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The influence of genetic factors and population dynamics on the mating system of the hermaphroditic freshwater snail *Biomphalaria pfeifferi*

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Although self-fertilization and its evolutionary consequences have been widely studied, the relative influence of genetic and environmental factors on the determination of mixed-mating systems remains poorly known. In 1999 and 2000, we surveyed the mating system, the population dynamics and some life-history traits of four populations of the freshwater snail *Biomphalaria pfeifferi*, the major intermediate host of *Schistosoma mansoni* in Africa, in two areas of Madagascar (Itasy and Antananarivo). We confirmed that *B. pfeifferi* is a predominant selfer, with selfing rates ranging between 80 and 100%. Temporal and geographical variation of the selfing rate was observed at both local and large spatial scale. Historical processes of colonization and invasion of *B. pfeifferi* in Madagascar could explain the geographical variation of the mating system observed at regional scale. Pure selfing has probably evolved in the two populations of Antananarivo area as a reproductive assurance strategy in a metapopulation where extinction is frequent and migration rare. The reproductive assurance hypothesis does not explain the spatio-temporal mating system variations observed in Itasy area. However genetic factors including inbreeding depression-the expression of which can be environmentally mediated-and metapopulation dynamics could influence the mating system in both populations sampled in Itasy and lead to different levels of evolutionary stable intermediate selfing rate in this region. Our results therefore highlight the influence of environmental heterogeneity and stochasticity on mating system.

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For more than a century, biologists have been concerned with self-fertilization and its evolutionary importance. As a consequence, the selfing rate has been estimated in a large number of species, particularly in plants, although several estimates are available in hermaphroditic animals (Jarne 1995). An interesting result is that most populations exhibit extreme selfing rates, and a few populations intermediate values (plants: Barrett 2002, animals: Jarne and Städler 1995). Several evolutionary models have been developed in order to explain these intermediate values as stable strategies. Classical, genetic models balancing the automatic advantage of selfing (cost of outcrossing) and inbreeding depression indeed do not generally predict that intermediate selfing rates can be evolutionary stable (reviewed by Jarne and Charlesworth 1993, Uyenoyama et al. 1993). Alternative

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models have included other genetic and environmental factors to account for these intermediate rates (Ronfort and Couvet 1995, Kirkpatrick and Jarne 2000, Cheptou and Schoen 2002, Tsitrone et al. 2003). Genetic factors concern population structure and its consequences on biparental inbreeding. When populations experience founding events (Uyenoyama 1986) or when gene flow is restricted (Ronfort and Couvet 1995), mating may involve genetically related individuals. This leads to biparental inbreeding which modifies both the cost of outcrossing and inbreeding depression and favours selfing. On the other hand, the occurrence of recurrent bottlenecks modifies inbreeding depression (Kirkpatrick and Jarne 2000). Environmental factors may also affect the cost of inbreeding depression and favour stable mixed mating system. Two main hypotheses have been described. First, reproductive assurance is one of the most longstanding and widely accepted hypotheses explaining how self-fertilization can be advantageous in highly outcrossing species with strong inbreeding depression (Baker 1955, Lloyd 1992, Jarne and Charlesworth 1993). When the probability of finding a mate is very low, a strategy of delayed self-fertilization can be adopted because producing progeny which suffers from inbreeding depression is favourable to producing no progeny at all (Lloyd 1992). This may occur in populations existing at low densities (Watkins and Levin 1990, Van Treuren et al. 1993, Routley et al. 1999, Mavraganis and Eckert 2001), or in colonizing species often experiencing founding events (Baker 1955, Cheptou et al. 2002). Using theoretical models, Pannell and Barrett (2001) showed that metapopulation dynamics may also favour selfing through the reproductive assurance hypothesis. When extinction is frequent and balanced by recolonization, high extinction rates and low immigration and patch occupancy rates select for reproductive assurance. Second, environmental variation may induce changes in individual fitness and affect the balance between the cost of outcrossing and inbreeding depression. This may occur for migrant individuals or in temporarily changing environments (snails: Negovetic and Jokela 2001). Theoretical models have shown that this can lead to intermediate selfing rates (Cheptou and Schoen 2002). Variation in inbreeding depression with environmental conditions has indeed been documented several times. In animals, Coltman et al. (1999) described the influence of parasitism on inbreeding depression and Meagher et al. (2000) focused on the enhancement of inbreeding depression with competition at high densities. However counter-examples can also be found (plants: Cheptou et al. 2000, animals: Dahlgaard and Loeschcke 1997, Armbruster et al. 2000, Dahlgaard and Hoffmann 2000). It is interesting to note that founding events and density may lead to stable mixed mating systems from both genetic and environmental modifications of the cost of inbreeding depression. The theoretical models mentioned in the three points above have generated predictions on the relationship between the selfing rate and genetic or ecological factors. Most of the studies evaluating these predictions have been conducted in plant species (Cheptou et al. 2000, Mavraganis and Eckert 2001, Cheptou et al. 2002, Herlihy and Eckert 2002), and data remain scarce in animals (Henry et al. 2003). A first problem is that the variation in the selfing rate has been appropriately evaluated in a limited number of hermaphroditic animal species, essentially snails (review by Jarne et al. 1993, illustrated by Jarne and Städler 1995, Viard et al. 1997, Jokela et al. 2003). For example, we have limited knowledge of the spatial and temporal variation of the selfing rate. Second, although inbreeding depression has been studied many times in animals, the role of other factors on mating system has been evaluated in few studies (Henry et al. 2003).

In this perspective, hermaphroditic freshwater snails (Basommatophora) constitute relevant biological models to study the evolution of the selfing rate (Jarne et al. 1993). Selfing and outcrossing occur within the same species. Populations occupy unstable or temporary habitats, are generally strongly structured, and exhibit wide spatial and temporal demographic variation (Brown 1994, Charbonnel et al. 2002c). The relative influence of genetic and environmental factors on the mating system can therefore be addressed (Jokela et al. 1997). More specifically, we focus on *Biomphalaria pfeifferi* populations from Madagascar, as part of a larger study on the population biology of this species (Charbonnel et al. 2002a,b,c). Four populations were studied over two years. Our study combines a genetic approach aimed at determining the selfing rate and population spatial and temporal structure (using microsatellite markers), a demographic approach aiming at estimating population size (using capture–mark–recapture CMR models) and estimates of fitness traits under laboratory conditions. The purposes of this study were to test for the stability of a mixed mating system in *B. pfeifferi* populations by evaluating the spatio-temporal variations of the selfing rate, and to analyse the potential factors involved in the mating system evolution of *B. pfeifferi*, including self-fertilization depression, metapopulation structure (patch size) and metapopulation dynamics. (i) We estimated self-fertilization depression by analysing how the traits related to fitness vary with the selfing rate. A negative relationship was expected between fitness and selfing rate, although the difference in fitness between selfed and outcrossed individuals might not be extremely large in selfing species such as *B. pfeifferi* (Doums et al. 1996). (ii) We evaluated the relationship between population size and the selfing rate, with population size taken as a proxy for the probability of finding a partner. Selfing should decrease with increasing population size under the

