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Stability of genetic structure and effective population size inferred from temporal changes of microsatellite DNA polymorphisms in the land snail *Helix aspersa* (Gastropoda: Helicidae)

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Temporal evolution of genetic variability may have far-reaching consequences for a diverse array of evolutionary processes. Within the polders of the Bay of Mont-Saint-Michel (France), populations of the land snail *Helix aspersa* are characterized by a metapopulation structure with occasional extinction processes resulting from farming practices. A temporal survey of genetic structure in *H. aspersa* was carried out using variability at four microsatellite loci, in ten populations sampled two years apart. Levels of within-population genetic variation, as measured by allelic richness, H_e or F_{IS}, did not change over time and similar levels of population differentiation were demonstrated for both sampling years. The extent of genetic differentiation between temporal samples of the same population established (i) a stable structure for six populations, and (ii) substantial genetic changes for four populations. Using classical *F*-statistics and a maximum likelihood method, estimates of the effective population size (N_e) illustrated a mixture of stable populations with high N_e, and unstable populations characterized by very small N_e estimates (of 5–11 individuals). Owing to human disturbances, intermittent gene flow and genetic drift are likely to be the predominant evolutionary processes shaping the observed genetic structure. However, the practice of multiple matings and sperm storage is likely to provide a reservoir of variability, minimizing the eroding genetic effects of population size reduction and increasing the effective population size.

ADDITIONAL KEYWORDS: extinction – *F*-statistics – gene flow – genetic diversity – genetic drift – metapopulation – population structure.

INTRODUCTION

Detailed knowledge about the distribution of genetic variation in wild, subdivided populations provides insights into a species' evolutionary history and its future continuity, because historical and extrinsic ecological events can leave signatures in spatial genetic structuring (e.g. Viard, Justy & Jarne, 1997; Davison & Clarke, 2000; Frankham, Ballou & Briscoe, 2002; Pfenninger, 2002; Raybould *et al*., 2002). In spite of the wealth of spatial genetic studies, there have been few short- and long-term studies on the evolution of genetic structure over time (Hossaert-McKey *et al*., 1996; Viard *et al*., 1997; Tessier & Bernatchez, 1999; Pertoldi *et al*., 2001). Nevertheless, many natural populations are unstable over time, and often constitute arrays of ephemeral local demes subjected to more or less frequent extinction and recolonization events through metapopulation dynamics (McCauley, 1995; Harrison & Taylor, 1997; Thrall, Burdon & Murray, 2000; Whitlock, 2001). Therefore, temporal surveys may give additional insight into the dynamics of inter-

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connected populations beyond those offered by punctual spatial studies (e.g. Pertoldi *et al*., 2001; Heath *et al*., 2002; Kinnison *et al*., 2002; Guinand *et al*., 2003).

This study focuses on the short-term evolution of genetic structure in the common garden snail *Helix aspersa*, within an unstable and agricultural environment. Native to the Western Mediterranean, *H. aspersa* has successfully colonized a large range of anthropogenically disturbed habitats across northwest Europe (agricultural and suburban areas, as well as domestic gardens) since the Holocene, mainly through passive dissemination following human displacements (Madec, 1991; Guiller, Madec & Daguzan, 1994). As a consequence, spatial and temporal heterogeneities in the *H. aspersa* species range are associated with a pronounced degree of phenotypic variation in combinations of life-history traits; this is particularly evident in reproductive traits, for which great plasticity can be observed even within the same population (Madec, Desbuquois & Coutellec-Vreto, 2000).

There is an increasing consensus among both ecologists and evolutionary geneticists that population dynamics should be addressed at the metapopulation level, because local populations have relatively short lifetimes resulting from ongoing extinction and recolonization processes (Harrison & Taylor, 1997; Thrall *et al*., 2000; Whitlock, 2001; Frankham *et al*., 2002). Recent studies (Arnaud, Madec & Daguzan 1999a; Arnaud *et al*., 1999b, 2001) characterized the processes shaping the genetic differentiation of *H. aspersa* in a recently (within the last 200 years) settled area, the polders of the Bay of Mont-Saint-Michel (Brittany, north-western France). In *H. aspersa*, the mosaic of populations inhabiting the agricultural area of the polders of the bay of Mont-Saint-Michel could be described as a 'classical' metapopulation, because many populations experience unpredictable extinction events that are confirmed by the presence of groups of empty shells in particular areas (Arnaud, 2003; see also Arnaud *et al*., 1999b). Indeed, in this intensive agricultural environment, several unpredictable mortality factors are commonly observed: herbicide and pesticide treatments, habitat destruction by agricultural machines and burning, and human predation (Madec *et al*., 2000; Arnaud *et al*., 2003). Whereas at very fine scales of observation (<900 m) the spatial genetic distribution clearly fits an isolation-by-distance model (Arnaud *et al*., 2001), a strong patchy genetic differentiation is demonstrated at the scale of the whole polder area (50 km^2) using allozyme and microsatellite markers (Arnaud *et al*., 2003). This pattern reveals trends compatible with a metapopulation functioning and involves both (i) interpopulation connectivity through environmentally induced migration pathways such as hedges or roadside embankments, and (ii) short-range gene flow sometimes associated with accidental long-distance dissemination and founder effects owing to farming practices (Arnaud, 2003).

However, whether or not this assemblage of populations really behaves as a metapopulation involves not only a spatial component, but also a temporal one. In order to determine how such a genetic patchiness evolves on a short time-scale, we aimed at further investigating the temporal evolution of the genetic composition within populations. To this end, changes at microsatellite loci were examined for ten temporally spaced population samples collected two years apart. Variations in allele frequencies were used (i) to estimate whether spatial differentiation varies with time, (ii) to estimate whether there are significant changes in classical population genetic parameters such as gene diversity or levels of inbreeding, and (iii) to evaluate the variance effective population size using both *F*-statistics and maximum likelihood methods (e.g. Ingvarsson & Olsson, 1997; Lehmann *et al*., 1998; Tarr, Conant & Fleischer, 1998; Funk, Tallmon & Allendorf, 1999; Jehle *et al*., 2001; Turner, Salter & Gold, 2001; Heath *et al*., 2002; Lenfant & Planes, 2002). Indeed, the effective population size N_e is another important population parameter driving the dynamics of genes in space and time (Wright, 1931; Nei & Tajima, 1981; Waples, 1989). When population size drastically declines, theory predicts a loss of genetic variability leading to an increased rate of inbreeding and fixation of deleterious alleles, especially for strongly isolated small populations (Wang & Caballero, 1999; Whitlock, 2001; Frankham *et al*., 2002). This is expected to limit the short- and long-term evolutionary potential of the population under consideration. Although knowledge of the parameter N_e is fundamental in understanding population structure, N_e is seldom estimated in natural populations because the required demographic (direct) parameters are often difficult to acquire (Ingvarsson & Olssen, 1997; Jehle *et al*., 2001; Berthier *et al*., 2002).

