Microsatellites and the Genetics of Highly Selfing Populations in the Freshwater Snail *Bulinus truncatus*

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ABSTRACT

Hermaphrodite tropical freshwater snails provide a good opportunity to study the effects of mating system and genetic drift on population genetic structure because they are self-fertile and they occupy transient patchily distributed habitats (ponds). Up to now the lack of detectable allozyme polymorphism prevented any intrapopulation studies. In this paper, we examine the consequences of selfing and bottlenecks on genetic polymorphism using microsatellite markers in 14 natural populations (under a hierarchical sampling design) of the hermaphrodite freshwater snail *Bulinus truncatus*. These population genetics data allowed us to discuss the currently available mutation models for microsatellite sequences. Microsatellite markers revealed an unexpectedly high levels of genetic variation with ≤41 alleles for one locus and gene diversity of 0.20–0.75 among populations. The values of any estimator of F_{is} indicate high selfing rates in all populations. Linkage disequilibria observed at all loci for some populations may also indicate high levels of inbreeding. The large extent of genetic differentiation measured by F_{st}, R_{st} or by a test for homogeneity between genic distributions is explained by both selfing and bottlenecks. Despite a limited gene flow, migration events could be detected when comparing different populations within ponds.

THE genetic structure of subdivided populations has classically been viewed as resulting from a balance between selection, genetic drift and migration in populations of finite and constant size. More recently, the influence of population extinction and recolonization processes has been modeled (SLATKIN 1985; BARTON and WHITLOCK 1995). However, real situations are even more complex, with diverse forces acting on the way genes are distributed within and among populations. Here, we will consider the effects of two of these forces, namely the mating system (self-fertilization) and population dynamics (bottlenecks), on the distribution of neutral variability.

Selfing is known to reduce the frequency of heterozygotes and to create inbred lines within populations (see *e.g.*, HARTL and CLARK 1989). What is probably less well known is that selfing leads to a loss of genetic variability because of genetic hitchhiking (HEDRICK 1980), background selection against deleterious mutations (CHARLESWORTH *et al.* 1993) and decreased effective population size (POLLAK 1987). These theoretical expectations have found an empirical basis in surveys of hermaphroditic plants (HAMRICK and GODT 1990; SCHOEN and BROWN 1991) and animals (JARNE 1995). Selfing also influences the distribution of variability among populations to an extent related to the amount of gene flow. Empirical studies indeed revealed that indices of population structure (F_{st} or equivalent) are much higher in groups of selfing populations than in groups of outcrossing populations (HAMRICK and GODT 1990; SCHOEN and BROWN 1991; JARNE 1995).

The dynamics of variability also depend on more ecologically determined processes. Temporal and/or geographical variation in ecological conditions may induce large variation in population size, or even population extinction, that may be followed more or less quickly by recolonization. Some authors have shown that the genetic differentiation among populations could increase with extinction/colonization processes (*e.g.*, WADE and MCCAULEY 1988; review in BARTON and WHITLOCK 1995). This may be true with population bottlenecks in general. Bottlenecks also drive neutral variability to very low levels though the extent of the loss in variability is a function of population size during the bottleneck and the population growth after the bottleneck (references in TAJIMA 1989). Overall, both selfing and bottlenecks are expected to strongly depress neutral variability within populations and to increase differentiation among populations.

When measured using allozymes, the variability of highly selfing populations was often seen to be reduced to almost zero, with often little variation among populations (examples in SCHOEN and BROWN 1991; JARNE 1995). Fine scale analyses of such populations to test

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the above mentioned predictions, as well as to understand the evolution of high selfing rates, therefore require more polymorphic markers. The most promising candidates are microsatellites, which exhibit the same desirable attributes as population genetic markers as allozymes (codominance, Mendelian inheritance, presumed neutrality), though are much more variable and occur in large numbers in eukaryotic genomes (STALLINGS *et al.* 1991; QUELLER *et al.* 1993; ARENS *et al.* 1995). Microsatellite loci consist of tandem repeats of a short nucleotide motif, and an allele is defined as a number of repeats. The mutational mechanisms are still debated, but strand slippage during replication could be a major mechanism for the formation of new alleles (WOLFF *et al.* 1989). On this basis, two mutation models have been proposed. First, the stepwise mutation model (SMM), originally developed for allozyme data (OHTA and KIMURA 1973), assumes that alleles increase or decrease in length by one repeat unit (VALDÈS *et al.* 1993). Second, the two-phase model (TPM) assumes that alleles can mutate according to the SMM with probability p; changes of more than one repeat occur with probability $1 - p$ (DI RIENZO *et al.* 1994). Although the TPM with $p \neq 1$ seems more appropriate (DI RIENZO *et al.* 1994), it has not been possible to reject the SMM (TPM with $p = 1$) or even the infinite allele model (IAM) (KIMURA and CROW 1964) in several examples (VALDÈS *et al.* 1993; ESTOUP *et al.* 1995). The mutation process in natural populations is mainly known through the comparison of expected and observed distributions of alleles (DI RIENZO *et al.* 1994; ESTOUP *et al.* 1995; see STRAND *et al.* 1993 for an exception). Thus, information on the genomic dynamics of microsatellites can be gained from population genetic studies. That microsatellites may evolve in a stepwise fashion has also to be considered when analyzing data because the mutation model and rate are instrumental parameters in population genetic theory. For example, the classical relationship between $\hat{F}_{st}$ and Nm may not hold with the SMM (SLATKIN 1995; ROUSSET 1996).

