Selfing, sexual polymorphism and microsatellites in the hermaphroditic freshwater snail *Bulinus truncatus*

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SUMMARY

Studies on the evolution of self-fertilization and sexual polymorphisms (the co-occurrence of several sexual morphs in a species) have focused on plants. Aphally, a sexual polymorphism occurring in gastropods, offers the opportunity to extend study of these issues to animals. We present progeny-array analyses of the selfing rate and correlated matings in the tropical freshwater snail *Bulinus truncatus*. This study is based on 447 offspring originating from 57 families and five natural populations. To overcome the lack of allozyme polymorphism, four polymorphic microsatellite markers were used. Selfing rates higher than 78% were detected in all populations, and no correlation with the aphally ratio (the proportion of individuals lacking the male copulatory organ per population) was evident. Outcrossing was detected in 17 families only, and individual outcrossing rates were variable and did not depend on the sexual morph of the mother. These results illustrate the power of microsatellites for detailed genetic studies, indicate that high selfing rates may have a strong genetic basis, and unexpectedly suggest that phally polymorphism may be neutral with respect to selfing.

1. INTRODUCTION

Hermaphroditism is a widespread trait of multicellular organisms, and understanding its evolution and its consequences is a major field of interest in evolutionary biology. Two obvious consequences of hermaphroditism are the possibility of self-fertilization and the occurrence of sexual polymorphisms, the latter being defined as the co-occurence of two (or more) sexual morphs within a given species (e.g. gynodioecy; see Jarne & Charlesworth 1993). Botanists have tried to understand the forces acting on the evolution of both selfing and sexual polymorphisms for over a century. However, hermaphroditism also occurs widely among animals (see Bell 1982). Selfing is possible in many large zoological groups (Jarne 1995), and numerous examples of sexual polymorphism have been described, giving the opportunity to extend these studies to animals.

Here, we focus on aphally, a sexual polymorphism known in pulmonate gastropods only, and with no equivalent in plants (Larambergue 1939). Phally polymorphism is characterized by the co-occurrence of regular hermaphroditic individuals (euphallic) and individuals lacking the male copulatory organ, referred to as aphallic. While the former can reproduce through outcrossing as both male and female, and through selfing as well, individuals of the latter cannot provide sperm to a mating partner. Aphally might therefore be related to the mating system both at the population and individual levels. Indeed, selfing is obligatory in populations entirely composed of aphallic individuals. A decrease in the selfing rate is expected when the aphally ratio (AR – the proportion of aphallic individuals per population) decreases, because euphallic individuals are expected to provide partly sperm to a female-acting snail. The selfing rate appears as a key-factor in models of the evolution of aphally, and more generally that of sexual polymorphisms (Jarne & Charlesworth 1993).

A primary goal is therefore to estimate precisely the selfing rate in self-fertile hermaphroditic species. Two methods are currently available. First, selfing may be inferred from the population inbreeding coefficient *F*. This parameter is indeed related to the selfing rate in a straightforward manner, assuming a population of large size at inbreeding equilibrium and reproducing through a mix of selfing and random-mating (mixed-mating model; see Brown 1990). A more direct method is based on the comparison of multilocus genotypes from sets of mothers and their offspring (progeny-array analysis; Ritland & Jain 1981; Brown 1990). The first method gives a mean value per population averaged over many generations, though yields no insight into the variance in selfing rates among individuals, an important characteristic when considering the evolution of the mating system. Moreover, it is not always obvious whether the assumptions of the mixed-mating model hold. Progeny-array analyses are in these respects much more accurate than indirect approach

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estimates, though they are more laborious and not commonly used; in animals, only one study is currently available thus far (Städler *et al*. 1995).

We estimate the selfing rate in five populations of the freshwater snail *Bulinus truncatus* using progeny-array analyses. As this species has been shown to exhibit basically no population genetic variability using allozymes, we base our work on four highly polymorphic microsatellite loci which exhibit the qualities required for progeny-array analyses, but are much more variable than allozymes (Queller *et al*. 1993; Viard *et al*. 1996). The five populations studied are characterized by various ARs, and we specifically analyse (i) the selfing rate at the population level and its variation among individuals; and (ii) the relationship between the selfing rate and aphally, at both the individual and population levels.

