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Lydia Bousset, P-y Henry, P Sourrouille, P Jarne. Population biology of the invasive freshwater snail *Physa acuta* approached through genetic markers, ecological characterization and demography.. *Molecular Ecology*, 2004, 13 (7), pp.2023-36. 10.1111/j.1365-294X.2004.02200.x . hal-02266290

HAL Id: hal-02266290

<https://hal.science/hal-02266290>

Submitted on 13 Aug 2019

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Population biology of the invasive freshwater snail *Physa acuta* approached through genetic markers, ecological characterization and demography

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Abstract

The respective role of factors acting on population functioning can be inferred from a variety of approaches, including population genetics and demography. We here investigated the role of four of these factors (mating systems, population size, bottlenecks and migration) in the hermaphroditic freshwater snail *Physa acuta*. Twenty-four populations were sampled either around Montpellier (local scale), or at the scale of France (global scale). At local scale, eight populations were sampled twice, before and after summer drying out. The genetic structure of these populations was studied using microsatellite loci. Populations were classified according to openness (ponds vs. rivers) and water regime (permanent vs. temporary) allowing predictions on genetic patterns (e.g. diversity within populations and differentiation). At local scale, progeny-arrays analysis of the selfing rate was conducted, and size distributions of individuals were followed over two years. Results with regard to the four factors mentioned above were: (i) Estimates of population selfing rates derived from inbreeding coefficients were only slightly higher than those from progeny-arrays. (ii) More variation was detected in rivers than in ponds, but no influence of water regime was detected. One reason might be that permanent populations are not going less often through low densities than those from temporary habitats at the time scale studied. (iii) There was limited evidence for genetic bottlenecks which is compatible with the fact that even marked reduction in water availability was not necessarily associated with demographic bottlenecks. More generally, bottlenecks reducing genetic variation probably occur at population foundation. (iv) Lower genetic differentiation was detected among rivers than among ponds which might be related to limitations on gene flow. Demographic and temporal genetic data further indicates that flooding in rivers is unlikely to induce marked gene flow explaining the strong genetic differentiation at short geographical scale in such habitats. Finally, the demographic data suggest that some populations are transitory and subject to recurrent recolonization, a pattern that was also detected through genetic data.

Keywords: demography, ecological characterization, genetic structure, microsatellites, snail

Introduction

Natural populations are influenced by both demographic and evolutionary factors. Although no unified framework has been proposed up to now to consider both groups of factors at once, it remains possible to adopt a pluralistic approach, that is to combine results from the analysis of population genetic structure (see, e.g. Slatkin 1985; Hartl & Clark 1997; Rousset 2001) with those from population demography (see, e.g. Ebert 1999; Caswell 2001). Further information can be gathered from environmental characterization of habitats or ecological approaches. In the simplest version of the pluralistic approach, even a simple characterization of habitats may help in interpreting genetic patterns. Giles & Goudet (1997) showed that population age explains the genetic differentiation of populations in a plant, as expected from metapopulation theory. Habitat openness predicts the amount of genetic variability maintained within populations of a freshwater snail (Charbonnel *et al.* 2002a). A more sophisticated approach includes the analysis of the same factor with different methods. The most obvious example is migration: it might be inferred under specific hypotheses from the spatial distribution of genetic variability (see, e.g. Hartl & Clark 1997), but also from cohort analysis (Ebert 1999) or capture–mark–recapture methodology (Schwarz & Seber 1999). This has been done in a few cases only and has sometimes provided surprisingly congruent estimates (Johnson & Black 1995; review in Ims & Yoccoz 1997; Rousset 2001). However, the pluralistic approach remains underrepresented in the literature (see References in Charbonnel *et al.* 2002c). We here tried to follow such an approach, focusing on four influent factors in population biology, namely mating systems, population size, demography (bottlenecks) and migration.

In this perspective, hermaphroditic freshwater snails (Gastropoda: Pulmonata) might constitute appropriate biological models (Brown 1994; Städler & Jarne 1997; Dillon 2000). We focus here on *Physa acuta*, also referred to as *P. heterostropha* or *P. integra* (Dillon et al. 2002). This preferentially outcrossing species self-fertilizes at population rates ranging from 0.1 to 0.3 (Wethington & Dillon 1997; Jarne et al. 2000; Tsitrone et al. 2003; Henry et al. submitted for publication). *P. acuta* occupies a variety of natural and artificial habitats which both are discontinuously distributed and exhibit wide temporal variation in water availability. Habitat openness (e.g. rivers vs. ponds) and water regime (e.g. permanent vs. temporary) can therefore be used to characterize populations. This environmental variation should be associated with marked variation in population census size (see Brown 1994; Dillon 2000) and therefore genetic drift, and potentially with variation in migration and extinction/recolonization processes (Charbonnel et al. 2002b,c; Chlyeh et al. 2002). *P. acuta* is an invasive species originating from North America which has widely enlarged its distribution area over the last two centuries. It is now found on all continents, and is indeed the most cosmopolitan freshwater snail (Dillon et al. 2002). This extension might have been associated with population bottlenecks during founding events, as detected in other snail species (Meunier et al. 2001; Charbonnel et al. 2002a). However, the demography of this species is hardly known (Brown 1979), and the distribution of genetic variability among populations has been documented only once (Dillon & Wethington 1995).

The goal of our study was to characterize the influence of the four factors mentioned above on *P. acuta* population biology, as part of a wider study on the demographic and evolutionary processes influencing mating system evolution (Jarne et al. 2000; Henry et al. 2003; Tsitrone et al. 2003). Genetic (microsatellite markers) and demographic and ecological data were used to interpret population patterns in the light of external data (e.g. habitat characterization) and compare estimates of population parameters (e.g. bottlenecks) derived from different sources. In total, the study included 25 populations from France. More specifically, the role of the four factors mentioned above was approached as follows:

1. The mating system was studied using a double approach. Estimates of selfing rates at the population level were obtained indirectly from the genetic survey of all populations. In a subset of populations, the selfing rate was also derived from progeny-arrays analyses, which returns individual estimates (Ritland 2002). At the population level, inbreeding is a source of deviation from Hardy–Weinberg equilibrium (HWE). However, other processes (e.g. population structure) can generate such deviations (see, e.g. Zouros & Foltz 1984). Mean individual estimates, therefore, help in the interpretation of population estimates.
- 2 Populations were characterized based on habitat openness and water regime. This allowed predictions on the amount of genetic variation within populations, i.e. evaluation of the influence of genetic drift. In 11 populations around Montpellier, the demography was monitored through monthly surveys of population density and individual size distributions (see Brown 1994; Ebert 1999) over a 2-year period. Again, this allowed predictions of density with regard to openness and water regime.
- 3 The occurrence of bottlenecks was analysed based both on tests of population genetic structure and on the demographic survey. Predictions were made on both genetic and demographic variation with regard to environmental descriptors. Although the genetic signals detected through genetic analyses are unlikely to take place on the same time scale as current demography (see Cornuet & Luikart 1996), it is interesting to evaluate whether the current demography is indicative of trends on a longer time scale.
- 4 Migration was indirectly evaluated using genetic markers, for example, testing for isolation-by-distance, and contrasted among populations with regard to environmental descriptors. Migration events were also analysed based on both sudden changes in size distribution and density, and temporal variation in allelic frequencies in eight populations that were sampled twice over time. As actual demographic estimates of migration were not derived, a direct comparison with genetic estimates was not possible (see Rousset 2001 for a review). However, our approach allows detection of massive migration events and this can be contrasted among populations and compared with genetic divergence.