Data on the population genetics of selfing animals have been obtained mainly in snails, especially freshwater species (JARNE 1995 for review). Tropical populations of this latter group occupy patchily distributed, transient habitats and regularly experience population size restriction. Their genetic structure is therefore appropriately analyzed in the framework of the theory of subdivided populations (JARNE and STÄDLER 1995). Populations experiencing both bottlenecks and high selfing rates basically have no allozyme variability. This is especially true in the widespread species *Bulinus truncatus*, which prompted us to characterize microsatellite loci (JARNE *et al.* 1994). Taking advantage of the variability uncovered at several microsatellite loci, the purpose of this study is to analyze the distribution of genetic variability at various geographic scales in *B. truncatus* and to test the metapopulation model proposed earlier (NJIOKOU *et al.* 1993). Our goal is therefore to estimate the prevalence of selfing and gene flow among populations characterized by different levels of habitat stability and geographical distance. Our data also allow us some suggestions about the evolution of microsatellites.

MATERIALS AND METHODS

Species studied and sampling localities: *B. truncatus* (Gastropoda, Pulmonata) is distributed over most of Africa and some Mediterranean islands where it occupies various kinds of freshwater habitats such as rivers, ponds and irrigation systems (BROWN 1994). Habitats are subject to annual cycles of drought and floods with large fluctuations in population size and water surface available for freshwater species (*e.g.*, VERA *et al.* 1995 in Niger). The type of habitat (permanent or temporary) is an indicator of the pond dynamic. *B. truncatus* is one of the major vectors of various species of schistosomes, agents of bilharzioses in humans and cattle. It is a simultaneous hermaphrodite that can self-fertilize. This species is characterized by the occurrence of two sexual morphs: regular hermaphroditic individuals, referred to as euphallic individuals, and aphallic individuals, which are deprived of the male copulatory organ but still can self-fertilize, or outcross as female when euphallics are also present (DE LARAMBERGUE 1939). Aphally is determined by both environmental and genetic factors (DE LARAMBERGUE 1939; SCHRAG and READ 1992; DOUMS *et al.* 1996). The aphally ratio (frequency of aphallic individuals) is highly variable among natural populations (SCHRAG *et al.* 1994). Such a sexual polymorphism has implications on the selfing rate, because selfing is obligatory in strictly aphallic populations. However, no association between aphally ratio and population selfing rate has yet been demonstrated. As both sexual morphs can self-fertilize and given the low variability observed using population allozyme data, *B. truncatus* has been assumed to be a highly selfing species (JELNES 1986; NJIOKOU *et al.* 1993). *B. truncatus* is also an allotetraploid species with disomic inheritance (GOLDMAN *et al.* 1983).

Fourteen population samples originating from Niger were collected in February 1994 (Figure 1). For each population, three to four persons collected individuals for a period of 30 min on average over a large area ($\sim$500 m^2) to avoid sampling only few families. Snails were then brought back to the laboratory and individuals were checked for their sexual morph. The aphally ratio was estimated in each sample as the ratio of aphallic over all individuals. Aphallic and euphallic individuals were then frozen separately until DNA extraction. Genotypes were obtained from a total of 399 individuals.

We collected these samples to illustrate the variation of different factors. Ponds were classified according to water availability as permanent, semipermanent or temporary. Besides covering different aphally ratios and types of habitat (Table 1), samples originated from various regions. In large ponds of more than several squared kilometers such as Boyze, Namaga and Mari, several samples were collected to contrast the variation within and among ponds (Figure 1).

Analysis of microsatellite loci: DNA extraction was performed according to JARNE *et al.* (1992). Four microsatellite loci (BT1, BT6, BT12 and BT13) were used among those previously characterized (respectively loci 1, 5, 11 et 12 in Table 1 in JARNE *et al.* 1994). BT1 and BT6 are dinucleotide repeat loci and BT12 and BT13 are tetranucleotide repeat loci. They were chosen because of their high polymorphism and unambiguous amplification pattern. DNA samples were amplified according to the protocol described in JARNE *et al.* (1994), slightly modified. The modifications were as follows:

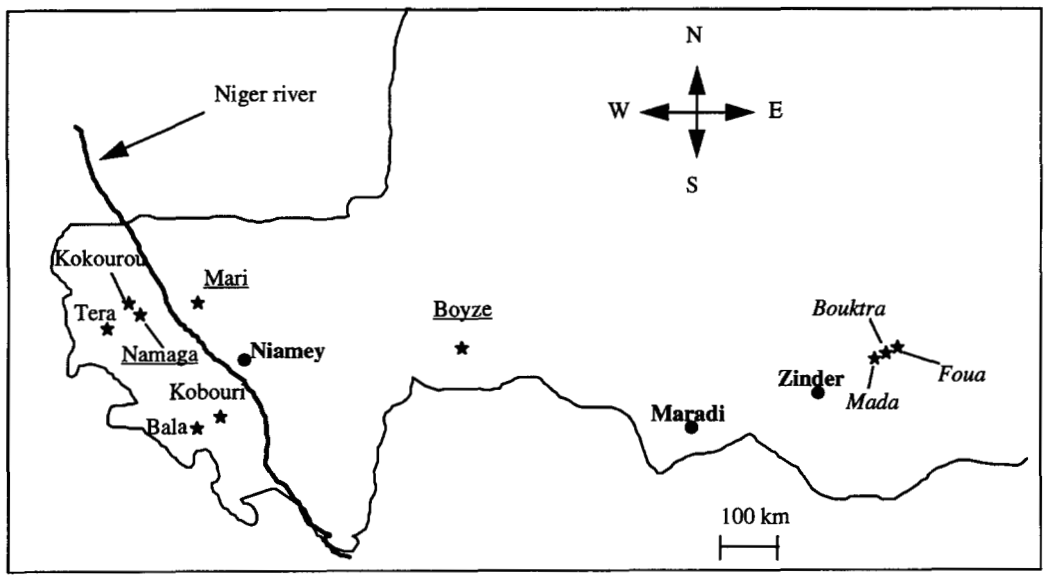

FIGURE 1.—Map of Southwestern Niger with sample collection sites indicated by stars. Underlined locality names indicate ponds where more than one sample was collected.

15 μM dATP were used instead of 6 μM, 1 μCi of [^{35}S]-dATP (Amersham) instead of 5 μCi, 2% BSA (bovine serum albumin) instead of 0.08% and, for BT1 1 mM $MgCl_2$. Amplification reactions were performed in a Biometra thermocycler. Initial denaturation was 4 min at 95° followed by 25 cycles [94° for 30 sec, 30 sec (40 sec for BT13) at 50° for BT1 and BT6, 52° for BT12 and 58° for BT13, and 72° for 30 sec] and 10 min at 72°. Each PCR product (8 μl) was then mixed with 7 μl of formamide loading dye and denatured by heating at 95° for 3 min before being loaded onto a denaturing sequencing gel (6% acryl-bisacrylamide and 8 M urea). Several M13 sequencing reactions (T7 Sequencing Kit, Pharmacia) were also loaded adjacent to the samples to serve as an absolute size control. Gels were run for 3–5 hr at 1800 V, dried and exposed to X-Ray film for 24–48 hr.

TABLE 1

Population characteristics

Population	*N*	AR	Habitat
Bala	30	0.36 (83)	T
Boyze I	30	0.88 (102)	P
Boyze II	25	0.94 (165)	P
Bouktra	22	0.89 (19)	T
Foua	14	0.88 (8)	T
Kobouri	19	0 (23)	P
Kokourou	32	0.67 (116)	T
Mada	36	1 (59)	T
Mari Sud	29	0.84 (129)	SP
Mari Nord	31	0.76 (37)	SP
Namaga PM	32	0.69 (64)	SP
Namaga B	24	0.50 (192)	SP
Namaga W	39	0.77 (47)	SP
Tera	36	0.74 (54)	P

Aphally ratio (AR) with the number of individuals checked for their sexual morph in parentheses; type of habitat (T, temporary pond; P, permanent pond; SP, semipermanent pond) of the natural populations and number of individuals studied. Boyze I and Boyze II are separated by 3 km, Mari Sud and Mari Nord by 0.7 km; Namaga W is separated from Namaga B and Namaga PM by 4 and 3.5 km, respectively, and Namaga B is 1.5 km from Namaga PM.

Statistical analysis: Genetic polymorphism in each population was measured as the mean number of alleles per locus, observed heterozygosity and gene diversity (NEI 1987). Allelic frequencies are not provided because of space limitation and are available upon request from P. JARNE.

Deviations from Hardy-Weinberg expectations, genotypic linkage disequilibria and differentiation among populations or groups of populations were tested using the package GENEPOP, version 1.2 (RAYMOND and ROUSSET 1995). The test of Hardy-Weinberg proportions for less than five alleles is an exact test. For five alleles or more, a Markov chain method is used to obtain an unbiased estimate of the exact probability. As the gametic composition of double heterozygotes were not available, the genotypic linkage disequilibria is defined in terms of two-locus genotypic counts (WEIR 1990, p. 102). The null hypothesis is that genotypes at one locus are independent from genotypes at the other locus. To test for genotypic linkage disequilibrium and genetic differentiation among populations, GENEPOP also uses a Markov chain method. In all cases, the Markov chain was set to 100,000 steps and 1000 steps of dememorization. Standard errors were always below 0.005. The overall significance of multiple tests was estimated by Fisher's combined probability test.

We estimated the selfing rates (S) from natural populations using the classical formula $F_{is} = S/(2 - S)$. Indeed, this relation holds whatever the mutation model (IAM or SMM) as demonstrated analytically by ROUSSET (1996). This assumes genetic equilibrium under a mixed-mating model (see, *e.g.*, HARTL and CLARK 1989). The estimator $\hat{f}$ of F_{is} values (WEIR and COCKERHAM 1984) were estimated using the software FSTAT (GOUDET 1995).

The number of alleles, the number of heterozygotes and the heterozygote deficiencies (via the $\hat{f}$ values) are related to the selfing rate. We analyzed the relationship between the aphally ratio and the selfing rate by regressing all these parameters on the aphally ratio. A Kruskal-Wallis test (SOKAL and ROHLF 1995, pp. 423–427) was performed to test for the relationship between the type of habitat and the two parameters cited above.

F-statistics were computed according to WEIR and COCK-

TABLE 2

Microsatellite polymorphism

	BT1	BT6	BT12	BT13
T^a	4 (1–3)	10 (1–4)	35 (2–11)	41 (2–15)
N_p^b	2.1 (0.9)	2.3 (1.0)	6.7 (3.1)	7.7 (4.6)
Size range (bp)	176–184	116–165	206–398	244–436

Total number (T), minimum (m) and maximum (M) number of alleles observed within populations and mean number of alleles per population (N_p). Size range refers to the size of the amplified product.