MATERIAL AND METHODS

Study site and sampled populations

All studied populations were sampled in the polders of the Bay of Mont-Saint-Michel, an intensive agricultural zone located in Brittany (north-western France) (Fig. 1). This anthropogenically disturbed area consists of a mosaic of cultivated fields delimited by a complex network of hedges, roads, small canals and dykes, the embankments of which serve as refuges for many animal species (Arnaud *et al*., 1999a, 2003). These polders were constructed from 1851 onwards and the last dyke, protecting land against encroachment of seawater, was completed in 1933. This study

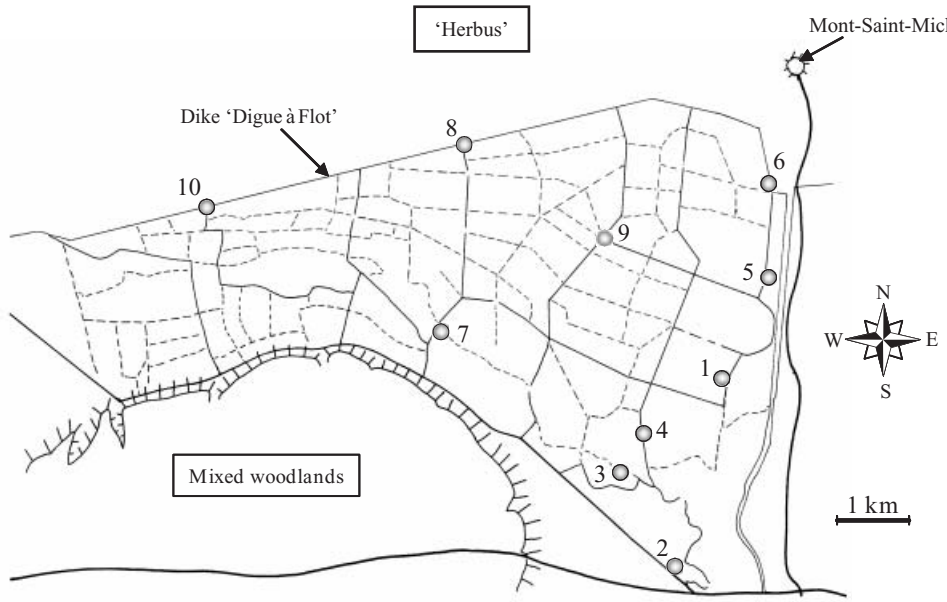

Figure 1. Map of sampled area and spatial location of ten populations of *Helix aspersa*. The polder area is bordered on its southern side by mixed woodlands and on its northern side by the dyke 'Digue à Flot'. This last dyke protects the polders against rising sea levels. The 'Herbus' area is a salt meadow pasture which is periodically covered by sea during major high tides. Full lines represent either local roads or overland routes. Dashes lines indicate dikes or hedges.

site is a recently colonized area by *H. aspersa* and, owing to farming practices (intensive weeding) and human predation (collecting), unpredictable extinction events are observed (Madec *et al*., 2000). Within such a context, the setting of *H. aspersa* populations is likely to have a complex short-term evolutionary history mirrored in a nonequilibrium mutation-drift state.

The stability of population structure was analysed for populations sampled two years apart. The initial collecting phase was performed in June 1998 and comprised 32 sampled localities (see Arnaud *et al*., 2003). Of these localities, ten sites were resampled in June 2000, thus providing a geographically representative subsampling of the whole study area. These localities, numbered from 1 to 10 in this study (see Fig. 1), respectively, corresponded to localities 7, 8, 9, 10, 14, 15, 21, 24, 29, 30 previously described in Arnaud *et al*. (2003). No striking environmental changes were observed at any locality between the two sampling periods. In order to minimize genetic artefacts of population differentiation when within-samples substructuring yields genetically distinct entities (the Wahlund effect), snails were collected from an area as small as possible, i.e. no more than 5×5 m. For both years of collecting, sampling areas were the same. Only sexually mature adult snails (characterized with a lip at the edge of the shell aperture) were collected. Because of the low density of adults for all sampled locations, the number of individuals sampled per collection site was small, ranging from 15 to 20.

MICROSATELLITE DNA GENOTYPING

Total DNA extraction was performed using a 10% Chelex-100 (Sigma) suspension. A total of 390 individuals were genotyped for four microsatellite loci (*Ha2*, *Ha5*, *Ha10* and *Ha11*) multiplexed in a single PCR reaction with unambiguous amplification patterns. Microsatellite polymorphisms were electrophoresed and analysed with fluorescent detection methods using an ABI 310 automated sequencer (Perkin-Elmer), as described in Guiller *et al*. (2000). Visualization and sizing of DNA fragments were performed using the ABI PRISM 310 collection and GENESCAN 3.1 software (Applied Biosystems).

STATISTICAL ANALYSES

Genotypic phase disequilibrium, adequacy to Hardy–Weinberg expectations, observed (H_o) and expected (H_e) heterozygosities, and intrapopulation fixation index (F_{IS}) were estimated within populations at each sampling date using GENEPOP version 3.1d (Raymond & Rousset, 1995). Heterogeneity in allelic frequencies between temporal samples of a given population was evaluated using exact probability tests implemented in GENEPOP. We calculated allelic rich-

ness, a measure of the number of alleles independent of sample size, using the rarefaction method described in El Mousadik & Petit, 1996). Pairwise genetic differentiation between populations was quantified following Weir & Cockerham's (1984) estimators of F_{ST} using FSTAT version 2.9.3 (Goudet, 1995). We evaluated the significance of pairwise F_{ST} by randomly permuting multilocus genotypes among samples (10 000 permutations), using the log-likelihood statistic G, as suggested by Goudet *et al*. (1996). The overall significance of multiple comparisons was assessed applying Bonferroni adjustments for simultaneous statistical tests (Rice, 1989). We tested differences in mean H_e, F_{IS} and allelic richness (i) between each pairwise temporally spaced population sample using a non-parametric Wilcoxon signed-rank test (Viard *et al*., 1997; Tessier & Bernatchez, 1999), and (ii) among the two sets of temporal samples using a permutation scheme (10 000 replicates) implemented in FSTAT 2.9.3. Using the same permutation procedure, we evaluated whether there was significant variation in overall spatial genetic differentiation (F_{ST}) between the two temporal sets of populations. Genetic relationships among spatial and temporal population samples were also depicted using an unrooted neighbour-joining dendrogram computed with the DCE genetic distance algorithm of Cavalli-Sforza & Edwards (1967) based on allele frequencies. Support for the observed topology was evaluated by calculating bootstrapped confidence values on branches using 1000 resampling replications over individuals.