reproductive assurance hypothesis (Bateman 1956), under the hypotheses of a decrease of the cost of self-fertilization depression at low density due to biparental inbreeding (Ronfort and Couvet 1995) or to environmental conditions that decrease the expression of inbreeding depression (e.g. competition, Meagher et al. 2000). (iii) We compared the magnitude of temporal and spatial genetic differentiation with the variation in the selfing rate. Significant spatial and temporal differentiation may indicate a metapopulation dynamics involving bottlenecks or extinction and restricted gene flow. This must be associated with large selfing rate under the reproductive assurance hypothesis (Pannell and Barrett 1998) or biparental inbreeding (Ronfort and Couvet 1995). On another hand, weak spatial and temporal genetic differentiation may reflect a metapopulation dynamics characterized by rare extinction events and important gene flow among populations. This must lead to low levels of selfing rates under the reproductive assurance hypothesis (Pannell and Barrett 1998) or biparental inbreeding (Ronfort and Couvet 1995).

Material and methods

Study organism and populations

Biomphalaria pfeifferi is a tropical freshwater snail. Its distribution spans over most of Africa, south of the Sahara, and has recently expanded to Madagascar, probably in association with human colonization (Angers et al. 2003). Experimental and genetic studies (Jelnes 1980, Mimpfoundi et al. 1986, Rupp and Woolhouse 1999), as well as population structure analyses (Charbonnel et al. 2002a,b), have shown that this simultaneous hermaphroditic species preferentially self-fertilizes. However, some variation in the selfing rate has been uncovered among populations from Madagascar (range: 0.79–1, Charbonnel et al. 2002a,b). An important point with regard to the reproductive assurance hypothesis is that populations of tropical freshwater snails experience frequent fluctuations in size, including bottlenecks, because of seasonal drought and flooding (Brown 1994, Charbonnel et al. 2002a,c), and founding events through habitat colonization after events of long-distance dispersal (Charbonnel et al. 2002a).

Four populations of *B. pfeifferi* were surveyed in Madagascar at several dates over two years. Two populations (MA and MB) are located in a village near Antananarivo. They occupy two ponds of 10 m^2 separated by less than 20 m and connected by a small canal. Preliminary CMR surveys have shown that snails can disperse through this canal following the water flow (N. Charbonnel, unpubl.). The water level varies annually in relation with the seasonal cycle of rain and human activities, and the ponds might even dry out. Both populations were sampled in January (rainy season), May (cold and dry season) and August (hot and dry season) 2000 for genetic, demographic and life history studies. The two other populations are located in the artificial lake of Itasy, about 150 km west of Antananarivo, respectively downstream (IC) and upstream (IM) a dam. They both correspond to a 10 m^2 area. They have been previously surveyed for a population genetic study (Charbonnel et al. 2002c). IC and IM were sampled at the end of the hot and dry season (October), in both 1999 and 2000. Genetic data collected in IM in May 1999 were also included (Charbonnel et al. 2002b). Lake Itasy shows water level fluctuation throughout the year, mainly noticeable in IC, but never dries out.

Estimating the outcrossing rate

The outcrossing rate was estimated in the four populations using two different methods, both relying on six highly polymorphic microsatellite loci (Charbonnel et al. 2002a,b,c). The first indirect method is based on the classical relationship linking the outcrossing rate t_f and Wright's inbreeding coefficient F_{is}: $t_f = (1 - F_{is})/(1 + F_{is})$. The unbiased estimator $\hat{f}$ of F_{is} was calculated according to Weir and Cockerham (1984) using a sample of at least 20 individuals per sample when possible. Less than 10 individuals were used in MA and MB because of very low snail abundance in these ponds (Table 1). F_{is} standard deviation was estimated using 500 bootstraps of individuals within population. The mean number of alleles (n_{all}), the observed heterozygosity (H_o) and gene diversity (H_e) were estimated from the same data. The second direct method is based on progeny-arrays following a methodology previously used in other snail species (Viard et al. 1997, Trouvé et al. 2003). Ten adults larger than five mm in shell length were collected in the field (less individuals were sometimes collected because of low abundance; Table 1), and isolated in plastic boxes. They were fed with frozen lettuce ad libitum, and water was changed twice a week. Egg capsules were collected over 20 days, after which adults were killed and stored in 95% alcohol for further genetic analyses. Juveniles from the first capsule laid (or from the two first when deposited the same day) were put into alcohol three days after hatching. This procedure insures that the estimated selfing rates are those of individuals just prior to field collection, since allosperm can be stored for several weeks after copulation (Viard et al. 1997). The outcrossing rate was first estimated as a minimum family multilocus outcrossing rate t_{mm} computed as the proportion of progeny exhibiting non-maternal alleles. Maximum likelihood estimates were also generated using MLTR (version 2.4, Ritland 2002) for estimating family (t_{ml}) and population (t_m and t_s, multilocus and single-locus estimates respectively) outcrossing rates. The

Table 1. Population characteristics, within-population polymorphism, estimates of the population and family outcrossing rate. N_s is the number of adults sampled, n_{all} is the mean number of alleles per population, H_o the observed heterozygosity, H_e gene diversity, and $\hat{f}$ the estimate of F_{is}. t_f is the indirect estimate of the outcrossing rate. Families and offspring refer respectively to the number of families per population and offspring per family studied. t_m and t_s are the multilocus and single-locus outcrossing rates. $t_m - t_s$ provides information on biparental inbreeding. The standard deviation of these values (in parentheses) was obtained using 500 bootstraps. – indicates that populations were monomorphic at all loci. No polymorphism was detected within MA and MB families in May and August, and the analyses were not conducted. MB went extinct in August 2000 because the pond dried out, and no data are available.

