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Description and Power Analysis of Two Tests for Detecting Recent Population Bottlenecks From Allele Frequency Data

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ABSTRACT

When a population experiences a reduction of its effective size, it generally develops a heterozygosity excess at selectively neutral loci, *i.e.*, the heterozygosity computed from a sample of genes is larger than the heterozygosity expected from the number of alleles found in the sample if the population were at mutation drift equilibrium. The heterozygosity excess persists only a certain number of generations until a new equilibrium is established. Two statistical tests for detecting a heterozygosity excess are described. They require measurements of the number of alleles and heterozygosity at each of several loci from a population sample. The first test determines if the proportion of loci with heterozygosity excess is significantly larger than expected at equilibrium. The second test establishes if the average of standardized differences between observed and expected heterozygosities is significantly different from zero. Type I and II errors have been evaluated by computer simulations, varying sample size, number of loci, bottleneck size, time elapsed since the beginning of the bottleneck and level of variability of loci. These analyses show that the most useful markers for bottleneck detection are those evolving under the infinite allele model (IAM) and they provide guidelines for selecting sample sizes of individuals and loci. The usefulness of these tests for conservation biology is discussed.

UNDERSTANDING the effects of population bottlenecks on genetic variation has become increasingly important in population genetics, speciation theory, and conservation biology. Conservation biologists widely agree that population bottlenecks should be avoided in threatened species because they can increase rates of inbreeding, loss of genetic variation and fixation of mildly deleterious alleles, and thereby reduce adaptive potential and increase the probability of extinction (FRANKEL and SOULÉ 1981; RALLS *et al.* 1988; HEDRICK and MILLER 1992; JIMINEZ *et al.* 1994; LANDE 1994; MILLS and SMOUSE 1994; VRIJENHOEK 1994; FRANKHAM 1995a,b; LYNCH *et al.* 1995; but see BRYANT *et al.* 1986 and GOODNIGHT 1987). Populations that have recently suffered a severe reduction in size are especially important to identify for conservation because they are most likely to suffer increased risk of extinction.

Bottlenecks or founder events may be important in some modes of speciation (MAYR 1954; CARSON 1971; KANESHIRO 1976, 1980; TEMPLETON 1980). For example, colonization of a new area by few individuals or a single mated female may cause extensive genetic changes that lead to reproductive isolation (CARSON 1971). Researchers are conducting field studies to detect natural colonization and founder events, and to evaluate the effects of bottlenecks on colonizing populations (CARSON 1992; THORNTON 1992 and references therein). These studies would benefit from molecular methods that can detect historical bottlenecks and help evaluate the magnitude and pattern of genetic change associated with colonization.

Many authors have invoked the "bottleneck hypothesis" to explain observations of low genetic variation in their study species (BONNEL and SELANDER 1974; PEMBERTON and SMITH 1985; MENKIN 1987; O'BRIEN *et al.* 1987, 1988 and citations therein; RANDI and APOLLONIO 1988; GOTTELLI *et al.* 1994). However, conclusions that a population has suffered a severe bottleneck and loss of genetic variation are usually inferential because researchers seldom know historical population sizes. Consequently, it has been suggested that the bottleneck hypothesis and the effect of bottlenecks on genetic variation have been overemphasized in the literature (PIMM *et al.* 1989; CARSON 1990; DINNERSTEIN and MCCRACKEN 1990).

Because historical population sizes and levels of genetic variation are seldom known, methods for detecting bottlenecks in the absence of historical data would be useful. ROGERS and HARPENDING (1992) have developed a method for detecting ancient historical bottlenecks using DNA sequence data and a genealogical analysis approach. The present paper describes and evaluates methods for detecting recent historical bottlenecks using allele frequency data. These methods take advantage of the high level of polymorphism detectable

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by recently discovered molecular markers (*i.e.*, micro and mini satellites) and of the well developed theory of the effects of bottlenecks on loss of alleles and heterozygosity from selectively neutral loci (NEI *et al.* 1975; DENNISTON 1978; WATTERSON 1984, 1986; MARUYAMA and FUERST 1985; ALLENDORF 1986).

We first discuss the principles of the method for detecting bottlenecks, and then determine the theoretically expected relationship between number of alleles and heterozygosity for a locus evolving under the infinite allele model (IAM) and stepwise mutation model (SMM) in a bottlenecked population. Next we describe two statistical tests for detecting bottlenecks using empirical data. Finally, we apply the two statistical tests to four example data sets from the literature and conduct a statistical power analysis to determine the sample sizes of loci and individuals necessary to detect bottlenecks using the two statistical tests.

PRINCIPLE OF THE METHOD

For selectively neutral loci, allele number and frequency distribution in natural populations result from an equilibrium between mutations and genetic drift. The parameters of this "mutation drift" equilibrium are the mutation rate and the effective population size. When a bottleneck occurs in a population and the effective population size is significantly reduced, it causes a correlative and progressive reduction of the allele number and heterozygosity. As noted by NEI *et al.* (1975; see also DENNISTON 1978), allelic diversity is reduced faster than is heterozygosity during a bottleneck. As a consequence, there is a transient deficiency in the number of alleles found in a sample of individuals (MARUYAMA and FUERST 1985), *i.e.*, the observed number of alleles in the sample is less than the number of alleles expected from the observed heterozygosity under the assumption that the population is at mutation drift equilibrium. Note that MARUYAMA and FUERST's computations rely on the assumption that loci evolve according to the IAM. With this model, each mutation produces a new allele that is different from all existing ones (KIMURA and CROW 1964).

The allele deficiency is a complex function of four parameters: the time since the beginning of the bottleneck (t), the effective population size ratio before/after the beginning of the bottleneck (α), the mutation rate (μ) of the locus and the sample size of genes (n). MARUYAMA and FUERST's computations have shown that the magnitude of an allele deficiency first increases with t, reaches a maximum and then decreases asymptotically toward zero, corresponding to a new mutation drift equilibrium. The value of the maximum as well as the time at which it is reached depend on the other three parameters (α, μ and n).

Since recently bottlenecked populations exhibit an allele deficiency, a potential test for detecting bottlenecks might be based on detecting an allele deficiency in a sample taken from the population under study. If a significantly high proportion of loci exhibit an allele deficiency, one might conclude that the population is not at mutation drift equilibrium due to a recent reduction of the effective population size. To test for an allele deficiency, one has to determine the relationship between the observed heterozygosity and the expected number of alleles. In theory, these two quantities are related through the parameter θ ($4N_e\mu$). However, it has been shown that the estimation of θ from the heterozygosity is biased (ZOUROS 1979) and that it is much more efficient to use the number of alleles for that purpose (EWENS and GILLESPIE 1974). A better solution would hence be to base a test on the difference between the observed heterozygosity and the heterozygosity expected from the observed number of alleles. Populations exhibiting a significant *heterozygosity excess* would be considered as having experienced a recent genetic bottleneck. Note that this heterozygosity excess should not be confused with the *excess of heterozygotes*. The former compares observed and expected *heterozygosities*, in the sense of NEI's (1978, p. 177) *gene diversities*, whereas the latter compares the *number of heterozygotes* with Hardy-Weinberg equilibrium expectation.

Heterozygosity excess or deficit can occur after a recent change of the effective population size but also if heterozygotes have a selective advantage or disadvantage. The comparison between observed and expected homozygosities (which is obviously equivalent to comparing heterozygosities) has already been used as a test for detecting selection (WATTERSON 1978). However, this test for homozygosity (or heterozygosity) deviation concerns specific loci whereas all loci are affected in the same way after a bottleneck. Therefore, tests for detecting a bottleneck should be based on the average behavior of a sufficiently large set of loci.