In addition to estimating classical population genetics parameters, we also evaluated the short-term effective population size of each of the ten temporally studied populations. Assuming that (i) all individuals mate randomly and have the same reproductive potential, (ii) generations are discrete, and (iii) mutation, migration and selection can be negligible, the increase of the Wright–Malécot inbreeding coefficient (Wright, 1931; Malécot, 1948) during t generations is equal to $F = 1 - (1 - 1/2N_e)^r$, where N_e will be equal to the effective population size. Obviously, in real populations, such underlying conditions do not hold, so that N_e is considered to be the size of an idealized population that experiences the same magnitude of genetic drift as the real population under consideration (Wright, 1931).

Several approaches have been proposed to estimate N_e from genetic data (e.g. Pollak, 1983; Waples, 1989; reviewed in Wang & Caballero, 1999). In this study, current short-term N_e was estimated from temporal changes in allelic composition. We applied traditional estimates based on F-statistics, corrected for reduced sampling size (Pollak, 1983), by using the term introduced by Nei & Tajima (1981), called F_{NT}, and another one derived from Reynolds' genetic distance (Reynolds, Weir & Cockerham, 1983; Laval, 2001), called F_R:

$$\hat{F}_{NT} = \frac{1}{k}\sum_{i=1}^{k}\frac{(x_{0,i} - x_{t,i})^2}{\bar{x}_i(1-\bar{x}_i)} - \left(\frac{1}{S_0} + \frac{1}{S_x}\right) \quad (1)$$

with $\bar{x}_i = (x_{0,i} + x_{t,i})/2$;

$$\hat{F}_R = \frac{\sum_{i=1}^{k}(x_{0,i} - x_{t,i})^2}{\left(1 - \sum_{i=1}^{k} x_{0,i}^2\right)} \quad (2)$$

in which $\sum_{i=1}^{k} x_i^2$ is replaced by the expression $\frac{\left(\sum_{i=1}^{k} x_i^2 - \frac{1}{S}\right)}{\left(1 - \frac{1}{S}\right)}$ for generations 0 and t. In these, expressions k is the number of alleles, $x_{0,i}$ and $x_{t,i}$ represent the frequency of allele i at generations 0 and t, respectively, and S_0 and S_t represent the sample sizes (twice the number of individuals) at generations 0 and t.

Recently, several maximum-likelihood methods have been proposed (e.g. Anderson, Williamson & Thompson, 2000; Berthier *et al*., 2002; Wang & Whitlock, 2003). Because of the large and prohibitive computation times required by these approaches when highly polymorphic markers are used, we applied the maximum likelihood estimator introduced by Laval (2001) and Laval, SanCristobal & Chevalet (2003). This method, based on the Dirichlet approximation of the intrapopulation allelic frequency distributions, is generally more accurate than F-statistics when highly polymorphic markers are used (Laval *et al*., 2003).

Data from multiple loci were combined by using the weighted arithmetic mean (Pollak, 1983):

$$\hat{F} = \frac{\sum_l (n_{0,l} - 1)\hat{F}_l}{\sum_l (n_{0,l} - 1)} \quad (3)$$

where $\hat{F}_l$ is given by eqns (1) and (2) and Laval's estimator, and $n_{0,l}$ is the number of observed alleles at locus l for generation 0.

The generation time is difficult to assess (e.g. Funk *et al*., 1999). Field studies in the polder area indicate that *H. aspersa* individuals born within a single breeding season (spring to autumn), hibernate and are sexually mature the following spring (Madec *et al*., 2000). Although snails can be long-lived, individual lifespan does not exceed one year in such an unstable and human-disturbed environment (L. Madec, pers. observ.). Thus, we conservatively estimated the number of generations between the two successive sam-

plings as equal to 2. Because t, the number of generations between successive samplings, is small, N_e was then simply derived from the approximated equation $\hat{F} \approx t/2Ne$. When the increase of inbreeding between two generations is very low, the estimated temporal variance of allele frequencies is smaller than expected based on sampling error alone [smaller than $\frac{1}{S_0}+\frac{1}{S_t}$ in eqn. (1), for example] and yields negative estimates of N_e (Waples, 1989; Lehmann *et al*., 1998). In such cases, N_e is assumed to be infinite.

Calculation of 95% confidence intervals for N_e followed Waples (1991) and Turner *et al*. (2001):

$$(1-\alpha)\ \text{CI for}\ \hat{F} = \frac{n\hat{F}}{\chi^2\alpha/2[n]}, \frac{n\hat{F}}{\chi^2(1-\alpha)/2[n]}, \quad (4)$$

where n is the degree of freedom associated with $\hat{F}$ and $\chi^2\alpha/2[n]$ is the critical $\alpha/2$-value of χ^2 for n degrees of freedom (Waples, 1991).

RESULTS

Microsatellite loci displayed high levels of polymorphism, with the observed number of alleles per locus varying from 5 (*Ha2*) and 9 (*Ha5* and *Ha10*) to 24 (*Ha11*) and the mean observed heterozygosity (averaged over all populations and generations) ranging from 0.61 (*Ha5*) to 0.87 (*Ha11*). For each population, the sample size, the allelic richness, the expected heterozygosity and the F_{IS} averaged over all loci are given in Table 1 for both sampling dates.

In general, mean observed and expected heterozygosities were high and of the same order of magnitude, with mean F_{IS} values close to zero (Table 1). Concordantly, no significant departure from Hardy–Weinberg expectations was detected by multilocus probability tests (two-tailed) in either the samples of 1998 or those of 2000, after Bonferroni correction. Only one significant ($P < 0.05$) single-locus departure from Hardy–Weinberg equilibrium, involving *Ha10*, was observed in sample 10 collected in June 2000. Similarly, out of 120 comparisons, only seven locus pairs showed genotypic linkage disequilibrium ($P < 0.05$), a proportion similar to that expected from type I error. Moreover, by using multiple probability tests, no significant disequilibrium was detected across all population samples, suggesting the absence of physical linkage of loci.