Sampling date	October 1999		October 2000		January 2000		May 2000		August 2000
Population	IC	IM	IC	IM	MA	MB	MA	MB	MA
N_s	26	12	24	24	10	9	11	7	10
n_{all}	3.00	2.80	3.40	4.00	2.00	2.2	1.00	1.00	1.00
H_o	0.06	0.00	0.07	0.16	0.00	0.03	0.00	0.00	0.00
H_e	0.31	0.35	0.44	0.52	0.18	0.09	0.00	0.00	0.00
$\hat{f}$	0.80 (0.07)	1.00 (0.00)	0.83 (0.06)	0.74 (0.11)	1.00 (0.00)	0.66 (0.37)	–	–	–
t_f	0.11	0.00	0.09	0.15	0.00	0.20	–	–	–
Families	10	6	10	11	6	8	9	7	6
Offspring	42	30	48	63	17	19	43	34	27
t_m	0.312 (0.124)	0.137 (0.050)	0.446 (0.092)	0.406 (0.078)	0.001 (0.000)	0.001 (0.000)	–	–	–
t_s	0.216 (0.098)	0.064 (0.026)	0.205 (0.049)	0.227 (0.051)	0.001 (0.000)	0.005 (0.001)	–	–	–
$t_m - t_s$	0.096 (0.039)	0.073 (0.026)	0.240 (0.049)	0.180 (0.054)	0.000 (0.000)	−0.004 (0.001)	–	–	–

difference between these two rates gives a minimum estimate of apparent selfing due to biparental inbreeding. The expectation-maximization (EM) algorithm was used as recommended for highly inbred species (Ritland 2002). The variance of the estimates was estimated using 500 bootstraps, with family as the unit of resampling for population estimates, and individual progeny for family estimates.

Relative fitness of adults and selfing rate

The fitness of sexually mature snails isolated in the laboratory was estimated in the four populations studied at each sampling date using the progeny-arrays protocol described above. Fitness was estimated based on two parameters, namely fecundity (number of eggs laid in the first capsule; N_{egg}) and offspring survival (three days post-hatching; S_u). Note that this includes environmental effects since populations were studied at different dates. A lowered fitness of preferentially selfing individuals compared to preferentially outcrossing ones may be due to inbreeding depression acting at early stages and/or to a low efficiency of selfing. These phenomena correspond to self-fertilization depression (Jarne et al. 1991) and may indicate that selection favours outcrossing. Differences between selfing and outcrossing snails were tested by comparing N_{egg} and S_u of selfed (null outcrossing rate) and outcrossed (non null outcrossing rate) capsules within populations using an analysis of variance (N_{egg}) or a logistic regression (S_u). Population was treated as a random effect and the mating system as a crossed, fixed effect. The residuals were checked for normality when necessary.

Size and temporal fluctuations of populations

Estimating population size

Population size was estimated in the four populations studied at each sampling date using closed population CMR models (Schwartz and Seber 1999). For technical reasons, this has not been done in Itasy in 1999. Assumptions of CMR models, as applied to freshwater snails (e.g. impact on survival), are fully discussed in Chlyeh et al. (2002). Each sampling session lasted for 20 min, after which snails were marked using numbered, coloured plastic tags and immediately released. Recaptures were performed four times in Itasy and three times in Antananarivo, with less than 15 h between recapture sessions. Recruitment, immigration, death and emigration could therefore be neglected. Data were analyzed using closed capture models (Otis et al. 1978) using Capture included in the MARK software (White and Burnham 1999). Population size (N) was estimated from the initial capture (p) and recapture (c) probability. The influence of time (model M(t)), individual (model M(h))

or behaviour (model M(b), because capture and recapture rates may differ for behavioural reasons) on capture probability was evaluated. The best model was selected on the basis of likelihood ratio tests.

Temporal stability of populations

Genetic temporal differentiation of *B. pfeifferi* populations was investigated using data from the indirect method of outcrossing rate estimation. Data previously obtained for individuals sampled in May 1999 in IM were also included. The temporal genetic differentiation was evaluated using a homogeneity test (Goudet et al. 1996) computed as an exact test for each population between two sampling dates. Temporal differentiation between successive sampling dates for a given population was estimated using the estimator $\hat{\theta}$ of F_{st} (Weir and Cockerham 1984). The calculations were performed using Genepop 3.1c (Raymond and Rousset 1995). Temporal differences in allelic frequencies were analyzed within populations following Waples (1989) in order to test whether significant temporal variation results from stochastic processes (sampling and genetic drift) or from migration. Selection was discarded here since we are concerned with neutral markers. This method allows estimating the maximal effective population size that explains temporal variation. If this size is far lower than the size that can be inferred from demographic surveys, migration becomes a likely candidate explaining temporal variation of allelic frequencies (described in Charbonnel et al. 2002c). We assumed six generations per year, an effective size of 10, 50, 100, 200 or 500 individuals and individual sampling before reproduction (sampling plan II). We tested for very low values (10, 50) to take into account the reduction of effective size due to selfing. Indeed, with a selfing rate of one, the effective size is halved (Pollack 1987).

Predictions on the mating system according to population dynamics

The mating system may depend on environmental demographic stochasticity. In particular, the reproductive assurance hypothesis predicts a positive correlation between the outcrossing rate and partner density, even under strong inbreeding depression (Jain 1976). This prediction was confirmed in species that exhibit high levels of self-fertilization (75%) and inbreeding depression (0.98; Herlihy and Eckert 2002). This was tested here in two ways. First, assuming that population size reflects the availability of sexual partners, a positive correlation was expected between population size and population outcrossing rate. Second, at larger time scale, the population temporal stability may reflect the probability of finding a mating partner (Henry et al. 2003). Indeed, temporal genetic instability reveals low effective size (N_e) that could be explained by the occurrence of bottlenecks or extinction-colonization events between successive samples. A negative correlation was expected between increasing temporal genetic differentiation and population outcrossing rate. Temporal genetic instability may also indicate important immigration from differentiated populations. This is expected to lower biparental inbreeding, and thus to favour selfing. Under this hypothesis, a negative correlation between temporal genetic differentiation and outcrossing rate was also expected.

The reproductive assurance hypothesis was also tested by analyzing the variability of T_{c1} (time between isolation of individuals and the first egg capsule laid; see protocol on relative fitness above) in the different populations. Under the hypothesis of reproductive assurance, selfing is expected when the availability of sexual partners is limited. In populations that are not subject to stochasticity, outbreeding should be selected. Thus, individuals isolated in the laboratory that have not stored allosperm before being captured should delayed selfing in the expectation of a partner. This analysis assumes that the individual variation at the time of their isolation in terms of time since their previous copulation is negligible compared to the differences of T_{c1} expected between individuals that have stored allosperm (whatever the time since their previous copulation) and individuals that have not stored allosperm. Inversely, in populations that are subject to stochasticity, selfing should be selected as reproductive assurance. No delayed selfing should therefore be observed here. This was tested by comparing T_{c1} between selfed (null outcrossing rate) and outcrossed (non null outcrossing rate) capsules using the same analysis as in the section on relative fitness.