Materials and methods

Populations sampled, demographic survey and ecological characterization

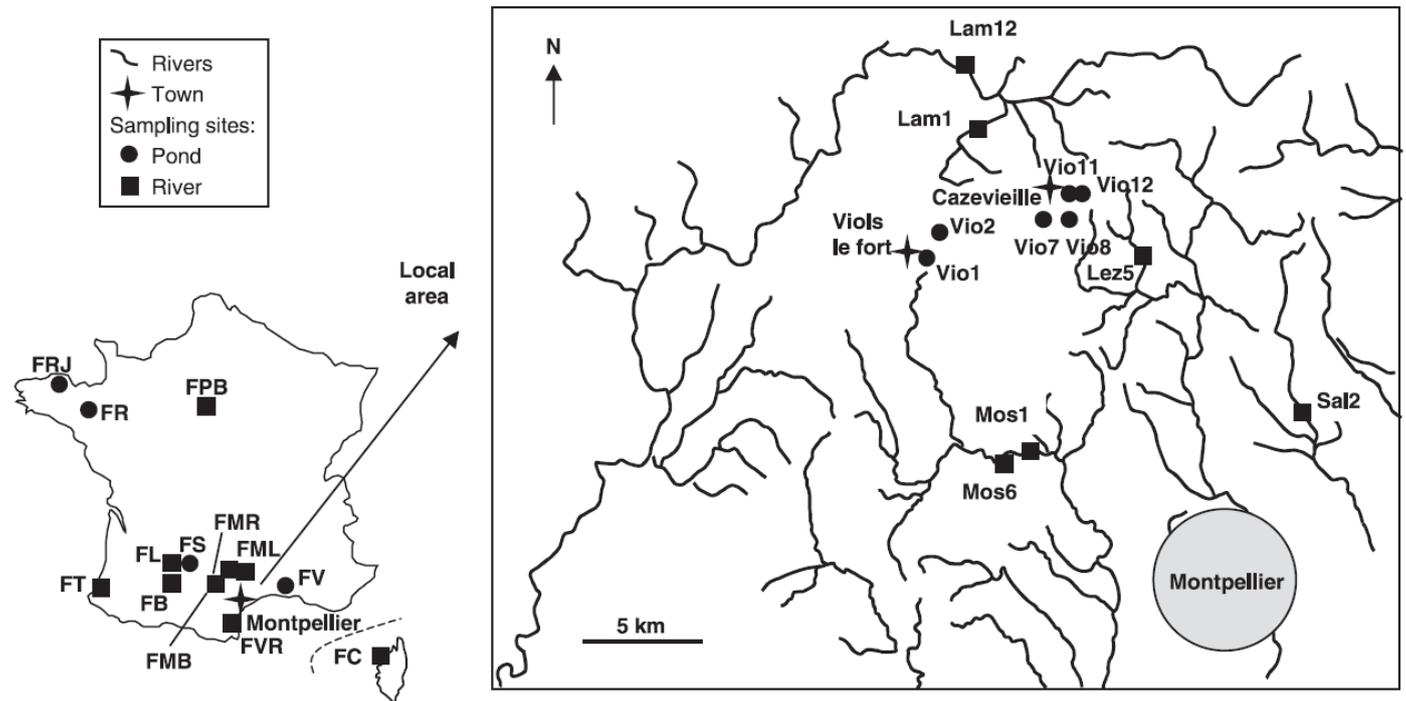
The location of the 25 populations studied is given in Table 1. A subsample of populations located near Montpellier was more thoroughly studied, and will be referred to as the local scale. Three hundred and twenty-nine individuals were sampled from 11 sites both in June (t_1) and October (t_2) 2000 (Table 1). Unless specified, t_1 and t_2 samples from a given population were analysed separately. These sites were separated by distances of 0.5–19 km (Fig. 1). They were surveyed monthly from July 2000 to July 2002 for demographic and ecological purposes (Henry 2002). On average, 32 (SE = 23) individuals were measured per visit and per site; size being the distance from the apex to the base of the spire (to the nearest 0.1 mm). Individuals were also marked with a paint dot to quantify survival, recruitment and migration based on the capture–mark–recapture methodology (Schwarz & Seber 1999). However, the recapture probability was too low and population size too high to obtain meaningful results (not shown; Henry 2002). Population density was visually scored (0, 1, 5, 10, 25, 50, 100 ind/m). Note that this is significantly correlated with a more classical measure of density, the number of individuals collected per minute (Spearman rank order correlation coefficient, $r_s = 0.827$, $N = 229$, $P < 10^{-4}$). Sites were classified according to hydrological conditions (Table 1) as temporary (drying out on at least one visit during the 2-year monthly survey) or permanent. Ponds were also distinguished from rivers. In rivers, the connectivity of successive water pools was noted (connected or disconnected). More details are given in Henry (2002).

Table 1. Information on the 25 populations of *Physa acuta* studied (see also Fig. 1). *Lez5* was considered in the study of allelic segregation only. The first 12 populations (local scale) in the table were followed for demographic purposes (see text for details)

Population	Locality	Coordinates		Date		Habitat	
		Latitude	Longitude	t_1	t_2		
Vio11	Cazevieille	N. 43°46'	E. 03°47'	06/00	10/00	Po	P
Vio2	Viols	N. 43°45'	E. 03°43'	06/00	10/00	Po	P
Vio7	Cazevieille	N. 43°45'	E. 03°46'	06/00	10/00	Po	P
Vio1	Viols	N. 43°44'	E. 03°42'	06/00	10/00	Po	T
Vio12	Cazevieille	N. 43°46'	E. 03°48'	06/00	10/00	Po	T
Vio8	Cazevieille	N. 43°45'	E. 03°47'	06/00	10/00	Po	T
Mos1	Mosson, downstream	N. 43°40'	E. 03°46'	06/00	10/00	R	P
Sal2	Salaison	N. 43°41'	E. 03°55'	06/00	10/00	R	P
Lam1	Lamalou, upstream	N. 43°47'	E. 03°43'	06/00	—	R	T
Lam12	Lamalou, downstream	N. 43°48'	E. 03°44'	06/00	—	R	T
Mos6	Mosson, upstream	N. 43°39'	E. 03°45'	06/00	—	R	T
Lez5	Yorgues	N. 43°44'	E. 03°50'	—	—	R	T
FMB	Montpellier, Buèges	N. 43°50'	E. 03°40'	07/98		R	nc
FML	Montpellier, Le Bay	N. 44°00'	E. 04°10'	07/98		R	nc
FMR	Montpellier, Rieufort	N. 43°90'	E. 04°00'	07/98		R	nc
FV	Var, Signes	N. 43°25'	E. 05°90'	09/96		nc	nc
FC	Corsica, Balagne	N. 42°60'	E. 08°75'	03/00		R	nc
FT	Landes, Tosse	N. 43°60'	W. 01°50'	12/01		R	nc
FS	Lot et Garonne, St Etienne de Fougère	N. 44°45'	E. 00°65'	09/01		Po	nc
FL	Lot, Clairac	N. 44°30'	E. 00°35'	09/01		R	nc
FB	Baïse, Vialle	N. 44°00'	E. 00°40'	09/01		R	nc
FPB	Essonne, Bièvres	N. 48°70'	E. 02°25'	05/02		R	nc
FVR	Pyrénées Orientales, Villeneuve de la Raho	N. 43°70'	E. 02°80'	07/02		R	nc
FR	Ille et Vilaine, Le Rheu	N. 48°15'	W. 01°75'	07/02		Po	nc
FRJ	Côtes d'Armor, La roche Jagu	N. 48°70'	W. 03°00'	07/02		Po	nc

Date is the date of sampling. — indicates that snails were not detected or absent. In the 'habitat' column, Po and R stand for ponds and rivers, respectively (FC was sampled in a dam lake), and P, T and nc for permanent, temporary and not characterized, respectively.

