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Partial selfing, ecological disturbance and reproductive assurance in an invasive freshwater snail

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Abstract

Although reproductive assurance (RA) might play a central role in the evolution of the selfing rate, this hypothesis has never been seriously investigated in an hermaphroditic animal. We studied the mating system of the freshwater snail *Physa acuta* in which the availability of mating partners might be highly variable, because this species is an efficient colonizer occupying unstable habitats. A total of 11 populations differing in ecological disturbance regime (water level, openness) and snail densities were monitored over 2 years. The outcrossing rate was estimated in *ca* 10 families per population using microsatellite markers and the progeny-array approach. Components of fecundity and survival were recorded for each progeny. Predominant outcrossing ($t_m=0.94$) was detected, with a few individuals (4%) purely selfing. The outcrossing rate did not explain among-family variation in fitness components. None of the predictions formulated under the RA hypothesis were verified: (i) selfing was related neither to disturbed habitats, nor to temporal density fluctuations, (ii) it was positively related to population density, (iii) it co-occurred with multiple paternity, and (iv) it did not induce delayed reproduction. Explanations for these negative results are discussed in light of other arguments supporting the RA hypothesis in *P. acuta*, as well as alternative theories explaining the occurrence of partial selfing, as either a genetically fixed or plastic trait.

Keywords: demography; density; mating system; *Physa acuta*; reproductive assurance; self-fertilization

Introduction

The capacity to self-fertilize is obviously advantageous: offspring can be produced even when mates are unavailable. Reproductive assurance (RA) has therefore been a popular hypothesis for explaining the evolution of the selfing rate (see Jarne and Charlesworth, 1993). Further investigations (eg Lande and Schemske, 1985) rationalized the understanding of the genetical processes driving the evolution of the selfing rate, converting the problem mainly into a trade-off between the automatic advantage (50%) of gene transmission under selfing and the lowered fitness of selfed relative to outcrossed progeny, that is, inbreeding depression. The influence of environmental constraints has since then been incorporated into several theoretical models that account for population structure and dynamics (eg Ronfort and Couvet, 1995; Pannell and Barrett, 2001; Cheptou and Dieckmann, 2002). Theoretical studies focusing specifically on the role of the reproductive assurance hypothesis (RAH) showed that delayed selfing is always advantageous when it occurs as RA (Lloyd, 1992; Tsitrone et al, 2003a), even in a metapopulational context (Pannell and Barrett, 1998; but see Pannell and Barrett, 2001).

The wide acceptance of the central role of RA in the evolution of selfing (eg Lloyd, 1980; Schoen et al, 1996; Barrett, 2002) basically relies on empirical evidence. Major arguments are the over-representation of self-compatible species among long-distance colonizing species (reviewed in Pannell and Barrett, 1998), the repeated evolution of selfing populations or species from outcrossing ancestors in constraining habitats (eg Dole, 1992), and the occurrence of autogamous characters in populations experiencing chronically low density (eg Dole, 1992; Städler and Jarne, 1997). More direct arguments are derived from studies on plant pollination showing that selfing correlates with pollen–vector unreliability and/or low plant population density (eg Lloyd, 1980; Watkins and Levin, 1990; Fausto et al, 2001). However, the evolutionary significance of the RAH has been recently questioned (eg Barrett, 2002), the advantage of selfing being outweighed by seed discounting and inbreeding depression (Herlihy and Eckert, 2002). Moreover, although widely studied in plants, RA has hardly been investigated in hermaphroditic animals.

We engaged in an ecological and genetical study aiming at evaluating the role of RA in a hermaphroditic freshwater snail, *Physa acuta* (Gastropoda: Pulmonata; Henry, 2002; Tsitrone et al, 2003b;

Bousset et al, 2004). Freshwater snails are good candidates for such a work (Jarne and Charlesworth, 1993; Städler and Jarne, 1997). *P. acuta* predominantly outcrosses, probably because of strong inbreeding depression (Jarne et al, 2000; Henry et al, 2003; Tsitrone et al, 2003b). Residual selfing in outcrossing animal species might constitute an adaptive strategy of RA (Tsitrone et al, 2003a), and there are some ecological reasons for thinking that it might be so in *P. acuta*. First, this species inhabits a wide range of more or less temporary habitats varying in openness (eg ponds *versus* rivers). As a consequence, population size is likely to vary dramatically through time because of drying out events or floods (reviewed in Städler and Jarne, 1997). Second, *P. acuta* is the commonest species in isolated habitats (ie ponds) of the area studied here (reviewed in Henry, 2002), in accordance with its invasive status (Dillon et al, 2002; Bousset and Jarne, unpublished data). As colonization has been shown to be associated with founding events in freshwater snails (Charbonnel et al, 2002; Meunier et al, 2004), this opens opportunities for selfing as a RA strategy. In this context, Tsitrone et al (2003a) have shown theoretically that a key-factor, if RA is to play a role in the evolution of selfing, is the waiting time – the time that an isolated individual should wait before selfing when compared to an outcrossing individual. Its occurrence has been experimentally documented, and this character proved to be heritable (Tsitrone et al, 2003b; Escobar, David and Jarne, unpublished data). This direct approach suggests that the RAH can explain residual selfing rates in outcrossing species.