Results

Estimating the outcrossing rate

Results on the within-population polymorphism are presented in Table 1. All populations at all sampling dates showed very low levels of variability, in terms of number of alleles and gene diversity. This was particularly true of MA and MB from May to August 2000 (during the dry season), with a single microsatellite genotype detected. Very few heterozygotes were observed. Estimates of F_{is} were accordingly high (0.66 to 1) which produced estimates of outcrossing rates ranging between 0.00 and 0.20. Note that the F_{is} standard deviation in MA January 2000 is very high (0.37) and makes this estimate weakly reliable (Table 1). In Itasy, the direct count of non-maternal alleles and the MLTR method gave very similar estimates of family outcrossing rates (Pearson correlation coefficient, $r = 0.985$, $n = 37$, $p < 0.0001$). The result of the second method only will be reported. In Antananarivo, the analyses revealed polymorphism neither within families, nor within populations. Both populations presented very low levels of

outcrossing rates (t_s: range 0.001–0.005, Table 1). In Itasy, both the single locus (t_s; range 0.064–0.227) and multiloci ($t_{m;}$ 0.137–0.446) estimates showed consistent levels of outcrossing rates, but the surprising result was the detection of mating system variation among sites, families and sampling dates. All $t_m - t_s$ values were significant in Itasy populations, as they were greater than twice the standard error (Rankin et al. 2002), indicating biparental inbreeding.

Relative fitness of adults and selfing rate

The outcrossing rates and estimates of life-history traits per family are presented in Appendix A. The number of eggs laid in the first capsule (N_{egg}) and juvenile survival (S_u) could not be surveyed in Itasy in October 1999 for technical reasons. Moreover, the analyses of the family outcrossing rates could not be conducted in Antananarivo populations as no polymorphism was detected within populations. Statistical tests revealed no significant effect of mating system on fecundity (Fig. 1, mating system: $F = 0.002$, $df = 1$, $p = 0.965$; population: $F = 0.085$, $df = 1$, $p = 0.773$; interaction: $F = 1.199$, $df = 1$, $p = 0.288$) although a lower fecundity was detected in purely selfed capsules than in the other capsules in IM families. Residuals were normally distributed (Shapiro–Will test: $p = 0.375$). Note that a marginally significant and positive correlation was observed between fecundity and family outcrossing rate in IM (Fig. 2: Kendall, $r = 0.434$, $p = 0.06$). On the other hand, a lower juvenile survival was observed in purely selfed than in the other capsules (Fig. 1 and 2, mating system: $LRT = 4.753$, $df = 1$, $p = 0.029$; population: $LRT = 0.263$, $df = 1$, $p = 0.607$). A significant and positive correlation was observed between juvenile survival and family outcrossing rate in IC (Fig. 2: Kendall, $r = 0.573$, $p = 0.038$). In Antananarivo, there was a significant effect of the sampling date on fecundity (date: $F = 5.923$, $df = 1$, $p = 0.022$; population: $F = 0.091$, $df = 1$, $p = 0.766$; interaction: $F = 0.111$, $df = 1$, $p = 0.741$), the highest values being observed in May. Residuals were normally distributed (Shapiro–Will test: $p = 0.082$) (Fig. 3). No significant effect was detected on juvenile survival (date: $LRT = 3.003$, $df = 2$, $p = 0.223$; population: $LRT = 1.777369$, $df = 1$, $p = 0.182$).

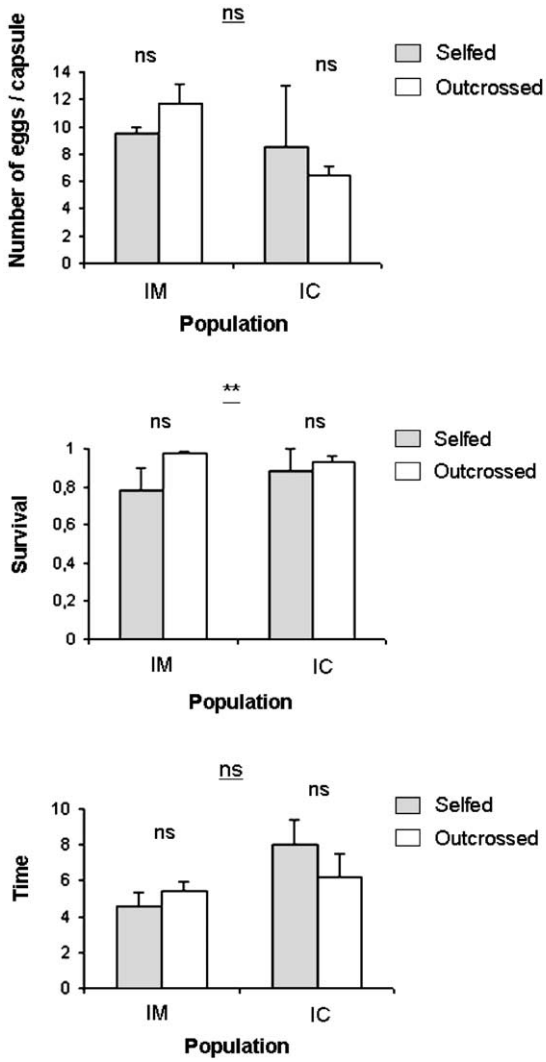

Fig. 1. Influence of the mating system on three life history traits in Itasy populations. Number of eggs in the first capsule N_{egg} (top panel), survival rate three days after hatching S_u (middle panel) and time between adult isolation and laying of the first capsule T_{c1} (bottom panel) are represented for selfed (grey bars) and mixed or outcrossed (white) capsules. All available sampling dates have been taken into account. Error bars represent standard errors. The significance of the mating system effect is indicated using ns (non significant) and stars (**: significant) for the whole sampling (underlined) and for each population considered separately (not underlined).