Spatial differentiation was significant over all the 10 populations for both sampling periods as shown by overall F_{ST} estimates (0.093 and 0.075 in 1998 and 2000, respectively, $P < 0.001$). For each population sample, neither H_o, H_e, mean number of alleles, F_{IS} nor allelic richness showed significant changes over time (Wilcoxon signed-rank tests, $P > 0.05$, data not shown). Similarly, when averaged over all populations, none of these parameters showed significant differences with time ($P > 0.05$).

Although no meaningful changes over time could be detected using measures of genetic diversity, allele frequency distributions did change. An example of the most striking changes in allele frequencies is illustrated for populations 6 and 9 in Figure 2. In order to evaluate the stability of allelic frequencies over time, we first applied pairwise exact tests of population differentiation (Table 2). In general, only the less variable locus (*Ha2*) suggested a strong allelic heterogeneity over time. Significant variations were also observed for loci *Ha5* and *Ha11* but we failed to detect

Table 1. Mean genetic characteristics for ten populations using four microsatellite loci in the land snail *Helix aspersa*. *S*, sample size (gene number); A_{rich}, mean allelic richness; *He*, mean gene diversity; F_{IS}, intrapopulation fixation index. Numbers 1998 and 2000 refer to the sampling year

	S		A_{rich}		H_e		F_{IS}	
Populations	1998	2000	1998	2000	1998	2000	1998	2000
1	40	40	5.85	6.09	0.74	0.75	–0.06	–0.13
2	40	40	5.68	6.29	0.70	0.73	0.04	–0.02
3	30	40	7.00	5.99	0.79	0.74	–0.04	–0.02
4	40	40	6.27	6.14	0.77	0.76	0.02	–0.03
5	32	40	6.88	6.56	0.74	0.77	–0.01	0.01
6	38	40	4.77	4.81	0.60	0.66	–0.07	–0.15
7	40	40	6.02	6.25	0.74	0.74	–0.01	0.09
8	40	40	4.73	5.04	0.64	0.63	–0.15	0.01
9	40	40	7.11	5.01	0.72	0.58	0.00	0.04
10	40	40	6.66	5.35	0.74	0.72	0.01	–0.13

Table 2. Temporal heterogeneity of allelic compositions at four microsatellite loci in ten populations of the land snail *Helix aspersa*: probability values for rejecting the null hypothesis of allelic frequency stability using exact tests of differentiation

	Locus					
Populations	*Ha2*	*Ha5*	*Ha10*	*Ha11*	Multilocus probability	
1	****	0.13	0.15	0.16	****	($\chi^2_8 = \infty$)
2	0.49	0.42	0.39	0.39	NS	($\chi^2_8 = 6.85$)
3	0.15	0.33	0.40	0.34	NS	($\chi^2_8 = 9.88$)
4	0.22	0.92	0.52	0.34	NS	($\chi^2_8 = 6.55$)
5	0.82	0.33	0.36	0.29	NS	($\chi^2_8 = 7.05$)
6	****	***	0.65	0.05	****	($\chi^2_8 = \infty$)
7	****	0.05	0.40	0.93	*	($\chi^2_8 = 29.76$)
8	1.00	*	0.21	0.28	NS	($\chi^2_8 = 11.70$)
9	****	0.37	0.16	****	****	($\chi^2_8 = \infty$)
10	0.20	0.09	0.29	*	NS	($\chi^2_8 = 17.81$)

*$P < 0.05$; ***$P < 0.001$; ****$P < 0.0001$; NS, non-significant, after Bonferroni correction.

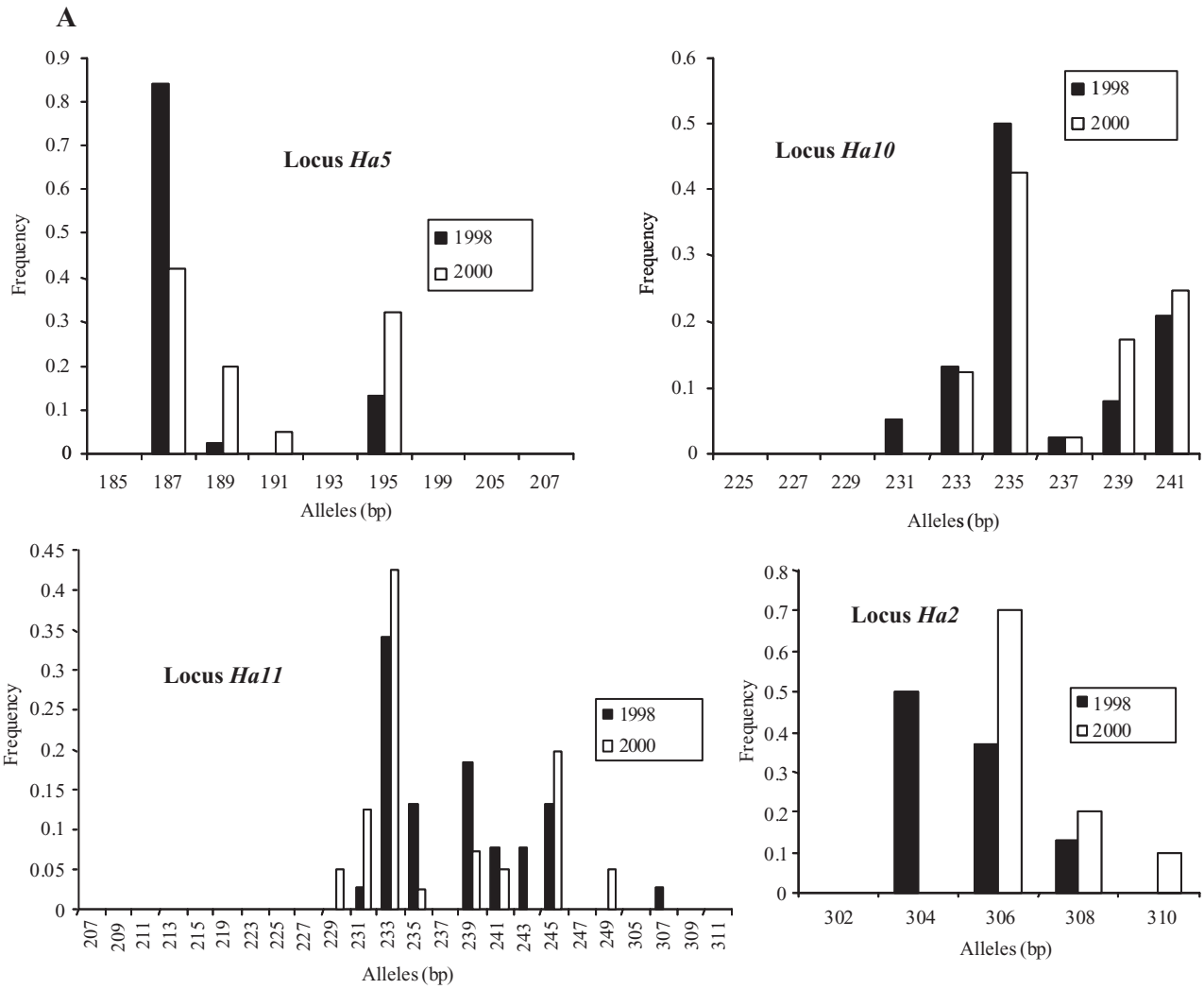

Figure 2. Temporal changes in allelic composition at four microsatellite loci in two populations of the land snail *Helix aspersa*. (A) and (B) refer to populations 6 and 9, respectively. Alleles are denoted by their total size in base-pairs (bp).