[a] Values in parentheses are range of m to M.

[b] Values in parentheses are standard deviation.

ERHAM (1984) using the software FSTAT, version 1.2 (GOUDET 1995) which allows the calculation of the estimator $\hat{\theta}$ of the F_{st} values between each pair of populations. $\hat{\theta}$ is classically used to infer gene flow (Nm) among populations. In a finite island model, $Nm = [(1/\hat{\theta}) - 1]/4$. However, SLATKIN (1995) pointed out that $\hat{\theta}$ may be inappropriate with microsatellite loci because of their mutation process. Using the coalescence theory, this author developed R_{st}, a statistic based on variance in allelic size. He showed that in a finite island model at equilibrium, R_{st} is related to Nm according to the formula $Nm = [(d_s - 1)/4d_s]\ [(1/R_{st}) - 1]$ where d_s is the number of populations sampled. MICHALAKIS and EXCOFFIER (1996) demonstrated that R_{st} is formally identical to φ_{st} originally developed to analyze differentiation among haplotypic data. For the purpose of comparing $\hat{\theta}$ and R_{st} in our data set, we computed φ_{st} (R_{st}) at different scales (intrapond and among all ponds) using the software WINAMOVA (EXCOFFIER *et al.* 1992). MICHALAKIS and EXCOFFIER (1996) developed a procedure to handle diploid data. Haplotypic distances required by WINAMOVA were calculated using a program developed by Y. MICHALAKIS (personal communication). Our data set corresponds to a total of 262 haplotypes. As WINAMOVA is currently dimensioned for 255 haplotypes, we estimated R_{st} among all ponds taking into account only one of three populations in Namaga, and one of two in both Boyze and Mari.

To test for isolation by distance (SLATKIN 1993), Mantel tests were performed to analyze the independence between geographical and genetic distances using the GENEPOP software. Ten thousand permutations were done. The statistic used is Spearman's rank correlation coefficient. Geographical distances among and within ponds were the shortest distances measured on a map and in the field respectively. Genetic distances were $\hat{\theta}$ values.

RESULTS

Microsatellite polymorphism: As *B. truncatus* is an allotetraploid species, we first tested for coamplification of homeologous loci from the two parental genomes. Alleles at the four loci were demonstrated to be inherited in a Mendelian fashion using selfed families maintained in the laboratory (data not shown). As coamplification never occurred, loci were subsequently analyzed as diploid.

A high polymorphism was observed, especially at tetranucleotide loci (Table 2). Alleles generally differed by multiples of 2 bp for BT1 and BT6 and by multiples of four bp for BT12 and BT13, with only few exceptions (alleles *179* at BT1, *116* at BT6, *223* at BT12, *244* and *254* at BT13). The total number of alleles observed in the 14 populations is especially high for the loci BT12 and BT13 (Table 2). Mean numbers of alleles per population vary among loci, with large values for BT12 and BT13 (Table 3). The variances of allele size are very large. For example at locus BT13, the minimum and maximum size are 244 and 436 bp, respectively, which corresponds to 26 and 74 (GATA) repeats assuming that changes occurred only by insertion or deletion of repeat units. At BT12, the minimum size is 206 bp $[(GATA)_{24}]$ and maximum size is 398 $[(GATA)_{72}]$. Distributions of allelic frequencies for each population vary among loci with monomodal patterns for BT1 and more complicated ones for the tetranucleotide repeats. Comparisons of allelic distributions for a given locus also indicated large differences among populations. For example, we observed at locus BT13 a monomodal distribution (Boyze II), a bimodal distribution (Mada) or more complex ones (Namaga PM and Mari Nord).

Hardy-Weinberg equilibrium, heterozygote deficiencies and selfing rate: Although some populations were monomorphic at loci BT1 and BT6, none of the 14 populations studied was monomorphic over all loci and all populations were highly polymorphic at loci BT12 and BT13 except those of Bala and Boyze (Table 3). Mean number of alleles per locus and average H_o and H_e for each population are given in Table 4. There was a significant correlation between the mean number of alleles per locus and observed heterozygosity (linear regression, $r = 0.618$, d.f. = 12, $P = 0.02$).

Gene diversity values were high and observed heterozygosity values always low in all populations. No heterozygotes were observed over all loci in Bala. At loci BT1 and BT6, no heterozygotes were observed in several populations (Table 3).

All loci showed highly significant deviations from genotypic proportions expected under Hardy-Weinberg equilibrium (Table 3), with significance level generally $<10^{-5}$ and always <0.014 (Tera, BT1). Combined probability tests over all loci (Table 4) revealed a highly significant departure from Hardy-Weinberg expectations for all populations. In five populations (Kokourou, Mari Nord, Namaga PM, Namaga B, Tera), there were enough individuals of both sexual morphs to compare aphallic individuals to euphallic individuals.