Size and temporal fluctuations of populations

Estimates of demographic population size

The capture probability in Itasy ranged between 0.14 and 0.71. The best model describing the data in IC was M(tb), including temporal variability in both capture (p) and recapture (c) probability (Table 2). The population size, corresponding to a number of individuals in the area prospected, was 104 ± 34 [78–250]. The best model in IM was M(b), and the probabilities of capture and recapture did not vary with time. The population size estimate was 197 ± 91 [116–543]. Sizes were so low in January and October 2000 in both MA and MB that no precise estimate could be obtained. In further analyses, we considered that MA and MB population size did not exceed ten individuals. The best model in May 2000 was M(h) (Table 2), suggesting individual variation in capture probability (Otis et al. 1978). The estimates of

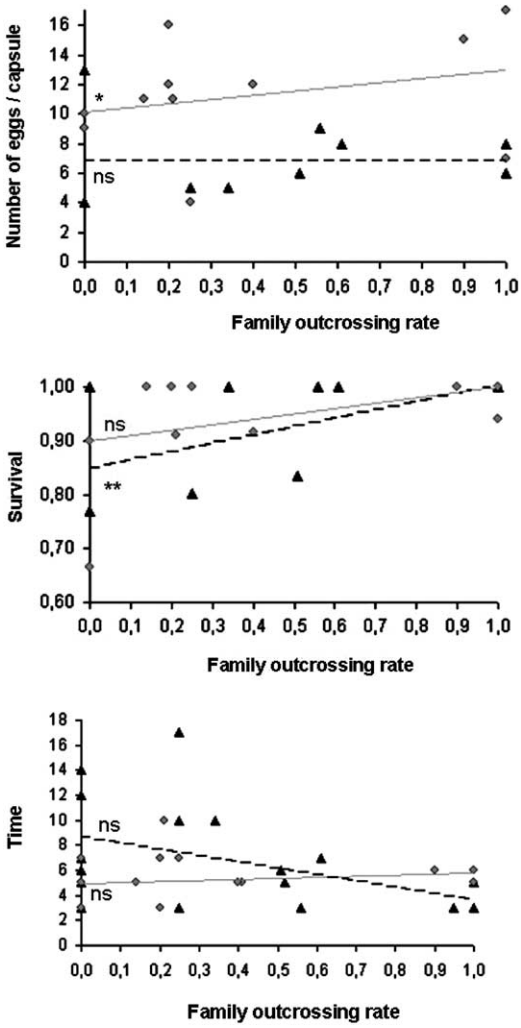

Fig. 2. Correlation between the estimated family outcrossing rate and N_{egg} (the number of eggs laid in the first capsule; top panel), S_u (the survival rate of progenies three days after hatching; middle panel) and T_{c1} (the time spent between adult isolation and the laying of the first capsule; bottom panel) in IM (grey circle, solid line) and IC (black triangle, dotted line) families. The significance of the correlation is indicated using ns (non significant) and stars (*: marginally significant, **: significant).

population size were 45 ± 6 [36–61] and 37 ± 6 [29–52] in MA and MB respectively.

Temporal stability of populations

The exact tests of genotypic differentiation per locus and pairs of temporal samples were all significant at the 0.05 level in Itasy. $\hat{\theta}$ ranged between 0.13 (IM May 1999 to October 1999 or 2000) and 0.29 (IC October 1999 to October 2000). The estimates of spatial differentiation were not significantly different from 0 (October 1999) or low (0.12 in October 2000) between IM and IC. The tests of genotypic differentiation were all significant in Antananarivo, and the associated estimates of temporal differentiation were extremely large (between 0.85 in MB from January to May 2000 and 0.90 in MA from January to May 2000). The spatial differentiation between MA and MB was highly significant and showed high values both in January ($\hat{\theta} = 0.84$) and May 2000 ($\hat{\theta} = 1.00$). Waples' method showed that, for three loci in IM and two in IC, the threshold effective size upon which drift only cannot explain the differences of allelic frequencies observed between two sampling dates was 50 individuals (Table 3). In Antananarivo populations, whatever the sampling dates considered, two loci (*Bpf2* and *Bpf9*) exhibited no significant change of the allelic frequencies between sampling dates, and three loci (*Bpf8*, *Bpf10* and *Bpf16*) exhibited drastic changes with new alleles appearing between January and May 2000. Considering these three loci, Waples' test indicated that even when under very small effective population size (10), drift alone cannot explain the observed temporal differentiation. Our demographic and genetic results provide information about the differences between Itasy (higher population sizes and outcrossing rates, lower temporal differentiation) and Antananarivo (lower population sizes and high selfing rates, higher temporal differentiation), and do not allow discriminating geographic variation from the reproductive assurance hypothesis. No statistical analysis could thus be performed to test the effect of population size or genetic stability on the mating system. Note however that IM had a larger population size and less temporal differentiation than IC.

Time before laying the first capsule and selfing rate

No significant effect of the mating system was detected on the time spent between adult isolation and first egg-laying in Itasy (main effects: mating system: $F = 0.621$, $df = 1$, $p = 0.437$; population: $F = 1{,}819$, $df = 1$, $p = 0.187$; date: $F = 0.007$, $df = 1$, $p = 0.933$; Fig. 1 and 2). Residuals were normally distributed (Shapiro–Will test: $p = 0.09$). When considering sampling dates separately, a significant effect of population was found in October 1999 (mating system: $F = 2.350$, $df = 1$, $p = 0.151$; population: $F = 4{,}758$, $df = 1$, $p = 0.041$; interaction: $F = 2.350$, $df = 1$, $p = 0.151$), lower values being observed in IM than in IC. Note that there was no significant correlation between T_{c1} and S_u whatever the population studied (Kendall: IC $r = -0.244$, $p = 0.325$; IM $r = -0.467$, $p = 0.641$). In Antananarivo, the time spent between adult isolation and first egg-laying varied according to interactions between seasons and populations (date: $F = 0.078$, $df = 1$, $p = 0.782$; population: $F = 0.412$, $df = 1$, $p = 0.526$; interaction: $F = 5.059$, $df = 1$, $p = 0.033$), the highest values being observed in MA families sampled in January, and the lowest in MA families sampled in May or MB families sampled in January (Fig. 3).

Table 2. IC and IM population size (N, with standard error and 95% confidence interval) estimated using closed CMR models. p_i and c_i are respectively the probability of capture and recapture at time i. M(tb) supposes that p and c differ and vary through time, M(b) that p and c are differ, and M(h) that p only varies. – indicates not enough data for estimating population size.

Population	Model selected	N	Capture probability
IC – October 2000	M(tb)	104 (34; 78–250)	$p_1 = 0.25$, $p_2 = 0.31$, $p_3 = 0.08$, $p_4 = 0.35$ $c_1 = 0.64$, $c_2 = 0.68$, $c_3 = 0.44$, $c_4 = 0.71$
IM – October 2000	M(b)	197 (91; 116–543)	$p = 0.14$, $c = 0.45$
MA – January 2000	–	–	–
MB – January 2000	–	–	–
MA – May 2000	M(h)	45 (6; 36–61)	$p = 0.18$
MB – May 2000	M(h)	37 (6; 29–52)	$p = 0.18$
MA – August 2000	–	–	–

