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# Ecological components and evolution of selfing in the freshwater snail *Galba truncatula*

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self-fertilization;
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## Abstract

The reproductive assurance hypothesis emphasizes that self-fertilization should evolve in species with reduced dispersal capability, low population size or experiencing recurrent bottlenecks. Our work investigates the ecological components of the habitats colonized by the snail, *Galba truncatula*, that may influence the evolution of selfing. *Galba truncatula* is a preferential selfer inhabiting freshwater habitats, which vary with respect to the degree of permanence. We considered with a population genetic approach the spatial and the temporal degree of isolation of populations of *G. truncatula*. We showed that patches at distances of only a few meters are highly structured. The effective population sizes appear quite low, in the order of 10 individuals or less. This study indicates that individuals of the species *G. truncatula* are likely to be alone in a site and have a low probability of finding a partner from a nearby site to reproduce. These results emphasize the advantage of selfing in this species.

## Introduction

The costs and benefits of biparental reproduction are old problems in evolutionary biology (Darwin, 1878) and substantial debates continue regarding most aspects of mating systems evolution (e.g. Hurst & Peck, 1996; Jokela *et al.*, 1997; Ayala, 1998; Agrawal & Lively, 2001; Cheptou & Mathias, 2001; Welch & Meselson, 2001; Cheptou & Dieckmann, 2002; Victoir & Dujardin, 2002). Self-compatible hermaphroditic organisms offer the opportunity to study the evolution of selfing vs. outcrossing with their associated benefits. Beside the gene transmission advantage (cost of outcrossing: Fisher, 1941), uniparental reproduction presents a major ecological benefit, the 'reproductive assurance'. This hypothesis of reproductive assurance proposes that selfing guarantees reproduction when potential mates (and/or pollinators) are scarce or absent (Darwin, 1877, 1878). Darwin (1878) considered reproductive assurance the chief reason for the evolution of selfing. This advantage of selfing was further investigated in theoretical as well as empirical work. These studies jointly indicated that self-fertilization should evolve in species with reduced mobility (Tomlinson, 1966; Ghiselin, 1969), low population sizes, or experiencing recurrent bottlenecks (Baker, 1955, 1967; Stebbins, 1957; Price & Jain, 1981; Pannell & Barrett, 1998).

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Here, we investigate which ecological factors affect the evolution and maintenance of self-fertilization in the freshwater snail, *Galba truncatula* (previously named *Lymnaea truncatula*). This hermaphroditic species is a preferential selfer, although some variations in selfing rate exist among individuals and between populations (Trouvé *et al.*, 2003; Meunier *et al.*, 2004). Here we will focus on the characteristics of the sites inhabited by this species, which seems to be (i) discretely distributed and (ii) ephemeral, and we will estimate the strength of these characteristics to investigate if they can favour the evolution of selfing.

The first evidence for the patchy distribution of *G. truncatula* is the strong differentiations among populations from the Old World separated by about 30–10 000 km (Meunier *et al.*, 2001), as well as among Swiss populations distant from 10 to 100 km (Trouvé *et al.*, 2003). However, the very low gene flow at this large spatial scale is not very surprising for snails. A question remains as to the spatial scale at which a significant reduction in the level of gene flow can be detected. At that point, we just have a fragmentary answer: Trouvé *et al.* (2003) found that two geographically close

populations, separated by 25 m, presented a high genetic differentiation ($F_{ST} = 0.5$, $P < 0.001$). Does this result constitute a rule or an exception? Answering this question is of importance because low levels of gene flow would mean a low probability for the snails to find a mate from a nearby population when alone in a site. In this context, selfing would present a major ecological benefit.

The second characteristic of the habitats colonized by *G. truncatula* is that they differ highly in water availability. Although this species can colonize ponds and streams (i.e. permanent habitats) that subsist during the entire year, it is most often found in puddles or wet meadows (temporary habitats) that are subject to drought in summer and frost in winter (LD and ST personal observation). Goumghar *et al.* (2001) investigated the influence of aestivation and pointed out that summer drought affects the survival rate, which may be reduced from 30 to 80% in, respectively, lowland and highland populations in central France. Low temperatures in winter are also known to increase development time and mortality (Roberts, 1950; Rondelaud, 1977). Together these empirical observations suggest an effect of temporary habitats on the demography of populations. While mortality increases under these stressful conditions, snails may nevertheless survive aestivation buried into the soil or covered by plants (Kendall, 1949; Rondelaud & Morel-Vareille, 1975; Moukrim & Rondelaud, 1992); and some snails might also survive in frozen ponds (Roberts, 1950). In this way even careful field observations may underestimate the occurrence of *G. truncatula*. One way to ascertain that population sizes are significantly reduced following harsh meteorological conditions, and thereby that demographic bottlenecks affect the evolutionary properties of populations of *G. truncatula*, is to investigate temporal variations in allele frequencies and effective population size. Indeed, decreases in census population sizes following events of drought and frost are expected to result in changes in allele frequencies over time and to reduce effective sizes in temporary habitats relative to permanent ones. If these effects are observed, they will reveal an important adaptive advantage of selfing in *G. truncatula*: uniparental reproduction would ensure progeny production when population sizes are reduced following drastic seasonal conditions. Furthermore, if bottlenecks are confirmed, they may affect the evolution of inbreeding depression (Kirkpatrick & Jarne, 2000), which constitutes the main genetic factor counterbalancing the advantage of selfing, and they may modify the evolution of selfing (Cheptou & Dieckmann, 2002).

Our goal is to identify, using a population genetic approach, the ecological factors (degree of isolation, low population size) that should affect the evolution of selfing in *G. truncatula*. To this end, we first examine the spatial population structure and address the following questions: At which spatial scale is genetic variance distributed? Which ecological factors prevent migration? Second, in order to investigate the consequences of habitat disturbance, we analyse the population structure over time, and the effective size of populations from temporary and permanent habitats. Results from this study lead to a discussion of the possible influence of various factors, including selfing, on the estimated genetic parameters. Finally, the results are considered in the context of mating system evolution in *G. truncatula*.

## Material and methods

### Sampling sites and genotyping

Sampling of adult snails for the micro-spatial and temporal studies took place in Western Switzerland, between the Lemanic plain and the Jura (Fig. 1). At each site an area of about 10 $m^2$ was sampled.