TABLE 3

Variability parameters for each population and locus

	Bala	BoyzeI	BoyzeII	Bouktra	Foua	Kobouri	Kokourou	Mada	Mari Sud	Mari Nord	Namaga PM	Namaga B	Namaga W	Tera
BT1														
N	60	60	44	44	28	36	64	70	58	58	64	48	78	72
N_{all}	1	1	1	1	2	1	3	3	3	2	3	3	3	2
SR	184	184	184	184	180–184	184	176–184	179–184	179–184	180–184	176–184	176–184	176–184	180–184
H_o	0	0	0	0	0	0	0	0.09	0.10	0.10	0.09	0.17	0.03	0
H_e	0	0	0	0	0.35	0	0.53	0.28	0.41	0.46	0.39	0.60	0.59	0.06
HW	—	—	—	—	0.001	—	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	0.014
BT6														
N	58	60	46	44	24	38	64	68	56	60	60	48	76	72
N_{all}	1	1	1	2	3	1	3	2	3	4	3	3	2	3
SR	116	163	163	157–165	157–165	155	143–157	157–165	116–157	116–159	143–157	143–157	143–157	143–161
H_o	0	0	0	0	0	0	0.13	0	0.07	0.13	0.10	0.17	0	0
H_e	0	0	0	0.24	0.51	0	0.67	0.44	0.56	0.64	0.52	0.60	0.37	0.42
HW	—	—	—	0.0002	0.0001	—	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$
BT12														
N	60	60	48	42	28	34	64	66	58	62	56	48	76	72
N_{all}	2	3	3	7	8	4	10	11	7	8	11	7	9	4
SR	258–262	278–286	278–286	206–398	214–394	278–290	242–286	206–298	250–318	246–318	238–282	238–278	226–278	238–298
H_o	0	0.10	0	0	0	0	0.19	0.09	0.21	0.19	0.20	0.13	0.03	0.03
H_e	0.36	0.53	0.44	0.80	0.87	0.48	0.86	0.82	0.81	0.86	0.84	0.85	0.85	0.62
HW	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$
BT13														
N	60	60	46	44	28	34	62	68	56	60	56	46	78	72
N_{all}	4	2	4	5	5	4	15	5	8	12	14	14	12	4
SR	276–288	424–428	424–436	256–276	248–296	328–340	252–420	244–272	256–368	256–384	254–424	256–424	254–408	344–384
H_o	0	0.20	0.13	0.05	0.07	0.12	0.26	0.03	0.36	0.23	0.04	0.17	0	0
H_e	0.43	0.43	0.67	0.43	0.73	0.74	0.93	0.71	0.81	0.87	0.87	0.92	0.86	0.48
HW	$<10^{-5}$	0.0058	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$

Number of alleles scored for each sample and locus (*N*), number of alleles (N_{all}), size range (*SR*), observed heterozygosity (H_o), gene diversity (H_e), and exact probability or unbiaised estimate of type-I error for departure from Hardy-Weinberg proportions (*HW*) per locus and population. —, irrelevant.

TABLE 4

Within-population polymorphism

	Bala	BoyzeI	BoyzeII	Bouktra	Foua	Kobouri	Kokourou	Mada	Mari Sud	Mari Nord	Namaga PM	Namaga B	Namaga W	Tera
N_{loc}	2.0	1.8	2.3	3.8	4.5	2.5	7.8	5.3	5.3	6.5	7.8	6.8	6.5	3.3
	± 1.4	± 1.0	± 1.5	± 2.8	± 2.7	± 1.7	± 5.9	± 4.0	± 2.6	± 4.4	± 5.6	± 5.2	± 4.8	± 1.0
H_o	0.00	0.08	0.03	0.01	0.02	0.03	0.14	0.05	0.18	0.16	0.11	0.16	0.02	0.01
	± 0.00	± 0.08	± 0.06	± 0.02	± 0.03	± 0.05	± 0.09	± 0.04	± 0.11	± 0.05	± 0.06	± 0.02	± 0.02	± 0.01
H_e	0.20	0.24	0.28	0.34	0.61	0.31	0.75	0.56	0.65	0.71	0.66	0.74	0.67	0.39
	± 0.23	± 0.28	± 0.34	± 0.36	± 0.23	± 0.37	± 0.18	± 0.25	± 0.20	± 0.20	± 0.24	± 0.17	± 0.23	± 0.24
All	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$

Mean number of alleles per locus (N_{loc}), observed heterozygosity (H_o) and gene diversity (H_e) averaged over loci for each population. "All" refers to overall significance for departure from Hardy-Weinberg proportions using Fisher's combined probability test. Values are means ± SD.

In the five populations the deviation from Hardy-Weinberg expectations was similar in both group (data not shown).

$\hat{f}$ values over all loci are very large, ranging from 0.69 to 1 and selfing rates ranged from 0.82 to 1 (Table 5).

Linkage disequilibrium: Exact tests for genotypic linkage disequilibrium resulted in 24 significant values ($P < 0.05$) out of 61 comparisons. In the populations Namaga PM, Boyze I and Boyze II, independence was rejected between BT12 and BT13 only ($P = 0.037$, 0.018, and 0.003, respectively). Only one significant value was observed in Foua and Bouktra (between BT6 and BT13 with $P = 0.007$ and 0.008, respectively). Six highly significant values out of six were observed in Kokourou. No pairs of loci appeared in linkage disequilibrium in the populations Bala, Kobouri, Mari Sud and Mari Nord. In the five populations for which aphallic and euphallic individuals were numerous enough, similar results were obtained for both sexual morphs and all individuals.