The RAH is here tested through an indirect, correlative approach, aiming at determining whether population and individual variation in selfing rate are affected by environmental, demographic and individual factors as predicted under the RAH. In all, 11 sites harbouring populations of *P. acuta* were studied representing four habitat types characterized by their water regime (temporary *versus* permanent) and openness (ponds *versus* rivers). Population density and water level were monitored monthly for 2 years. The outcrossing rate was estimated in *ca* 10 families per population using microsatellite markers and the progeny-array approach (Ritland, 2002).

Predictions from the RAH were formulated under two models for the genetic basis of selfing. First, selfing was assumed to be a genetically fixed trait, with natural selection acting on the selfing rate. The selfing rate would be adapted to the past history of mate availability. The RAH predicted that mate availability and the outcrossing rate covary across ecological disturbance regimes (water regime and openness), both being lower in temporary than in permanent habitats, and in ponds than in rivers. Second, selfing was assumed to be a genetically plastic trait, with natural selection acting on the adjustment of the selfing rate to environmental conditions (which is possibly the case in *P. acuta*; Tsitrone et al, 2003b). The selfing rate would thus be adapted to the instantaneous mate availability. Predictions from the RAH were: the outcrossing rate increases with increasing density, multiple paternity is positively associated with the outcrossing rate, and the time to first egg laying of isolated individuals under laboratory conditions is negatively associated with the outcrossing rate.

Materials and methods

Populations studied and progeny arrays

A total of 11 populations of *P. acuta* were monitored monthly from July 2000 to 2002 in the Montpellier area, France. Ecological details of these populations are given in Table 1 (see also Henry, 2002). Analysis of population genetic structure is presented in Bousset et al (2004). Populations were distinguished according to habitat openness (rivers=open *versus* ponds=closed) and water regime (permanent *versus* temporary). Temporary sites dried out at least once during the 2-year survey. The snail density was visually scored (*ca* 0, 1, 5, 10, 25, 50 or 100 ind/m²) at each visit. When snails were spatially aggregated, we reported the range of density (d_{sampl} in Table 1), and the range mean was used in statistical analyses.

Table 1: Ecological characterization, population and individual covariates, and mating system parameters estimates (with SD in parentheses) for 11 populations of *Physa acuta*

Population	Sal2	Mos1	Lam1	Lam12	Mos6	Vio2	Vio7	Vio11	Vio1	Vio8	Vio12
Openness	O	O	O	O	O	C	C	C	C	C	C
Water regime	P	P	T	T	T	P	P	P	T	T	T
d_{sampl}	10–25	25	5–100	100	1	1	100	25	50–100	10	25
d_{ham}	4.73	0.66	0.36	0.23	0.45	0.61	0.95	0.54	11.22	15.45	0.84
d_{freq}	0.12	0.00	0.56	0.00	0.36	0.52	0.28	0.26	0.04	0.00	0.24
p_{fert}	0.900	0.789	0.941	0.850	0.833	0.526	0.842	0.500	0.833	0.875	0.895
Size	7.6 (0.8)	8.0 (1.1)	8.5 (0.5)	8.9 (0.7)	10.8 (3.8)	8.5 (1.0)	8.6 (0.6)	8.6 (1.0)	8.1 (0.9)	9.7 (0.8)	10.7 (3.0)
N_{egg}	12.6 (5.3)	18.5 (8.8)	25.0 (11.9)	12.5 (6.23)	36.7 (14.6)	22.6 (13.1)	25.2 (8.8)	29.8 (4.1)	15.0 (5.5)	25.0 (9.1)	28.9 (9.8)
t_{laying}	2.6 (1.9)	8.9 (3.5)	5.6 (3.8)	6.7 (4.8)	2.3 (0.6)	4.4 (5.3)	6.5 (5.8)	5.5 (2.3)	4.6 (4.4)	8.3 (3.6)	3.0 (1.1)
t_{incub}	5.8 (0.8)	6.5 (1.1)	8.1 (1.1)	5.8 (0.7)	5.3 (0.6)	6.0 (1.0)	8.4 (2.7)	6.4 (0.9)	5.7 (0.6)	6.4 (1.1)	8.1 (2.9)
S_{7d}	0.739 (0.250)	0.814 (0.219)	0.821 (0.143)	0.819 (0.282)	0.878 (0.144)	0.832 (0.232)	0.635 (0.311)	0.676 (0.277)	0.884 (0.142)	0.760 (0.211)	0.537 (0.295)
N_{fam}	13	11	10	12	3	7	11	6	11	10	12
NG_0	19	18	16	17	4	18	19	18	17	14	17
NG_1	80	76	70	75	21	44	71	43	76	70	79
F_{is}	0.040 (0.047)	0.047 (0.044)	0.006 (0.020)	0.007 (0.022)	0.047 (0.066)	0.210 (0.076)*	0.088 (0.069)*	0.001 (0.005)	0.098 (0.065)*	0.115 (0.051)*	0.011 (0.022)
t_{Fis}	0.923	0.910	0.988	0.986	0.910	0.653	0.838	0.998	0.821	0.794	0.978
t_m	0.991 (0.011)	0.977 (0.026)	0.990 (0.013)	0.834 (0.100)*	0.998 (0.045)	0.957 (0.060)	0.731 (0.135)*	0.974 (0.042)	0.847 (0.083)*	0.998 (0.011)	0.997 (0.015)
t_s	0.970 (0.025)	0.926 (0.042)*	0.935 (0.043)*	0.876 (0.108)*	0.998 (0.045)	0.926 (0.094)	0.664 (0.157)*	0.945 (0.048)*	0.726 (0.108)*	0.987 (0.023)	0.971 (0.019)*
$t_m - t_s$	0.021 (0.020)	0.051 (0.036)	0.055 (0.039)*	−0.042 (0.057)	0.000 (0.000)	0.031 (0.050)	0.067 (0.039)*	0.029 (0.026)*	0.120 (0.045)*	0.011 (0.023)	0.025 (0.015)*
r_s	0.063 (0.028)*	0.253 (0.098)*	0.078 (0.038)*	0.845 (0.158)*	0.104 (0.041)*	0.618 (0.273)*	0.652 (0.270)*	0.063 (0.122)	0.406 (0.286)*	0.103 (0.010)*	0.104 (0.011)*
r_p	0.321 (0.142)*	0.200 (0.117)*	0.279 (0.093)*	0.629 (0.207)*	0.028 (0.052)*	0.445 (0.362)	0.290 (0.171)*	0.038 (0.099)*	0.125 (0.062)*	0.175 (0.093)*	0.084 (0.048)*