Discussion

Mating system variability

This study confirms that *B. pfeifferi* is a predominant selfer, with average estimates of selfing rate higher than 80%. Progeny-arrays analyses detected variation among individuals, with some outcrossing in 40 to 80% of the families studied depending on the population. More surprisingly, they suggested lower levels of selfing within populations than the indirect method (based on F_{is} estimates). Such a discrepancy between the direct and indirect method has not been observed in previous studies of highly selfing freshwater snails (Viard et al. 1997, Trouvé et al. 2003), although slightly more selfing has been detected with the indirect than with the direct method. It is unlikely to result from the fact that PCR on juveniles were conducted using small amounts of DNA, since maternal alleles were always detected in the progenies. It is also not explained by high variance in estimates of allele frequencies due to small number of individuals genotyped as the F_{is} standard deviation estimates do not exceed those concerning the family outcrossing rates. Only the indirect estimate of the selfing rate of MB in January 00 is not reliable (Table 1). Different biological hypotheses may then be considered. The first hypothesis assumes that F_{is} estimates reflect other processes than selfing such as biparental inbreeding and spatial variance in allele frequencies (Walhund effect). These factors enhance the indirect estimations of the selfing rate and may explain a part of the discrepancies observed between these indirect estimates and the selfing rate estimates based on the progeny arrays (Städler and Jarne 1997). However other biological processes than biparental inbreeding and Walhund effect must be involved in these differences as the indirect estimates of the selfing rates exceed the single locus direct estimates ($1 - t_s$). These processes may rely on the spatial and temporal variation of the selfing rate. Indeed the indirect method reflects the selfing rate in the parental generation, whereas the direct method provides an estimate in the progeny generation. Temporal variation in both the selfing rate and inbreeding depression has been demonstrated in plants (Cheptou et al. 2000, 2002, Rankin et al. 2002) and animals (Keller et al. 2002), and might explain the results observed in *B. pfeifferi* populations. Moreover, the indirect method reflects the selfing rate under field conditions, whereas the direct method provides an estimate under laboratory conditions. Selfing rates and inbreeding depression may vary with environmental or stressful conditions and this could explain the differences observed between the direct and indirect estimates of *B. pfeifferi* mating system. Both phenomena have been observed in the preferentially outcrossing freshwater snail species *Physa acuta* (Jarne et al. 2000, Henry et al. 2003).

This study also illustrates the high spatial variability of the selfing rate at large and local geographical scales which is in agreement with previous population genetic analyses (Charbonnel et al. 2002a,b). At a large geographical scale, populations from Itasy exhibited lower levels of selfing than populations from Antananarivo. Only one family over 36 produced an outcrossed progeny in Antananarivo. Such geographical pattern of variation in the selfing rate had also been observed in the preferentially selfing slug *Arion fasciaticus* in Europe (Jordaens et al. 2000). This may result from ecological differences between regions or from historical processes but these possibilities have not further been investigated in this slug. In *B. pfeifferi*, there are genetic indications that populations from Antananarivo result from a recent, unique colonization wave, while those from Itasy represent a mixture between two genetically differentiated colonization waves (Charbonnel et al. 2002b). Ecological differences are also obvious, as Antananarivo populations have been sampled in ponds belonging to a complex irrigation system, which means a highly anthropic, disturbed habitat, whereas Itasy populations have been sampled on the bank of a large lake.

At a local scale (within a metapopulation), both spatial and temporal variations of the selfing rate were detected in Itasy. In October 1999, the selfing rate estimated in IC was greater than the one in IM whereas in October 2000 selfing rates were similar in both populations. This is true whatever the method considered. On another hand, IC exhibited similar levels of selfing in October 1999 and October 2000 whereas the selfing rate estimated in IM was slightly greater in

Table 3. Results of Waples' test for successive samples for each locus in Itasy (a) and Antananarivo (b). Results are shown for six generations per year assumed. Note that this number had little influence on the results. – indicates that no sample was available, and NP indicates no polymorphism. NS indicates that the test was not significant; other results were significant ($p < 0.01$) assuming the effective population size given in the Table. 10 was the lowest population size value tested.

a)

	May 1999–October 1999						October 1999–October 2000					
Population/locus	Bpf2		Bpf8	Bpf9	Bpf10	Bg16	Bpf2		Bpf8	Bpf9	Bpf10	Bg16
IM	50		NS	NS	NS	50	50		50	NS	100	NS
IC	–		–	–	–	–	50		50	NS	NS	NS

b)

	January 2000–May 2000						January 2000–August 2000						May 2000–August 2000			
Population/locus	Bpf2		Bpf8	Bpf9	Bpf10	Bg16	Bpf2		Bpf8	Bpf9	Bpf10	Bg16	Bpf2		Bpf8	Bpf9	Bpf10	Bg16
MA	NP		10	NS	10	10	NP		10	NS	10	10	NP		NP	NP	NP	NP
MB	NP		10	NS	10	10	–		–	–	–	–	–		–	–	–	–

October 2000 than in October 1999. Such temporal variations have rarely been documented. Changes of the inbreeding coefficient between successive generations have been measured to estimate inbreeding depression in plants (Dole and Ritland 1993). At larger time scale, variation of selfing rate among years was described in the plant *Schiedea menziessi*, and explained by a recent shift in the habitat (Rankin et al. 2002). A single animal study related the influence of environmental heterogeneity on temporal variation of mating system and was conducted in *Daphnia magna*, a crustacean reproducing by cyclical parthenogenesis. Experiments revealed monthly changes in the frequencies of inbred clones, resulting from variation of the level of inbreeding depression, itself relying on the presence/absence of competition with outbred clones (Haag et al. 2002). The mating system variations observed in Itasy populations: IC presents higher but constant levels of outcrossing rates whether IM exhibits lower but varying levels of outcrossing rates, could be related to genetic factors. Indeed, self-fertilization depression seemed stronger in IC than in IM when considering juvenile survival in October 2000. However both populations exhibited similar selfing rate estimates at this time. The difference of a stable vs an unstable mixed mating system in IC and IM may thus rely on ecological constraints such as environmental variations of the cost of self-fertilization depression or of the advantages of outcrossing in IM (reproductive assurance, adaptation to a temporally varying environment). Further works concerning habitat characterization and self-fertilization depression are now required to test these hypotheses. In Antananarivo, the significant effects of sampling dates on the number of eggs laid in the first capsule or of the interaction between seasons and populations on the time spent between the laying of the first capsule may reflect such spatio-temporal variation of ecological conditions. This does not seem to affect the mating system but we must confirm this result as the temporal variation of the mating system could not be studied because of an absence of within population polymorphism.

Better to outcross sometimes?