Trouvé *et al.* (2003) described two types of habitats (permanent and temporary). Here we defined more accurately the types of habitats and recognized three categories: temporary, semi-permanent and permanent. Temporary habitats include ponds that dry out or freeze completely many times throughout the year. At the other extreme, permanent habitats never experience such drought or frost events and continue to exist during the entire year. Between these two extremes, semi-permanent habitats may dry or freeze but only during very harsh periods, which generally occur once or twice a year.

Snails were genotyped at six microsatellite loci (loci 16, 20, 21, 29, 36 and 37) following the procedure described in Trouvé *et al.* (2000).

### Micro-spatial analyses

Sampling for the spatial study took place in October 1999 in six localities (Fig. 1), consisting of two permanent ($Chevenez_p$ and $Corcelles_p$), two semi-permanent ($Suchy_{sp}$ and $Taulard_{sp}$) and two temporary localities ($Dizy_t$ and $Bioley_t$) (the subscripts *t*, *sp* and *p* indicate, respectively, *temporary*, *semi-permanent* and *permanent*). Four patches were sampled in each permanent locality and five patches were sampled in each nonpermanent locality (i.e. temporary and semi-permanent). We collected about 15 snails in each patch, and measured the straight-line distance between patches. Additionally we noted the distribution of patches and the presence/absence of physical barriers between them (Fig. 2). Piles of stones, raised paths or fields together form a network of physical barriers that potentially separate patches.

Gene flow within and among patches was analysed in the context of hierarchical genetic structure, and isolation by distance vs. isolation by barriers. More details are given in the following sections.

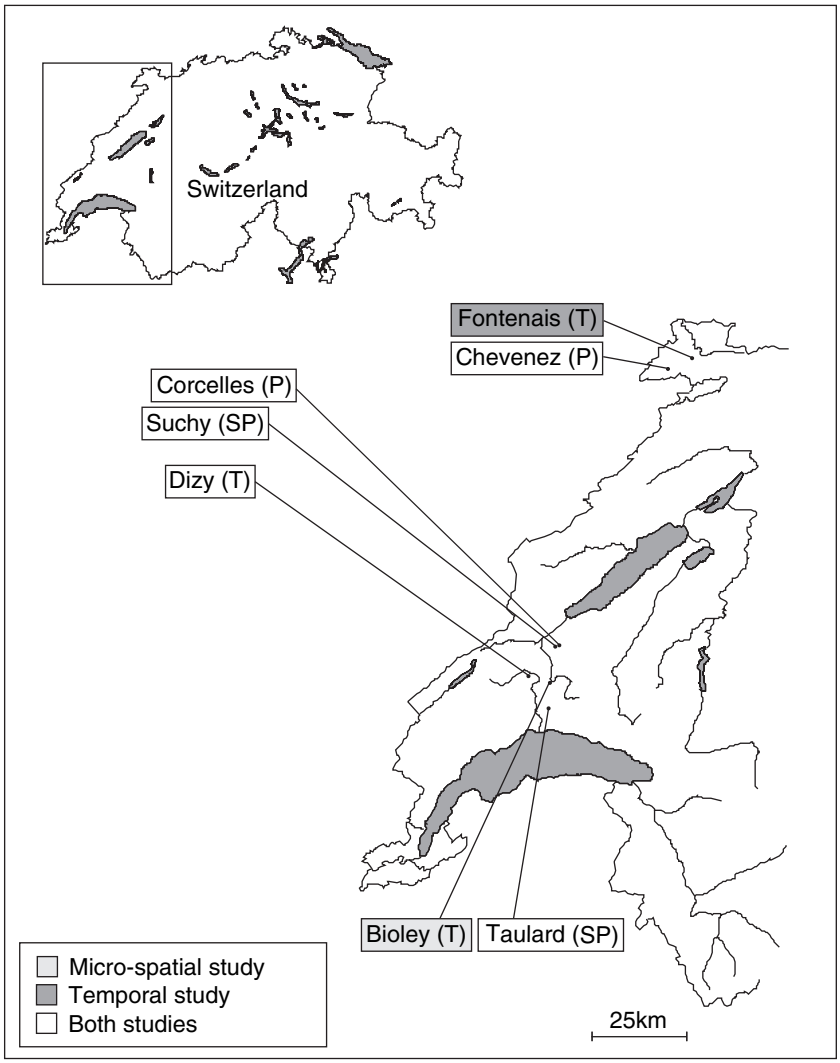


**Fig. 1** Geographic localities of the populations studied in Switzerland for the micro-spatial and temporal studies. T, temporary; SP, semi-permanent; P: permanent.

*Hierarchical analysis*

Hierarchical analysis of the genetic structure was performed to detect the structure at two different levels: patches and localities. The classical *F*-statistics terminology (i.e. $F_{ST}$, $F_{IS}$) was modified using the following notation: subscript *Ind* refers to individual, *Patch* to patch, *Loc* to locality, *Total* to total. Consequently, $F_{PatchTotal}$ is the correlation of genes within patches relative to the total, whereas $F_{LocTotal}$ is the correlation of genes within localities relative to the total. $F_{PatchLoc}$ estimates the correlation of genes within patches relative to localities, and $F_{IndPatch}$ measures the correlation of genes within individuals relative to patches. The hierarchical estimates of *F*-statistics were obtained from variance components of gene frequencies (Weir & Cockerham, 1984; Weir, 1996; Yang, 1998). The variance components were computed with the algorithm described in Yang (1998) using the statistical package R (Ihaka & Gentleman, 1996) and were defined as follows: within individuals: $\sigma_w^2$, among individuals within patches: $\sigma_c^2$, between patches within localities: $\sigma_b^2$, and among localities: $\sigma_a^2$. Per locus and overall estimators of genetic differentiation were obtained from these components as $F_{PatchTotal} = (\sigma_a^2 + \sigma_b^2)/(\sigma_a^2 + \sigma_b^2 + \sigma_c^2 + \sigma_w^2)$, $F_{LocTotal} = \sigma_a^2/(\sigma_a^2 + \sigma_b^2 + \sigma_c^2 + \sigma_w^2)$, $F_{PatchLoc} = \sigma_b^2/(\sigma_b^2 + \sigma_c^2 + \sigma_w^2)$, and $F_{IndPatch} = \sigma_c^2/(\sigma_w^2 + \sigma_c^2)$. The significances of the deviation from zero of all *F*-values were tested with permutation procedures: For $F_{IndPatch}$, alleles were permuted among individuals within patches. For $F_{PatchLoc}$, individuals were permuted among patches but kept within their locality of origin. For $F_{LocTotal}$, whole patches were permuted among localities. Finally, for $F_{PatchTotal}$, individuals were permuted among patches and localities. When the permutation procedure was available in FSTAT v 2.9.3 (Goudet, 2001), this software was used (for $F_{IndPatch}$ and $F_{PatchTotal}$), while the statistical package R was used for testing the significance of $F_{PatchLoc}$ and $F_{LocTotal}$. Code for carrying these tests is available