The ecological variables are habitat openness (Open versus Closed), water regime (Permanent versus Temporary), density at sampling (d_{sampl} in individual/m²; intervals are given when *P. acuta* were spatially aggregated), harmonic mean of population densities (d_{ham}), and frequency of occurrence of population densities of one ind/m² or less (d_{freq}). A population covariate is the proportion of fertile individuals (p_{fert}). The individual covariates are the size of G_0 individuals (mm), number of eggs per capsule (N_{egg}), egg-laying delay (t_{laying} in days), incubating time (t_{incub} in days), and survival 7 days after first hatching (S_{7d}). N_{fam} , NG_0 and NG_1 are the number of families, of G_0 and G_1 individuals genotyped. F_{is} is the inbreeding coefficient, and t_{Fis} is the population outcrossing rates based on F_{is} . t_m and t_s are the multilocus and single-locus outcrossing rates, and $t_m - t_s$ the amount of apparent selfing accounted for by biparental inbreeding. r_s and r_p are the correlations of selfing among families and of outcrossed paternity within families. Stars indicate estimates significantly higher than 0 (F_{is} , $t_m - t_s$, r_s) or lower than 1 (t_m , t_s , r_p).

In July 2000, 20 mature individuals (denoted G_0) were collected in each population. They were measured (length) and isolated in 75 ml plastic boxes less than 6 h after field collection. They were maintained in the laboratory at 25°C under a 12:12 photoperiod regime and fed *ad libitum* with boiled lettuce. Water was changed every 3 days. Plastic boxes were checked daily for egg capsules, until the first capsule was laid, over a period of 21 days. The egg-laying delay (t_{laying} ; mean: 5.1±3.9 days) was calculated as the time elapsed between isolation and first egg laying. The fraction of fertile individuals (p_{fert}) was the fraction of individuals that laid eggs over these 21 days. When several capsules were laid by a given snail, only the oldest capsule, based on embryo development, was analysed. Its number of eggs (N_{egg}) was recorded. Hatching was checked daily until the first juvenile (denoted G_1) was detected, from which we derived the minimum incubation time (t_{incub}), that is, the time elapsed between egg laying and first hatching. Survival (S_{7d}) was defined as the number of surviving G_1 7 days after first hatching divided by the number of eggs per capsule.

A G_0 mother and its G_1 offspring constituted a family. Since *P. acuta* stores sperm after copulation (eg Wethington and Dillon, 1997), preferentially outcrosses (Jarne et al, 2000; Tsitrone et al, 2003b; Bousset et al, 2004) and G_0 individuals were placed in optimal conditions for egg laying, we assumed that the estimated outcrossing rate reflects that under natural conditions.

Estimating the outcrossing rate

G_0 and G_1 individuals were stored at −80°C and in 95% alcohol, respectively, prior to DNA extraction. DNA extraction, PCR amplification and allele size scoring were performed according to Sourrouille et al (2003). The seven loci used are described in Monsutti and Perrin (1999) and Sourrouille et al (2003). Loci were coamplified by PCR (Pac1, Pac2 and Pac5; Pac4 and Pac7; AF108762 and AF108764; Bousset et al, 2004) and products were run simultaneously on ABI Prism Genetic Analysers (310 or 3100). PCR amplification and allele size scoring were performed simultaneously for the G_0 mother and its progeny. In all, 10 families per population and seven juveniles per family were genotyped. When less than seven juveniles were available, at least one family was added in order to genotype the same overall number of juveniles per population (Table 1). Families and juveniles were randomly chosen. Mendelian segregation of alleles at the loci used was checked elsewhere (Bousset et al, 2004). When null alleles were detected (loci Pac4, Pac7), data from these families and loci were omitted from the analysis. Estimates of allelic frequencies per locus and per population

are given in Web Table 1, available online. The mean number of polymorphic loci per population ranged between 3 and 6, and the mean number of alleles per loci and population varied between 1.8 and 4.2 (Bousset et al, 2004).