Before devising statistical tests for detecting a heterozygosity excess, it is important (1) to verify that the conclusion that bottlenecks cause a heterozygosity excess under the IAM can be extended to other mutation models and (2) to understand the theoretically expected relationship between magnitude of heterozygosity excess and observed number of alleles as a function of the time elapsed since the beginning of the bottleneck. Because the relationship between heterozygosity and allele number depends on the mutational process, we evaluate two classical models of mutation. We first evaluate the IAM because all the theoretical developments cited above have been based on this model. We evaluate also the Stepwise Mutation Model (SMM) because some important loci such as microsatellites may more closely follow this model (SMM, SHRIVER *et al.* 1993; VALDES *et al.* 1993). Under the strict SMM, mutations change the state of an allele by one step forward or backward with equal probability (OHTA and KIMURA 1973). Thus the SMM allows for mutation to existing

allelic states (homoplasy) and thereby results in fewer distinct allelic states than the IAM for a given mutation rate. Another reason for evaluating the SMM is because this model and the IAM are considered as two extremes over the range of possible mutation models (CHAKRABORTY and JIN 1992).

Infinite allele model: WATTERSON (1984) provided formulae for determining the relationship between heterozygosity and number of alleles at loci evolving under the IAM in a bottlenecked population. WATTERSON's formulae are based on the following assumptions: (1) the population evolves according to the Wright Fisher model of random mating; (2) before the bottleneck occurs, the population of N diploid ($2N$ haploid) individuals is at mutation drift equilibrium. From a given generation onward (corresponding to $t = 0$), the size of the population is changed to N_1 diploid ($2N_1$ haploid) individuals.

Following WATTERSON (1984) and noting $\theta = 4N\mu$ and $\theta_1 = 4N_1\mu$, the number of different alleles, K_o, in a sample of n genes taken in the population at time t measured in units of $2N_1$ generations has the following expectation:

$$E(K_o) = \sum_{k=0}^{n} P_k \left[\sum_{j=1}^{k} \theta/(j + \theta - 1) + \sum_{j=k+1}^{n} \theta_1/(j + \theta_1 - 1) \right], \quad (1)$$

where

$$P_k = \sum_{j=k}^{n} e^{-j(j+\theta_1-1)t/2} \times (-1)^{j-k}[(2j + \theta_1 - 1) \times (k + \theta_1)_{(j-1)} n_{[j]}]/[k!(j - k)!(n + \theta_1)_{(j)}],$$

$$x_{(j)} = x(x + 1) \cdots (x + j - 1) \quad \text{and} \quad x_{[j]} = x(x - 1) \cdots (x - j + 1)$$

Similarly, the mean sample heterozygosity is given by

$$H = 1 - E(F) \quad (2)$$

where

$$E(F) = \sum_{i=1}^{n} (i/n)^2 E(\Gamma_i)$$

$$E(\Gamma_i) = [i(\theta_1 + n - 1)_{[i]}]^{-1} \sum_{k=0}^{n} P_k \left\{ \sum_{l=0}^{A} [\theta + (\theta_1 - \theta)\delta_{l,0}] \times C_i^l k_{[l]}(n - k)_{[i-l]}(\theta_1 + k - 1)_{[l]}[(\theta_1 + n - 1)_{[l]}]^{-1} \right\},$$

A = smaller of k and i,

$\delta_{l,0} = 0$ if $l \neq 0$ and $\delta_{l,0} = 1$ if $l = 0$.

The unbiased observed heterozygosity in the bottlenecked population is equal to $(1 - F)n/(n - 1)$ and its expectation is $Hn/(n - 1) = H_o$. To compute the corresponding expected heterozygosity, H_1, in a population at mutation drift equilibrium, we first look for the value of M that satisfies EWENS's (1972) formula,

$$E(K_o) = \sum_{j=0}^{n-1} M/M + j),$$

and compute $H_1 = M/(1 + M)$.

The difference $H_o - H_1$ measures the heterozygosity excess (if $H_o > H_1$) or deficiency (if $H_o < H_1$) and $E(K_o)$ is the (average) observed number of alleles in the sample. Figure 1A provides an example of the relationship $H_o - H_I$ as a function of $E(K_o)$ for a sample of 50 genes and various times after a bottleneck has occurred. In this example, the population size was reduced 100-fold ($\alpha = 100$) at the initiation of the bottleneck. The curves linking points in Figure 1A correspond to a given time after the bottleneck began. These curves have been established with a set of different values of θ corresponding to a geometric series (ratio $2^{0.5}$) starting at 0.25 and ending when the average number of alleles is larger than 10. This figure shows that (1) all values of $H_o - H_I$ are positive (a heterozygosity excess exists) as expected for a bottlenecked population, (2) for a given time after the bottleneck began, there is a rapid increase of the heterozygosity excess as a function of the number of alleles, until the curve reaches a maximum and then slowly decreases back to zero, and (3) a maximum heterozygosity excess of 0.14 is obtained around N_I ($0.5 \times 2N_I$) generations after the bottleneck began. Note that the largest heterozygosity excess occurs for values of θ ranging from 1 (for $t = 0.005$) to 8 (for $0.5 \leq t \leq 2.5$). These values of θ are reasonnable for microsatellite data but are larger than those usually found with allozyme loci.

Stepwise mutation model: CHAKRABORTY and NEI (1977) provided formulae describing the change of heterozygosity for loci evolving under the SMM in a population following a bottleneck. However, no explicit formula is available for calculating the expected number of alleles in a sample. So we employed a computer simulation approach based on the coalescent process of a sample of n genes (see HUDSON 1990), with a slight modification in the computation of the times $T(j)$ during which j lines of descent are present in the genealogy of the sampled genes. With a bottleneck in which population size immediately changes from N to N_I at time τ, $T(j)$ were computed as follows.

Starting from $j = n$ to $j = 2$, let $S(j) = T(n) + T(n - 1) + \ldots + T(j + 1)$ and $S(n) = 0$ and let q be an outcome of an exponential law with parameter $c = j(j - 1)/2$ as above. We took

$$T(j) = q \quad \text{if } S(j) + q < \tau$$

$$T(j) = (N/N_1)q \quad \text{if } S(j) > \tau$$

$$T(j) = (\tau - S(j)) + (N/N_1)(S(j) + q - \tau) \quad \text{if } S(j) < \tau \text{ and } S(j) + q > \tau.$$