Figure 1 Location of the 13 French populations sampled outside the Montpellier area. The populations sampled near Montpellier are indicated in the enlarged panel. Information on openness and water availability is given in Table 1.



The population genetic structure was also analysed on a global scale, using an additional sample of 13 populations (157 individuals; Table 1). Three are located around Montpellier and 10 elsewhere in France (Fig. 1). These 13 populations were either ponds or rivers (Table 1). Prior to genetic analysis, all individuals were stored in 90° alcohol or frozen.

Generating genetic data

Prior to the analysis of population structure, allelic segregation was checked at 10 microsatellite loci (Monsutti & Perrin 1999; Sourrouille *et al.* 2003). No significant deviation from Mendelian expectations was observed (see online electronic supplementary material, Web Table 1). DNA extraction and characterization of variability were performed as described in Sourrouille *et al.* (2003). The distribution of genetic variability in natural populations was analysed using 8 of these 10 loci, i.e. excluding *Pac14* (limited variation) and *AF108761* (some ambiguous scoring at global scale). Polymerase chain reaction (PCR) co-amplification was possible for some groups of loci (*Pac1*, *Pac2* and *Pac5*; *Pac4* and *Pac7*; *AF108762* and *AF108764*). PCR products were run in two groups (*Pac1*, *Pac2*, *Pac5*, *Pac4*, *Pac7*, *AF108762* and *AF108764*; *AF108758*) on an ABI Prism 310 genetic analyser. Some null homozygotes were detected, but they were concentrated at two loci (*Pac4* and *Pac7*). The results presented here were obtained without these two loci. However, including *Pac4* and *Pac7* influenced our results to a limited extent (see online electronic supplementary material).

Genetic markers were also used to estimate the population selfing rate (averaged over families) using progeny-arrays (Ritland 2002) and seven of the eight loci mentioned above. The study was conducted at local scale (11 populations), and 6–13 families were studied per population. On average 6.7 (SE = 1.0) progenies were studied per family. The methodology and results are fully detailed in Henry (2002).

Analysing the mating system

The selfing rate was first estimated based on population structure. Deviation from the genotypic proportions expected at HWE at each locus was tested in each population using exact tests (Rousset & Raymond 1995), and departure from HWE over all loci was evaluated using Fisher's method for combining probabilities. The unbiased estimator \hat{f} of Wright's F_{IS} was calculated according to Weir & Cockerham (1984) using genepop 3.1 (Raymond & Rousset 1995). Selfing rates were computed as $\hat{s} = 2\hat{f}/(1 + \hat{f})$ (Pollak 1987). This assumes that selfing is the sole source of deviation from HWE. Population selfing rates derived from progeny-arrays were estimated using the maximum likelihood procedures implemented in mltr 2.4 (Ritland 2002).

Genetic diversity within populations and population size

The genetic variability was described using the mean number of alleles (N_{all}), mean observed heterozygosity (H_O) and mean gene diversity (H_E) per population over all loci (Nei 1987). Estimates of allelic frequencies per locus and population are given as online electronic supplementary material (Web Table 2). Deviations from genotypic equilibrium between pairs of loci were tested in each population using exact tests (Rousset & Raymond 1995). Predictions were made on genetic variation within populations, based on habitat openness and water regime (Table 2; see also Viard *et al.* 1997b,c; Charbonnel *et al.* 2002a,b,c). (i) Migration and colonization should maintain larger effective population size in rivers than in ponds; and (ii) genetic drift and extinction should be more common in temporary than in permanent habitats. More variation was therefore expected in rivers than in ponds and in permanent than in temporary habitats (Table 2). The harmonic mean density over sampling events was estimated in each population from the demographic survey, and higher values were expected in permanent than in temporary habitats (Table 2). No specific predictions were derived with regard to openness, because potentially stronger demographic bottlenecks in ponds during droughts might be counter-balanced by low density after flooding in rivers. We also tested whether current density is related to longer term population size, and could therefore predict the amount of genetic variation within populations.

Table 2. Predictions on the genetic structure (within-population polymorphism, occurrence of bottlenecks, genetic differentiation among populations, isolation-by-distance and temporal variation) and on demography based on the ecological characterization of habitats. Predictions on the genetic structure are based on the idea that (i) migration and colonization maintain larger effective population size in rivers (R) than in ponds (Po); (ii) genetic drift and extinction are more common in temporary (T) than in permanent (P) habitats

	Openness	Water regime
Genetic structure		
Polymorphism (N_{all}, H_E)	Po < R	P > T
Bottlenecks (probability)	Po > R	P < T
Differentiation (F_{ST})	Po > R	P < T
Isolation-by-distance		
Mantel (P)	Po > R	P > T
Regression slope (a)	Po > R	P < T
$D\sigma^2$	Po < R	P > T
Temporal variation (F_{ST})	Po = R*	P < T
Demography		
Density (harmonic mean)	—†	P > T
Low-density events (probability)	Po > R	P < T
Extinction (probability)	Po > R	P < T

> and <, higher and lower values, respectively. =, we have no specific reason to expect differences.

* Sampling drift only is considered, because no migration occurred between the two sampling events.

† No specific expectation

Population demography: bottlenecks

The demographic history of populations was analysed using the method of Cornuet & Luikart (1996), which is based on the heterozygosity expected in a population which size has suddenly varied. This does not require HWE. Observed heterozygosity is compared with the heterozygosity expected under mutation–drift equilibrium given the number of alleles present in the population. An excess of heterozygosity is generally expected after a bottleneck, whereas a deficit is expected in growing populations. We performed the Wilcoxon signed ranks test implemented in bottleneck (Piry *et al.* 1999) in each population under the three available models of mutation (infinite allele (IAM), two-phase (TPM) and stepwise mutation (SMM) models), because there is no consensus on which model is the most appropriate for microsatellites. The occurrence of bottlenecks was also searched for in the demographic data. The three parameters used were the harmonic mean density (see above), the occurrence of low-density events (when one individual per m² or less was observed)

and the probability of extinction which are expected to be low, high and high, respectively, when bottlenecks are frequent. Predictions with regard to environmental descriptors on both the genetic and demographic approaches are given in Table 2. The genetic approach detects bottlenecks at a much larger time scale (of the order of N_e generations, with N_e the effective population size; Cornuet & Luikart 1996) than the two years of our demographic survey. However, we reasoned that current bottlenecks in specific environments (e.g. ponds) might be indicative of historical bottlenecks.