Juvenile survival increased with the outcrossing rate, especially in IC, and a marginally significant effect was detected on fecundity in IM (Fig. 1 and 2; the analysis was not conducted in Antananarivo because of lack of variation). How can such a result be explained, taking into account that there was limited difference in the mean selfing rate and its variation between the two populations? We note first that our fitness estimates could be interpreted in terms of inbreeding depression under restrictive conditions only, as we had

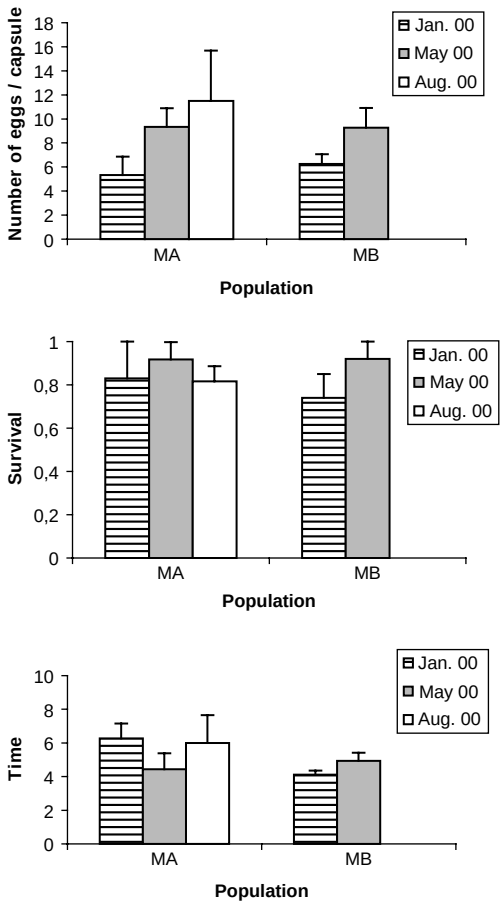

Fig. 3. Estimates of three life-history traits in MA and MB at three sampling dates: January 2000 (Jan. 00, black hatched bars), May 2000 (May 00, grey bars) and August 2000 (Aug. 00, white bars). Top panel: number of eggs in the first capsule N_{egg}; middle panel: survival rate of the first-capsule progenies three days after hatching S_u and bottom panel: time spent between the isolation of adults and the first capsule laid T_{c1}. No data were available in MB in August 2000 as the pond had dried out.

no information on variation of the selfing rate between egg-laying and the stage studied (three days old juveniles). One such condition is to assume that mortality affected selfed offspring only, and difference in fitness with the selfing rate would reflect the expression of inbreeding depression in early life stages. Our result would then be consistent with previous studies conducted in snails reporting some inbreeding depression, even in highly selfing species, mainly expressed as juvenile survival (Doums et al. 1996). Of course this condition is not guaranteed since outbreeding depression has also been reported several times (Henry et al. 2003). However this seems unlikely since fitness traits did not increase with the selfing rate in our study. More generally, the difference between IC and IM could reflect an environment-dependent expression of inbreeding or outbreeding depression, as has been demonstrated in many species (Cheptou et al. 2000, Dahlgaard and Hoffmann 2000, Keller et al. 2002, Henry et al. 2003). The two populations indeed strongly differ in environmental conditions and water availability, even if they are connected by strong gene flow. This could allow the maintenance of intermediate selfing rates in Itasy populations of *B. pfeifferi* (Cheptou and Mathias 2001).

Metapopulation dynamics and reproductive assurance strategy

The genetic and demographic surveys revealed different metapopulation dynamics in Itasy and Antananarivo which may influence the mating system. Indeed, recent theoretical models have shown that the strength of selection for selfing as a reproductive assurance strategy in a metapopulation increases with the colony extinction rate, and decreases with the number of immigrants and the proportion of sites occupied (Pannell and Barrett 1998). In Antananarivo, low population size was detected. The threshold effective population sizes estimated using Waples' method were similar to that obtained from the demographic survey. This reflects both scarce events of migration between ponds, which is confirmed by the high levels of spatial genetic differentiation observed, and high levels of genetic drift that are revealed by the strong temporal genetic differentiation detected. These dynamics are explained by high population extinction rates and foundation events occurring during the dry season as ponds dry out. The detection of new alleles at three loci during the temporal survey in Antananarivo populations confirms this dynamics. Colonisation events following extinction may involve distant patches not sampled in this study. These events may be related to animal movements or human activities. Birds and zebus may cover long distances, accidentally carrying snails with the dry mud fixed on their legs and foot. Farmers may also carry water from one watershed to another for irrigation. Also note that *B. pfeifferi* occupied only a few of the numerous ponds and canals composing the irrigation system in Antananarivo (E. Sellin, unpubl.). This metapopulation dynamics, coupled with the recent colonization of Antananarivo region by *B. pfeifferi* (Charbonnel et al. 2002b), should favour selfing as a reproductive assurance strategy, whatever the level of inbreeding depression expressed in these populations. This is in agreement with the high values of selfing rates estimated in MB and MA (January 2000) and the absence of polymorphism detected in these populations in May and August. Pure selfing and founding events can both account for this last result.

Lower levels of spatial and temporal genetic differentiation and higher estimates of population size (convergent with results from Waples' test which also suggest limited effect of genetic drift) were observed in Itasy.

Moreover populations of *B. pfeifferi* can be found all around Lake Itasy. These may indicate that gene flow among populations was more important and that foundation events and bottlenecks were less frequent in Itasy than in Antananarivo. There is therefore little reason to call for reproductive assurance as a major force shaping the evolution of the selfing rate (Pannell and Barrett 1998). Larger selfing rates were estimated in IM than in IC in 1999, and this also seemed at variance with the predictions of the reproductive assurance hypothesis. Indeed, although we could not test it statistically, IM has a larger demographic size and shows less temporal genetic differentiation than IC. However it remains possible that population size and the occurrence of drift, as described here, do not appropriately estimate the probability of finding a partner, or alternatively that snails are able to detect partners even at very low density. Rare events of population extinction could be the only situation under which the reproductive assurance strategy plays a role in the evolution of selfing, as suggested by results in a preferentially outcrossing freshwater snail (Henry, pers. comm.). The last argument refuting the hypothesis of reproductive assurance in Itasy is the absence of delayed selfing observed in IM. Indeed, temporal genetic data reveal that this population is stable and presents a large effective size. Under the assumption of reproductive assurance only, outbreeding should be selected in this population, and isolated individuals without allosperm should delay selfing in the expectation of a partner. Again, other ecological hypotheses than reproductive assurance, such as environmental heterogeneity in space and time and local adaptation, could explain the evolution of the mating system in Itasy.

