ID coefficients based on O_1 generation genotypic frequencies were positive and significantly greater than zero (values varied from 0.397 to 0.610 for PHC-0012 and PHC-0035, respectively) as expected for a partially selfing species. Therefore, the excess of homozygotes observed and the intermediate outcrossing rates estimates (mean = 34 %) indicated relatively frequent outcrossing events. There was a wide variation in the rate of outcrossing among populations (ranged from 24 to 44 %). The differences in homozygosity and outcrossing rates found could indicate different selection pressures affecting to the runner bean genetic material. Different farming practices could be involved in maintaining these populations. Previous studies have also noted significant Wright's ID variation among founder populations (Pray and Goodnight 1995; Dudash et al. 1997). Therefore, our study highlights the importance of the mating system (likely influenced by environmental fluctuation) in the population diversity of runner bean populations from the Iberian Peninsula. Instability in environmental parameters can influence the mating system and help maintain intermediate rates of selfing in these mixed-mating populations.

Inbreeding depression: consequences and evolution of self-fertilization

In this work, a substantial portion of individuals failed to germinate and flower through the inbreeding process, suggesting that deleterious alleles responsible for ID can be present at embryonic and adult stages of development. Several studies have reported embryo mortality owing to inbreeding (Meinke 1991; Seavey and Carter 1994; Husband and Schemske 1996), and found also an association between ID and a reduction in plant vigor (Charlesworth and Charlesworth 1987; Husband and Schemske 1996), which could explain the results observed. The results evidenced genetic variation in ID among families and generations. However, most populations showed $\delta < 0.5$ for fitness traits (germination and survival), while the half of the populations showed values of $\delta > 0.5$ for seed yield. In this context, if ID is greater than 50 %, complete outcrossing is predicted to evolve, and if ID is less than 50 %, complete selfing should evolve (Husband and Schemske 1996; Goodwillie et al. 2005; Morgan and Wilson 2005). Hence, the genetic variation found in ID suggests that the mixed mating system of runner bean populations might be evolutionary stable.

Differences in inbreeding effects among populations and plant growth stages can reflect differences in either the number or the type of deleterious mutations. Two main types of deleterious mutations are usually considered to be responsible for ID: (1) deleterious mutations of large effect (lethal), typically found in a few loci, and (2) mildly deleterious mutations in many loci across the genome (Husband and Schemske 1996; Charlesworth and Charlesworth 1999; Fox et al. 2008). Thus, at one extreme, can be genes of the highest order, such as lethals or near-lethals. The viability results are consistent with this type of major deleterious recessives because S_1 and S_2 generations were much less viable than the outbred populations from which they were derived. During the first selfing generations, these deleterious recessive alleles are exposed to selection as homozygotes and are eliminated. Therefore, high values of viability and survival in S_5 generation were observed, in which both rates were up to 90 %. Further, the viability data showed differential expression of deleterious genes in populations such as PHC-0035 and PHC-0012, with the lowest viability values, regardless of the level of inbreeding. One of the explanations for the differences among populations is that progenies vary in the number of recessive alleles that they carry (Koelewijn 1998), surely as a result of their different history of inbreeding or differences in the accumulation of mutations (Schultz and Willis 1995). These results highlight that ignoring the genetic information on the germplasm may underestimate the extinction risk of some runner bean populations with a high number of lethal or near-lethal alleles and thus lead to inappropriate conservation measures of these populations.

Quantitative characters as seed yield and size are determined by a large number of genes having small, more or less equal effects. These genes are recessives of low-intermediate effect, only slightly less favorable than their dominant alleles. The additive effect of these multiple mildly deleterious alleles might deteriorate the development (metabolism and physiology) of selfed progeny, thus decreasing seed weight (Husband and Schemske 1996; Charlesworth and Charlesworth 1999). In this case, the better families during selfing process were better, in part, because they had improved in combining value during the progress of selfing. These results reflect the importance of

selecting inbreeds carrying the largest complements of favorable genes. The inbreeds carrying the most favorable genes, such as PHC-0016, would provide the most promising material for future breeding programs.

The fact that the populations had deleterious alleles of large and small effects to a greater or lesser extent could explain the differences among-populations found in this work for seed yield, survival and germination (Koelewijn 1998; Fox et al. 2008). This variation is also reported in other species (Dudash et al. 1997; Carr and Dudash 1997; Koelewijn 1998; Daehler 1999; Kelly 2005).

We analyzed short-term forces acting on the genetic diversity through the selfing process and five loci showed a significant deviation from neutrality. Genetic drift is expected to cause fixation of different genes, while the general effect of inbreeding could result in an important physiological duress to which the organism responds in a standard manner. Two loci (BM154 and BM187) are co-located with QTLs for seed yield (Blair et al. 2006; Rodríguez-Suárez et al. 2007; Checa and Blair 2008) and showed homology with genes related to embryonic development, suggesting that the variability observed among populations and generations could be due to genetic differences for their capability to inbreed. A maize study revealed that 25 % of the genes differentially expressed between inbred lines and hybrids were associated during early embryo development with signal transduction (Meyer et al. 2007). Our results showed that the mechanisms involved in embryonic development could have played an important role in the adaptive response of plants to self-pollination. Detailed expression profiling experiments will be need to further refine the insight of genes that might be differentially expressed between inbred and outcrossed individuals and, therefore, play a role during ID manifestation. The integration of genomic tools might also allow to map and clone genetically-related genes of ID.

Consequences for runner bean crop improvement ad conservation programs

Knowledge of the runner bean mixed-mating system is essential for determining a suitable genetic germplasm conservation and efficient breeding. There is enough variation within and among population to select for tolerance to inbreeding. Several lines that have been self-pollinated for many generations became homozygous at almost all gene loci and produced a uniform population of true breeding progeny and acceptable performance. The results confirmed that runner bean displays mating system variation, characterized by almost equal levels of outcrossing and selfing, and that may be evolutionary stable over a long period of time. The fixation of the runner bean inbred lines that suffer only moderately ID could avoid the complete outcrossing, although inbreeding effects in several fitness traits could be difficult to purge. This type of mating system is reported in an increasing number of plants (Goodwillie et al. 2005) and supports predictions that despite the potential for decreased fitness of progeny due to severe ID, self-fertilization is likely to be an important mode of reproduction of this species.

Acknowledgments Support was provided by Spanish Government (AGL2008-02091 and AGL2011-25562), EU-FEDER, JAE-Doc contract from National Spanish Research Council (CSIC) to A. M. González. The authors thank the Diputación Provincial de Pontevedra (Spain) for farm facilities.

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