The mating system parameters were estimated using maximum likelihood procedures (software MLTR 2.4; Ritland, 2002). Both population-level multilocus (t_m) and single-locus (t_s) outcrossing rates were estimated. Family-level multilocus outcrossing rates are noted t_{mf} . The difference between t_m and t_s gives a minimum estimate of apparent selfing due to biparental inbreeding (Ritland, 2002). Correlations of selfing (r_s) document the among-family variation in selfing, with a non-null correlation indicating significant heterogeneity (Ritland, 2002). Correlations of paternity (r_p) represent the proportions of full-sibs (*versus* half-sibs) among outcrossed offspring (Sun and Ritland, 1998; Ritland, 2002) with r_p significantly lower than 1 indicating multiple paternity. The Expectation–Maximization method and the ‘parent chosen in probability’ option were used. SD of estimates were calculated based on 500 bootstraps assuming Normal distributions. The resampling unit was, respectively, the family and the individual within family for population- and family-level estimates. SD for averages over all population or family estimates were computed with the δ -method (Mood et al, 1974). F_{is} , $t_m - t_s$ and r_s were considered as significantly higher than 0, and t_m , t_s and r_p as lower than 1 when more than 95% of the 500 resampled estimates were, respectively, higher than 0 or lower than 1. Assuming that selfing is the sole source of inbreeding, we calculated the selfing rate corresponding to F_{is} values according to: $t_{Fis} = (1 - F_{is}) / (1 + F_{is})$ (Pollak, 1987).

Selfing rates and fitness traits

Since juveniles were genotyped 7 days after hatching, t_m and t_{mf} estimates are expected to be biased positively, because of inbreeding depression (Jarne and Charlesworth, 1993). This is particularly likely since inbreeding depression is strong in juvenile *P. acuta* (Wethington and Dillon, 1997; Jarne et al, 2000; Henry et al, 2003; Tsitrone et al, 2003b). The t_m value should therefore explain population-level variation in the proportion of fertile G_0 individuals (p_{fert}), and t_{mf} should explain family-level variation in the number of eggs per capsule (N_{egg}), incubating time (t_{incub}) and survival at 7 days (S_{7d}).

Tests of the RAH

Predictions from the RAH depend on the assumed genetic basis of the selfing rate (see Introduction; Table 2).

(i) The selfing rate could be a genetically fixed trait (predictions prefixed by F) evolving under the selective pressure of temporal fluctuation in mate availability. Mate availability was expected to differ with habitat. Episodes of low density should be less frequent in permanent than in temporary habitats, because population bottlenecks associated with drying-out events are expected to be more frequent in temporary habitats (Charbonnel et al, 2002; Trouvé et al, 2003; Bousset et al, 2004). They should also be more frequent in closed than in open habitats, because immigration more easily refills populations after a population bottleneck in open than in closed habitats (Bousset et al, 2004). The probability of low density episodes was estimated based on the harmonic mean density (d_{harm}) and the proportion of visits when density was one individual per m^2 or less (d_{freq}) over our 2-year survey. This threshold was chosen because we were technically unable to estimate density precisely at lower values. In response to these expected variations of mate availability, the outcrossing rate t_m was predicted to be higher in permanent than in temporary habitats (F1), in open than in closed habitats (F2), positively correlated to d_{harm} (F3), and negatively correlated to d_{freq} (F4).

(ii) The selfing rate could be a plastic trait adapted to realise the best compromise between inbreeding depression and instantaneous mate availability (predictions prefixed by P). (P1) At the population level, the probability of outcrossing should be positively correlated with partner availability, estimated as local density during genetic sampling (d_{sampl}). (P2) Under delayed selfing, partner availability should not only affect the fraction of selfed ovules, but also the number of outcrossing partners. The probability of outcrossing should thus be negatively correlated to the proportion of half-sibs among outcrossed offspring, r_p . (P3) Previous work has shown the existence of a waiting time in *P. acuta*, defined as the difference in age at first egg-laying between outcrossing and selfing virgin individuals (Tsitrone et al, 2003a, 2003b). By analogy, the individual egg-laying delay (t_{laying}) was expected to negatively correlate with the outcrossing rate.