2. MATERIALS AND METHODS

B. truncatus is a simultaneous hermaphrodite, freshwater snail (Gastropoda: Pulmonata) which has a continuous reproduction over all its lifespan. It is one of the major vectors of various species of *Schistosoma*, agents of bilharziasis in humans and cattle in Africa (Brown 1994). *B. truncatus* is also an allotetraploid species. It is distributed over most of Africa, several Mediterranean islands and part of the Middle East. In the Sahelian area, its habitats (natural ponds) are subjected to annual cycles of droughts and floods, which impose wide fluctuations in snail density and reduce the genetic variability through population bottlenecks (Viard *et al*. 1996). We performed progeny-array analyses on five populations sampled in January and February 1995 from Niger (table 1). These populations are characterized by a range of densities (number of snails collected per unit time and per person) and ARs (table 1; see map in Doums *et al*. 1996*a*). ARs are from Doums *et al*. (1996*a*) where the populations Mari Sud, Bala, Doubalma, Ligido and Kotaki were referred to as Mari Sud-3, Bala-3, Doubalma-3, Ligido-1 and Kotaki-1 respectively. Individual genotypes (adults and progenies) were assessed using four highly polymorphic microsatellite loci. The Mendelian inheritance of alleles and the absence of linkage between loci have been demonstrated (Viard *et al*. 1996). The four loci were analysed as described in Viard *et al*. (1996).

First, we studied the within-population polymorphism using samples from February 1995. These snails were additional to those used for progeny arrays. We calculated the mean number of alleles (n_{md}), the observed heterozygosity (H_o) and the gene diversity (H_e). The estimator of F_{is} values was computed using the software Genepop 2.0 (Raymond & Rousset 1995). From these values, we estimated the outcrossing rates (t_f) from natural populations using the classical formula:

$$F_{is} = (1-t_f)/(1+t_f).$$

The populations Mari Sud, Bala and Doubalma have previously been analysed (F. Viard, F. Justy & P. Jarne, unpublished data).

The progeny-array analyses were performed as follows. Just after collection in the field, the sexual morph of several adults per population was checked. These individuals were thereafter isolated in plastic boxes, and their first egg capsules collected over 6 d. This experimental design relies on the ability of freshwater snails to store allosperm obtained from prior copulations as females, and to use it for several days or weeks after copulation (Jarne *et al*. 1993). Sperm storage is well documented in *B. truncatus* (Larambergue 1939). Adults were then frozen in liquid nitrogen, before being brought back to the laboratory in France for microsatellite analysis, except for snails from Kotaki which were brought back just after sampling in the field and before egg collection. Egg capsules were brought back to the laboratory in France where juveniles grew up. The number of families and offspring analysed per population is given in table 1. The hatching rate was low, and juvenile mortality high, when compared with previous studies (Doums *et al*. 1996*b*), surely as a consequence of the egg capsules being transferred from Niger to France. However, inbreeding depression, which would bias our estimates of the selfing rate, is an unlikely cause, based on the results of Doums *et al*. (1996*b*) who found limited inbreeding depression in two populations of *B. truncatus* from Niger.

Single locus and/or multilocus estimates of the proportion of progenies due to outcrossing were computed in two ways.

Table 1. *Population and family characteristics*

(Aphally ratios are given together with the number of individuals checked in parentheses. The density is given as the collection yield per person and per minute. Key statistics for the within-population polymorphism: N, n_{md}, H_o and H_e are the sample size, the mean number of alleles per locus, the observed heterozygosity and the gene diversity, respectively, $\hat{f}$ is the inbreeding coefficient over all loci and t_f is the outcrossing rate estimated from $\hat{f}$ values. For the progeny-array analysis, date, families and offspring refer to the sampling date, the number of families with aphallic (A) and euphallic (E) mothers, and the number of offspring studied, respectively; n_{all} is the number of alleles in the progeny arrays at loci BT1, BT6, BT12 and BT13, respectively.)

Population	Mari Sud	Bala	Doubalma	Ligido	Kotaki
aphally ratio	0.71 (480)	0.30 (202)	0.59 (254)	0.84 (51)	0.48 (830)
density	5.9	1.8	3.5	3.2	1.8
N	36	36	30	41	32
n_{md}	8.5	2.8	2.0	3.3	3.3
H_o	0.14	0.01	0.02	0.05	0.04
H_e	0.74	0.20	0.16	0.32	0.23
$\hat{f}$	0.81	0.93	0.88	0.85	0.84
t_f	0.10	0.04	0.06	0.08	0.09
date (1995)	24 Feb.	20 Feb.	22 Feb.	11 Jan.	16 Jan.
families (A+E)	8+8	3+7	6+2	4+8	6+5
offspring	102	85	55	108	97
n_{all}	2, 3, 10, 12	1, 1, 3, 4	1, 2, 2, 5	1, 1, 4, 3	1, 1, 6, 7