Influence of aphally and habitat: No correlation was found between the aphally ratio and the number of alleles (linear regression, $r = 0.14$, d.f. = 12, $P = 0.64$), observed heterozygosity ($r = 0.057$, d.f. = 12, $P = 0.85$) and selfing rate estimated with the $\hat{f}$ values ($r = -0.14$, d.f. = 12, $P = 0.63$), as well as between the type of habitat and either the aphally ratio (Kruskal-Wallis test, $H = 0.64$, d.f. = 2, $P = 0.58$) or the observed heterozygosity ($H = 4.61$, d.f. = 2, $P = 0.10$). However, the correlation was significant between habitat and number of alleles ($H = 7.17$, d.f. = 2, $P = 0.03$). More alleles were observed in temporary and semipermanent ponds than in permanent ponds.

TABLE 5

Fixation index and selfing rate estimates

Population	$\hat{f}$	$S(\hat{f})$
Bala	1.00	1.00
BoyzeI	0.69	0.82
BoyzeII	0.89	0.94
Bouktra	0.97	0.98
Foua	0.97	0.98
Kobouri	0.91	0.95
Kokourou	0.81	0.90
Mada	0.91	0.95
Mari Sud	0.72	0.84
Mari Nord	0.77	0.87
Namaga PM	0.83	0.91
Namaga B	0.79	0.88
Namaga W	0.98	0.99
Tera	0.98	0.99

$\hat{f}$ values according to WEIR and COCKERHAM (1984) and selfing rate S estimates using $\hat{f}$ values.

Among population structure: The overall differentiation among populations was highly significant ($P < 10^{-5}$) and corresponded to a $\hat{\theta}$ value of 0.375 (Table 6). At any scale, populations appeared structured. Indeed, intrapond analysis for Boyze, Mari and Namaga showed a highly significant differentiation over all loci with $\hat{\theta}$ values of 0.3, 0.01 and 0.07, respectively. When considering $\hat{\theta}$ values for each pair of populations, both high values (*e.g.*, 0.71 between Bala and Boyze I, 0.60 between Mada and Tera) and small values (*e.g.*, 0.12 between Kokourou and Foua, 0.05 between Kokourou and Namaga) were observed. $\hat{\theta}$ values were always smaller or equal to R_{st} values (Table 7). The number of migrants estimated from those parameters was small for Boyze and among all ponds. We also tested whether a difference was detectable between aphallic and euphallic individuals within the same population. A significant differentiation ($P < 0.05$) was observed in NamagaPM only.

A slight but significant pattern of isolation by distance was found. Indeed, the probability value of the Mantel's test value for rejecting independence between genetic and geographic distances was 0.048.

DISCUSSION

Evolution of microsatellite sequences: The difference in size between the shortest and the largest allele observed (49 bp for BT6 and 192 bp for both BT12

TABLE 6

Analysis of the genetic structure at the general (among all populations) and intrapond level

	General		Intrapond					
			Boyze		Mari		Namaga	
	P	$\hat{\theta}$	P	$\hat{\theta}$	P	$\hat{\theta}$	P	$\hat{\theta}$
BT1	$<10^{-5}$	0.50	—	—	0.119	0.00	$<10^{-3}$	0.10
BT6	$<10^{-5}$	0.58	—	—	0.010	0.03	$<10^{-5}$	0.08
BT12	$<10^{-5}$	0.25	$<10^{-5}$	0.35	0.009	0.00	$<10^{-5}$	0.05
BT13	$<10^{-5}$	0.27	$<10^{-5}$	0.31	0.024	0.00	$<10^{-5}$	0.06
Total	$<10^{-5}$	0.38	$<10^{-5}$	0.30	$<10^{-3}$	0.01	$<10^{-5}$	0.07

P is the exact probability of Fisher exact test. $\hat{\theta}$ is calculated according to WEIR and COCKERHAM (1984). —, monomorphic locus.

and BT13) as well as the size of some alleles appears unusually large when compared with others studies (AMOS *et al.* 1993; MORGANTE *et al.* 1994; TAYLOR *et al.* 1994; BANCROFT *et al.* 1995; GERTSCH *et al.* 1995; ISHIBASHI *et al.* 1995; PAETKAU *et al.* 1995), especially for tetranucleotide loci (WALL *et al.* 1993). Our results raise questions about the constraints acting on microsatellite size. Population genetic studies may be biased toward alleles of small size because screening methods and PCR are usually optimized for these alleles (<500 bp). However, it is clear from interspecies comparisons that microsatellites are limited in size (STALLINGS *et al.* 1991; FITZSIMMONS *et al.* 1995; GARZA *et al.* 1995). This may be explained by either asymmetrical mutation with higher mutation rates towards shorter length alleles (though no empirical data are available) or by a selective constraint on the maximal size that can be attained.

That allelic sizes are multiples of the basic motif (two for dinucleotide loci and four for tetranucleotide loci) indicates that slippage, adding or substracting one, two or more repeats is the basic process modifying microsatellites, which could favor a SMM or TPM. Indeed, sequencing of allele 420 from the population Namaga PM at locus BT13 revealed that 420 is a perfect $(GATA)_n$ repeat (the allele originally sequenced is 256). We observed some exceptions to this rule meaning that nucleotide insertion or deletion within or outside the microsatellite array are possible though occurring at a lower frequency. Further insights into the mutation model could be gained by comparing observed distributions with theoretical distributions generated under various assumptions (DI RIENZO *et al.* 1994; ESTOUP *et al.* 1995). Self-fertilization and fluctuating population dynamics have yet to be incorporated into these theoretical approaches before interesting comparisons may be done in *B. truncatus*.