Table 2: Seven predictions (numbered as in text) relating the outcrossing rate (t) to ecological characteristics, and fitness traits, as derived from the reproductive assurance hypothesis

Number	Level	Prediction
F1	Population	Contrast $t_{\text{permanent}} > t_{\text{temporary}}$
F2	Population	Contrast $t_{\text{open}} > t_{\text{closed}}$
F3	Population	Correlation $(t, d_{\text{harm}}) > 0$
F4	Population	Correlation $(t, d_{\text{freq}}) < 0$
P1	Population	Correlation $(t, d_{\text{saml}}) > 0$
P2	Family	Correlation $(t, r_p) < 0$
P3	Family	Correlation $(t, t_{\text{laying}}) < 0$

Predictions were derived under either fixed (noted F) or plastic (noted P) genetic determination of selfing. Habitats were characterized as permanent or temporary, and as open or closed. The outcrossing rate (and therefore the prediction) is either at the population, or at the family level.

d_{saml} : density at sampling; d_{harm} : harmonic mean of population densities; d_{freq} : frequency of occurrence of population densities of one ind/m² or less; r_p : correlation of outcrossed paternity within families; t_{laying} : time elapsed between isolation and first egg laying.

Statistical analyses

The probability of outcrossing was analysed using logistic regression models, followed by likelihood ratio tests (LRT). To convert the normal ML estimates of outcrossing rate t_m into a binomial variable, that is, the probability of being an outcrossed offspring, the number of genotyped progeny per population N_{G1} was multiplied by t_m and $1-t_m$ (Table 1). This produced the estimated number of outcrossed and selfed juveniles per population. Using logistic regressions, with t_m as the dependent variable and the reconstructed number of juveniles as sample size, accounted for the binomial variance of t_m estimates. Note that this is not the case under analyses of variance (ANOVA) with classical transformation (eg arcsin) of t_m which, however, provided similar results (results not shown). The explanatory variables were habitat openness, water regime and density variables (d_{harm} , d_{freq} and d_{saml}). The effects of openness, water regime and their interaction were tested in a single model, and that of density variables in separate models.

The dependent variables d_{freq} , p_{fert} and S_{7d} were also analysed using logistic regression models: d_{freq} was modelled in relation to openness, water regime and their interaction, and p_{fert} in relation to t_m . Family-level $\text{Ln}(t_{\text{laying}}+1)$, $\text{Ln}(N_{\text{egg}})$, and $\text{Ln}(t_{\text{incub}})$ were analysed using ANOVAs. The explanatory variables for S_{7d} , $\text{Ln}(t_{\text{laying}}+1)$, $\text{Ln}(N_{\text{egg}})$ and $\text{Ln}(t_{\text{incub}})$ were the log-transformed individual size, $t_{\text{laying}}+1$, N_{egg} , t_{incub} and t_{mf} (see Table 3 for more details on each model). The homogeneity of d_{harm} among habitats was tested using a Kruskal–Wallis test, followed by Mann–Whitney tests of the predicted relationships with habitat openness and water regime. Logistic regression and ANOVA models were built with JUMP 3.2.1 (S.A.S. Institute Inc.), and nonparametric tests with Statistica 5.5 (Statsoft Inc.).

Table 3: Analysis of fitness components and outcrossing rate in *P. acuta*

Fitness component	$\text{Ln}(t_{\text{laying}}+1)$		$\text{Ln}(N_{\text{egg}})$		$\text{Ln}(t_{\text{incub}})$		S_{7d}	
	F(1,96)	P	F(1,96)	P	F(1,92)	P	F(1,91)	P
$\text{Ln}(\text{size})$	0.014	0.906	0.001	0.972	0.169	0.682	0.382	0.538
$\text{Ln}(N_{\text{egg}})$	0.030	0.863	—	—	0.257	0.614	2.824	0.096
$\text{Ln}(t_{\text{laying}}+1)$	—	—	0.605	0.439	0.001	0.976	—	—
t_{mf}	0.460	0.499	0.169	0.682	0.383	0.537	0.556	0.458
$\text{Ln}(\text{size}) * \text{Ln}(N_{\text{egg}})$	0.205	0.651	—	—	0.452	0.503	1.906	0.171
$\text{Ln}(\text{size}) * \text{Ln}(t_{\text{laying}}+1)$	—	—	0.225	0.637	0.131	0.718	—	—
$\text{Ln}(\text{size}) * t_{\text{mf}}$	0.036	0.851	0.044	0.834	0.119	0.731	0.710	0.402
$\text{Ln}(N_{\text{egg}}) * \text{Ln}(t_{\text{laying}}+1)$	—	—	—	—	0.254	0.615	—	—
$\text{Ln}(N_{\text{egg}}) * t_{\text{mf}}$	2.104	0.150	—	—	0.301	0.584	0.366	0.547
$\text{Ln}(t_{\text{laying}}+1) * t_{\text{mf}}$	—	—	1.827	0.180	0.273	0.603	—	—

The table reports the F -values and their associated probability (P). The dependent variables (first row) are the log-transformed egg-laying delay (t_{laying} ; since individuals laid eggs within 1 day, the dependent variable was $\text{Ln}(t_{\text{laying}}+1)$), number of eggs per capsule (N_{egg}), minimum incubating time (t_{incub}) and survival at 7 days (S_{7d}). The first three variables were analysed with ANOVA models and S_{7d} with a logistic regression model. The explanatory variables were the log-transformed individual size, $\text{Ln}(N_{\text{egg}})$, $\text{Ln}(t_{\text{laying}}+1)$ and the family-level multilocus outcrossing rate t_{mf} . Interaction terms are denoted by a star. — indicates that the effect was not included in the model.