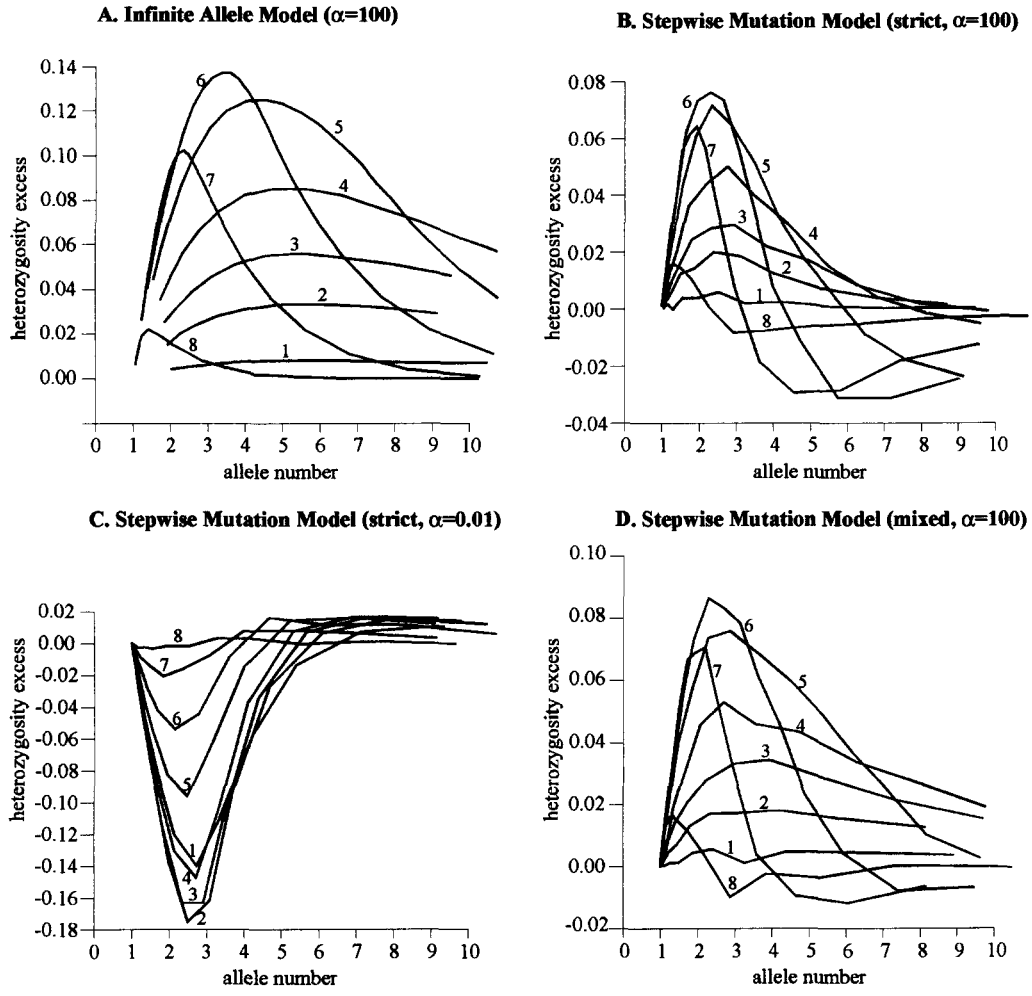

FIGURE 1.—Theoretical relationships between heterozygosity excess ($H_o - H_e$) and number of alleles in a population at different times (t) after an immediate change of the population size in units of $2N_e$ generations (N_e = postchange effective population size). In cases A, B and D, the size was *reduced* by a factor of 100 (α = 100) whereas in case C, it was *increased* by the same factor (α = 0.01). In case A, loci evolve according to the IAM model. In cases B and C, they follow the strict SMM. In case D, loci evolve under a mixed SMM model (90% one-step and 10% multistep SMM). A results from direct computations based on WATTERSON's (1984) paper. B–D result from computer simulations (10,000 replicates, see text for details).

A gene phylogeny is obtained by randomly coalescing pairs of genes. Mutations on the edges of the phylogeny are simulated according to a Poisson law with parameter $2N_f\mu = \theta_1/2$. Starting with an arbitrary state (allelic class) in the ancestor, the states of all nodes are determined, taking into account all mutations along the edges. Under the strict one step SMM, a mutation changes the allelic state by +1 or −1 with equal probability. Eventually, allelic states of the n genes of the sample are determined and the number of different alleles and the heterozygosity in the sample are deduced.

For a given value of θ_1, the average number of different alleles [equivalent to $E(K_o)$] and the average heterozygosity (equivalent to H_o) are computed. The latter is then compared to the heterozygosity (H_1) expected under mutation drift equilibrium and corresponding to the observed number of alleles. We obtained this expected heterozygosity using the same simulation process in which the effective population size does not change ($N = N_1$, $\alpha = 1$). More precisely, we established the relationship at equilibrium between the number of alleles and the heterozygosity for a sample of the same size (n = 50) and by varying θ_1 according a geometric progression (ratio $2^{0.5}$) starting at 0.0001 and increasing up to a value producing on average (over 20,000 replicates) >12 different alleles. Expected equilibrium heterozygosities were then estimated from the average number of alleles by linear interpolation.

Figure 1B shows the average relationship between heterozygosity excess and number of alleles under the strict SMM for a sample of 50 genes. As in Figure 1A, each curve corresponds to a different time following the initiation of the bottleneck. These lines have been established by varying θ_1 according to a geometric progression of ratio 2 starting at 0.01 and ending when the number of alleles exceeds 10. Every point is the average of 5000 simulation replicates. The shapes of the curves for the SMM (Figure 1B) are similar to those for the IAM (Figure 1A). The main differences are the heterozygosity excess is lower under the SMM than the IAM, the maxima of the curves are obtained for lower values

of the number of alleles and negative values (heterozygosity deficiency) occur with larger values of θ_1.

The fact that heterozygosity deficiency can occur with loci evolving under the strict one step SMM presents two potential problems for the detection of bottlenecks when using tests for heterozygosity excess. First, the test may not be significant even in a bottlenecked population because some loci, evolving under the strict SMM and with large θ_1, exhibit heterozygosity deficiency. Second, by symmetry, heterozygosity excess might occur for SMM loci when the population is expanding. As for the latter problem, Figure 1C strongly suggests that expanding populations produce a virtually undetectable heterozygosity excess under the strict SMM. [Note: Figure 1C results from the same simulation process as Figure 1B but with a population expansion instead of a population size reduction ($\alpha = 0.01$ instead of $\alpha = 100$)]. Consequently, a significant heterozygosity excess for selectively neutral markers should be detected only in populations having experienced a recent size reduction. Note that in the case of a population expansion, the heterozygosity deficiency reaches much larger values than after a population size reduction.

However, since population size reduction results in both heterozygosity excess and deficiency for markers evolving under the strict SMM, the power of a potential test for detecting bottlenecks might be largely reduced for these kinds of markers. It is then useful to study intermediate mutation models to evaluate in which conditions bottlenecks can be more easily detected by testing for heterozygosity excess. Figure 1D illustrates the results obtained with a model in which 90% of mutations follow the strict SMM (one step SMM) and the remaining 10% produce multistep changes (between 1 and 20 steps, according to a uniform distribution). This model still produces heterozygosity deficiency, but at an undetectable level whereas the maxima (heterozygosity excess) slightly increase.

Since (1) the *strict* SMM is a rather unrealistic model for most of genetic markers, (2) a small deviation from this model reduces drastically the heterozygosity deficiency in favor of the heterozygosity excess (Figure 1D), and (3) even under the strict SMM, the heterozygosity excess resulting from a population size *expansion* is virtually undetectable, a significant heterozygosity excess at a set of selectively neutral loci can still be taken as a strong indication that a recent population size reduction has occurred.

DESCRIPTION OF THE TWO TESTS

Now that the expected relationship between number of alleles and degree of heterozygosity excess in a bottlenecked population has been established, we describe two statistical tests for detecting a heterozygosity excess in data from natural populations.

Test 1: Because population bottlenecks induce a transient excess of heterozygosity, finding an observed heterozygosity that is higher than the expected (equilibrium) heterozygosity for a large majority of loci in a population suggests that this population may have recently experienced a genetic bottleneck. A possible statistical test for excess of heterozygosity would simply be a sign test on the difference (observed − expected) heterozygosity across all loci in a population sample.

Assume that a sample of n individuals has been scored for L polymorphic loci (monomorphic loci are useless since the observed and expected heterozygosities are identical, *i.e.*, both are equal to zero). If mutation drift equilibrium is assumed (*i.e.*, no bottleneck), there is approximately an equal probability of getting a positive or a negative difference between the observed and the expected heterozygosities. In contrast, if there has been a recent bottleneck in the population, a positive difference (heterozygosity excess) should be observed more often than is a negative difference. Hence, we could simply test if the number of loci for which there is a heterozygosity excess is significantly larger than $L/2$, assuming an *a priori* binomial distribution of parameters L and $1/2$. However, in a finite sample, heterozygosity can have only a finite number of values and its probability distribution is discrete and asymmetric. Consequently, for any locus there is a specific probability (slightly different from 0.5) of heterozygosity excess. This probability can be computed if we know the theoretical distribution of the heterozygosity in a sample of n individuals assuming mutation drift equilibrium. As shown below, the latter distribution can be established, using computer simulations under both the IAM and SMM mutation models.