The temporal variation in population size was further studied based on temporal genetic variation. Temporal variation of neutral genetic patterns across generations in a given population may result from stochastic processes (sampling and genetic drift) or migration (see, e.g. Waples 1989; Charbonnel *et al.* 2002c). In our study, variation between t_1 and t_2 can be interpreted in terms of stochastic sampling of individuals only. Migration can be ruled out because water pools in rivers remained disconnected between t_1 and t_2 . Analysis of the size distributions also suggests that the individuals collected at t_1 and t_2 (that is before and after summer drought) belonged to the same spring cohort. Indeed, mature snails were collected at t_1 at a mean size of 8.7 ± 1.6 mm over all populations, and the increase in size by t_2 ranged from 4.0 to 8.1 mm per population. Massive recruitment between the two sampling dates would have resulted in a bimodal size structure and a decrease in mean size. Population temporal differentiation was tested using homogeneity tests (Goudet *et al.* 1996). The estimator θ (θ -temporal) of F_{ST} (Weir & Cockerham 1984) was calculated over all loci in each population.

Migration

The estimator θ of F_{ST} (Weir & Cockerham 1984) was calculated over all loci, both over all populations and all pairs of populations (Web Table 3) using genepop 3.1. The estimates of pairwise spatial differentiation (θ -spatial) were compared with θ -temporal (see above) using Mann–Whitney U -test. Less temporal than spatial variation was expected given the short time scale and the fact that sampling drift only was acting. Isolation-by-distance was tested using the method of Rousset (1997), under which a correlation is expected between the logarithm (in a two-dimensional habitat) of the geographical distance and $F_{ST}/(1 - F_{ST})$. This correlation was tested using matrix correlation methods based on Mantel test. A reduced major axis (RMA) regression was performed (see Sokal & Rohlf 1981; Bohonak 2002). Estimates of $D\sigma^2$ were calculated from the slope of the regression, and confidence intervals on the slope were calculated by bootstrapping using ibd 1.2 (Bohonak 2002). The geographical distance between two sampling points was computed as a linear distance (see Viard *et al.* 1997a). Predictions were produced with regard to environmental descriptors. More differentiation and isolation-by-distance was expected in ponds than in rivers, because of the expected difference in effective population size, and in temporary than in permanent habitats. Migration among populations was also approached from genetic data using assignment and exclusion methods (Cornuet *et al.* 1999). The main results, consistent with those of the F_{ST} -based analysis, are reported as online electronic supplementary material.

Immigration was analysed based on size distributions. From the distribution of individual size classes, it is possible to infer the number of cohorts occurring in a population at a given time (Ebert 1999). The temporal variation of this distribution allows inferring demographic events such as recruitment of new cohorts. However, such histograms are often hard to interpret when size classes overlap over cohorts and sample size is limited. Persat & Chessel (1989) developed a method based on correspondence analysis aimed at smoothing such distributions. This method is explained fully in Charbonnel (2001) and was used in another snail species (Charbonnel *et al.* 2002c). In our study, immigration was expected to translate into the sudden appearance of modes that cannot be explained by growth or recruitment. This assumes that immigrants and residents had different sizes. Furthermore, the method can only detect massive immigration events. This is unlikely in ponds, because potential vectors cannot move a full cohort of subadult to adult snails, and data from closed habitats were not analysed. Immigration events were expected to be more frequent in temporary than in permanent habitats. Although this approach detects very specific events of migration, their occurrence might be used to interpret genetic patterns.

All predictions presented in Table 2 were tested using nonparametric tests (Siegel & Castellan 1988). Note also that the comparisons of genetic and demographic patterns with regard to water regime are reported as online electronic supplementary material only (Web Table 5), as no test was significant.

Results

Analysing the mating system

Fifteen of 32 multilocus tests of Hardy–Weinberg equilibrium were significant with low P -values (Table 3). Heterozygote deficiencies were detected as often in populations around Montpellier as in other populations (Table 3). The estimates of S corresponding to non-null estimates of F_{IS} ranged from 0.12 to 0.61 (15 populations; Table 3). At the local scale, no selfing was detected in six populations (F_{IS} not significantly different from zero). In two populations, some selfing was detected at either t_1 , or t_2 . Using progeny-arrays showed that selfing rates significantly differed from zero in three of the populations studied at local scale only. Both approaches indicated no selfing in six populations, partial selfing in two populations and conflicting results for the last three populations. The distributions were not correlated (Spearman rank order correlation coefficient, $r_s = -0.065$, $P = 0.92$), although the mean values were not very different (0.06 and 0.07).

Table 3. Variation at six microsatellite loci in the 24 populations sampled. Date refers to the date of sampling when populations were sampled twice. N is the sample size and N_p the number of polymorphic loci. N_{all} , H_O and H_E are, respectively, the mean number of alleles, the mean observed heterozygosity and Nei's unbiased gene diversity over all loci. Their standard deviations are indicated into parentheses. P is the probability associated with exact tests (heterozygote deficiency) of Hardy–Weinberg proportions. \hat{f} and \hat{s} (F_{IS}) are the estimates of Wright's F_{IS} and the selfing rate, respectively. S was not estimated for negative values of \hat{f} , or in populations at Hardy–Weinberg equilibrium \hat{s} (PA), selfing rate estimated from progeny-arrays (at local scale only)