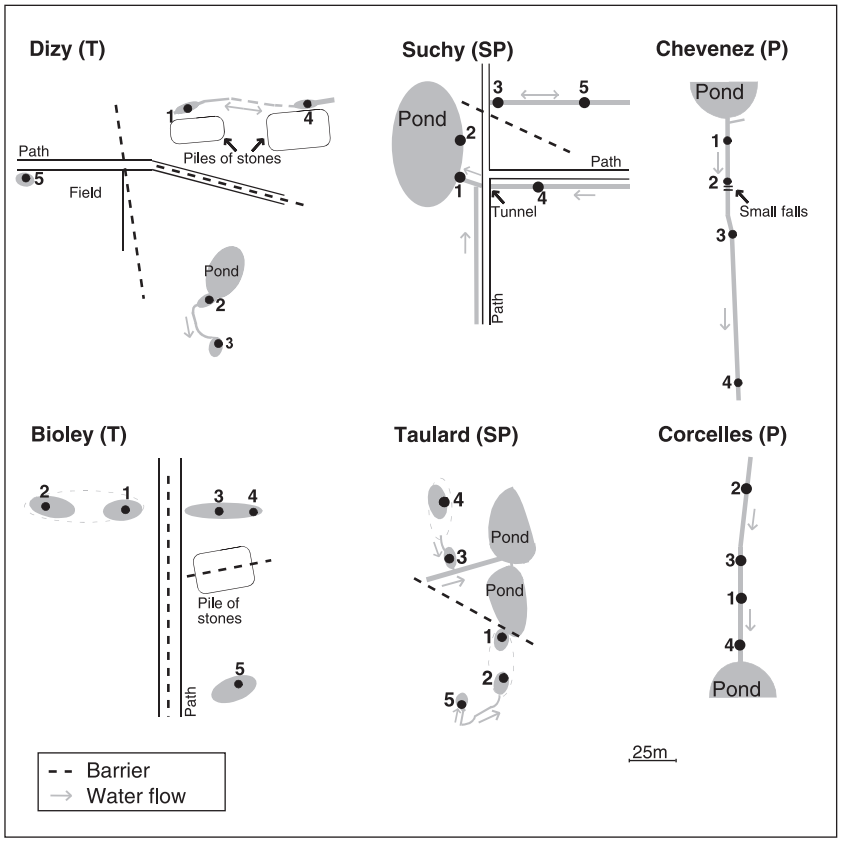


**Fig. 2** Patch and barrier locations within each locality for the micro-spatial study.

from the R library HIERFSTAT available from http://www.unil.ch/popgen/softwares/hierfstat.htm. This library also contains the data set for the hierarchical structure analysis (data set gtrunhier).

*F*-statistics were also estimated independently for each locality. To test for departure from panmixia within each patch and to test whether patches are differentiated within each locality, Weir & Cockerham (1984) estimators of *F*-statistics ($\hat{\theta}$ estimates $F_{ST}$, $\hat{f}$ estimates $F_{IS}$) were calculated with FSTAT version 2.9.3 and tested with randomization procedures (Goudet *et al.*, 1996). Due to multiple testing, sequential Bonferroni corrections (Rice, 1989) were applied to reduce the likelihood of false significant results.

Assuming that inbreeding only results from self-fertilization, selfing rates ($s$) have been indirectly estimated using the classical equation $s = 2\hat{f}/(1 + \hat{f})$, where $\hat{f}$ is the equilibrium inbreeding coefficient for a neutral locus. We have shown (Trouvé *et al.*, 2003) that these indirect estimates are concordant with progeny array analyses and therefore provide accurate estimates of selfing rate.

Allelic richness (El Mousadik & Petit, 1996; Petit *et al.*, 1998), as well as observed and expected heterozygosities within patches following Nei (1987) were also estimated with FSTAT.

*Isolation by distance vs. isolation by barriers*

We tested for isolation by distance by analysing the relation between the index of genetic differentiation $\hat{\theta}/(1 - \hat{\theta})$ and the logarithm of the geographic distance (Rousset, 1997) using classical Mantel tests (Manly, 1997) in permanent localities. Because nonpermanent habitats are by definition small (e.g. puddles), they are fragmented, and barriers potentially preventing migration can usually be detected between patches. Therefore, for nonpermanent localities, partial Mantel tests were performed to analyse the relative importance of geographic distance and natural barriers in explaining the matrix of genetic distances among pairs of patches. Matrices of presence/absence of natural barriers separating the patches were created (see Fig. 2 for barrier positions). These matrices are filled with either 0 or 1; the number 1 is entered if the two patches under consideration are separated by a barrier and 0 if they are not. Mantel tests were performed in FSTAT. A total of 10 000 randomizations were carried out for classical and partial Mantel tests.

## Temporal analyses

Temporal changes in allelic composition were studied in six localities (Fig. 1), two permanent (Chevenez$_p$ and

Corcelles$_p$), two semi-permanent (Suchy$_{sp}$ and Taulard$_{sp}$) and two temporary (Dizy$_t$ and Fontenais$_t$). One patch per locality was analysed. Permanent localities were sampled during three different seasons, autumn 1998 (A98: September to mid-October), spring 1999 (S99: end of May to mid-June) and autumn 1999 (A99: October). Nonpermanent localities (i.e. temporary and semi-permanent) were sampled in A98 and S99. About 50 snails were collected from a patch in each of these localities in each season. A total of about 629 snails were thus analysed, 294 from A98, 273 from S99 and 62 from A99.

Because both temporary and semi-permanent habitats are subject to variation in water availability, they should both show fluctuations in population size throughout the year. In this respect, they are in contrast with permanent populations, which should be more stable. Accordingly, these temporal analyses were based on comparisons of nonpermanent vs. permanent populations. We used two complementary approaches, one based on the estimation of temporal population structure and the other on effective population size.