First, as the occurrence of a non-maternal allele in a progeny indicates an outcrossing event, the proportion of progeny exhibiting non-maternal alleles provided a minimum multilocus estimate of the actual outcrossing rate (Städler *et al*. 1995). Second, we used the procedure of Ritland (1986) run with MLTR software (version 0.9; K. Ritland, unpublished data). MLTR generates maximum likelihood estimates (ML) of single and multilocus outcrossing rates (t_s and t_m respectively). The comparison of single and multilocus estimates gives some insight into the amount of inbreeding occurring from processes other than selfing, such as biparental inbreeding (Ritland & Jain 1981). The MLTR program also estimates the correlated mating parameters, namely the correlation of selfing (r_s) and the correlation of outcrossed paternity (r_p) within progeny arrays (Ritland 1989; Städler *et al*. 1995). A lack of correlation of selfing ($r_s = 0$) indicates that the selfing rate does not vary among families, whereas a correlation of 1 suggests that the sibships are either all selfed, or all outcrossed. More generally, large values of r_s indicate substantial heterogeneity of family outcrossing rates. The correlation of outcrossed paternity, r_p, can also be understood as the proportion of full sibs among outcrossed sibs (Ritland 1989). Of the two algorithms available in MLTR for the recursions, namely the expectation–maximization (EM) and the Newton–Raphson (NR) methods, we chose the EM method which is more suited for highly inbred species (Ritland 1986). The maximum number of iterations allowed was used and all variances of the estimates were estimated using 500 bootstraps. MLTR is currently dimensioned for a maximum of eight alleles per locus. The population of Mari Sud, which exhibits more than eight alleles at the BT12 and BT13 loci (ten and 12 alleles respectively), was analysed by pooling the less frequent alleles (those with a frequency lower than 0.05), so that the loci BT12 and BT13 exhibited only seven alleles each.

In order to analyse the relationship between the AR and the selfing rate at both the family and population levels, we first calculated the Pearson product-moment correlation coefficient between these parameters after arcsine transformation. Second, we tested for an effect of the population, the sexual morph and their interaction on the family selfing rate using an analysis of deviance on proportional data, which assumes that the error term of the model follows a binomial distribution. This analysis was performed using the software GLIM (Baker & Nelder 1985). To account for overdispersion, the significance of each term of the model was tested using an *F*-test according to Crawley (1993, p. 278).

3. RESULTS

Key statistics describing the within-population polymorphism are given in table 1. The five populations, especially Mari Sud, exhibited a high genetic variability. They were also characterized by a low number of observed heterozygotes and large $\hat{f}$-values, indicating the occurrence of pronounced heterozygote deficiencies. The selfing rates estimated from $\hat{f}$-values were above 0.90. The high variability was reflected in the large number of alleles found among progeny arrays (table 1). For example, Mari Sud exhibited 12 alleles at locus BT13 over the 16 families analysed.

With the exception of two families in both Bala and Kotaki, maternal genotypes were known, so that a direct count of non-maternal alleles was possible (table 2). In the 53 families for which the maternal genotypes were known, non-maternal alleles were found in 20.6, 14.9, 14.6, 2.8 and 19.8 % of the offspring in Mari Sud, Bala, Doubalma, Ligido and Kotaki respectively. Using MLTR, 17 families out of the 57 studied had outcrossed progenies (table 2). The direct count and MLTR method gave very similar family outcrossing rates (Pearson correlation coefficient $r = 0.993$, $n = 17$, $p < 10^{-5}$). Moreover, no additional families were found to have outcrossed progenies using MLTR. Using the inferred maternal genotype of the four unknown mothers, the outcrossing rates were 12.9 and 16.5 % in Bala and Kotaki, respectively.

Table 2. *Estimates of family outcrossing rates*

(Family refers to the name of the family followed by the sexual morph of the mother (A, aphallic, E, euphallic). Estimates are given for the families exhibiting some outcrossing only. They are based on the direct count of non-maternal alleles (t_{mm}) and on the maximum-likelihood procedure (ML) for which standard deviations obtained from 500 bootstraps are given in parentheses. *N* is the number of offspring per family.)

population/ family	*N*	t_{mm}	ML
Mari Sud			
5E	4	0.50	0.50 (0.23)
6A	6	0.17	0.17 (0.17)
7E	4	0.75	0.76 (0.16)
8E	5	0.20	0.20 (0.17)
14A	4	1.00	1.00 (0.00)
15A	8	1.00	1.00 (0.00)
19A	13	0.15	0.16 (0.15)
Bala			
21E	6	0.67	0.71 (0.20)
23E	6	0.17	0.17 (0.14)
29E	6	1.00	1.00 (0.00)
Doubalma			
7A	9	0.11	0.11 (0.12)
28A	8	0.63	0.74 (0.19)
34E	6	0.33	0.35 (0.20)
Ligido			
3A	7	0.43	0.44 (0.20)
Kotaki			
10A	8	0.75	0.89 (0.14)
58E	10	0.80	0.80 (0.13)
62E	8	0.25	0.25 (0.15)