Population	Date	N	N_p	N_{all}	H_O	H_E	P	\hat{f}	\hat{s} (F_{IS})	\hat{s} (PA)
Vio11	t_1	18	4	1.8	0.39 (0.33)	0.29 (0.24)	1	-0.32	—	0.03
	t_2	18	4	2.3	0.24 (0.19)	0.30 (0.23)	0.05	0.22	0.37	—
Vio2	t_1	18	3	2.3	0.30 (0.33)	0.32 (0.35)	0.21	0.11	—	0.04
	t_2	20	3	2.5	0.33 (0.33)	0.33 (0.33)	0.07	0.04	—	—
Vio7	t_1	19	3	2.2	0.25 (0.25)	0.26 (0.27)	0.01	0.07	0.14	0.27*
	t_2	20	4	2.3	0.28 (0.30)	0.30 (0.28)	0.02	0.07	0.13	—
Vio1	t_1	17	5	3.3	0.39 (0.32)	0.41 (0.22)	0.01	0.08	0.15	0.15*
	t_2	19	5	2.8	0.35 (0.19)	0.39 (0.20)	0.002	0.13	0.22	—
Vio12	t_1	17	6	2.3	0.38 (0.15)	0.39 (0.16)	0.46	0.05	—	0.00
	t_2	18	5	2.3	0.36 (0.26)	0.36 (0.24)	0.21	0.03	—	—
Vio8	t_1	14	3	2.0	0.21 (0.22)	0.22 (0.24)	0.03	0.12	0.21	0.00
	t_2	19	3	2.0	0.23 (0.23)	0.24 (0.26)	0.23	0.08	—	—
Mos1	t_1	17	6	3.2	0.55 (0.15)	0.55 (0.16)	0.34	0.04	—	0.02
	t_2	21	6	3.3	0.55 (0.15)	0.57 (0.15)	0.17	0.06	—	—
Sal2	t_1	19	6	4.2	0.51 (0.21)	0.53 (0.16)	0.01	0.06	0.12	0.01
	t_2	18	6	3.8	0.40 (0.18)	0.46 (0.22)	0.05	0.16	0.28	—
Lam1	t_1	16	6	2.7	0.53 (0.24)	0.50 (0.19)	0.22	-0.04	—	0.01
Lam12	t_1	17	6	3.0	0.46 (0.24)	0.44 (0.23)	0.13	-0.01	—	0.17*
Mos6	t_1	4	6	2.8	0.63 (0.41)	0.53 (0.18)	0.74	-0.06	—	0.00
FMB		12	6	4.0	0.46 (0.11)	0.51 (0.15)	0.02	0.14	0.24	—
FML		12	6	3.3	0.29 (0.22)	0.49 (0.19)	10^{-4}	0.44	0.61	—
FMR		13	5	2.7	0.42 (0.25)	0.41 (0.23)	0.43	0.01	—	—
FV		12	5	3.3	0.43 (0.31)	0.53 (0.24)	0.002	0.23	0.37	—
FC		12	5	3.0	0.35 (0.28)	0.47 (0.25)	2.10^{-4}	0.29	0.45	—
FT		12	6	3.7	0.67 (0.19)	0.57 (0.14)	0.81	-0.14	—	—
FS		12	6	3.7	0.53 (0.21)	0.57 (0.18)	0.01	0.12	0.22	—
FL		12	6	4.0	0.69 (0.17)	0.59 (0.15)	0.67	-0.13	—	—
FB		12	5	3.7	0.50 (0.22)	0.52 (0.22)	0.07	0.08	—	—
FPB		12	6	3.3	0.51 (0.19)	0.52 (0.13)	0.19	0.05	—	—
FVR		12	6	4.0	0.39 (0.15)	0.49 (0.20)	0.004	0.25	0.40	—
FR		12	6	2.8	0.43 (0.28)	0.43 (0.24)	0.20	0.04	—	—
FRJ		12	5	2.7	0.26 (0.30)	0.37 (0.28)	0.002	0.33	0.49	—

* Estimate differs from 0 ($P < 0.05$) based on bootstrap analysis.

Genetic diversity within populations and population size

None of the tests of genotypic disequilibrium over all populations were significant. The genetic variation within populations is reported in Table 3 and Web Table 2. The mean number of alleles ranged from 1.8 to 4.2, and the average gene diversity from 0.22 to 0.59 (Table 3). The number of alleles and gene diversity were higher in rivers than in ponds both at local and global scale. However, no difference was observed when comparing harmonic mean densities of individuals (Table 4).

Table 4. Comparisons of the genetic and demographic parameters (defined in Table 3) between ponds and rivers. Values are given as means (SD). The ‘test’ column indicates either Mann–Whitney U-test (n_1 ; n_2 ; U) or Fisher’s exact test F (n_1 ; n_2), and P is the associated probability. ‘nt’ holds for no test, either when testing was irrelevant (Mantel and $D\sigma^2$) or not possible (no demographic data at global scale, not enough data for bottlenecks and extinctions). $>$, $<$ and $=$ are followed by #when the predictions of Table 2 were fulfilled. Values in the ‘bottlenecks’ row indicate the number of significant tests under the infinite allele model (IAM), two-phase model (TPM) and stepwise mutation model (SMM), and the total number of tests performed. km in the ‘differentiation’ row gives the mean geographical distance (standard deviation) between population pairs. Slope (a) values in the isolation-by-distance row are provided with their 95% confidence intervals generated by bootstrapping

	Openness							
	Local scale				Global scale			
	Ponds	Rivers	Test	P	Ponds	Rivers	Test	P
Genetic structure								
Polymorphism								
N_{all}	2.36 (0.40)	<# 3.29 (0.54)	U (7; 12; 6)	1.10^{-3}	2.50 (0.50)	<# 3.42 (0.50)	U (15; 16; 23)	4.10^{-5}
H_E	0.32 (0.06)	<# 0.51 (0.05)	U (7; 12; 0)	4.10^{-5}	0.35 (0.09)	<# 0.51 (0.05)	U (15; 16; 17)	8.10^{-6}
Bottlenecks								
(IAM/TPM/SMM/total)	0/0/0/12	3/3/2/7	nt		1/0/0/17	5/3/0/16	nt	
Differentiation								
paired F_{ST}	0.33 (0.10)	># 0.10 (0.06)	U (11; 30; 18)	1.10^{-6}	0.33 (0.12)	># 0.14 (0.08)	U (99; 118; 1082)	2.10^{-6}
km	4.2 (2.6)	< 15.5 (7.7)	U (10; 15; 23)	3.10^{-3}	217 (301)	< 245 (221)	U (99; 118; 3933)	3.10^{-5}
Isolation by distance								
Mantel (P)	10^{-4}	5×10^{-4}	nt		0.03	10^{-4}	nt	
slope (a) [95% CI]	0.32 [0.25; 0.49]	># 0.06 [0.03; 0.20]	No overlap		0.23 [0.19; 0.27]	># 0.11 [0.08; 0.16]	No overlap	
$D\sigma^2$	0.25	1.35	nt		0.35	0.72	nt	
Temporal variation								
paired F_{ST}	0.01 (0.01)	=# 0.001 (0.00)	U (2; 6; 2)	0.29	no data	no data	nt	
Demography								
Density (harmonic mean)	4.93 (6.64)	= 1.12 (1.77)	U (6; 6; 6)	0.06	no data	no data	nt	
Low density (% events)	24%	< 40%	F (134; 101)	1%	no data	no data	nt	
Extinction (nb)	0	3	nt		no data	no data	nt	

Population demography: bottlenecks

The null hypothesis of mutation–drift equilibrium was not rejected in most populations, whatever the mutation model (Web Table 6). One heterozygosity deficit was detected under SMM, which may indicate expansion or bottleneck (Cornuet & Luikart 1996). Heterozygosity excesses (indicating bottlenecks) were detected in five populations only, and the test was significant for the three models in Mos1 and Lam1 only.

Estimates derived from the demographic survey are reported in Table 4. Low-density events were more frequent in rivers than in ponds (Table 4). Three extinction events were detected. All occurred in Lam1 (October 2000 to May 2001, October to November 2001, January to April 2002; Table 4), a site in which a strong genetic signal of bottleneck was also detected. No extinction event was observed in ponds despite five summer drying-out events.

The pairwise estimates of temporal F_{ST} ranged from 0.00 to 0.04 (Fig. 3). As expected, there was no more differentiation in open than in closed habitats (Table 4).

Figure 2 Differentiation between pairs of populations in rivers and ponds at local scale. Multilocus estimates of pairwise differentiation are plotted against the logarithm of the linear distance (D ; in km). The corresponding RMA regression lines and the percentage of variation explained (r^2) are indicated, with $F_{ST}/(1 - F_{ST}) = 0.32 \ln(D) + 0.55$ and $F_{ST}/(1 - F_{ST}) = 0.06 \ln(D) + 0.07$ in ponds and rivers, respectively.