To compute the probability ($\mathrm{pr}_L[l]$) of having l loci among L for which there is a heterozygosity excess, we take the usual rationale in which the drawing of an additional locus is considered as a Markovian process in which states are the number of loci showing an heterozygosity excess $[0, 1, 2, \ldots, L]$. Starting with zero locus and a probability distribution $\mathrm{pr}_0[l]$ equal to [1, 0, 0, . . . , 0], we used the following recurrence relationships for $m = 1$ to L

$$\mathrm{pr}_m[l] = \{(1 - p_{1m})\ \mathrm{pr}_{m-1}[l]\} + \{p_{1m}\ \mathrm{pr}_{m-1}[l-1]\},$$

where $p_1 m$ is the probability of heterozygosity excess at locus m.

$$\text{Let} \quad Q = \sum_{l=l_0}^{L} \mathrm{pr}_L\,[l]$$

Q is the probability of getting at least l_o loci with heterozygosity excess. If Q is lower than (say) 0.05, the null hypothesis (mutation drift equilibrium) is rejected in favor of the hypothesis of an overall heterozygosity excess and a recent genetic bottleneck.

Test 2: Test 1 does not take into account the magnitude of the heterozygosity excess/deficiency. Under the

null hypothesis, the difference between the observed (H_o) and the expected heterozygosity (H_e) is the output of a random variable with an expectation equal to zero for all loci. If we divide these differences by the standard deviation of the corresponding distributions of heterozygosities, we will get standardized deviates, *i.e.*, random variables following different distributions but all having null expectation and standard deviation equal to one. The sum of these L standardized deviates approaches a Gaussian distribution with null expectation and variance equal to L, when L increases by virtue of the Central Limit theorem. Therefore, Test 2 consists in comparing the statistics T_2 to a $N(0,1)$ distribution,

$$T_2 = L^{-0.5}\sum_{l=1}^{L}\{\Delta_l/\sigma_l\},$$

with $\Delta l = (H_o - H_e)$ for the lth locus and σl is the standard deviation of the distribution of H at the lth locus. If the alternative hypothesis is only a heterozygosity excess (one-tailed test), we will reject the null hypothesis (population at mutation drift equilibrium) at the 5% level if T_2 is larger than or equal to 1.645 (as indicated in any table of the normal distribution).

Distribution of heterozygosity under the null hypothesis: For both tests, we need to establish probability distributions of the heterozygosity under the null hypothesis (population at mutation drift equilibrium). These probability distributions are actually conditioned by the observed number of alleles. Consequently, we cannot follow the previous approach that assumes that the parameter θ is known, since a range of values of θ are compatible with the observed number of alleles. Because the IAM and SMM have different properties, a different solution was applied to each model to take into account these new requirements.

For the IAM, the number of alleles is a nondecreasing function of the number of mutations in the genealogy of the sampled genes, *i.e.*, a new mutation increases the number of different alleles by zero (when it hits an edge already bearing a mutation) or one (when it hits a new edge). Therefore, to obtain a simulated sample with exactly k_o alleles, we first simulate a genealogy of the n genes in a population at mutation drift equilibrium (with edge lengths following the usual exponential law). Then we add one mutation at a time until there are exactly k_o different alleles in the sample. Since mutations are distributed according to a Poisson law, the probability of one mutation affecting a given edge is proportional to the relative length of this edge. The distribution of heterozygosities in samples simulated as explained above, is θ-free.

The same rationale cannot be applied to the SMM, because the number of alleles can increase, decrease or remain unchanged when adding a new mutation. Therefore, we used the classic simulation process of the coalescent process (assuming mutation drift equilibrium) with two modifications: (1) only iterations leading to the observed number of alleles (*i.e.*, the number, k_o, actually observed in the sample) were considered and (2) for each iteration, the value of θ was taken at random following a probability distribution defined according to the following Bayesian approach. Assuming a uniform prior distribution of θ, the condition $k = k_o$ is accounted for by considering a posterior distribution of θ proportional to the probability of getting k_o alleles given θ. This was achieved by simulating the coalescent process of a sample of n genes using a set of values of θ and counting, for each θ, the proportion of iterations in which $k = k_o$. In practice, the following steps were performed.

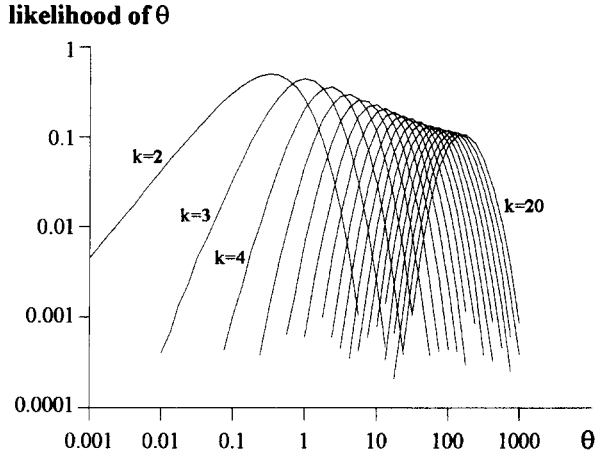

FIGURE 2.—Likelihood of θ given the number of alleles (k) for a locus evolving under the SMM. Curves have been obtained through computer simulation (200,000 replicates per value of θ) for a sample of 50 genes. The coalescent process was simulated using 49 values of θ ranging from 0.001 to 1000 and following a geometric progression (ratio $10^{1/8}$).

Step 1: Find extremes of the distribution of the likelihood of θ given k_o and n. For instance, starting with $\theta_{min} = 10^{-7}$, 200 iterations are performed. If none of them produces the correct number of alleles, a new θ_{min} is taken by multiplying the previous one by 10. This process is repeated until at least one iteration (out of 200) produces the observed number of alleles. The final minimum is then taken as the last θ_{min} divided by 10. An analogous procedure is conducted to get the maximum.

Step 2: Define a step between two successive values of θ. Preliminary studies have shown that an almost symmetric distribution of θ is obtained by varying θ along a logarithmic scale (Figure 2). Consequently, a multiplicative step between two successive θ's was applied in such a way that the range $\theta_{min} - \theta_{max}$ was covered with 12 different values of θ. The choice of 12 is arbitrary but it provides an acceptable compromise between precision and speed of execution.

Step 3: For each of the 12 θ's, the proportion of iterations (out of 1000) giving exactly k_o alleles is computed.