any significant changes for *Ha10*. Nonetheless, multilocus probability values revealed an overall significant heterogeneity in allelic composition for four populations (Table 2). The extent of genetic differentiation between temporal samples of the same population, quantified by pairwise F_{ST}, corroborated (i) a stable structure for six populations, supported by F_{ST} values close to zero, and (ii) substantial genetic changes for four populations with highly significant F_{ST} values, sometimes higher than 0.1 (Table 3). Despite this significant 'self-differentiation' over time, there was a tendency for all but one (sample 9) temporal replicate to cluster together by site of origin, as indicated by a Neighbour-Joining dendrogram depicting the population relationships (Fig. 3). Such a result suggests a partial stability of genetic variability, at least on a short time-scale. Accordingly, overall partitioning of genetic variance among populations (F_{ST}) between the two sampling dates did not differ significantly ($P = 0.42$, see also Table 3).

These results were translated into various estimates of effective population size (N_e), presented in Table 4. Populations 2, 3, 4 and 5, which exhibited non-significant temporal F_{ST} values (see Table 3), were all characterized by relatively high N_e values, ranging from 30 to infinity, depending on the estimate used (Table 4). By contrast, populations 1, 6 and 9 have modest effective size, with N_e values ranging from 4 to 11. A third category of populations exhibited intermediate N_e estimates, close to 15–30 individuals (e.g. populations 7, 8, 10). Whereas classical *F*-statistics yielded in some cases negative values of N_e, indistinguishable from infinity, the application of a maximum likelihood approach allowed biologically more realistic results (e.g. population 2). N_e values based on F_{ML} (maximum likelihood estimates) yielded no infinite

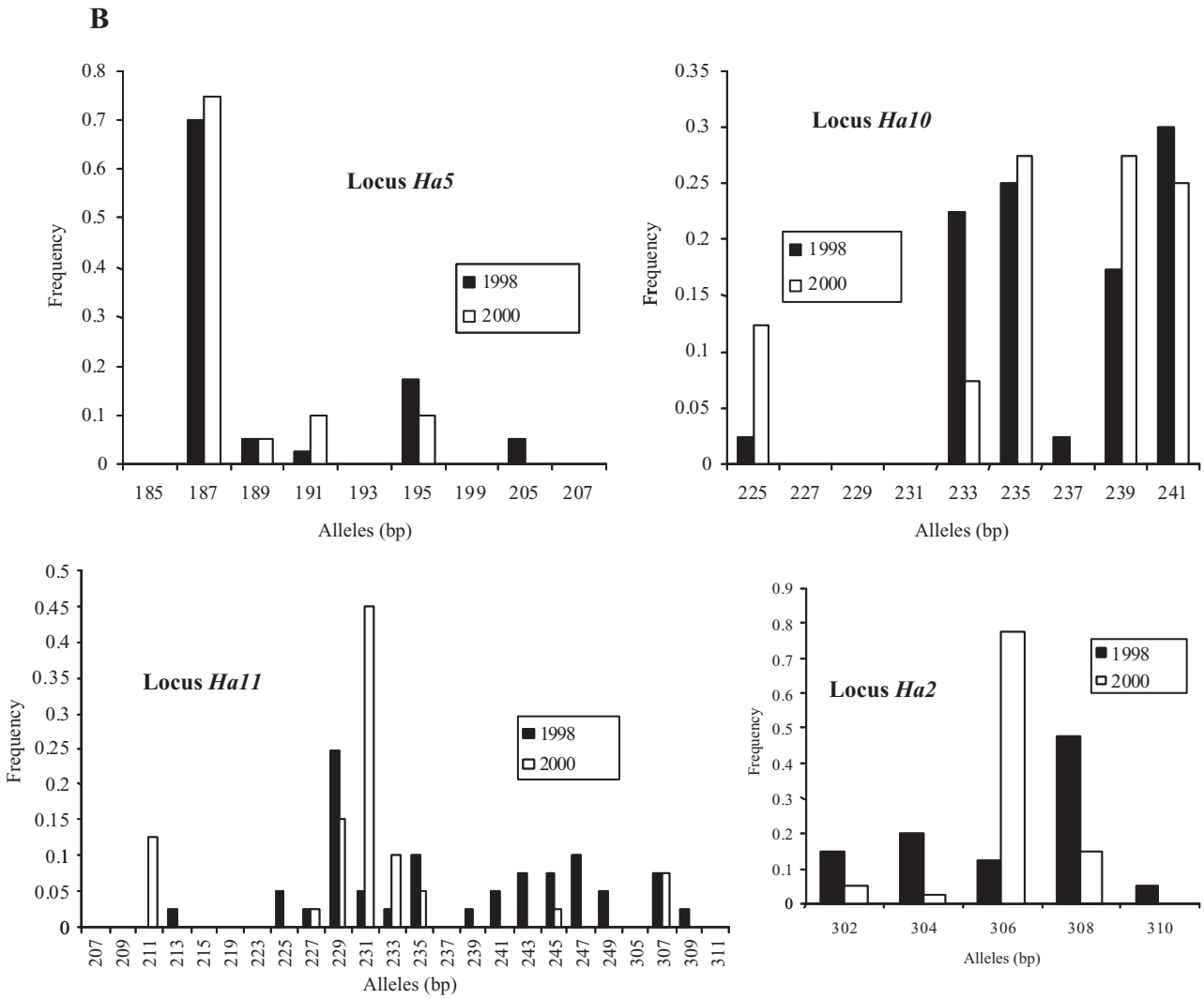

Figure 2. *Continued*

Table 3. Pairwise F_{ST} estimates calculated following Weir & Cockerham (1984) (above diagonal) and Cavalli-Sforza & Edwards' (1967) chord distance DCE values (below diagonal). Non-significant pairwise F_{ST} values after Bonferroni correction are indicated in bold characters. A and B after populations correspond to the year of sampling (1998 and 2000, respectively)