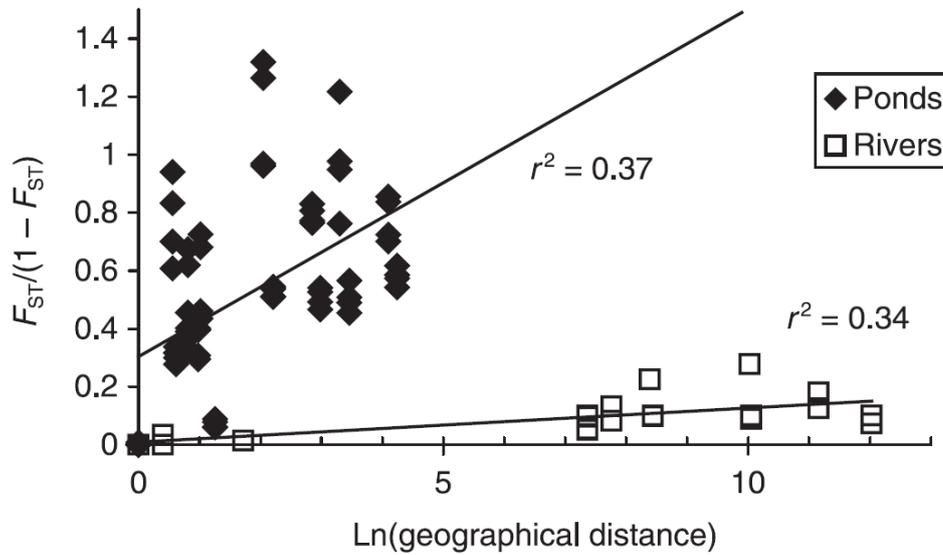
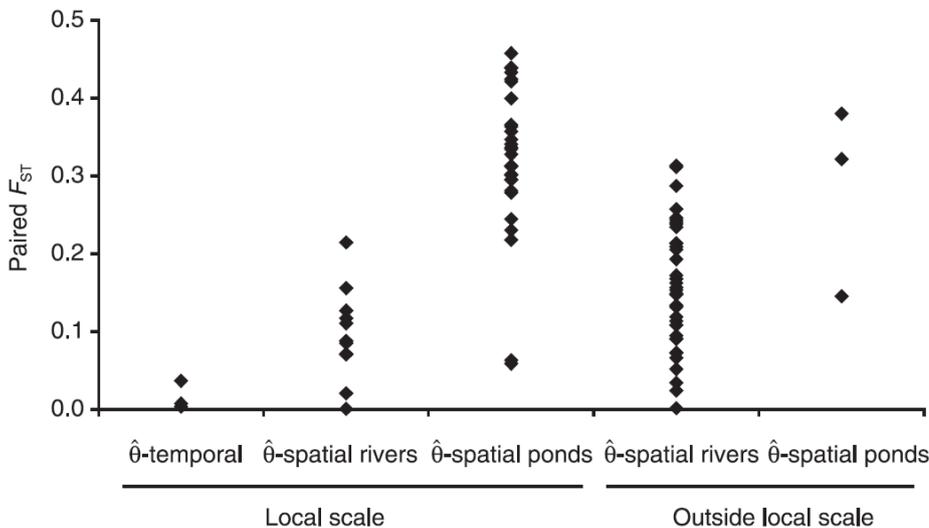


Figure 3 Estimates of temporal differentiation in populations sampled twice (θ -temporal; eight points) in the Montpellier area (local scale), and estimates of spatial differentiation between pairs of populations in rivers and ponds. Spatial differentiation is indicated both for the local scale (θ -spatial rivers, 11 points; θ -spatial ponds, 30 points) and outside the local scale (θ -spatial rivers, 45 points; θ -spatial ponds, 10 points).



Migration

The multilocus estimates of pairwise F_{ST} are reported in Web Table 3. Spatial estimates were higher than temporal estimates, both in rivers (Mann-Whitney $U = 5.0$, $n_1 = 8$, $n_2 = 10$, $P = 3 \times 10^{-4}$) and in ponds ($U = 0.0$, $n_1 = 8$, $n_2 = 25$, $P = 4 \times 10^{-6}$). At a local scale, isolation-by-distance was detected when considering all populations (Mantel test, $P = 0.01$; note the 95% CI: -0.34 to 0.35), as well as among both ponds and rivers (Fig. 2). At global scale, isolation-by-distance was significant in both ponds ($P = 0.03$) and rivers ($P = 10^{-4}$). At both scales, more differentiation was observed in ponds than in rivers (Fig. 3), and the patterns of isolation-by-distance were significantly different (no overlap of slopes; Table 4).

Size distributions were poorly informative because of the frequent occurrence of adverse climatic events (inducing high water level or turbidity) and low population densities (and thus small sample size). As immigration could be documented only if >10 individuals are sampled in successive visits, this limited our

This file is the post-print of: Bousset et al. *Molecular Ecology* (2004) 13:2023-2036 doi: 10.1111/j.1365-294X.2004.02200.x dataset to 42 visits (of 132). Although the evolution of modes was hardly identifiable, four massive immigration events were detected, and all occurred in temporary rivers (*Lam1* and *Lez5*).

Discussion

For the sake of clarity, the four factors studied (mating systems, population size, bottlenecks and migration) are discussed separately. For each factor, we discuss what has been gained from each approach, trying to provide an integrated view at the end of each subsection. The last subsection evaluates how genetic, ecological and demographic approaches might be integrated when studying population biology.

Mating system

The hypothesis of HWE was rejected in most populations tested (Table 3). As *Physa acuta* is a hermaphroditic species, these results may be explained by partial selfing (Dillon & Wethington 1995; Jarne *et al.* 2000). Assuming that heterozygote deficiencies are indeed due to selfing, selfing rates ranged between 0.12 and 0.61 in populations departing from HWE. Lower values were obtained at local scale. This confirms that *P. acuta* practises both outcrossing and selfing, although Jarne *et al.* (2000) reported lower values. Several forces may generate heterozygote deficiencies and it is worth comparing these values with more direct estimates derived from progeny-arrays. At the local scale, there was a slight difference only in the mean selfing rate over populations when comparing results from progeny-arrays (0.06) and population structure (0.07). A similar difference has been reported in the other highly selfing freshwater snails studied (Viard *et al.* 1997a; Charbonnel 2001; Trouvé *et al.* 2003). However, estimates from the two methods were congruent (either no or partial selfing) in 8 of the 11 populations studied.

Possible explanations for the difference between the two methods include temporal variation in the selfing rate, population substructure (Wahlund effect), biparental inbreeding and technical problems (miscoring and null alleles). We have no information on temporal variation in selfing rate. Some population differentiation may be detected even at short distances within the same river (see below), and the role of Wahlund effect remains possible. The progeny-array analysis indeed revealed some biparental inbreeding at local scale. Surprisingly, this occurred in small (size < 10 m) isolated ponds (Henry 2002), suggesting population structure at a very small spatial scale. However, this was in populations with selfing rates not significantly different from zero. Some miscoring was detected in progeny-arrays and segregation analyses (e.g. one allele was not detected in heterozygous individuals). However, this concerned fewer than 1% of the situations studied, and multilocus F_{IS} estimates were hardly affected (results not shown). As already mentioned, null alleles are segregating in the populations studied, but this hardly affected the estimated selfing rates (see online electronic supplementary material). On the whole, estimates derived from population structure seem to only slightly over-estimate mean selfing rates.