After completing these three steps, the coalescent process was simulated taking at random one of the 12 preceeding θ's with a probability proportional to the propor-

TABLE 1

Example of computations used to test for an heterozygosity excess at nine polymorphic loci in the Epping population of the hairy-nosed wombat

Locus	105	102	25CA	51CA	54CA	55A	67CA	68CA	71CA
Empirical data									
Sample size (haploid genomes)	48	40	32	56	56	56	56	56	56
Heterozygosity observed (H_o)	0.496	0.409	0.653	0.195	0.659	0.382	0.520	0.673	0.299
No. of alleles observed (k_o)	2	2	3	2	3	2	3	3	2
IAM									
Average heterozygosity (H_e)	0.22	0.23	0.40	0.20	0.35	0.21	0.36	0.35	0.21
Standard deviation (SD)	0.17	0.17	0.16	0.16	0.17	0.17	0.18	0.18	0.17
Standard deviate ($[H_o - H_e]/SD$)	1.72	1.08	1.50	−0.07	1.69	1.08	0.85	1.76	0.56
Probability ($H > H_e$)	0.45	0.41	0.54	0.42	0.51	0.41	0.52	0.52	0.43
SMM									
Average heterozygosity (H_e)	0.26	0.26	0.48	0.24	0.43	0.24	0.44	0.43	0.25
Standard deviation (SD)	0.17	0.17	0.14	0.17	0.16	0.17	0.16	0.16	0.17
Standard deviate ($[H_o - H_e]/SD$)	1.41	0.88	1.25	−0.26	1.41	0.88	0.50	1.52	0.30
Probability ($H > H_e$)	0.46	0.51	0.59	0.46	0.58	0.46	0.59	0.58	0.49

Data from TAYLOR *et al.* (1994). H_e and SD are the average and standard deviation of the distribution of the heterozygosity (H) obtained in simulated samples with a size and number of alleles identical to the empirical data, assuming mutation-drift equilibrium (null hypothesis). Prob ($H > H_e$) is the probability that the heterozygosity (H) is larger than the average (H_e) under the null hypothesis [Note that if the distribution is symetrical, Prob ($H > H_e$) will be 0.5]. All estimates are based on computer simulations with 1000 replicates. All heterozygosities (H_o, H_e and H) are computed according to the formula of NEI (1987): $[n/(n-1)][1 - \Sigma(p_i)^2]$ in which n is the number of haploid genomes and p_i the allele frequencies.

tion computed in step 3, disregarding all iterations that produce a number of alleles different from k_o.

For each locus, the average heterozygosity and standard deviation can be estimated through the above simulation processes with a level of precision controlled by the number of simulation replications.

EXAMPLES

Next we give examples of how to apply Test 1 and Test 2 to each of four empirical data sets published in the literature. The four data sets were chosen because they correspond to three situations of historical evolution of population size (reduction, expansion or stationarity).

An example of a population having experienced a recent bottleneck is the endangered species *Lasiorhinus krefftii,* the northern hairy-nosed wombat. The only remaining population (Epping Forest population in Queensland, Australia) has been reduced to fewer than 100 individuals. TAYLOR *et al.* (1994) have scored 16 microsatellite loci, of which nine are still polymorphic in a sample of 16–25 individuals. Data are summarized in Table 1.

For each polymorphic locus, simulations were performed to simulate gene genealogies with a sample size and a number of alleles identical to those in the data. One thousand simulation replicates were conducted under both mutation models and assuming mutation drift equilibrium. In each replication, and under each mutation model, the unbiased heterozygosity (NEI 1978) was computed. The average and standard deviations of these distributions were then estimated, as well as the proportion of heterozygosity values that were larger than the average (Table 1). Note that the latter proportion, which provides an estimate of the probability of heterozygosity excess under the null hypothesis for each locus, varies around 0.5 (between 0.4 and 0.6).

Under both mutation models, the observed heterozygosity exceeds the average of the corresponding distribution of heterozygosities expected at equilibrium for eight of the nine loci in the sample from the Epping wombat population. The probability of getting at least eight loci with heterozygosity excess in an equilibrium population is obtained by summing the last two columns of Table 2 (eight and nine loci). This sum is 0.011 and 0.026, under the IAM and the SMM, respectively, suggesting that the population significantly deviates from equilibrium expectations.

The statistic T_2 is equal to 3.43 and 2.64, respectively, under the IAM and the SMM. The corresponding probabilities are equal to 0.00030 and 0.0042. Thus, both Test 1 and Test 2 reject the hypothesis of mutation-drift equilibrium in the Epping population of wombats. Since, all loci except one exhibit a heterozygosity excess, these data support the conclusion of a recent bottleneck in this population.

For the second example, we applied both tests to a population of southern hairy-nosed wombats (*L. latifrons*) that is not known to have been bottlenecked recently (Brookfield population, data from TAYLOR *et al.* 1994). We found that under the IAM model, all 14 polymorphic microsatellite loci show a heterozygosity excess: this is highly significant ($P = 0.00037$). Test 2 results in the same conclusion ($T_2 = 3.54$, $P = 0.00020$).

TABLE 2

Probability distribution of the number of loci with a heterozygosity excess assuming mutation-drift equilibrium in the Epping wombat population

	No. of loci with a heterozygosity excess									
	0	1	2	3	4	5	6	7	8	9
IAM (Prob × 10,000)	33	269	955	1968	2594	2266	1313	487	105	10
SMM (Prob × 10,000)	12	120	539	1404	2335	2573	1877	875	237	28

This table is obtained by combining the Probabilities ($H > H_e$) from Table 1. For instance, the probability of getting nine loci with a heterozygosity excess is simply the product of the probabilities ($H > H_e$) for all nine loci (*cf.* text for the general rationale). This table shows that the probability of finding a heterozygosity excess at at least eight loci in the sample of the Epping wombat population is 0.0105 + 0.0010 under the IAM and 0.0237 + 0.0028 under the SMM.

Under the SMM, 10 loci show a heterozygosity excess. Ten of 14 loci is not significant under Test 1 ($P = 0.25$), but Test 2 provides a significant result ($T_2 = 1.81$, $P = 0.035$). Three tests out of four reject the hypothesis of mutation drift equilibrium in this population, suggesting here too a recent reduction in population size.

The next example is drawn from the microsatellite study of the Sardinian human population (DI RIENZO *et al.* 1994). The genetic variability of this population has been well documented using traditional markers, nuclear DNA polymorphisms and sequences of the D loop of mitochondrial DNA (DI RIENZO *et al.* 1994 and references therein). These studies suggest that this population has undergone a long period of size increase without immigration. If the Sardinian population has been expanding in size during the last few thousand generations, then an allele excess, *i.e.*, heterozygosity *deficiency*, is expected (MARUYAMA and FUESRT 1984) in contrast to the allele deficiency/heterozygosity excess as was seen in the wombat examples. Applying the present tests to the microsatellite data of DI RIENZO *et al.* (1994), the results were highly significant in all four combinations of test × model of mutation; *all* 10 microsatellite loci of the Sardinian population showed a *large* heterozygosity *deficiency* for the IAM and SMM ($P = 0.000093$ for the IAM and $P = 0.00014$ for the SMM) and hence negative standard deviates ($T_2 = 15.1$ under the IAM and $T_2 = 47.7$ under the SMM).

The last example is drawn from a microsatellite survey of continental and island populations of bumble bees (ESTOUP *et al.* 1995a). No genetic differentiation among the continental populations was detected, suggesting large scale genetic exchanges across the European continent. In contrast, Mediterranean island populations were clearly differentiated, indicating that migration of bumble bees is almost impossible over large distances across the sea. If the island population sizes have not been pertubated in recent history, then rapidly evolving loci, such as microsatellites, should be near mutation drift equilibrium. Our tests, applied to the population from Corsica island, showed no significant departure from equilibrium in all four combinations of tests, as expected for populations at mutation-drift equilibrium.

POWER ANALYSIS

A statistical power analysis was conducted to assess the ability of Tests 1 and 2 to detect bottlenecks under various conditions. Power is defined here as the probability of detecting a heterozygote excess when a recent bottleneck has occurred. Once more, the analysis was performed by computer simulation of the coalescent process as described above, varying the following parameters:

- sample size (20, 40, 60, 80 and 100 sampled genes)
- time since the beginning of the bottleneck (0.01, 0.025, 0.1, 0.25, 1, 2.5 and 10 multiplied by $2N_e$ generations)
- number of scored loci (5, 10, 15, and 20 polymorphic loci)
- effective population size ratio before/after the bottleneck ($\alpha = 10, 100, 1000$)
- mutation model (IAM or SMM).