*Temporal population structure*

Genetic characteristics (allelic richness, gene diversity…) of each patch for each season were estimated as described above. Temporal $\hat{\theta}$-values were calculated for each patch among samples collected in A98 and S99 for nonpermanent habitats and among samples collected in A98, S99 and A99 for permanent habitats. The significances of these estimators of differentiation were tested using permutations as described above.

Because nonpermanent populations are likely to undergo variations in population numbers (either once or numerous times per year), one might expect them to show higher temporal $\hat{\theta}$-values relative to permanent ones. Temporal $\hat{\theta}$-values calculated for each patch were thus compared between the two types of habitats using a Wilcoxon rank test.

*Effective population size*

The effective population size ($N_e$) can be estimated through instantaneous measure based on temporal variations in allele frequencies. Estimation of $N_e$ has classically been made assuming migration is unimportant in changing allele frequencies compared with genetic drift (e.g. Pollak, 1983; Waples, 1989; Williamson & Slatkin, 1999; Anderson *et al.*, 2000). This assumption is unlikely for most organisms. Ignoring migration may substantially bias, either upward or downward, $N_e$ estimates (Wang & Whitlock, 2003). Here, we estimate the variance effective population sizes ($N_e$) based on temporal variation in allele frequencies obtained from the temporally spaced genetic samples of patches originating from permanent, semi-permanent and temporary habitats. We used the algorithm described in Wang & Whitlock (2003) and implemented in the MLNE program, which jointly estimates the maximum likelihood of the immigration rate ($m$) and the effective population size ($N_e$). This program assumes an 'infinite-source' model, characterized by an infinitely large source population sending migrants to a focal population from which $N_e$ and $m$ are estimated. We estimated immigration rate and effective size for each of the six patches considered in turn; the allele frequencies from the infinite source were estimated from the remaining five populations, with their temporally spaced samples pooled following the strategy advocated by Wang & Whitlock (2003). To investigate the impact of immigration on the estimation of $N_e$, we also ran the program assuming complete isolation between patches (i.e. $m = 0$). This corresponds to the maximum likelihood estimates of $N_e$ developed by Wang (2001). The $N_e$ estimates obtained assuming that the populations are connected by gene flow were compared with those obtained when populations are assumed to be isolated, using a Wilcoxon signed rank test.

For nonpermanent patches, $N_e$ was computed with two sampling events (A98 and S99), whereas for permanent ones, $N_e$ was computed with an additional third sampling event (A99). Similar results (not shown) were obtained by using only the first two temporal samples for permanent populations (A98 and S99). The temporal approach for estimating $N_e$ requires the number of generations between sampling events to be known. The literature suggests that *G. truncatula* presents two to three generations per year (Rondelaud & Mage, 1990; Dillon, 2000 and references therein). We assumed that one generation separates the first (A98) from the second (S99) sampling event, and that three generations separate the first (A98) from the third (A99) sampling event. We verified that the results obtained are robust to the number of generations considered between the sampling events. Furthermore, we tested the $N_e$ estimates over populations, to determine whether permanent populations present higher values compared with nonpermanent ones, by a Wilcoxon test.

The program of Wang & Whitlock (2003) assumes that the source populations are clearly identified and is based on the approximation of an infinite-source population providing migrants to a focal population from which $N_e$ is estimated. In our temporal study, only one patch was sampled per locality; hence several populations that are potential sources of immigrants are missing from the data set. In this context, it is reasonable to ask whether the sampling scheme used can influence the results. Considering that the *a priori* identification of source populations is difficult, we modified the constitution of the source population in two ways. (i) We added the temporal samples of the focal population to the samples of the source populations. This was done in order to make the source population more similar to the focal patch, because close-by patches are missing from our data set. Patches from the same locality are potential source of migrants, and are likely to have a more similar genetic

composition than those of the other localities (see $\hat{\theta}$-values per locality in the results part). (ii) We ran the MLNE program using only the initial pooled temporal samples as a source population. In doing, this we assume that the genetic constitution of the migrants that might modify the allelic frequencies in the focal patch between the two sampling dates should be more similar to that of the first temporal sample. This assumption relies mainly on the sampling interval, which was estimated to be only one or two generations.

## Results

### Micro-spatial analyses

*Hierarchical analyses*

The combined estimate (over loci) of spatial differentiation among patches relative to the whole sampling area is very large ($F_{\text{PatchTotal}} = 0.64$; Table 1). The genetic variation in *G. truncatula* has a hierarchical structure. There is a strong differentiation even at a very small scale ($F_{\text{PatchLoc}} = 0.26$) that increases as the spatial scale increases ($F_{\text{LocTotal}} = 0.52$). Values of $F$ are highly significant ($P < 0.001$) for all but $F_{\text{PatchLoc}}$ at locus 37 ($P < 0.05$).

When considering the different localities separately, it can be seen that most of the global $\hat{\theta}$-values per locality are relatively high and significantly different from 0 ($\text{Bioley}_t$: $\hat{\theta} = 0.32$, $\text{Dizy}_t$: $\hat{\theta} = 0.1$, $\text{Taulard}_{sp}$: $\hat{\theta} = 0.15$, $\text{Suchy}_{sp}$: $\hat{\theta} = 0.45$, $\text{Chevenez}_p$: $\hat{\theta} = 0.32$: $P < 0.05$). The only exception is $\text{Corcelles}_p$, for which the among-patch differentiation is low and nonsignificant ($\hat{\theta}_{\text{global}} = 0.02$). Additionally for this locality and $\text{Dizy}_t$, no significant spatial differentiation could be observed among any pair of patches (Table 2). In the other localities, high and significant genetic divergences could be observed among most patches, even if separated by only a few meters (Table 2). This is the case, for instance, between patches 1 and 3 in $\text{Bioley}_t$, 50 m apart. In contrast, in $\text{Corcelles}_p$, populations from patches 2 and 4 separated by more than 100 m do not differ genetically. In $\text{Chevenez}_p$, allele frequencies and $\hat{\theta}$-values strongly suggest differentiation between patches 1–2 and 3–4, that is between upstream and downstream patches. These two zones are separated by small waterfalls and locally strong current. The result of a *post-hoc G*-test confirms the occurrence of upstream–downstream genetic structure ($\hat{\theta}_{\text{Up-Down}} = 0.17$, $P = 0.05$).