Low population-level outcrossing rates were found (table 3), though some variation was evident, from 2.8 % in Ligido to 21.8 % in Mari Sud. Single locus (t_s) and multilocus estimates (t_m) were almost identical except in Mari Sud, and the difference between t_m and t_s was generally positive (except in Doubalma). The large discrepancy between the t_s value (12.8 %, see table 3) and the direct count (20.6 %) in Mari Sud can be explained as an artefact of pooling alleles. Indeed, the outcrossing rate in Mari Sud decreased to 9.8 % when pooling rare alleles before the direct count. The very large values of *F* obtained with MLTR may be related to the large number of homozygous mothers for all the loci (50 mothers out of 53). The correlation of selfing (r_s) exhibits variability among populations, with large to very large values in Bala, Kotaki and Mari Sud, and low values in Ligido and Doubalma. Very large values of r_p were observed in all populations, except in Doubalma, suggesting that more than 90 %

Table 3. *Estimates of population-level outcrossing rates and correlated mating*

(Estimates of parental inbreeding coefficient (F), multilocus (t_m) and single locus (t_s) outcrossing rates, correlated selfing (r_s) and correlated paternity (r_p). Values are given with their standard deviation in parentheses, which were obtained from 500 bootstraps. Estimates in Mari Sud were obtained after pooling some rare alleles.)

	F	t_m	t_s	r_s	r_p
Mari Sud	0.931 (0.033)	0.218 (0.094)	0.128 (0.064)	0.669 (0.054)	0.901 (0.283)
Bala	0.990 (0.001)	0.144 (0.086)	0.102 (0.067)	0.810 (0.375)	0.990 (0.411)
Doubalma	0.857 (0.152)	0.205 (0.114)	0.217 (0.134)	0.367 (0.190)	0.302 (0.156)
Ligido	0.990 (0.001)	0.028 (0.027)	0.026 (0.026)	0.386 (0.022)	0.903 (0.029)
Kotaki	0.990 (0.001)	0.178 (0.101)	0.172 (0.103)	0.670 (0.151)	0.990 (0.001)

Table 4. *Effects of population, sexual morph and their interaction (population × morph) on the family selfing rate*

factors	scaled deviance	degrees of freedom	F-test	p value
population	19.8	4	$F_{[4,47]} = 1.19$	0.33
morph	4.5	1	$F_{[1,47]} = 1.08$	0.30
population × morph	14.8	4	$F_{[4,47]} = 0.89$	0.48
error	195.3	47		

of the outcrossed sibs are full sibs. This result agrees with a direct observation of the multilocus genotypes (data not shown) as, despite the large number of alleles, the outcrossed progeny always exhibited the same non-maternal allele within-family for all but one family. These observations, and the r_p values taken together, strongly suggest single paternity.

The AR of natural populations and the population-level outcrossing rate are not correlated ($r = -0.48$, $n = 5$, $p = 0.41$). The analysis of deviance showed that neither the factors tested (population and morph), nor their interaction, had a significant effect on the selfing rate (table 4). The full model, including the population and morph effects as well as their interaction, explained only 16.7 % of the total deviance. Both sexual morphs indeed exhibit a wide variation (from 0 to 1) in selfing rates (table 2).

4. DISCUSSION

A main result of our study is that *B. truncatus* is a highly selfing species, with average values of the selfing rate being higher than 80 % in the five populations studied, whatever their AR. Our analysis also showed that this results from most individuals only self-fertilizing, and the remaining individuals exhibiting any value of the selfing rate, whatever their sexual morph. Moreover, the high level of correlated paternity indicates that most of the outcrossed offspring are full sibs. Progeny-array analyses have previously been used in one animal species only, and showed large among-family differences together with high selfing rates for one population of the freshwater snail *Ancylus fluviatilis* (Städler *et al.* 1995). Our study also furnishes the first precise estimates of the selfing rate in those highly selfing species that typically exhibit such a low genetic variability that allozymes are almost useless for studying their mating system. Excellent examples of such a situation can be found in the aphallic land snail *Chondrina clienta*, (Baur & Klemm 1989), and in two annual monoecious herbs, *Begonia hirsuta* and *B. semiovata* (Ägren & Schemske 1993). Our results therefore show the power of microsatellites for genetic studies in populations submitted to recurrent bottlenecks, or in endangered species that exhibit little allozyme polymorphism (Barrett & Harder 1996).