For each set of parameters, 500 bottleneck simulation replicates were performed and the number of times Test 1 or Test 2 was significant ($P < 0.05$) and in the "right" direction (overall heterozygosity excess) was recorded. For each mutation model, two different ranges of heterozygosities [0.0 − 0.3] and [0.3 − 0.8] (at mutation drift equilibrium) were taken to compare the performance of each test for different types of genetic markers. At each iteration, loci were assigned a random *pre*bottleneck heterozygosity from a uniform distribution within the range of [0.0 − 0.3] or [0.3 − 0.8] and the corresponding value of $\theta(H/[1 - H]$ under the IAM or $0.5\{[1 - H]^{-2} - 1\}$ under the SMM) was used to simulate mutations along the edges of the gene phylogeny. Only loci that were polymorphic in the sample (*i.e.*, after the bottleneck) were retained. To save computer time, the three parameters necessary for the tests (average heterozygosity, probability of finding a heterozygosity above the average and standard deviation of the heterozygosity, assuming mutation drift equilibrium) were tabulated for all necessary combinations of sample sizes and number of alleles, according to the same simulation procedures described earlier (distribution of heterozygosity under the null hypothesis). Fig-

ure 3, A and B, summarizes the results of the power analysis for the IAM and SMM, respectively.

(1) The power is much higher when loci evolve under the IAM than under the SMM. For example, power is 0.8–1.0 for the IAM, but only 0–0.2 for the SMM when $0 < H < 0.3$, $\alpha = 10$, $\tau = 1$, and 20 loci and 60 genes are sampled. Thus to detect a bottleneck with a high probability, one needs more loci and larger sample sizes when using markers evolving under the SMM than with markers following the IAM.

(2) With stronger bottlenecks (larger α), the power increases, the time window during which detection is highly probable increases, and the period of maximum detectability (maximum power) is delayed to a longer time after the bottleneck is initiated. This is true under both models.

(3) The power increases with the number of loci and with the sample size. However, increasing the number of loci is generally more effective than increasing the sample size.

(4) Using markers with high heterozygosity ($0.3 < H < 0.8$) instead of low heterozygosity ($0.0 < H < 0.3$) slightly increases the overall power under the IAM but the reverse occurs under the SMM. Also, it moves the time window of maximum power toward the beginning of the bottleneck under both models. For example, comparable power is obtained at time 0.25 ($0.25 \times 2N_e$ generations after the bottleneck begins) with moderately variable markers and at $0.1 \times 2N_e$ with highly variable markers. This seems logical because more variable markers have higher mutation rates and thus return to equilibrium more rapidly after a bottleneck.

As expected from the difference in nature of the two tests (Test 1 is nonparametric, whereas Test 2 is parametric), the overall power of Test 2 was generally higher than that of Test 1. However, in the above combinations of parameters, the difference rarely exceeded 0.2.

The same computer program was used to estimate the average level of type I error by setting $\alpha = 1$ (*i.e.*, no bottleneck). Type I errors should occur in ~5% of the tests for heterozygosity excess when no bottleneck occurs in the simulation and when the level of significance used is 0.05. In over 35,000 replicate significance tests based on 20 loci, we found error rates of 4.8% (IAM) and 2.2% (SMM) for Test 1 and 6.7% (IAM) and 3.3% (SMM) for Test 2. Hence, both tests are slightly more conservative in rejecting the null hypothesis when the SMM is assumed.

DISCUSSION

We have presented a method for detecting a recent reduction of the effective population size in a population. This method is based on the property that bottlenecked populations generally develop a transient heterozygosity excess. However, if loci evolve under the strict one-step SMM, we observed that both heterozygosity excess and deficiency can occur depending on the variability of the locus and the time that has passed since the bottleneck began (Figure 1B). We have evaluated the IAM and the SMM because they represent two extreme models of mutation (CHAKRABORTY and JIN 1992). Most loci probably evolve in an intermediate way and we have shown that intermediate models of mutation reveal very little or no heterozygosity *deficiency* in bottlenecked populations (Figure 1D). Furthermore, population expansions cause almost exclusively heterozygosity deficiency. Thus detecting a significant heterozygosity excess under the SMM can be taken as evidence that a population has been recently bottlenecked.

A possible explanation of the unexpected postbottleneck heterozygosity deficiency encountered under the strict SMM is the following. At mutation-drift equilibrium, SMM loci have generally contiguous allelic states (*i.e.*, allele lengths for microsatellite loci). Following a bottleneck, some "gaps" in the distribution of allelic lengths can occur due to limited random sampling of gametes and loss of alleles having intermediate lengths. These gaps are much more likely with higher numbers of alleles. If these gaps are progressively "filled in" by mutations, there can be a transient excess of alleles (*i.e.*, deficiency of heterozygosity) compared to a stationary population in which the allelic state distribution contains far fewer gaps if any.

The strict SMM is obviously the most conservative model for testing for a significant heterozygosity excess caused by bottlenecks because in some conditions it can produce a heterozygosity deficiency and because the heterozygosity excess is always lower than under other mutation models. The SMM may also be more conservative than the IAM because the type I error rate for our statistical tests is slightly lower than that of the IAM.

The lower statistical power of the tests under the assumption of SMM compared to IAM (Figure 3, A and B) was consistent with the observation that the SMM generates a smaller heterozygosity excess than the IAM (Figure 1, A and B). Likewise, the larger power obtained under the SMM with low variability markers compared to highly variable ones is consistent with the fact that for a given time after the bottleneck started, a larger heterozygosity excess is observed at loci with fewer alleles (Figure 1B).

The tests for heterozygosity excess can detect bottlenecks for only a given window of time after a bottleneck has been initiated. For example, power analyses (Figure 3, A and B) and theoretical models (Figure 1) suggest that a bottleneck of $N_e = 50$ is likely to be detectable for ~25–250 generations (0.25–2.5 times $2N_e$) after the initiation of a population reduction. Thus only recent historical populations declines are detectable.

Test assumptions: Both tests rely on the assumption that each sample is representative of a well defined population, *e.g.*, with no immigration and no population substructure, and that loci are selectively neutral.