Over all patches and localities, $F_{\text{IndPatch}}$ values for all loci are very large (overall $F_{\text{IndPatch}} = 0.76$, $P < 0.001$; Table 1). When considering the different patches independently, 20 of 27 patches have a $\hat{f}$-based selfing rate estimate higher than 80%, which confirms the predominance of self-fertilization in this species, as observed in a previous work (Trouvé *et al.*, 2003). The selfing rates are consistent among patches within each locality, except for the locality of $\text{Corcelles}_p$ for which selfing rate ranges from 32 to 77%.

The genetic variability, estimated either with mean allelic richness (*Rs* based on sample size of seven individuals: range: 1–2.85) or mean gene diversity (*He*: range: 0–0.54), is low for all the patches, and is relatively homogeneous among patches within each locality. The

**Table 1** Per locus and overall hierarchical fixation indices.

| Locus | $F_{\text{IndPatch}}$ | $F_{\text{PatchLoc}}$ | $F_{\text{LocTotal}}$ | $F_{\text{PatchTotal}}$ |
|---|---|---|---|---|
| 16 | 0.77 | 0.24 | 0.50 | 0.62 |
| 20 | 0.66 | 0.27 | 0.62 | 0.72 |
| 21 | 0.72 | 0.39 | 0.40 | 0.63 |
| 29 | 0.80 | 0.25 | 0.47 | 0.60 |
| 36 | 0.85 | 0.27 | 0.46 | 0.60 |
| 37 | 0.70 | 0.06 | 0.70 | 0.71 |
| All | 0.76 | 0.26 | 0.52 | 0.64 |

All $F$-values are highly significant ($P < 0.001$), except $F_{\text{PatchLoc}}$ at locus 37 ($P < 0.05$).

**Table 2** Spatial differentiation. Pairwise $\theta$-values ($\theta$) were estimated among patches within each locality.

| | $\theta$ | | | |
|---|---|---|---|---|
| | 2 | 3 | 4 | 5 |
| *Temporary* | | | | |
| Dizy | | | | |
| 1 | 0.118 | 0.297 | 0.079 | 0.238 |
| 2 | | −0.005 | −0.061 | −0.048 |
| 3 | | | 0.023 | −0.005 |
| 4 | | | | 0.001 |
| Bioley | | | | |
| 1 | 0.040 | 0.218* | 0.309* | 0.415* |
| 2 | | 0.275* | 0.378* | 0.343* |
| 3 | | | 0.054* | 0.477* |
| 4 | | | | 0.545* |
| *Semi-permanent* | | | | |
| Suchy | | | | |
| 1 | 0.190* | 0.654 | 0.1 | 0.652* |
| 2 | | 0.608* | 0.098* | 0.610* |
| 3 | | | 0.483* | 0.157 |
| 4 | | | | 0.473* |
| Taulard | | | | |
| 1 | 0.005 | 0.138 | 0.284* | 0.303* |
| 2 | | 0.064 | 0.166* | 0.145 |
| 3 | | | −0.018 | 0.177* |
| 4 | | | | 0.195 |
| *Permanent* | | | | |
| Chevenez | | | | |
| 1 | 0.03 | 0.311* | 0.151 | |
| 2 | | 0.169 | 0.035 | |
| 3 | | | −0.024 | |
| Corcelles | | | | |
| 1 | 0.086 | −0.006 | −0.002 | |
| 2 | | 0.081 | −0.004 | |
| 3 | | | −0.014 | |

*Pairwise significance after sequential Bonferroni test.

values of these genetic parameters are similar to those found by Trouvé *et al.* (2003) and are thus not presented in this paper. The data set is available from http://www.unil.ch/popgen/softwares/data/.

*Isolation by distance vs. isolation by barrier*

For the permanent populations, the matrix $\hat{\theta}/(1-\hat{\theta})$ is not correlated to the matrix of geographic distance (Chevenez$_p$: $r^2 = 0.01$, $P = 0.87$; Corcelles$_p$: $r^2 = 0.12$, n.s.). In nonpermanent populations, partial Mantel tests were conducted to analyse the relative importance of geographic distance and natural barriers in explaining the genetic variation among patches. In Suchy$_{sp}$ and Taulard$_{sp}$, natural barriers appear to be a major cause of population differentiation; they explain a large and significant part of the variance of genetic distances among patches once geographic distance has been taken into account (Fig. 3). In Dizy$_t$, no effect of geographic distance or barrier can be detected. The percentage of variance explained by these two factors together is low ($r^2 = 0.07$, Fig. 3), certainly because of the low level of genetic variation among patches. In Bioley$_t$, although our model explains a large part of the variance, natural barriers explain none of the variability in genetic distance, once geographic distance matrix has been accounted for. This most likely results from the fact that, in this locality, geographic distance and natural barriers are highly correlated (Kendall rank correlation: $\tau = 0.6$, $P < 0.05$).

## Temporal analyses

*Temporal population structure*

The values of the genetic characteristics (allelic richness, gene diversity, $\hat{f}$) for all the patches and the three seasons are not reported here because (i) the genetic characteristics of the A98 samples for all the patches are those presented in Trouvé *et al.* (2003) and (ii) the values of these genetic features for S99 and A99 (when relevant) are similar to those of A98 (data set can be retrieved from http://www.unil.ch/popgen/softwares/data). The only exception is Dizy$_t$ for which genetic diversity increased between A98 and S99 ($Rs_{A98} = 1$; $Rs_{S99} = 2.02$; $He_{A98} = 0$; $He_{S99} = 0.08$). This allowed the estimation of $\hat{f}$ and $s$ in S99 for this population; the values are, respectively, 0.94 and 0.97. These values are available upon request. In brief, the genetic polymorphism expressed by the allelic richness (*Rs* based on sample size of 19 individuals: range: 1–2.74) and gene diversity (*He*: range: 0–0.48), is low for microsatellite markers. The selfing rate based on the inbreeding coefficient is always higher than 80% except for Corcelles$_p$.