A last ecological hypothesis explaining the difference in selfing rate between IC and IM is the parasitic pressure. Parasitism has been described as a possible evolutionary determinant of the maintenance of sexual reproduction and genetic polymorphism (theoretical predictions by Hamilton 1980, Agrawal and Lively 2001) and may influence the fitness cost of different mating strategies (Levri and Real 1997). Temporal genetic studies have also previously revealed spatially heterogeneous parasite-mediated selection in clonal populations of *Daphnia* (Little and Ebert 1999). Higher exposition to parasites or higher genetic heterogeneity of parasites in IC could favour outcrossing as a strategy facilitating the occurrence of resistant mutants in *B. pfeifferi*. Spatio-temporal variation of the prevalence of the trematode *Schistosoma mansoni* has previously been observed in *B. pfeifferi* at a local geographical scale in southern Madagascar (Charbonnel et al. 2002a). Further studies are thus required in Itasy populations to test this hypothesis.

Summary

The evolution of *B. pfeifferi* mating system is under the control of a complex system of both genetic and ecological factors that need to be explored more deeply. This study reveals marked spatio-temporal variability of selfing rate and life-history parameters, and highlights the influence of environmental heterogeneity and stochasticity on mating system. Large and local geographical scale studies of the population selfing rates are yet required to provide conclusions on the different ecological constraints such as parasitism, competition, and on the mechanisms of local adaptation that could underlie the evolution of *B. pfeifferi* mating system.

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Appendix A

Estimates of family outcrossing rates and fitness traits in four populations of *Biomphalaria pfeifferi* in Madagascar. N_{off} is the number of offspring per family, t_{mm} the minimum multilocus estimate of the outcrossing rate based on the count of non-maternal alleles and t_{ML} the family outcrossing rate estimated using maximum likelihood. Standard deviations obtained from 500 bootstraps are indicated in parentheses. T_{c1}, N_{egg} and S_u are respectively the number of days between isolation and the first capsule laid, the number of eggs deposited in the first capsule and the survival rate three days after hatching in the first egg capsule. – indicates no data. (a) and (b) refers respectively to the Itasy and Antananarivo populations.

a)

Population/family	N_{off}	t_{mm}	t_{ML}	T_{c1}	N_{egg}	S_u
IC October 1999						
IC1	5	1.00	0.95 (0.27)	3	–	–
IC2	5	0.80	1.00 (0.00)	3	–	–
IC3	4	0.00	1.00 (0.00)	12	–	–
IC4	4	0.00	1.00 (0.00)	3	–	–
IC5	4	0.25	0.25 (0.22)	3	–	–
IC6	4	0.00	0.00 (0.00)	12	–	–
IC7	4	0.50	0.52 (0.25)	5	–	–
IC8	4	0.00	0.00 (0.00)	5	–	–
IC9	4	0.00	0.00 (0.00)	5	–	–
IC10	4	0.00	0.00 (0.00)	14	–	–
IM October 1999						
IM1	5	0.00	0.00 (0.00)	5	–	–
IM2	5	0.20	0.20 (0.18)	3	–	–
IM5	5	0.40	0.61 (0.23)	5	–	–
IM7	5	0.00	0.20 (0.19)	3	–	–
IM8	5	0.00	0.00 (0.00)	3	–	–
IM9	5	0.00	0.00 (0.00)	3	–	–
IC October 2000						
ICJ86	4	0.25	0.25 (0.22)	10	5	0.80
ICJ88	4	0.25	0.25 (0.22)	17	5	0.80
ICJ99	5	0.60	0.61 (0.23)	7	8	1.00
ICV87	4	0.00	0.00 (0.00)	7	4	1.00
ICV73	5	0.00	0.00 (0.00)	6	13	0.77
ICV57	5	1.00	1.00 (0.00)	5	8	1.00
ICV62	6	0.33	0.34 (0.21)	10	5	1.00
ICV63	6	0.50	0.51 (0.21)	6	6	0.83
ICV64	7	0.33	0.56 (0.28)	3	9	1.00
ICV61	2	1.00	1.00 (0.00)	3	6	1.00
IM October 2000						
IMJ28	5	0.20	0.21 (0.21)	10	11	0.91
IMJ32	4	0.00	0.00 (0.00)	5	9	0.67
I8MJ16	5	1.00	1.00 (0.00)	5	7	1.00
IMV40	5	1.00	0.90 (0.00)	6	15	1.00
IMV38	5	0.00	0.00 (0.00)	7	10	0.90
IMV50	4	0.25	0.25 (0.22)	7	4	1.00
IMV8	5	0.20	0.20 (0.18)	3	12	1.00
IMV32	8	0.38	0.40 (0.18)	5	12	0.92
IMV34	8	1.00	1.00 (0.00)	6	17	0.94
IMJ33	5	0.20	0.20 (0.18)	7	16	1.00
IMV1	9	0.11	0.14 (0.22)	5	11	1.00

(b)

Population/family	N_{off}	t_{mm}	t_{ML}	T_{c1}	N_{egg}	S_u
MA January 2000						
MA NM1	3	0.00	0.00	12	2	0.00
MA NM3	2	0.00	0.00	5	4	1.00
MA NM5	1	0.00	0.00	8	1	1.00
MA NM6	6	1.00	1.00	6	6	1.00
MA NM7	2	0.00	0.00	5	8	1.00
MA 5	3	0.00	0.00	4	11	1.00
MB January 2000						
MB NM7	2	0.00	0.00	4	5	1.00
MB NM10	3	0.00	0.00	3	4	1.00
MB NM1	3	0.00	0.00	4	9	0.22
MB 13	2	0.00	0.00	4	5	0.40
MB NM6	3	0.00	0.00	3	9	1.00
MB NM2	1	0.00	0.00	6	4	1.00
MB NM8	3	0.00	0.00	4	9	0.67
MB NM5	2	0.00	0.00	5	5	0.60
MA May 00						
MA 36	5	–	–	2	11	1.00
MA 66	5	–	–	3	10	1.00
MA NM1	3	–	–	9	3	1.00
MA NM2	5	–	–	9	7	1.00
MA NM3	5	–	–	3	16	1.00
MA NM4	5	–	–	3	7	1.00
MA NM5	5	–	–	2	8	0.25
MA NM6	5	–	–	6	5	1.00
MA NM7	5	–	–	3	17	1.00
MB May 00						
MB 3	5	–	–	10	11	0.91
MB NM1	5	–	–	6	6	0.83
MB NM2	5	–	–	6	8	1.00
MB NM3	5	–	–	2	9	1.00
MB NM4	5	–	–	5	8	0.88
MB NM5	4	–	–	6	5	1.00
MB NM6	5	–	–	6	18	1.00
MA August 00						
MA 80	5	–	–	4	6	0.83
MA 90	5	–	–	4	10	0.80
MA NM1	5	–	–	5	29	0.93
MA NM2	2	–	–	14	2	0.50
MA NM3	5	–	–	3	18	0.83
MA NM4	5	–	–	6	4	1.00