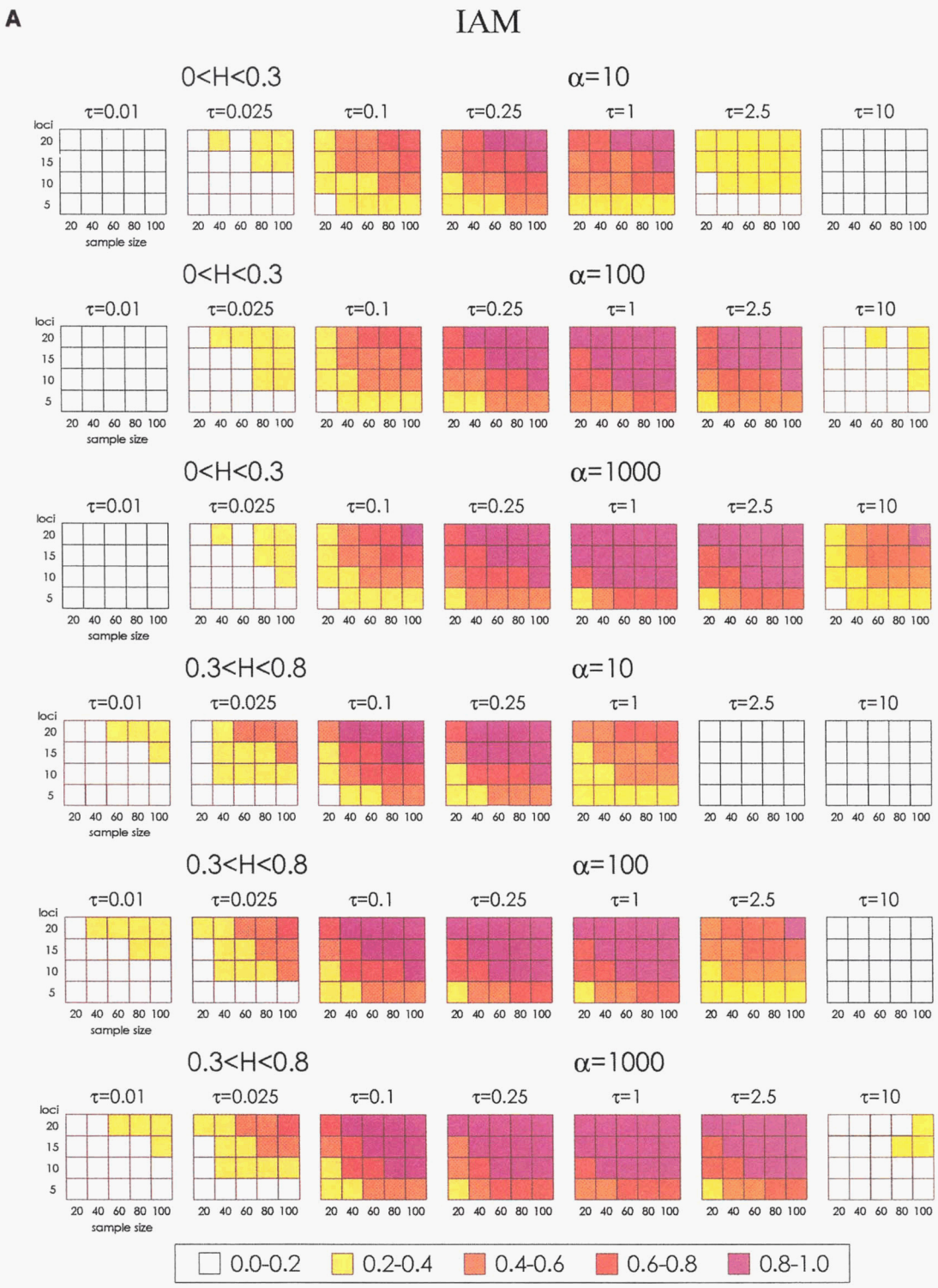

FIGURE 3.—Combined power analysis of the sign test and regression test for loci evolving under the IAM (A) and the SMM (B). Each of the six rows correspond to a given range of heterozygosity (0–0.3 or 0.3–0.8) and a given strength of the bottleneck (α = ratio of effective population size before/after the bottleneck). For each such combination, the power was computed for five different sample sizes (20, 40, 60, 80 and 100 genes, *i.e.,* 10, 20, 30, 40 and 50 diploid individuals) and four different numbers of *polymorphic* loci (5, 10, 15 and 20). The combined power, computed over 500 replicates in every specific combination, is represented by a color scale (bottom of figure). Power is defined as the proportion of bottleneck simulation replicates for which at least one of the two tests was significant and in the expected direction (heterozygosity *excess*).

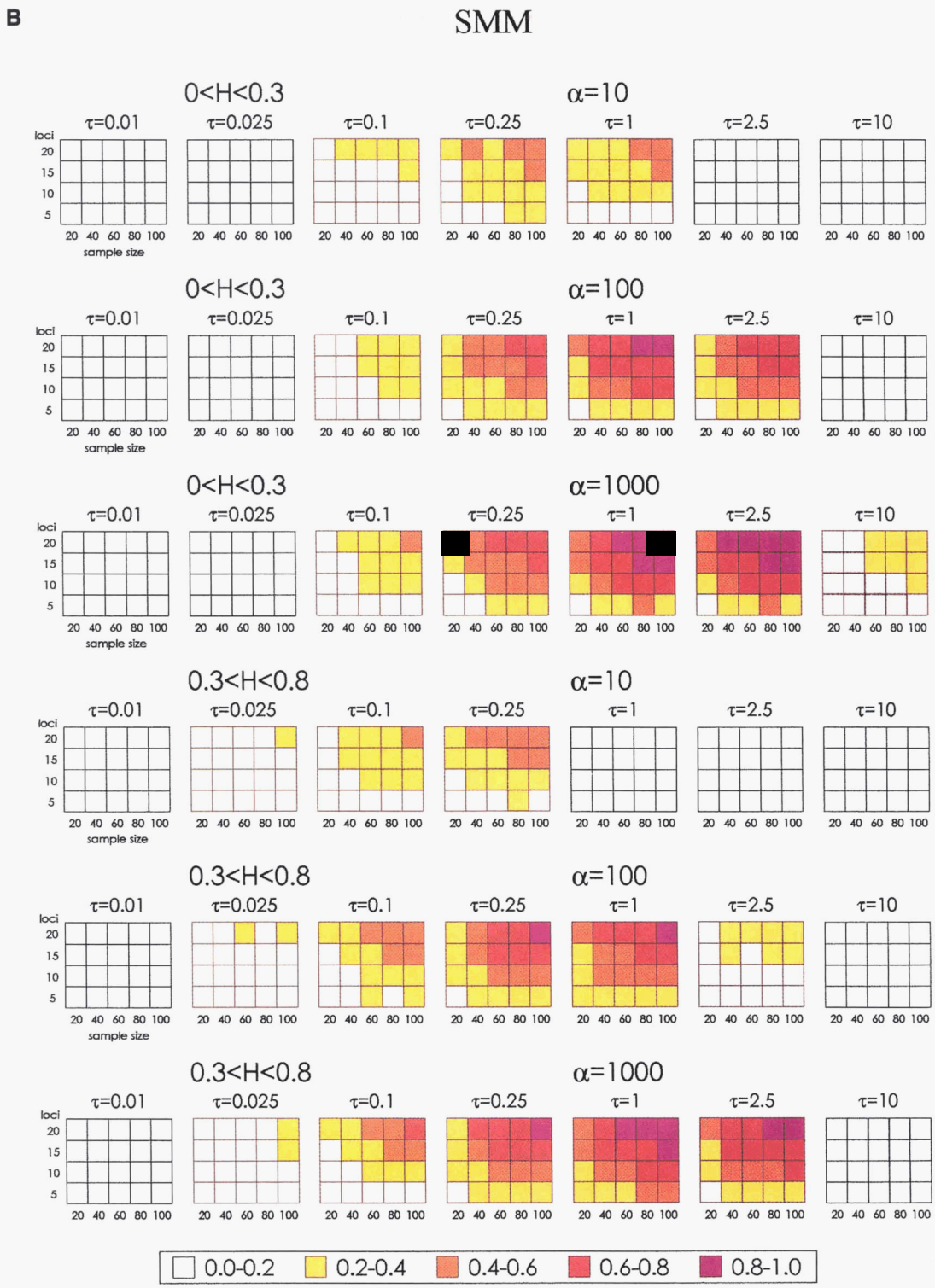

FIGURE 3.—*Continued*

Recent immigration may be particularly misleading. This is especially true if immigrants come from a population that is genetically divergent, because these immigrants could quickly increase the number of rare alleles in the population without substantially affecting the heterozygosity, mimicking an increase (or hiding a decrease) of the population size. This is why in selecting examples, we chose human or bumble bee populations in which recent immigration was unlikely. A similar bias can arise if the sample includes individuals from two or more actual populations (population substructure) or hybrids between populations. Another (minor) source of error is the presence of undetected (null) alleles at some loci. All such situations can be generally detected

by testing for Hardy-Weinberg equilibrium or the significance of the F_{is} statistic. These tests should be performed systematically before applying tests for heterozygosity excess.

Another assumption is the independence of the results among pairs of loci. This is achieved if all loci scored on a given sample are genetically independent, although this condition may not be necessary. Our preliminary simulation studies suggest that, in a population at equilibrium, correlations between numbers of alleles or between heterozygosities are negligible whenever loci are not tightly linked (*e.g.*, percentage of recombination >0.1). However, to be conservative, one should use only one locus of a pair of loci in linkage disequilibrium.