Genetic diversity within populations and population size

The microsatellite loci used are moderately polymorphic, with gene diversity ranging from 0.2 to 0.6. However, these values are higher than those obtained at much more limited geographical scale using allozymes (Dillon & Wethington 1995; Jarne *et al.* 2000). The mean gene diversity (0.43) is not very different from that reported in *Biomphalaria glabrata*, the other outcrossing freshwater snail species studied using microsatellites (Mavárez *et al.* 2002a,b), but is higher than values reported in selfing species (see Table 4 in Mavárez *et al.* 2002a). This is expected because the effective population size decreases with the selfing rate (review in Städler & Jarne 1997).

Estimates of genetic diversity were predicted to correlate with habitat openness and water regime (Table 2). Indeed, we observed more variation in rivers than in ponds. However, this might not be due to local population size because higher population densities were observed year-round in ponds. Note that this assumes that density correlates with local population size, which may not be true. A plausible explanation is that population size in rivers should be considered at the level of whole rivers. Similar estimates of genetic diversity were unexpectedly detected in permanent and temporary habitats (Web Table 5). One explanation suggested by the demographic survey is that large population size might be maintained in temporary habitats, despite drying out in summer. Of course the standing level of variation also depends on the past population size which is now discussed.

Population demography: bottlenecks

Genetic signals of bottlenecks were detected in one, three and five populations under the SMM, TPM and IAM, respectively (Web Table 6). However, the tests were significant for the three models in two populations only (*Lam1* and *Mos1*), both sampled around Montpellier. There is, therefore, limited evidence of bottlenecks: the populations studied probably did not experience a sudden decrease in size (with subsequent constant size) at the appropriate time scale for the tests performed (about $2N_e$ generations). However, it remains unclear how these tests are affected by fluctuating population size or migration, both likely in the situations studied. Fast increase after bottlenecks (see Appleton & Branch 1989; for data in *P. acuta*) may also dilute the bottleneck signal (Nei *et al.* 1975). This might explain why no genetic signal of demographic variation was detected at global scale. At first glance, this might be considered surprising because introduced species are expected to exhibit limited variation due to bottlenecks at introduction. *P. acuta* was first described in Europe in 1805 (Garonne River, France), supposedly spread towards the Mediterranean region, and then slowly towards northern Europe (Dillon *et al.* 2002). Our data, therefore, suggest invasion by a large number of propagules, in agreement with some theories of biological invasions (Williamson 1996), or successive invasions from the same genetic origin, presumably North America (L. Bousset & P. Jarne unpublished data).

The low number of significant genetic tests precluded a comparison with regard to habitat openness and water regime. However, comparisons were possible with demographic data: at the time scale considered, populations from ponds are no more likely to experience periods of low density than those from rivers. This contradicts genetic results on within-population polymorphism (based on the opposite demographic predictions). The sole extinction events were observed in a river (*Lam1*), where the flat, cemented riverbed did not offer refuges to snails during floods (Henry 2002). Interestingly this is one of the two populations in which a strong genetic signal of bottlenecks was detected. The demographic survey also indicates that populations from temporary habitats did not experience marked bottlenecks. No extinction followed drying out in summer. The absence of demographic difference between temporary and permanent habitats (Web Table 5) was confirmed using a specifically designed capture-recapture monitoring of density in *Lam12* during summer 2001 (Henry 2002). The site dried out completely, and up to 99% of individuals died between June and October. Following the first autumn rains, the density was still high (17 individuals/m). This is probably a consequence of snail behaviour: they buried themselves in the muddy waterbed allowing significant survival during drought (Henry 2002). Migration is unlikely to explain this result since the closest source of immigrants was 1.2 km upstream. In agreement with these results, the temporal genetic analysis indicated limited fluctuation in allelic frequencies between samples taken before and after summer drought. This further suggests that marked local environmental variation does not imply strong genetic fluctuation through sampling drift.

In conclusion, the environmental descriptors are poor predictor of bottlenecks, whether analysed through genetic or demographic variation. The main demographic difference, especially in relation to habitat openness, might be the occurrence of bottlenecks at population foundation (explaining the lower genetic variation) and low migration rates in closed habitats (see Discussion).

Migration

The range of pairwise F_{ST} estimates at the local scale (0.01–0.51) is consistent with values reported by Dillon & Wethington (1995) at similar geographical scale. At the local scale, isolation-by-distance was not detected when considering all populations. However, much more population differentiation was detected in ponds than in rivers (as expected), and estimates of $D\sigma^2$ are about five times higher in rivers than in ponds (the regression slopes were significant different). An explanation might be that dispersal is more limited in ponds where it most likely derives from rare opportunities of transport by humans or mammals (sheep or wild boar *Sus scrofa*, bird vectors being unlikely here; Henry 2002). In rivers, downstream dispersal along the water current (Clampitt 1974) or active upstream migration (Appleton & Branch 1989; Pointier *et al.* 1998) are probably more efficient ways of dispersing. A similar explanation might hold at the global scale at which more differentiation was also detected in ponds than in rivers. Birds might play a more significant role at this scale (Santamaría & Klaassen 2002).

What can be gained here from demographic data? From the monthly survey of snail size distributions (in rivers only), major events of immigration were detected in one, temporary population only (*Lam1*). Indeed *Lam1* is best considered as a transient population with recurrent extinction-recolonization on a yearly basis,

because the substrate offers no protection during floods. In other populations, immigration events could not be documented. Although the technique is limited to specific migration events (see Materials and methods), this is a first indication that migration proceeds through the exchange of a small number of migrants at a time in rivers. Complementary capture-mark-recapture analyses during flooding events (Henry 2002) are indicative of the same trend. In one experiment, 98% of marked individuals were displaced by flood but were recaptured at short distance (< 28 m), which presumably could be compensated for by active upstream dispersal. Even flooding in rivers does not necessarily cause massive displacement of populations, apart in peculiar habitats such as *Lam1*.

Conclusion

Combining approaches in population biology

Our study indicates that a pluralistic approach combining genetic and demographic data with environmental descriptors can be helpful when analysing the role of the four important factors considered here (see also, e.g. Whitlock & McCauley 1990; Peacock & Ray 2001; Charbonnel *et al.* 2002c; Chlyeh *et al.* 2002). The mating system can be inferred from both direct and indirect approaches. Mean selfing rates did not differ much, and it is likely that most inbreeding is due to selfing, especially at the local scale. However, the direct approach provides interindividual variation (Henry *et al.* submitted for publication) that might be of prime interest when studying the evolution of the selfing rate (see, e.g. Schultz & Willis 1995). Habitat openness influences the amount of genetic variation within populations, as already shown by Charbonnel *et al.* (2002a) in another freshwater snail, suggesting that even rough environmental characterization can be used to predict population variability. However, water regime can apparently not be used to predict this variation (but see Charbonnel *et al.* 2002a), perhaps because this trait does not capture essential features of variation in population size. Surprisingly, populations from permanent habitats do not pass through low densities less often than do those from temporary habitats at the time-scale studied. That limited evidence of genetic bottlenecks was gathered is compatible with the fact that even marked a reduction in water availability is not necessarily associated with demographic bottlenecks. A major conclusion with regard to the population biology of invasive species, such as *P. acuta*, is that those bottlenecks reducing genetic variation probably occur at population foundation, and that further demographic variation in population size might affect genetic variation to a limited extent. This is consistent with our most striking result, which is the lower genetic differentiation in rivers than in ponds. Bottlenecks (here at foundation) indeed increase genetic differentiation. A similar result was obtained in *B. glabrata* populations in Venezuela (Mavárez *et al.* 2002b), or in a marine snail when contrasting continuous and discontinuous habitats (Johnson & Black 1995, 1998). Similarly, Giles & Goudet (1997) observed less differentiation among old than among young populations of a plant. The demographic data allowed us to refine the picture in rivers: flooding does not induce marked gene flow and massive events of migration were not detected which might explain why genetic differentiation is observed at short geographical scale. These data also revealed that some populations are transitory with recurrent recolonization, a pattern that cannot be detected through classical surveys of population genetic structure.