Another issue is that the target species is allotetraploid having probably been formed through hybridization involving the ancestor of the extant species *B. tropicus* (GOLDMAN *et al.* 1983). Among the loci screened and amplified, we never observed coamplification of both sets of parental genomes. For reasons that are not clear the divergence between genomes may be large enough such that homologous sets of loci are never coamplified. Further insights would be gained by analyzing loci used in diploid species closely related to *B. truncatus* such as *B. tropicus*.

Estimating the selfing rate: The high variability uncovered in this study allows indirect estimation of selfing rate. $\hat{f}$ values lead to high selfing rate estimates. These results agree with experimental crossings performed in laboratory conditions: selfing rate, estimated on progeny arrays, were 0.78 and 0.84 for Kobouri and Mari Sud, respectively (C. DOUMS, F. VIARD, A.-F. PERNOT, B. DELAY and P. JARNE, unpublished data).

We observed large heterozygote deficiencies at all loci and populations studied. Heterozygote deficiencies constitute a recurrent theme in population genetics, and many plausible explanations have been proposed (ZOUROS and FOLTZ 1984). However, deficiencies of

TABLE 7

Estimates of the number of migrants from $\hat{\theta}$ and R_{st}

	d_s	$\hat{\theta}$	R_{st}	$Nm(\hat{\theta})$	$Nm(R_{st})$
Boyze	2	0.30	0.30	0.58	0.29
Mari	2	0.01	0.06	24.75	1.96
Namaga	3	0.07	0.10	3.32	1.50
All ponds	10	0.42	0.64	0.35	0.13

For analysis among all ponds data from only one population from Boyze, Mari and Namaga were used (see MATERIALS AND METHODS). d_s is the number of samples (populations).

the magnitude observed here (*e.g.*, Boyze I, BoyzeII and Kobouri) can only be explained by the high selfing rates estimated in *B. truncatus* species.

The lack of correlation between the aphally ratio and the selfing rate suggests that the aphally ratio is a poor indicator of the selfing ability in a given population. A note of caution is necessary because the aphally ratio is estimated in a given generation and the selfing rate is that of the previous generation. However, the aphally ratio in this species is stable over generation in natural populations (C. DOUMS, personal communication). Overall, our data from natural populations and laboratory crossings indicate that the individuals of *B. truncatus* populations preferentially self-fertilize whatever the aphally ratio in the population. This has consequences for some of the theories explaining the evolution of phally polymorphism. Assuming an approximately linear relationship between the aphally ratio and the selfing rate, SCHRAG *et al.* (1994) suggested that this evolution may be driven by parasites through the Red Queen process. If, as indicated here, this relationship does not hold, there is no reason to believe that parasites influence the aphally ratio through selection for recombination.

Within-population variability: Using allozymes in *B. truncatus,* various authors have shown that the mean number of alleles is 1–1.07, the average heterozygosity is 0 and the gene diversity is 0–0.005 (references in JARNE 1995), whereas values obtained here are much higher (Tables 3 and 4). This loss of variability observed in freshwater snails was explained by selfing and population dynamics as the eroding forces (JARNE and STÄDLER 1995). The difference is probably due to the higher mutation rates, by a factor 10 to 10,000 of microsatellites when compared with allozymes (DALLAS 1992; EDWARDS *et al.* 1992, ELLEGREN 1995). Given the strength of selfing and bottlenecks as forces reducing the variability in Niger populations of *B. truncatus,* it seems that mutation rates in between those of allozymes and microsatellites (say 10^{-5}–10^{-4}) would be necessary to maintain some intrapopulation variability. The variability we observed also depends on the loci studied, with similar levels of variability for dinucleotide repeat loci on one hand, and tetranucleotide repeat loci on the other hand. Relative estimates of the mutation rate for the two kind of loci can be obtained using a procedure developed by CHAKRABORTY and NEEL (1989). Assuming an equilibrium between migration and genetic drift under the IAM, these authors generalized EWENS' (1972) sampling formula. As this method assumes IAM, crude estimates of the mutation rates are obtained. However, this is of little concern since we are interested in relative mutation rates. To use the method of CHAKRABORTY and NEEL (1989) in selfing species, SCHOEN and BROWN (1991) assumed that the number of genes sampled equals the number of individuals, because in selfing populations individuals are highly inbred so that genes sampled from single individuals are likely to be identical by descent. Using these procedures for our data, the more polymorphic loci (BT12 and BT13) show a mutation rate about seven times higher than those of BT1 and BT6. Similar results have been previously found by ELLEGREN (1995). An alternative, though less likely, explanation of the lower variability of dinucleotide loci is genetic hitchhiking acting only on those loci (and over all populations) and not on tetranucleotide loci. This could be tested by extending the number of loci studied.

Comparisons of our results with previous studies using microsatellite markers are hampered by the lack of data in gastropods and detailed data sets in the literature. Despite a high variability when compared with allozymes, we observed a lower variability than has been found with microsatellites in species with small effective population size (black bear: PAETKAU and STROBECK 1994; polar bear: PAETKAU *et al.* 1995) or haplo-diploid insects (HUGHES and QUELLER 1993).