Populations	1A	2A	3A	4A	5A	6A	7A	8A	9A	10A	1B	2B	3B	4B	5B	6B	7B	8B	9B	10B
1A		0.093	**0.042**	0.069	0.063	0.098	0.081	0.179	0.094	0.093	0.071	0.056	0.094	0.062	0.083	0.113	0.101	0.175	0.171	0.141
2A	0.079	–	**0.072**	**0.061**	**0.024**	0.157	0.168	0.197	0.150	0.106	**0.046**	**–0.001**	0.049	**0.042**	0.066	0.059	0.099	0.180	0.079	0.111
3A	0.079	0.065	–	**0.031**	**0.051**	0.095	**0.049**	0.114	0.070	**0.037**	0.089	**0.033**	**0.012**	**0.018**	**0.050**	0.118	**0.048**	0.091	0.105	0.062
4A	0.076	0.061	0.047	–	**0.044**	0.133	0.097	0.104	0.065	**0.045**	0.064	**0.040**	**0.031**	**0.000**	**0.035**	0.082	**0.036**	0.093	0.075	**0.055**
5A	0.073	0.064	0.075	0.061	–	**0.105**	0.124	0.155	**0.072**	0.061	**0.027**	**0.013**	**0.047**	**0.021**	**–0.001**	**0.033**	**0.039**	0.148	**0.042**	0.068
6A	0.081	0.121	0.104	0.109	0.087	–	0.088	0.171	0.084	0.070	0.149	0.117	0.141	0.125	0.121	0.103	0.081	0.161	0.136	0.137
7A	0.106	0.133	0.066	0.087	0.102	0.080	–	0.137	0.087	0.066	0.144	0.123	0.119	0.098	0.108	0.156	0.063	0.123	0.181	0.108
8A	0.146	0.150	0.098	0.083	0.127	0.117	0.095	–	0.081	0.044	0.177	0.172	0.138	0.118	0.126	0.172	0.056	**–0.002**	0.161	0.081
9A	0.079	0.117	0.090	0.072	0.062	0.070	0.069	0.078	–	**0.048**	0.082	0.118	0.118	0.084	0.048	0.121	**0.027**	0.081	0.121	0.073
10A	0.091	0.095	0.066	0.060	0.071	0.073	0.063	0.059	0.053	–	0.112	0.075	0.052	**0.037**	0.054	0.092	**0.014**	**0.034**	0.073	**0.012**
1B	0.057	0.061	0.094	0.076	0.055	0.102	0.133	0.139	0.079	0.099	–	**0.048**	0.093	0.069	**0.038**	0.065	0.072	0.176	0.125	0.128
2B	0.061	0.022	0.050	0.050	0.058	0.091	0.097	0.138	0.093	0.079	0.058	–	0.032	**0.013**	0.048	0.053	0.065	0.155	0.068	0.093
3B	0.085	0.048	0.031	0.041	0.070	0.116	0.097	0.106	0.106	0.065	0.090	0.046	–	**0.019**	**0.047**	0.104	0.061	0.115	0.074	0.061
4B	0.073	0.050	0.035	0.018	0.059	0.102	0.078	0.086	0.076	0.051	0.076	0.040	0.028	–	**0.025**	0.066	**0.038**	0.109	**0.049**	**0.039**
5B	0.072	0.078	0.063	0.043	0.026	0.105	0.091	0.097	0.052	0.060	0.059	0.070	0.055	0.042	–	0.062	**0.030**	0.132	0.064	0.049
6B	0.101	0.098	0.117	0.075	0.062	0.061	0.114	0.102	0.077	0.072	0.078	0.086	0.106	0.076	0.073	–	0.075	0.178	0.077	0.114
7B	0.105	0.103	0.059	0.060	0.069	0.075	0.029	0.070	0.055	0.051	0.085	0.074	0.075	0.055	0.059	0.080	–	0.047	**0.059**	**0.043**
8B	0.123	0.113	0.067	0.065	0.110	0.103	0.066	0.024	0.069	0.043	0.118	0.096	0.077	0.068	0.091	0.090	0.051	–	0.139	0.071
9B	0.092	0.067	0.088	0.052	0.049	0.088	0.102	0.097	0.071	0.068	0.065	0.050	0.075	0.045	0.049	0.059	0.060	0.080	–	0.067
10B	0.143	0.115	0.084	0.084	0.096	0.141	0.072	0.087	0.086	0.041	0.138	0.103	0.077	0.063	0.073	0.117	0.067	0.064	0.079	–