We analysed temporal differentiation among seasons for nonpermanent and permanent patches. Allelic frequencies of the four nonpermanent patches differ significantly between A98 and S99 (Dizy$_t$: $\hat{\theta} = 0.034$, Fontenais$_t$: $\hat{\theta} = 0.175$, Taulard$_{sp}$: $\hat{\theta} = 0.034$, Suchy$_{sp}$: $\hat{\theta} = 0.024$; Fig. 4) once the probability level has been adjusted according to the sequential Bonferroni proce-

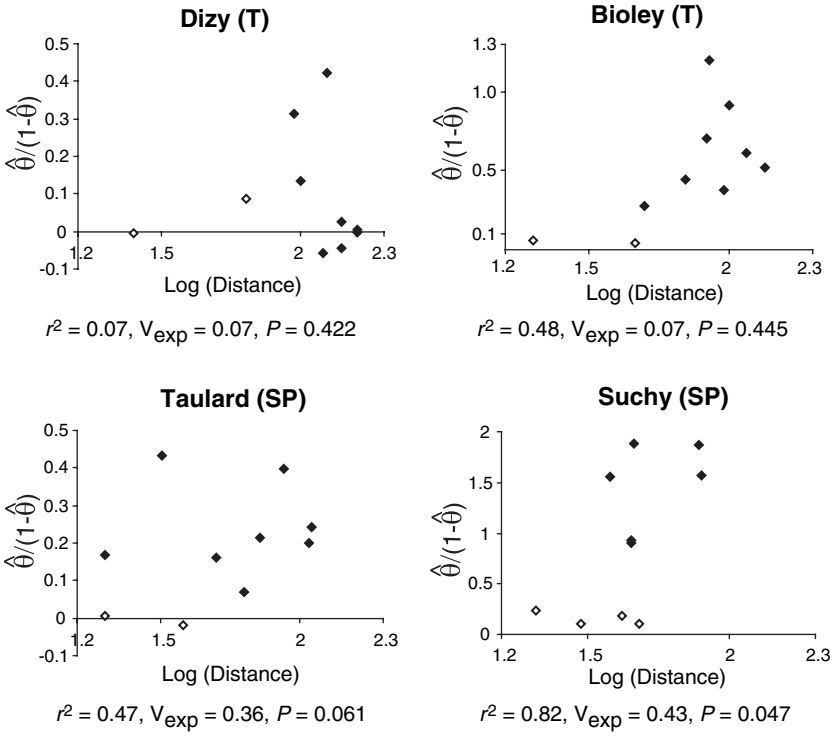


**Fig. 3** Genetic differentiation as a function of geographic distance. Empty squares represent distances among connected populations, whereas solid squares indicate distances among patches separated by natural barriers. T, temporary; SP, semi-permanent; $r^2$, amount of variance in genetic distance explained by our model; $V_{exp}$, proportion of genetic variance explained by the presence of barrier once geographic distance is taken into account; *P*, probability that this variance is different from zero.

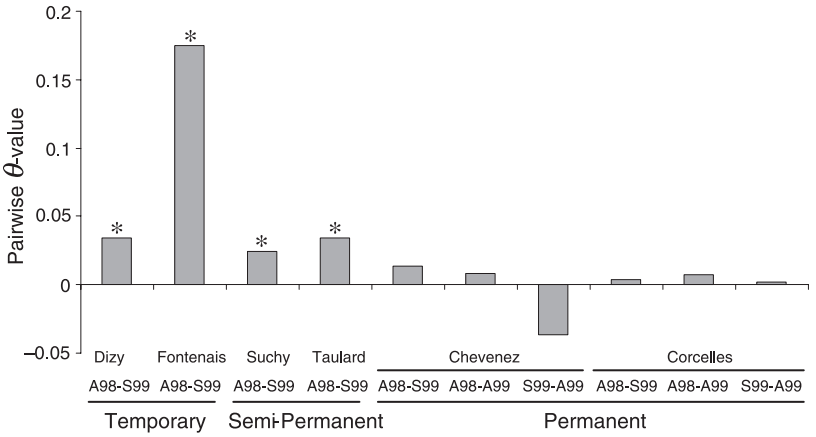


**Fig. 4** Temporal differentiation. Multilocus pairwise $\hat{\theta}$-values were estimated among two sampling dates, autumn 1998 (A98) and spring 1999 (S99), for temporary and semi-permanent populations. For permanent populations, three sampling dates, autumn 1998 (A98), spring 1999 (S99) and autumn 1999 (A99), were considered. *Pairwise significance after sequential Bonferroni test.

dure. For permanent patches three sampling dates (A98, S99 and A99) were compared and no genetic structure is detected among seasons for any comparisons (Fig. 4), although sample sizes were similar for the two types of habitats. Temporal pairwise $\hat{\theta}$-values are all lower than 0.014 for permanent patches, which is significantly lower than those of nonpermanent ones (Wilcoxon test: $W = 16$, $P = 0.01$).

*$N_e$ estimations*

Figure 5 shows the relative profile log-likelihood curves for the effective size, when populations are considered connected by gene flow (A) and when they are considered as isolated (B). The estimated effective population sizes are strikingly low, with all the values less than 13, when the populations are considered connected by gene flow (Fig. 5a). If no immigration is assumed ($m = 0$), the $N_e$ estimates (Fig. 5b) are significantly larger than those allowing for immigration but still small (Wilcoxon signed rank test: $V = 0$, $P < 0.05$). The 95% confidence intervals for all but one estimate of $N_e$ are relatively narrow. The exception is $\text{Dizy}_t$ when considered isolated. Our results indicate that populations from nonpermanent habitats present smaller effective population size (e.g. in $\text{Fontenais}_t$ $N_e = 3.89$) compared with populations from permanent ones (e.g. in $\text{Corcelles}_p$ $N_e = 12.63$). The difference is marginally significant (Wilcoxon test: $W = 8$, $P = 0.066$). The relative profile log-likelihood curves for the immigration rate are presented in Fig. 5c. Some among population variation in immigration rates is also observed (range: 0.006–0.1; Fig. 5c). These are generally low.

To investigate whether the $N_e$ estimations are sensitive to our sampling scheme, we modified the constitution of the source population (i) by adding the temporal samples of the focal population to the samples of the source population and (ii) by using only the initial pooled temporal samples as a source population. These two modifications of the source population give very similar estimates of $N_e$ and $m$ (results not shown) to those presented in Fig. 5. This indicates that our estimations are robust to modifications of the genetic composition of the source sample and, therefore, the method used seems pertinent for our biological system with a hierarchical population structure.