Because of its nonparametric nature, Test 1 does not require any further assumptions and can be used with a small number of loci. In contrast, the application of Test 2 is more restrictive since the statistics T_2 approaches a Gaussian distribution only as the sample size of loci becomes large (*i.e.*, >20). Note that most data sets, such as those presented as examples, may not have enough polymorphic loci for this test to be valid. Results in which the set of polymorphic loci is small (say <20) and only Test 2 is significant should be considered with caution. Such is the case of the southern hairy-nosed wombat population (second example above) when all loci are assumed to fit the SMM. Because microsatellite loci, such as those scored in the southern hairy-nosed wombat example, are likely to evolve predominantly under the SMM (Shriver *et al.* 1993), it seems reasonable to only consider the results of the tests under the SMM and not to reject the mutation drift equilibrium null hypothesis for this population.

Both tests can be applied assuming that all loci in a given sample fit the same mutation model. However, if there is evidence that some loci fit the IAM, whereas others fit the SMM, combining both kinds of loci is straightforward for both Test 1 and Test 2.

Choice of marker loci: When considering the results of the power analysis, there is no definite advantage in using highly variable markers: power is slightly increased under the IAM and decreased under the SMM for highly variable markers relative to moderately variable markers. However, this computation was based only on polymorphic loci, *i.e.*, all monomorphic loci arising in the simulated samples were disregarded. Obviously, the abundance of polymorphic loci in a postbottleneck sample will be larger when using highly variable marker loci. Consequently, more loci will have to be scored to find a sufficient number of polymorphic loci with moderately variable markers than with highly variable markers. Moreover, if the population has suffered a severe size reduction, only highly variable markers will still be polymorphic. However, using the most variable markers may not be the best solution in all cases, since these loci evolve rapidly and hence return back to equilibrium relatively quickly. Thus rapidly evolving markers (*e.g.*, microsatellites) will be more useful for detecting relatively recent bottlenecks, whereas less rapidly evolving markers (*e.g.*, allozymes) will be more useful for detecting less recent bottlenecks.

Allozyme markers may be less appropriate for testing for bottlenecks because they often exhibit relatively few alleles and low heterozygosity, especially in large mammals and birds. They are also less likely to be selectively neutral than are DNA level markers (Sing *et al.* 1973; Watt 1977; Karl and Avise 1992; Pogson *et al.* 1995). It is possible that balancing selection could promote high heterozygosity (with few alleles) and thereby cause a heterozygosity excess in the absence of a population bottleneck. However, it seems unlikely that balancing selection would affect a majority of allozyme loci and thereby cause a significant overall heterozygosity excess in a large sample of loci.

Micro- and mini-satellite loci (VNTRs or variable number of tandem repeats) are probably the best markers currently available for detecting recent bottlenecks because of their generally high level of variability. Microsatellites with 3- to 5-bp repeats are thought to evolve predominantly under the single-step SMM (Shriver *et al.* 1993) and therefore should be less suitable than microsatellites with shorter repeats for which the mutation model usually includes multiple-step mutation events (Di Rienzo *et al.* 1994) and is hence closer to the IAM. Interrupted microsatellites (Estoup *et al.* 1995b) or compound microsatellites with motifs of different lengths (Estoup *et al.* 1995c) are likely to fit the IAM better than pure repeat microsatellites, and hence to be among the most useful markers for detecting bottlenecks.

Sampling more loci *vs.* individuals: The statistical power of the bottleneck tests can be increased by screening more loci or by sampling more genomes (*i.e.*, individuals). However, there is a higher power benefit of analyzing more loci than more genomes for both tests. For instance, the only way for Test 1 to be significant with five loci is for all loci to have an heterozygosity excess, whereas by using three more loci, the test can be significant with either seven or eight loci having a heterozygosity excess. On the other hand, increasing the sample size of genomes will only increase the precision of the estimate of heterozygosity excess at each individual locus. Also, because Test 2 requires a minimum number of loci to be valid, sampling more loci is more important than sampling more individuals. Although sampling more loci provides the greatest power benefit, it is still important to sample at least 40 haploid genomes (20 diploid individuals) to achieve reasonably high power (Figure 3, A and B).

Applications of bottleneck tests: In conservation biology, the most important type of bottleneck to detect is a severe and rapid decline from large N_e. Severe population declines are also the type of bottleneck most

likely to be detected by our bottleneck tests. Severely bottlenecked populations are important to identify for conservation because they are likely to suffer from inbreeding depression, loss of genetic variation, fixation of deleterious alleles as well as increased demographic stochasticity all of which can reduce adaptive potential and the probability of population persistence (LANDE 1994; MILLS and SMOUSE 1994; FRANKHAM 1995a and citations therein).

Many wild populations around the world are suffering demographic bottlenecks (reduction of census size) and genetic bottlenecks (reduction of N_e) resulting from habitat fragmentation and insularization. It is important to recognize that populations suffering a reduction in census size may not suffer a severe reduction of N_e (a genetic bottleneck) if historical N_e has always been low due to fluctuations in population size, mating system dynamics (*e.g.*, polygyny or inbreeding), or metapopulation structure involving local extinctions and recolonizations (PIMM *et al.* 1989). Our analysis of the northern hairy-nosed wombat data suggests that the Epping population did have a large historical N_e and has recently suffered a severe genetic bottleneck along with the well documented demographic bottleneck. Thus, the tests can help identify which populations have suffered a severe reduction of N_e along with a reduction of census size, and thereby help identify populations at high risk of extinction due to genetic factors in addition to risks due to demographic factors.

The tests may also help detect bottlenecks associated with natural colonization events, and help resolve the debate over which models of founder speciation are most consistent with data from natural populations. Several different models of founder speciation have been proposed (for reviews see Harrison 1991 and HOWARD 1993). Some models involve a substantial reduction of allelic variation and heterozygosity (MAYR 1954; CARSON 1959). Other models (CARSON 1971; TEMPLETON 1980) involve severe but short-term founder events that reduce allele variation (mainly rare alleles) without substantially reducing heterozygosity. Because tests for a heterozygosity excess can detect severe, short-term founder events, they may help determine which models of founder speciation are consistent with colonization events observed in nature.

As shown in example three (Sardinian human population), populations increasing in size from a small N_e can also be detected with our statistical tests, because expanding populations are characterized by loci exhibiting a heterozygosity deficiency (MARUYAMA and FUERST 1984, our Figure 1C). When testing for a recent increase of population size, the most conservative mutation model is obviously the IAM because it provides the lowest estimate of expected equilibrium heterozygosity for a given number of alleles observed in a sample.

Incidentally, Test 1 and Test 2 can be used to determine which mutation model (IAM or SMM) best fits a set of genetic markers. For example, if a population is assumed to be at mutation-drift equilibrium for a set of markers, then it is possible to compare the observed heterozygosity with the theoretical distribution of heterozygosity under each model and possibly reject one of the two mutation models. We provide then an approach that is complementary to previous analyses for evaluating mutation models relative to microsatellite data (DEKA *et al.* 1991; SHRIVER *et al.* 1993; VALDES *et al.* 1993; DI RIENZO *et al.* 1994). As already noted, our tests, based on the comparison between observed and expected heterozygosity, use an approach similar to the EWENS-WATTERSON test (WATTERSON 1978) that compares homozygosities. But the latter tests for neutrality assuming mutation-drift equilibrium [or a change in population size for which characteristics are perfectly known as in WATTERSON (1986)] for a single marker locus and is limited to the IAM; whereas our approach tests for mutation drift equilibrium assuming neutrality and it bases conclusions on the statistical behavior of an entire set of markers under the IAM or the SMM.

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