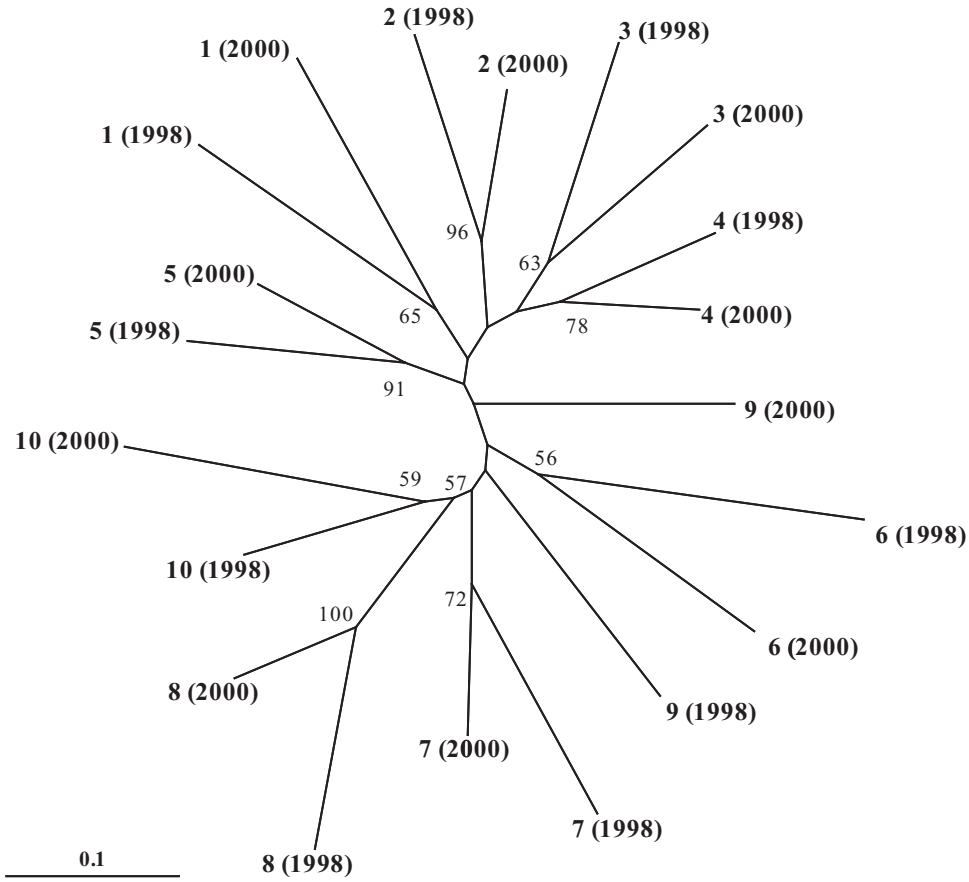

Figure 3. Neighbour-joining tree based on the DCE distance (Cavalli-Sforza & Edwards, 1967) using information from four microsatellite loci and depicting the genetic relationships among samples from ten populations of *Helix aspersa* collected two years apart (1998 and 2000). Only percentage bootstrap values >50% are indicated.

Table 4. Mean estimates of current effective population size (N_e) in ten populations of *Helix aspersa* using information from temporal changes at four microsatellite loci. K: number of independent alleles (Σ [number of alleles–1]) observed in the founder generation. S_0 and S_2 are the sample size (gene number) with subscript 0 and 2 denoting the first and second temporal samples, respectively. N_{eNT} and N_{eR} refer to the N_e estimates following Nei & Tajima (1981) and Reynolds *et al*. (1983), respectively. N_{eML} corresponds to the N_e estimates applying a maximum likelihood approach developed by Laval (2001)

Populations	K	S_0–S_2	N_{eNT} (C. I. 95%)	N_{eR} (C. I. 95%)	N_{eML} (C. I. 95%)
1	25	40–40	11.45 (6.01–18.61)	9.63 (5.05–15.66)	10.60 (5.56–17.24)
2	26	40–40	∞	∞	39.98 (21.29–64.47)
3	27	30–40	88.94 (47.99–142.28)	62.32 (33.63–99.69)	31.20 (16.84–49.91)
4	25	40–40	∞	693.46 (363.94–1127.60)	148.48 (77.92–241.40)
5	27	32–40	10 949.23 (5929.99–17 578.34)	400.63 (216.19–640.87)	55.66 (30.04–89.05)
6	20	38–40	9.50 (4.54–16.17)	6.00 (2.83–10.08)	6.00 (2.87–10.22)
7	23	40–40	16.51 (8.39–27.34)	9.00 (4.57–14.88)	23.06 (11.72–38.18)
8	21	40–40	503.83 (246.61–851.14)	∞	19.67 (9.63–33.24)
9	28	40–40	8.13 (4.44–12.91)	4.65 (2.54–7.38)	6.45 (3.69–10.54)
10	27	40–40	35.55 (19.18–56.87)	38.80 (20.93–62.07)	12.73 (6.87–20.36)

estimates and were, in general, smaller than those based on F_{NT} or F_R using F-statistics (e.g. populations 3, 5, 8 and 10; see Table 4).

DISCUSSION

Genetic variability in *Helix aspersa*

The overall spatial genetic differentiation estimated among all populations was not purely stochastic, instead reflecting the existence of a stable temporal genetic arrangement and genetically distinct populations. Furthermore, the population structure of *H. aspersa* can be best described by an isolation-by-distance model: significant positive relationships between geographical and genetic distances ($P < 10^{-5}$; data not shown) are observed for both time points. Given the same general patterns of genetic variation between the two points in time, we could conclude that a geographical survey at one time point is sufficient to resolve the main characteristics of *H. aspersa* populations (e.g. Viard *et al*., 1997; Tessier & Bernatchez, 1999). In spite of anthropogenic disturbances and contrary to expectations, the overall temporal genetic structure of *H. aspersa* appeared quite stable. Few significant temporal differences were found and within-population levels of genetic variation remained undetectable, whether measured by mean number of alleles, allelic richness, H_o, H_e, or F_{IS}. Keeping in mind that only four loci were used, our results illustrated neither a consistent level of inbreeding, nor a loss of genetic variation with time.

However, conversely to the absence of noticeable changes in overall spatial genetic features over time, a great proportion (4 of 10) of the sampled populations (populations 1, 6, 7 and 9) can be considered unstable, given their striking changes in allele frequencies. Hence, it should be noted that significant temporal changes in allelic composition do not necessarily imply a concomitant change in intrapopulation genetic parameters, as also documented by Viard *et al*. (1997) and Heath *et al*. (2002) (but see Kinnison *et al*., 2002). In addition, the time frame of this study may have been too short to reveal more marked genetic changes. As temporal variation was measured between only two approximated and perhaps overlapping generations, it is difficult to detect more subtle changes in time and space, the latter being the result of several generations of differentiation (Hossaert-McKey *et al*., 1996).

Population size in *Helix aspersa*

The low density of adults during the sampling phase as well as localized extinction events led us to predict small current N_e. It has been argued that using highly polymorphic microsatellite loci is expected to make the temporal method of estimating N_e particularly robust (Turner *et al*., 2001; Heath *et al*., 2002). Nonetheless, because the estimations of the variance effective size are known to be rather imprecise (Waples, 1989), we chose to use different approaches. Whatever the estimation used, small values of N_e were detected in populations that exhibited significant temporal changes in allelic composition, i.e. populations 1, 6, 7 and 9. The variance of N_e estimates can be small when the genetic differentiation over time is important ($t/2N_e \geq 0.1$, which reflects a small effective size of 10 with $t = 2$), leading to the same range of N_e values for all methods. Moderate to large values of N_e, however, were found for six populations. In this case, estimations of the variance effective size are known to be relatively imprecise when $t/2N_e$ is small (Waples, 1989; Laval *et al*., 2003). For instance, populations 4 and 5 were characterized by great differences between the different estimators of N_e that we used, such disparities being the result of a large variance in N_e estimates. In this respect, these results may (i) confirm the large size of these populations, and (ii) indicate that the maximum likelihood method provides more biologically meaningful results because no infinite N_e values were found (see Table 4).

Large populations may be maintained by individuals that were not sampled. Usually, not all snails present in an area are active on the surface, even under suitable weather conditions (Greenwood, 1974; Baur, 1993; Pfenninger, Bahl & Streit, 1996). For instance, Lamotte (1951) observed that only about 10% of individuals in a colony of *Cepaea nemoralis* could be collected on any one visit, snails being presumably buried in the soil. Alternatively, large populations may be maintained because of low-density demes spread over a larger geographical area than that considered during the sampling (e.g. Lehmann *et al*., 1998). Direct ecological studies are, however, required to test these hypotheses in *H. aspersa*.