## Discussion

### High differentiation and low effective size

Two results are particularly striking in this study. First, while genetically differentiated at a large spatial scale (tens of kilometres), patches of *G. truncatula* only a few meters apart are also highly structured. If two main factors, namely geographic distance and natural barriers, can hinder gene flow in *G. truncatula*, our study points to natural barriers as a major explanation of genetic divergence between patches. Second, the estimated effective population sizes are quite low. Given that the highest estimate of effective population size is about 13 individuals, and that census sizes are often large (of the order of 500 individuals or more: LD and ST personal observation) this gives clear evidence that major demographic crashes should occur at some point. These population characteristics (low effective size and strong genetic differentiation) indicate that individuals of the species *G. truncatula* are very likely to be at low density or alone in a site and have a low probability of finding a partner from a nearby site to reproduce. Selfing must, therefore, constitute a major advantage in assuring gene transmission to the next generation, as predicted by the reproductive assurance hypothesis (e.g. Darwin, 1878; Ghiselin, 1969; Pannell & Barrett, 1998).

Some of these conclusions are at odds with our current biological knowledge of *G. truncatula*. The reduced effective population size contrasts with the high numbers of individuals often observed in natural populations (of the order of 500–1000 individuals: LD and ST personal observation). Similarly, the reduced gene flow at a micro-spatial scale of a few metres stands in sharp contrast with the high potential for colonization known

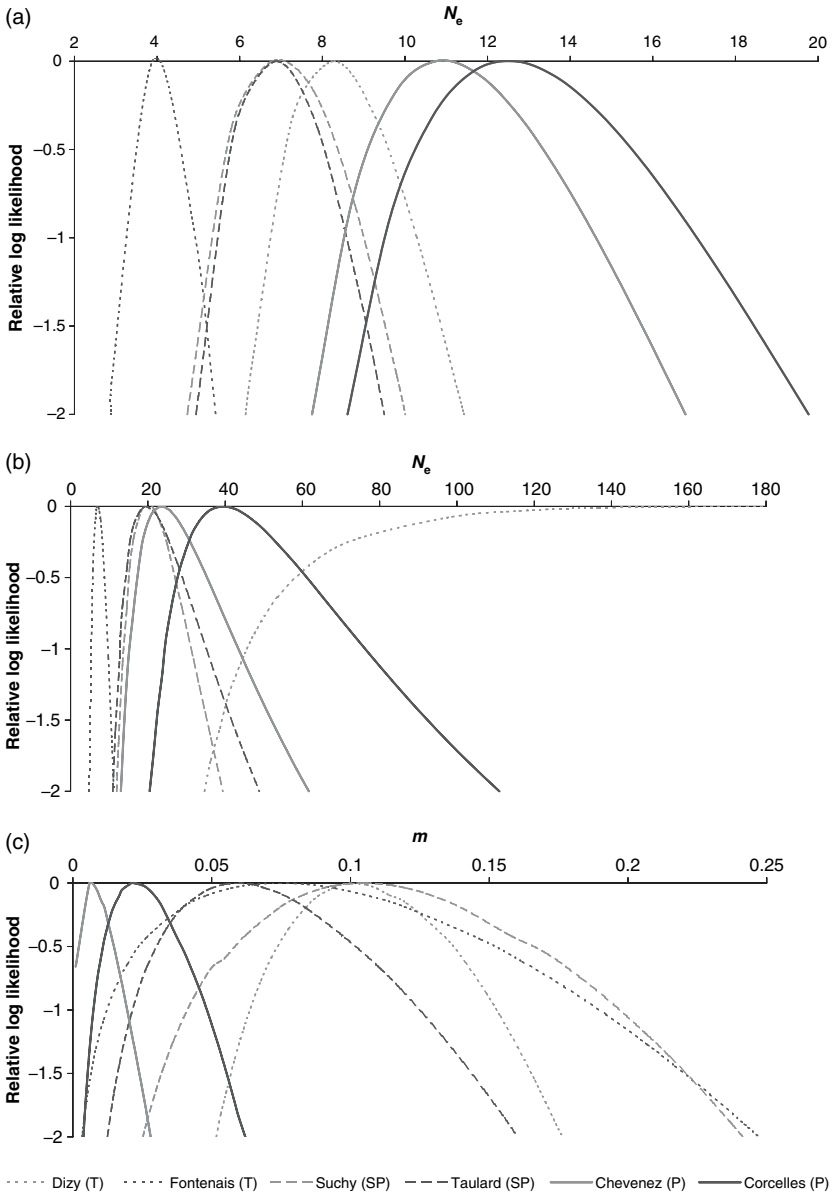


**Fig. 5** Relative profile log-likelihood curves for the effective population size when populations are considered as connected by gene flow (a) and when they are considered as isolated (b). Relative profile log-likelihood curves for the migration rate (c). T, temporary; SP, semi-permanent; P, permanent.

to exist in this species. The spread of *G. truncatula* in new habitats is exemplified (i) by its recent introduction in South America from Europe and its rapid invasion of the Bolivian Altiplano, as suggested by electrophoretic and molecular techniques (Bargues *et al.*, 1997; Jabbour-Zahab *et al.*, 1997; Meunier *et al.*, 2001) and (ii) its ability to recolonize very quickly habitats from which it has been excluded for long periods (Kendall, 1949 and references therein).

Several biologically realistic situations can be put forward to resolve these apparent paradoxes. The first explanation that comes to mind is the high selfing rate observed in this species. It is widely known that inbreeders present reduced effective population size compared with outbreeders (Pollak, 1987; Jarne, 1995). Selfing decreases $N_e$, reduces the expected coalescence time within subpopulations and therefore inflates the proportion of between-population variability (Maruyama & Tachida, 1992; Nordborg, 1997). In an island model with partial selfing, population differentiation converges to $F_{ST} = 1/[1 + 4\ Nm(1\text{-}s/2)]$, where, $N$ is the effective population size, $m$ the migration rate and $s$ the selfing rate (Maruyama & Tachida, 1992; Goudet, 1993; Charlesworth *et al.*, 1997; Nordborg, 1997). Following this expression, for the same number of migrants, $F_{ST}$ is expected to be higher when selfing occurs compared with situations of random mating. In some way, selfing reduces the effective number of migrants (Whitlock &

McCauley, 1990). However, exclusive self-fertilization would, at most, halve the effective population size and, therefore, hardly explains the observed extremely small effective sizes and the high $\hat{\theta}$-values. Other processes must be operating. The evidence that inbreeding populations generally have lower effective population sizes than outbreeders also suggests a possible role for hitchhiking and background selection (Charlesworth, 2003). Both of these processes imply selection and are expected to reduce the effective size over the two-fold reduction resulting from selfing. They also affect the coalescence time and enhance $F_{ST}$. These different genetic effects are magnified in species with a high selfing rate because of the reduced effective recombination rate (Charlesworth *et al.*, 1997; Nordborg, 1997; Charlesworth, 2003).