We are aware that the pluralistic approach followed here remains rather crude. More precise studies should conjointly estimate parameters. This has, for example, been done in a few studies for gene flow, as mentioned in the Introduction (see Rousset 2001). Alternatively, it would be interesting to have models of population genetic structure incorporating demographic parameters (e.g. population size, dispersal rate) in a more realistic way, or frameworks under which the likelihood of different models could be fitted to data and compared. Progress is made in this area (e.g. Estoup *et al.* 2001; Leblois *et al.* 2003). Our approach might also be improved if environmental variables were continuous, rather than binary (e.g. rivers vs. ponds). More sophisticated approaches of the relationship between genetic and environmental variables are also possible (see, e.g. Angers *et al.* 1999). Finally, the pluralistic approach has sometimes been perceived as problematic, because of the variety of time scales at which the different methods are working. For example, tests of bottlenecks on genetic data are concerned with events occurring a few N_e generations ago, whereas current estimates are derived from demography. The problem is not so much acute, as shown by results on dispersal (Rousset 2001). A more optimistic perspective is that the pluralistic approach is an improvement over purely genetic or demographic approaches when analysing evolutionary forces acting on populations at various time scales. It remains though that a general framework reconciling population genetics and demography has still to be developed.

Acknowledgements

We thank C. Chevillon, P. David, R.T. Dillon, F. Nijokou and J.-P. Pointier for snail sampling, C. Debain for help with the molecular analysis and J.-D. Lebreton for statistical advices. L. Bernatchez, N. Charbonnel, P.-A. Crochet and three anonymous referees provided insightful comments on the manuscript that significantly improved its quality. This project was supported by funds from INRA to L. Bousset, from CNRS and Bureau des Ressources Génétiques to P. Jarne, from the INVABIO program from the French Ministry for Ecology and Sustainable Development (MEDD) to P. Jarne and a fellowship from the French Ministry for Higher Education (MENRT) to P.-Y. Henry.

Supplementary material

The following material is available from <http://www.blackwellpublishing.com/products/journals/suppmat/MEC/MEC2200/MEC2200sm.htm>

Appendix A1

Table S1 Analysis of allelic segregation at ten microsatellite loci in 13 selfed families (seven offspring). Some (L2, L3, L8, L10, L12, L15, L18) were second laboratory generation offspring of individuals collected in March 2002 in a pond located on the CNRS campus in Montpellier. Other individuals were collected in March 2002 at the egg stage either in streams (Lam1a, Lam1b, Lez5), or in ponds (Vio8a, Vio8b, Vio12) located within a radius of 20 km around Montpellier (Table 1, Fig. 1 of main text). The parental genotype (allele sizes) is given per family and locus on the first line. The second line indicates allelic segregation among offspring (homozygotes for the smallest allele : homozygotes for the largest allele: heterozygotes). ***indicates significant deviation from Mendelian expectations.

Table S2 Allelic frequencies at eight microsatellite loci in the 24 populations of *Physa acuta* analysed. Values at Pac4 and Pac7 were obtained once null homozygotes were discarded. For locus AF108758, allele 407 indeed represents two bands of size 140 and 147 respectively (see 1 in the text above for details). Sampling dates are given in Table 1 of main text.

Table S3 Estimates of Wright's F_{ST} (below the diagonal) and associated P -values of homogeneity tests (above the diagonal; Goudet *et al.* 1996) at six microsatellite loci among population pairs of *Physa acuta* (24 populations were studied). Sampling dates are given in Table 1 of main text. 96% of these tests were significant at the 0.05 level, and 93% remained significant after a sequential Bonferroni correction.

Table S4 Relationship between the mean (standard deviation) proportions of individuals excluded and reassigned per population and ecological characterization of habitats. See main text for details on habitat characterization. The 'Test' column indicates Mann-Whitney U test (n_1 ; n_2 ; U); P is the associated probability and = is followed by '#' when the predictions were fulfilled. Samples in the eight populations sampled twice (at t_1 and t_2) were pooled prior to analysis.

Table S5 Comparisons of the genetic and demographic parameters (defined in Table 3) between permanent and temporary habitats. Values are means with standard deviations into parentheses. See main text for details on the parameters estimated and habitat characterization. The tests were conducted at local scale only, since water regime was not determined at global scale. The 'test' column indicates either Mann-Whitney U test (n_1 ; n_2 ; U) or Fisher exact test F (n_1 ; n_2), and P is the associated probability. 'nt' holds for no test, either when testing was irrelevant (Mantel and $D\sigma^2$) or not possible (no demographic data at global scale, not enough data for bottlenecks and extinctions). $>$, $<$ and $=$ are followed by '#' when the predictions of Table 2 of main text were fulfilled. Values in the 'bottlenecks' row indicate the number of significant tests under the infinite allele model (IAM), two-phase model (TPM) and stepwise mutation model (SMM), and the total number of tests performed. 'km' in the 'differentiation' row gives the mean geographic distance (standard deviation) between population pairs. Slope (a) values in the isolation by distance row are provided with their 95% confidence intervals generated by bootstrapping.

Table S6 Tests of mutation-drift equilibrium under the infinite allele model (IAM), the two-phase model (TPM) and the stepwise mutation model (SMM). The table gives the probability associated with Wilcoxon signed ranks test of the null hypothesis of equilibrium. ‘Excess’ and ‘Deficit’ (when $P \leq 0.05$) indicate respectively a bottleneck and an expansion.

Fig. S1 Probability of exclusion/reassignment in populations studied at local scale, located within a radius of 20 km around Montpellier (Table 1, Fig. 1 of main text). Full bars indicate individuals not excluded from the population in which they were sampled, empty bars indicate individuals excluded and not reassigned to the populations studied, and dashed bars indicate individuals excluded and reassigned to one of the populations included in the study. Samples from June (t_1) and October (t_2) were pooled before analysis.

Supporting Information

Lydia Bousset, currently a researcher at the Institut National de la Recherche Agronomique in Rennes (France), worked as a postdoctoral fellow on snail population genetic structure and invasion biology. Pierre-Yves Henry's PhD focused on the evolution of mating systems in snails, and is currently studying invasive populations of birds (Princeton, USA). Patricia Sourrouille is laboratory engineer with expertise in DNA analysis. Philippe Jarne works on the evolution of subdivided populations, largely using DNA markers, and of mating systems.

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