Results

Mating system characterization

P. acuta preferentially outcrossed in all populations. Population-level multilocus outcrossing rates ranged from 0.731 to 0.998 (average: 0.936 ± 0.019). The selfing rate significantly differed from 0 in three populations out of 11 (Lam12, Vio7, Vio1; Table 1). However, the correlation of selfing (r_s) among families indicated that outcrossing rates significantly varied among families in 10 populations out of 11 (Table 1). The family-level outcrossing rates ranged from 0.001 to 1 (average: 0.885 ± 0.002). Partial selfing was detected in at least one family per population (except in Mos6), and purely selfed families occurred in three populations only (Lam12, Vio7 and Vio1). $t_m - t_s$ ranged from -0.042 to 0.12 (average: 0.041 ± 0.011) with significant biparental inbreeding occurring in five populations (significant $t_m - t_s$ in Table 1). F_{is} differed from zero in four populations out of 11 (range: 0.088 – 0.210). However, no obvious relationship could be established with inbreeding since F_{is} was significant in Vio2 and Vio8 where neither selfing, nor biparental inbreeding was detected. Population-level correlations of paternity (r_p) ranged from 0.028 to 0.629 (Table 1), indicating multiple paternities within progenies in all populations but Vio2. The family-level average was 0.260 ± 0.613 (range: 0.001 – 1) corresponding to a mean number of 3.8 fathers per family (ie $1/r_p$; Sun and Ritland, 1998).

Selfing rates and fitness traits

The logistic regression model for S_{7d} did not fit the data ($\chi^2=576.3$, d.f.=91, $P < 10^{-4}$). Deviances were thus divided by a variance inflation factor \hat{c} (deviance/d.f.=6.333) before testing effects with LRTs. No effect of the outcrossing rate on any of the four reproductive parameters studied was detected among families (Table 3). This also holds for the proportion of fertile individuals (LRT=0.189, d.f.=1, $P=0.664$). None of the individual traits expected to confound the effect of the mating system significantly explained variation in fitness components (Table 3).

Tests of the RAH

Mate availability depended on the disturbance regime. The harmonic mean density (d_{harm}) differed among the four habitats ($H=7.833$, d.f.=3, $N=11$, $P=0.050$) with slightly higher values in closed temporary than in other sites (Table 1). When habitat types were tested separately, there was no detectable effect for either habitat openness ($U=6$, $Z=-1.643$, $P=0.100$), or for water regime ($U=13$, $Z=0.365$, $P=0.715$). The probability of occurrence of densities of one individual or less per m^2 (d_{freq}) varied among habitats (interaction Openness*Water regime, LRT=29.818, d.f.=1, $P < 10^{-4}$). However, it did not follow the predicted pattern: low densities were more frequent in open temporary (45%, $N=42$ visits) than in closed permanent (36%, $N=72$), closed temporary (10%, $N=62$) and open permanent sites (8%, $N=40$).

None of the predictions of the RAH (Table 2) were supported by our results. Although variation in outcrossing probability could be explained by habitat characteristics (interaction Openness*Water regime, Table 4), they did not fit with variation in d_{freq} , at variance with predictions from the RAH: the overall outcrossing rate was lower in closed permanent (0.86) than in open temporary (0.92), closed temporary (0.95) and open permanent (0.98). Moreover, this variation was no more significant when tested in the presence of d_{sampl} (results not shown; F1 and F2 not fulfilled). d_{harm} and d_{freq} did not explain the among-population variation in outcrossing probability (Table 4; F3 and F4 not fulfilled).

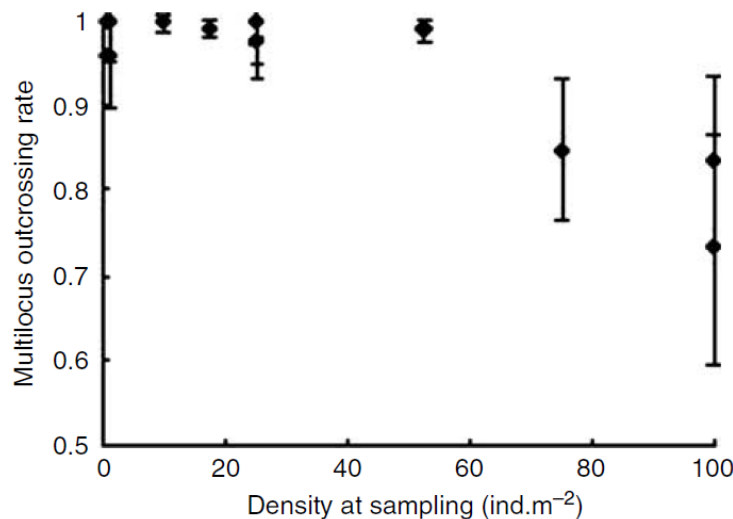
Table 4: Likelihood-ratio tests (LRT) for ecological correlates of the population-level outcrossing probability