Besides selfing, the occurrence of bottlenecks may explain the within-population structure. Indeed, *B. truncatus* populations exhibit seasonal changes in density (VERA *et al.* 1995) and are likely to experience regular bottlenecks, as most populations of tropical freshwater snails (BROWN 1994). When we tested the influence of habitat on some genetic parameters, a significant value was observed only once (between the type of habitat and the number of alleles). Unexpectedly the highest and almost the lowest number of alleles were observed in Kokourou and Bala, respectively, both of which are temporary ponds. However, a low value, although not significant, showed a relationship between the type of habitat and the observed heterozygosity. A temporal analysis including a regular survey of the ponds would be worthwhile to analyze the stability of this relationship between bottlenecks and genetic parameters. Under both IAM and SMM (NEI *et al.* 1975; CHAKRABORTY and NEI 1977; SIRKKOMAA 1983), the average heterozygosity and number of alleles are expected to strongly decrease when a population goes through a bottleneck. The influence of the mutation rate was not investigated and these authors assumed very low values. This may be of concern for microsatellites given their high mutation rate. Moreover, the respective influence of bottlenecks, hitchhiking and background selection is difficult to evaluate because they all induce a decrease of neutral genetic variability.

Self-fertilization and bottlenecks may also interact, though to our knowledge no mathematical analysis has been developed to test whether the consequences of a bottleneck are more marked in selfing populations. This may be important because selfing species are quite often colonizing species that experience founder events.

From our data, large genotypic disequilibria were observed between different pairs of loci over a large num-

ber of populations. This may be a consequence of several factors including physical linkage between the loci studied, mating system, finite population size or migration events (HEDRICK 1985). As the genomic sites of the microsatellite loci studied is unknown, we cannot reject the hypothesis of physical linkage. However, this is unlikely as the four loci were screened at random out of the 72 chromosomes of *B. truncatus*. An alternative explanation is selfing (HEDRICK 1980), which is already supported by empirical data (ALLARD 1975). The larger the selfing rate, the longer the gametic disequilibrium will be maintained in the population (HEDRICK 1985). The reason is that with high selfing rates the proportion of double heterozygotes is decreased so that few recombinants are formed (HEDRICK 1985, p. 347). We here had access to genotypic disequilibria. However, the gametic and the genotypic disequilibria have very similar values in highly selfing species because genes sampled from single individuals are likely to be identical-by-descent. The high genotypic disequilibria observed in some populations of *B. truncatus* may reflect familial structures created by high selfing rate (STEBBINS 1957; WALLER 1993). Bottlenecks may induce important gametic disequilibria, which could subsequently be maintained by nonrandom mating (HEDRICK 1985). This could explain the large disequilibria observed for all pairs of loci for the Kokourou population, which experiences regular bottlenecks (temporary pond). Finally mutation is generally neglected when considering the creation of linkage disequilibria (HEDRICK 1985). This may have to be reevaluated for microsatellites.

Genetic variability among populations: Our results indicate that the populations of *B. truncatus* studied here cannot be considered as drawn from the same gametic pool, even when considering populations within a pond (*e.g.*, Boyze, Namaga, Mari). This suggests that gene flow is limited. However, though $\hat{\theta}$ values are generally high among ponds when calculated for each pair of populations (0.3–0.7), they are small (<0.1) within ponds (*e.g.*, Namaga or Mari) or even among ponds within the same geographic area (*e.g.*, 0.08 and 0.05 between Kokourou and Namaga PM or Namaga B, respectively, 0.17 between Foua and Mada). The migration events may mainly depend on the history of the pond (human activities, contraction and expansion of the ponds).

From our data, the estimates of gene flow (Nm) based on $\hat{\theta}$ are higher than those based on R_{st} though always of the same order (except for Mari). SLATKIN (1995) demonstrated that under a SMM or TPM $\hat{\theta}$ overestimate genetic similarity for small Nm values. There is currently no consensus on how best to estimate gene flow among populations when using microsatellites. However, SLATKIN (1995) pointed out that under a TPM, R_{st} must generally be preferred to $\hat{\theta}$, which depends on both the mutation rate and process. Analytical analyses (ROUSSET 1996) are in agreement with these simulations. However, the performance of $\hat{\theta}$ increases as the TPM allows larger changes in the number of repeats, *i.e.*, when the TPM converges toward IAM.

In highly selfing populations, these formulas underestimate the actual amount of gene flow because both the effective population size and migration rate (if migration occurs at the diploid stage as is usually the case in snails) are lower than in the corresponding random-mating populations (MARUYAMA and TACHIDA 1992; JARNE 1995). Selfing populations appear spuriously more structured than they are when taking R_{st} or F_{st} results at face value, at least in intrapond studies. It is therefore not clear whether the higher values of population structure estimators (estimators of F_{st} or G_{st}) reported by HAMRICK and GODT (1990), HEYWOOD (1991) and JARNE (1995) for selfers *vs.* outcrossers actually indicate that the extent of gene flow is limited in selfing populations.

B. truncatus occupies transient habitats in which water availability (volume and surface) varies greatly across seasons. Exchange between populations may occur during the contraction-expansion cycles so that the dynamics of the ponds may partly explain the structurating between close ponds or within ponds. For example, Kokourou and Namaga communicate during the rainiest years, which could provide opportunities for migration of individuals. Similarly, the reduction in size of the temporary ponds Namaga and Mari during the dry season may allow some contacts between subpopulations and, in this case, extinction and recolonization processes may be a source of gene flow. In contrast, Boyze is a permanent pond and the two populations studied are separated by 3 km. This may prevent any passive exchanges between subpopulations though aquatic birds may contribute to the spread of genes among populations. Selfing could conceivably increase the differentiation between the two sampling sites.

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