A second possible explanation for the extreme values of the genetic parameters $N_e$ and $\hat{\theta}$ is linked to ecological and demographic processes: recurrent fluctuations in population sizes and, most likely, extinctions are frequent events for local *G. truncatula* populations owing to the frequent variations in water availability. Although the effect of extinction recolonization dynamics on the partitioning of genetic diversity has been extensively studied (Slatkin, 1977; Nei, 1987; Wade & McCauley, 1988; Whitlock & McCauley, 1990; Whitlock & Barton, 1997), the interplay of selfing and metapopulation dynamics was only investigated recently (Ingvarsson, 2002). Ingvarsson (2002) pointed out that extinction and recolonization reduce genetic diversity and that this effect may be more pronounced in selfing than outcrossing species. His model also showed that these population turnovers generally lead to higher $F_{ST}$-values irrespective of which colonization model is used (propagule or migrant pool models). However, the model of Ingvarsson (2002) specifically applies to plants because of the distinction between pollen and seed migration; its equivalent for animals, with no male gametes specific dispersal phase, remains to be done.

Thirdly, variance in reproductive success can also result in a serious reduction in effective population size. There may be a nonpoisson distribution of progeny per parent because of chance, environmental or genetic factors. For instance, owing to harsh meteorological conditions in summer and winter, few adult *G. truncatula* may survive locally and may participate in the pool of founders the following favourable season. Additionally, this snail species constitutes the main intermediate host involved in transmission of numerous digenetic parasites (Platyhelminthes), known to be extremely harmful to the snail. These parasites castrate and sometimes kill their hosts (Malek, 1980; Sorensen & Minchella, 1998; Trouvé *et al.*, 2003). Whole families of snails could be infected because they present a higher probability to encounter the parasites or to develop the infection because of their genotypes. If these intuitive evidences are appealing, Laporte & Charlesworth (2002) argued that in self-fertilizing species, variance in reproductive success should theoretically alter $N_e$ to a lesser extent than in outcrossing species. The overall conclusion is that it is difficult to know which of the possible factors reviewed above (selfing, population dynamic, variance in reproductive success) is the most important one causing the observed low effective sizes and strong genetic structures. Most likely several genetic and environmental processes are operating jointly.

### Temporal analyses in permanent vs. nonpermanent populations

Although a short temporal scale (one generation only) is considered, nonpermanent populations showed large variation in allele frequencies over time. On the contrary, in permanent populations, no temporal genetic structure was detected over a whole year (first sample: autumn 1998, last sample: autumn 1999). The corollary of this is the somewhat higher effective size of permanent populations relative to nonpermanent ones, despite the lack of statistical power to test for differences (four nonpermanent and two permanent populations). Temporal changes in allele frequencies and lower effective sizes of nonpermanent populations could be caused by constantly lower census sizes of those populations. However, this is unlikely to be true since during field collections, we never noticed lower census size of nonpermanent populations when the sampling sites were wet. More likely is that these findings are consistent with bottlenecks during harsh weather conditions. A differential importance of genetic drift in nonpermanent and permanent populations was already suggested when higher levels of allelic richness and gene diversity were found in permanent relative to temporary populations (Trouvé *et al.*, 2003). Most likely enough water is available in permanent habitats to prevent summer drought as well as winter frost, whereas nonpermanent habitats seem to present a higher sensitivity to extreme meteorological conditions.

These inferences on the population dynamics additionally help our understanding of the inbreeding depression and genetic load in this species. Kirkpatrick & Jarne (2000) showed that inbreeding depression decreases and the genetic load increases following a bottleneck, especially if bottlenecks are smaller than 10 individuals. In nonpermanent populations of *G. truncatula*, the temporal variation in allele frequencies and the strikingly low effective sizes suggest that bottlenecks have much chance to occur and to affect the depression and load. Consistent with this, we previously showed two results suggesting that within-population inbreeding depression is low and/or drift load is high in *G. truncatula* (Trouvé *et al.*, 2003). First, we did not detect any difference in selfing rate estimates obtained from hatchlings and adults indicating similar survival to adulthood of inbred and outcrossed individuals. Second,

we found that the infection is not related to the heterozygosity level of host genotypes. Nonetheless, low depression and/or high load could also result from constant small population sizes, as pointed out by Lynch *et al.* (1995) and Bataillon & Kirkpatrick (2000). This hypothesis might be more relevant for permanent populations, which appear more stable.

### $N_e$ estimations

Our estimates of $N_e$ when the effect of migration is included are different from those obtained when populations are considered as isolated. Despite the triviality of this result – it is well known that migration affects allelic frequencies (e.g. Nei & Tajima, 1981) – the direction of deviation is not obvious. In our study, omitting migration led to larger estimates of $N_e$. This might happen if populations are at equilibrium between migration and drift, resulting in a slowdown of changes in alleles frequencies over time. The effective size measured in one population would then represent that of the set of interconnected populations rather than of the focal population. In this situation, the effective size of the focal population, when migration is ignored, would be overestimated (Wang & Whitlock, 2003). Alternatively, higher $N_e$ when no migration is assumed could result if the focal population receives migrants from a large population with relatively stable allelic frequencies over time (M. Whitlock, pers. comm.). However, neither argument seems tenable for *G. truncatula*, considering the high $\hat{\theta}$-values observed and the population instabilities as suggested by ecological and genetic evidences.

## Conclusions

The results presented here indicate that *G. truncatula* have reduced dispersal, and extremely small effective population sizes. These characteristics strongly suggest that individuals of this species present at some point a high probability to be alone in a site and have few chances to meet a partner from a nearby site to reproduce. These ecological circumstances strongly select for the evolution of selfing, as a mean to ensure reproduction. A high rate of selfing is also favoured by intrinsic genetic advantage, derived from a 3 : 2 excess of successful gametes for selfers compared with outcrossers (Fisher, 1941). Given the ecological and genetic advantages of selfing an intriguing question remains: Why does some outcrossing still occur in *G. truncatula*? It could be that only partially selfing populations subsist and that obligate selfers quickly disappear, because of the accumulation of deleterious mutations. However, this argument appears unrealistic given that a low rate of outcrossing cannot greatly elevate the time to extinction of a partially selfing population, especially when population size is small (Schultz & Lynch, 1997). In consequence, if the advantage of selfing is now relatively well understood in *G. truncatula*, further research is needed to investigate how a low rate of outcrossing is maintained.

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