	LRT	d.f.	P
Openness	5.958	1	0.015
Water regime	0.313	1	0.576
Openness*water regime	13.849	1	0.001
d_{harm}	0.364	1	0.546
d_{freq}	1.570	1	0.210
d_{sampl}	64.680	1	$< 10^{-4}$

The dependent variable was the reconstructed binomial data corresponding to the estimated multilocus outcrossing rate and sample size per population (see Materials and methods). The ecological covariates were openness (closed *versus* open), water regime (permanent *versus* temporary), the harmonic mean density (d_{harm}), the frequency of occurrence of densities ≤ 1 ind m^{-2} (d_{freq}) and the density at sampling (d_{sampl}). All effects were tested against the null model.

The population-level probability of outcrossing was negatively dependent on the density at sampling (Table 4; Figure 1), contrary to the predicted positive correlation (P1). This effect remained strongly significant when using a more conservative statistical approach under which the sample size per population was reconstructed such as to be proportional to the SD of t_{mf} (results not shown). At the population level, multiple paternities occurred in the three populations in which significant selfing was detected (Table 1). At the family level, there was no relationship between t_{mf} and r_p ($r=0.088$, d.f.=98, $P=0.385$; P2 not fulfilled). The egg-laying delay did not depend on the family-level multilocus outcrossing rate (Table 3; prediction P3), although essentially selfing individuals tended to exhibit long egg-laying delays.

Figure 1 Relationship between population-level multilocus outcrossing rate ($t_m \pm SD$) and population density at sampling (d_{sampl}) in *P. acuta* ($N=11$ populations).



Discussion

The mating system of *P. acuta*

P. acuta reproduced primarily through outcrossing, with a population-level average outcrossing rate of 0.936. Over all families, the average outcrossing rate was 0.885. These results are in agreement with those in a natural population from the same area (0.921 in Jarne et al, 2000). Wethington and Dillon (1997) found a rather lower value (0.385), but this was under laboratory conditions with North-American individuals. Our estimates based on F_{is} (average tF_{is} : 0.89) were close to those derived from progeny arrays.

Examining fitness components showed that the variation in the proportion of fertile individuals, number of eggs per capsule, incubation time, and offspring survival were not explained by variation in outcrossing rate. This might be interpreted as evidence of limited inbreeding depression. However, this would be surprising since strong inbreeding depression has been consistently detected in *P. acuta* (Wethington and Dillon, 1997; Jarne et al, 2000; Henry et al, 2003). Moreover, in the partially selfing population Lam12, F_{is} did not differ from zero, suggesting the occurrence of inbreeding depression between the juvenile and adult stages. Alternatively, this might be due to imprecision in family-level estimates of t_{mf} which is not accounted for in our analyses. Discounting the estimation error on t_{mf} blurs the acceptance of the null hypothesis. To circumvent this general problem, a comprehensive statistical framework is required that would allow comparative analyses of mating system parameters, and in which the contribution of individual and population covariates could be directly built in the Maximum Likelihood procedure and tested through comparisons of nested models.

Tests of the RAH

The RAH has been repeatedly invoked to explain the maintenance of selfing in sessile or poorly mobile organisms. Its role was suggested in freshwater snails because these organisms often inhabit unpredictable temporary habitats (eg Jarne and Charlesworth, 1993; Städler and Jarne, 1997; Charbonnel et al, 2002). Tsitrone et al (2003a) clarified the conditions under which RA is likely to play a role in mainly outcrossing

animal species (see Introduction). Their model has been supported by experimental work conducted in *P. acuta* that showed that the waiting time is heritable (Tsitrone et al, 2003b). There is therefore evidence from a direct approach that the RAH explains residual selfing in highly outcrossing species.

The RAH was here evaluated using an indirect, correlative approach. None of the tests performed (Table 2) support the hypothesis that *P. acuta* self-fertilizes in response to a low probability of mate encounter. At the population level, the ecological constraints on the mating system related to habitat, via population density, were not those expected: temporary or closed habitats were no more likely to go regularly through low-density periods than permanent or open habitats (the opposite trend was indeed found). A conservative interpretation of our results is that the outcrossing rate was not related to those environmental variables that might influence the probability of mate encounter, that is, water regime, habitat openness, temporal fluctuations of density, and occurrence of low-density events. The density of G_0 individuals at sampling did explain a significant fraction of the among-population variation in outcrossing rate. However, the relationship was opposite to the prediction: increasing population density was associated with decreasing outcrossing rate. Another surprising result under the RAH is that multiple paternity and the selfing rate were positively associated: why did individuals self their own ovules when mate density was high enough to ensure multiple paternities in some offspring (see also Cheptou et al, 2002)? On the whole, the indirect approach developed here did not provide any evidence in favour of the RAH as a major determinant of the selfing rate. This is compatible with results from another indirect approach in which the probability of mate encounter was experimentally manipulated (Henry, 2002).