A very satisfying result is that our estimates of the selfing rate at the population level are in agreement with previous crossing experiments (Doums *et al.* 1996*b*) and population genetic analyses (estimates from F_{is} values; Viard *et al.* 1996). Crossing pairs of euphallic individuals from Mari Sud, and analysing offspring genotypes with microsatellites, we previously obtained a selfing rate of 0.84, versus 0.78 here (Doums *et al.* 1996*b*). However, pairing mature individuals in the laboratory suffers from some limitations. For example, copulation is somewhat enforced, and little environmental variation is allowed. In natural populations, the actual selfing rate will depend on a suite of environmental conditions (e.g. the opportunity to find a partner), which are taken into account in estimates from both the inbreeding coefficient method and progeny-array analyses. Second, the selfing rates derived from population genetic analyses were always high and close to those obtained with progeny arrays (table 1; Viard *et al.* 1996; F. Viard, F. Justy & P. Jarne, unpublished data). This method can therefore certainly be used to form an idea on the selfing rate, even in highly selfing species. However, it gives no idea about the selfing rates at the family level.

Our results are also consistent with studies on inbreeding depression. The evolution of the selfing rate is indeed driven by various factors, among which inbreeding depression features prominently. Based on the partial dominance model, theoretical studies have suggested that the magnitude of inbreeding depression should decrease with increased inbreeding (reviewed in Jarne & Charlesworth 1993). Husband & Schemske (1996) substantiated these predictions in a survey of 54 plant species for which both the selfing rate and data on inbreeding depression were available. The inbreeding depression in selfers was found to be 43 % of that in preferential outcrossers. Although such a large data set is not available in animals, a similar trend has been found (Doums *et al.* 1996*b*). Little inbreeding depression was observed in *B. truncatus* (Doums *et al.* 1996*b*), in agreement with the high selfing rates observed here.

The technique used here to estimate the selfing rate also provided some information on the number of fathers contributing to outcrossed sibships (multiple paternity). In four out of five populations, very high values of the correlation of paternity indicated single paternity. This may be explained first by a limited number of copulations, for example resulting from selection for low copulation rates in highly selfing species (Doums *et al.* 1996*b*) or because the density of snails is too low. The latter hypothesis can probably be rejected since we failed to detect a correlation between density and the outcrossing rate ($r = 0.33$, $n = 5$, $p = 0.59$). A second explanation is that copulation is not necessarily followed by sperm transfer, and a third one is competition among sperm from various partners. Studies of mate choice including genetic markers designed to distinguish between these two explanations have not been performed in *B. truncatus*. However, evidence of sperm competition is available in freshwater snails (Vianey-Liaud 1997). We also note that *B. truncatus* can store sperm for periods probably longer than the time-lag between two copulation events. The high values of r_p, together with the high values of r_s and the fact that most inbreeding is due to selfing in the populations studied (indicated by the limited differences between single and multilocus estimates of the selfing rate), also suggest that Ritland's correlated mating model may be appropriate to describe the functioning of our populations (Ritland 1989), at least more appropriate than the single mixed-mating model.

Large among-family variations in the selfing rate were also observed in the five populations studied. Using a phenotypic ESS-type model, Jarne *et al.* (1992) showed that the equilibrium AR depends on the differences in selfing rate between aphallic and euphallic individuals. But the variations observed here do not appear to be related to any behavioural differences between the two sexual morphs, since no morph effect was detected in the family selfing rate. These among-family differences may result from ecological variations (e.g. variable period between the most recent mating event as female and sampling, and aggregative distribution of snails). However, individuals in *B. truncatus* highly self-fertilize even when they are given the opportunity of a partner. This indicates that the selfing rate may have a genetic basis.

We failed to observe any correlation between the selfing rate and the AR. This trend was previously observed on a larger data set in which selfing rates were estimated from *F* values on 14 populations from Niger. This runs counter to the intuition that the AR and the selfing rate are linearly related (Schrag *et al.* 1994). More generally, any theory explaining the evolution of phally polymorphism has to cope with high selfing rates, whatever the AR. This also gives some credence to the idea that phally polymorphism may evolve, not only under selective pressure (Schrag *et al.* 1994), but also because of stochastic factors, as previously suggested by population genetic surveys (Viard *et al.* 1996). In light of these results, the role of stochastic factors as a major force in the evolution of both selfing and sexual polymorphisms might have to be reconsidered.

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