Estimates of effective population size in land snails are relatively scarce. Using mark–release techniques, Crook (1980) estimated *H. aspersa* neighbourhood sizes to range from 15 to 215 individuals, an order of magnitude equivalent to those observed in this study using indirect methods via genetic data. Similarly, Selander & Kaufman (1975) suggested a harmonic mean deme size of 15 individuals in *H. aspersa* from field observations. All these estimates of N_e are all considerably smaller than those estimated for other helicids such as *C. nemoralis* (Murray, 1964; Greenwood, 1974). Precise knowledge of population structure is essential for understanding evolutionary processes within land snail populations (Baur, 1993; Davison & Clarke, 2000; Bellido *et al*., 2002; Pfenninger, Eppenstein & Magnin, 2003). However, within

such an unstable mosaic landscape, we cannot assume a 'consensus' N_e in *H. aspersa*. Local populations are likely to display different and independent demographic histories including bottlenecks, immigration events and changing degrees of connection to neighbouring populations, depending on their own demographic and ecological characteristics (Arnaud, 2003).

Hence, N_e estimated from temporal changes in allelic frequencies yielded an assemblage of mixed populations, some characterized by moderate to large N_e and three colonies exhibiting N_e averaging only ten individuals. All things being equal, the general features of the metapopulation functioning in *H. aspersa* may be envisioned as a mixture of local, stable colonies with high N_e, associated with more unstable ones experiencing ephemeral situations and suggesting a nonequilibrium dynamic. Indeed, the populations sampled evolve in an unstable environment in which stressful conditions include burning and poisoning (Madec *et al*., 2000; Arnaud *et al*., 2003) as well as human harvesting, pressures little different from destructive sampling methods. As such, the variation detected in allelic frequencies could be a good illustration of the impact of unpredictable harmful conditions in agricultural environments.

The case of populations 6 and 8 is interesting given their spatial localization within the polder area: both are situated on the most recently constructed dyke that protects this agricultural area against ingress of seawater. This dyke constitutes the most inhospitable habitat for snail survival because of (i) constant exposure to the wind (i.e. desiccation of snails) and (ii) the scarcity of hospitable refuges for aestivating or hibernating (e.g. scattered trees, loose stones). Direct observations on this dyke suggest that isolated trees could act as transient reception habitats containing only migrant adults, but only a few stable populations situated in dyke interruptions (big, loose stones that act as resting sites) are able to persist and export colonizing individuals. Hence, site 6, localized around an isolated tree and characterized by a population with very small N_e, may be envisioned as a temporary 'transition' habitat during individual migration. This hypothesis is also suggested by the allelic frequency changes at loci *Ha2*, *Ha5* and *Ha11* (see Fig. 2); the pattern suggests a recent influx of immigrants rather than mutation events that may require a longer time frame to create novel alleles. Conversely, site 8, characterized by several refuges formed by rocks and a high juvenile density (J.-F. Arnaud, pers. observ.) could be viewed as a stable 'donor' habitat (*sensu* Hansson, 1991) owing to the relatively large N_e values observed (see Table 4). Indeed, the observation of donor, transition and reception habitats have been documented in a closely related species, *Helix pomatia*, for which both dispersing, resident and homing individuals were observed using mark–recapture studies (Hansson, 1991). This asymmetrical pattern of migration is commonly observed and sometimes described as source–sink systems (Harrison & Taylor, 1997; Frankham *et al*., 2002). Such a structure is likely to cause a very strong reduction in effective population size of the metapopulation as a whole, increasing the probability of fixation of deleterious alleles as well as reducing the overall level of genetic variance relative to a symmetrical island model (Whitlock, 2001).

The genetic effective size of a population is also affected by among-population processes such as the source of colonizers and the frequency of subsequent gene flow (McCauley, 1995; Thrall *et al*., 2000; Whitlock, 2001). Given that temporal methods estimate N_e by measuring the pace at which allele frequencies change, migration causing a rapid change in allele frequencies will result in an estimate of N_e that is too low (Heath *et al*., 2002; Wang & Whitlock, 2003). An interesting feature in our data is the relatively high gene diversity (60–70%) observed for populations 1, 6 and 7 which is not consistent with their low N_e estimates. This conservation of genetic diversity suggests the possibility of intermittently low levels of gene flow from nearby populations, effectively comprising a genetic connectedness through a metapopulation structure. The dispersal features of this metapopulation functioning are likely to involve displacements resulting from farming practices (Arnaud *et al*., 2003) and could explain, at least partially, the persistence of *H. aspersa* in this agricultural environment compared to other closely related land snail species with low dispersal capabilities, such as *Cepaea nemoralis* or *C. hortensis*, that are unable to survive in this man-disturbed area.

Moreover, the demographic functioning of a metapopulation creates intrinsic emergent properties that influence the evolution of major biological traits (the 'metapopulation effect' see Olivieri & Gouyon, 1997). Hence, the large phenotypic plasticity found in *H. aspersa* (Madec *et al*., 1998, 2000) could also play a role in its high colonizing capacity for all anthropogenically disturbed habitats. In this latter respect, the selective regime of the polders seems to result in an exceptionally high fecundity and a reduced life span of adult snails (Madec *et al*., 2000). Moreover, Murray (1964) argued that the practice of multiple matings and sperm storage over long periods is likely to buffer the genetic effects of marked population fluctuations and, consequently, to increase the effective population size. The land snail *H. aspersa* is a simultaneously hermaphroditic animal where self-fertilization is unknown. Ongoing paternity analyses in *H. aspersa* (G. Evanno, L. Madec & J.-F. Arnaud, unpubl. data) demonstrate that, in a single clutch, offspring from several matings are produced. This multiple paternity,

together with long-term storage of sperm and inbreeding avoidance, would provide a reservoir of variability and minimize the genetic effects of population size reduction. As also suggested by Murray (1964), the evolution of the mating system in land snails may be directed towards multiple matings and sperm storage precisely to protect organisms with moderate dispersal capabilities against genetic depauperacy, especially those living in changing environments with stochastic disturbance. *Helix aspersa* is closely associated with people (Guiller *et al*., 1994; Arnaud *et al*., 1999a,b), and as a consequence is found preferentially in disturbed and transient habitats, suggesting that situations of nonequilibrium dynamics might be the rule in this land snail species.

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