It must be emphasized that our predictions on the probability of mate encounter were based on short-term ecological trends and population dynamics (over a 2-year survey). This did not capture the occurrence of much rarer events of low mate encounter probability, that is, due to abnormal disturbance events (eg, prolonged drying out) or very rare founding events. Those events might have indeed been the ecological factors maintaining selfing as a RA mechanism. Founding events very likely occur in *P. acuta* since this species displays high dispersal abilities (reviewed in Henry, 2002) and is the most successful worldwide invasive freshwater snail (Dillon et al, 2002; Bousset and Jarne, unpublished data). Such founding events might be scarce in the studied area, since neither demographic, nor genetic signatures were detected (Henry, 2002; Bousset et al, 2004). The mating system would rapidly revert to preferential outcrossing when mates become available, because of inbreeding depression (Stephenson et al, 2000; Pannell and Barrett, 2001), and signals of RA would no more be detectable.

Very few studies in animals have evaluated the RAH, and most failed. In predominantly selfing snails, Trouvé et al (2003) and Charbonnel et al (2005) concluded that the among-population variation in selfing rate is inconsistent with this hypothesis. The only supporting results come from comparative analyses showing that selfing gastropods are better colonizers (Selander and Ochman, 1983; Bengtsson and Baur, 1993). Less controversial arguments come from studies in plants, where negative relationships between self-pollination (and morphological or incompatibility-related traits) and density (of plants or of pollination vectors) have been repeatedly documented (eg Rankin et al, 2002 and references therein; but see eg Cheptou et al, 2002 for opposite results). However, it is worth noting that most results are derived from correlative observations of fitness components (the indirect approach above). Moreover, the only satisfactory quantitative test dismissed the evolutionary importance of RA because the advantage of autogamy was outweighed by seed discounting and inbreeding depression (Herlihy and Eckert, 2002). On the whole, the significance of selfing as a RA strategy, particularly in animals, remains to be demonstrated.

So, why do P. acuta and other snail species partially self-fertilize?

Although several studies have dealt with the reproductive biology of *P. acuta*, the maintenance of some selfing remains unexplained (Henry, 2002; but see Tsitrone et al, 2003b). We briefly review several theories, which are alternatives to the RAH, which may be consistent with the empirical evidence from *P. acuta*. These explanations can be extended to other freshwater snail species with a mating system similar to that of *P. acuta* (see Henry et al, 2003 for a list). A first view assumes that partial selfing in *P. acuta* is the expression of a mixed-mating system, that is, a polymorphism with genetically fixed selfing rates. Three evolutionary scenarios can be envisaged. (i) The selfing rate greatly varies among families (Jarne et al, 2000; this study; see Trouvé et al, 2003 and references therein for results in other snail species), as do selfing propensity and

inbreeding depression (Jarne et al, 2000; Henry et al, 2003; Tsitrone et al, 2003b). The required conditions for a coevolution between loci determining the selfing rate and fitness driving to mixed mating therefore seem to be met (Uyenoyama et al, 1993). However, the observation that no genetic covariation between selfing propensity and inbreeding depression was found in *P. acuta* (Escobar, David and Jarne, unpublished data) weakens this hypothesis. (ii) Biparental inbreeding, due to restricted dispersal in association with density-dependent recruitment (Uyenoyama, 1986; Ronfort and Couvet, 1995), could also be relevant and was detected in five populations out of 11 in this study. However, significant selfing rates were not systematically associated with biparental inbreeding, and density-dependent recruitment, although likely, remains to be investigated in *P. acuta*. (iii) Mixed mating can evolve in structured populations (eg Pannell and Barrett, 2001). The highly fragmented, potentially unstable populations of *P. acuta*, in association with preferential outcrossing, high dispersal abilities and high inbreeding depression, nicely fit the situation modelled by Pannell and Barrett (2001): in the case of extinction–colonization, preferential outcrossing should have a selective advantage over preferential selfing thanks to a higher number of viable propagules.

A second view envisages the selfing rate as a plastic trait, and plasticity itself as the target of natural selection, adjusted to environmental conditions (Stephenson et al, 2000; Pannell and Barrett, 2001). Tsitrone et al (2003a) demonstrated that delaying selfing according to mate availability increases both the probability of mate encounter and future reproduction through resource reallocation towards growth. A side prediction is that, if the waiting time before selfing is positively related to survival, the propensity to self-fertilize should be higher when survival is low. If high population density represents a high mortality risk, this would explain why the outcrossing rate was here negatively related to population density. Also, it could explain within-individual temporal variation, like age dependency, in selfing rate (Watkins and Levin, 1990; Stephenson et al, 2000; Rankin et al, 2002), which remains to be documented in hermaphroditic animals.

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Supplementary information

Web Table 1 *Estimated allelic frequencies (output from the MLTR software) at seven microsatellite loci in the 11 populations of Physa acuta analysed. Values at loci Pac 4 and Pac7 were obtained once null homozygotes were discarded. Frequencies in Mos6 are raw data from G0 individuals because the small sample size prevented computing estimations. Alleles are designated by their size in base pairs.*

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