This PDF version contains corrections to the original print version and that found on the Springer webpage.

p. 311 line 8 When $n < N_e$ (original version has >)
p. 311 line 9 when $n \approx N_e$ (original version has <)

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Evaluation of the Linkage Disequilibrium Method for Estimating Effective Population Size

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Abstract Data on linkage disequilibrium at unlinked loci provide an estimate of the inbreeding effective population size of the parental generation of the sampled cohort. The inbreeding effective population size, N_e, is the reciprocal of the probability that two gametes, selected at random without replacement from those that produced the sampled cohort, derive from the same parent. Effective population size is an important parameter for measuring the rate of inbreeding in a population. We detail the construction of the linkage disequilibrium estimator of N_e, and evaluate its performance by simulation. We simulate populations which are dioecious and non-selfing. We use the simulations to examine the effects of several types of deviation from ideal population conditions, and of sample size, genotyping errors, number of loci typed, and polymorphic loci. We find substantial bias in the N_e estimator when there have been recent fluctuations in census population size, when the index of breeding variability is greater than one, and when the ratio of sample size to effective population size differs substantially from one. Due to high variability, estimators that have low bias for the reciprocal of N_e can present substantial bias when used as estimators of N_e itself. We consider a recent small sample size bias correction proposed for the method, and find that it improves bias in the reciprocal, but at the expense of increased bias for N_e. The improvements in the bias of the reciprocal are usually small, but are substantial when sample size is much less than N_e, while the increase in bias for N_e is often substantial. We test the method on two exhaustively sampled rat populations, and find it performs as expected from simulation. For practitioners, we recommend that resources are spent first in ensuring that the sample size is likely to be greater than the effective population size, and only then that the number of loci is increased to improve the precision of the estimate.

Keywords Burrow's composite disequilibrium measure · Effective population size · Non-ideal populations · Rats · *Rattus* · Squared correlation coefficient

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1 Introduction

1.1 Census Population Size

Population size is a fundamental parameter of interest in ecological systems. Classical statistical methods have been developed for estimating the census population size (N_c) from ecological capture data over a period of sampling (e.g. Seber 1982; Borchers et al. 2002). For population modeling, we are often interested in the total number of breeding individuals, often differentiating between breeding males and females (Caswell 2001; Buckland et al. 2004). Only the breeders will contribute to the next generation. The number of potentially breeding individuals (adults) can be determined from census data using knowledge of the age structure of the population, or by using an appropriate surrogate such as the size of animals.

1.2 Effective Population Size

From a genetic perspective, concepts of population size are related to the rates of loss of genetic variation, fixation of deleterious alleles, and inbreeding. In an infinite population without mutation, migration, or selection, allele frequencies are constant over the generations. However, for a finite population, a random process of genetic drift operates to change allele frequencies from one generation to the next (Caballero 1994). This occurs because each generation is formed by taking a finite sample of the gametes produced by the parental generation. The sampled allele frequencies will not match the parental frequencies exactly, and departures become greater as the sample size becomes smaller. The magnitude of genetic drift between generations therefore contains information about the sampled number of gametes, and hence the population size. Small populations also increase the chance of an individual possessing two copies of the same allele (homozygosity), because both copies were inherited from the same ancestor. This can cause inbreeding depression.

Measures of genetic change are not reflected directly by the census population size, but are related to the breeding population size, life history characteristics such as variable individual breeding success and biased sex ratios, and fluctuations in population size over the generations. The effective population size, N_e, is a surrogate size that is related directly to the genetic change being experienced by the population (Crow and Denniston 1988). The effective population size is the size of an ideal population that would experience the same amount of genetic change as that observed in the population under study (Wright 1931; Crow and Kimura 1970). The ideal population meets the three conditions of equal sex ratio, random mating, and constant census population size over generations (Crow and Denniston 1988; Caballero 1994). The notion of effective size provides a yardstick for understanding the rate of genetic change for any population, irrespective of its life history and other characteristics. When generations do not overlap, the effective population size estimate refers to the size of a single generation (Waples 2005).

To maintain constant population size in the ideal population, N adults must produce $2N$ gametes. The numbers of gametes contributed by adults 1,. . ., N are k_1,. . ., k_N, where these values sum to $2N$. Random mating in the ideal population means that, for each required gamete, the contributing adult is selected randomly and independently from the N adults available, so the joint distribution of (k_1,. . ., k_N) is Multinomial ($2N$; $1/N, \ldots, 1/N$) and the marginal distribution of k_i is Binomial ($2N$; $1/N$). This model is also termed binomial mating. In a population of constant size, the mean number of gametes contributed per adult is therefore $\mu_k = 2$, and the variance is $\sigma_k^2 = 2(1 - 1/N)$. As $N \to \infty$, the distribution tends to Poisson with $\sigma_k^2 = \mu_k = 2$. The model assumes that a gamete can unite with any other gamete, and hence the ideal population is capable of selfing (mating with oneself) and sex is not taken into account (Crow and Denniston 1988).

Effective population size (N_e) quantifies the size of an ideal population that would undergo a given level of genetic change. In the ideal population, regardless of which measure of genetic change is considered, N_e is equal to the census population size of a generation. For most real populations, however, the ideal conditions do not hold and N_e is smaller than the census population size. This is largely because real mating is not binomial and some individuals have greater breeding success than others. Frankham (1995) suggested that N_e may be as small as a tenth of the census population size for many species. It is rare for N_e to exceed the census size, but this can occur if variability in the number of gametes contributed by each parent is less than that expected from binomial chance, i.e. $\sigma_k^2 < \mu_k(1 - 1/N)$. This is termed minimal inbreeding and can be produced in managed populations (Caballero 1994).

When considering deviations from the ideal population, it is necessary to specify what measure of genetic change underlies the definition of N_e. This leads to different notions of effective population size, the most common of which are inbreeding effective size and variance effective size (Waples 2005). For inbreeding effective size, genetic change refers to the rate of increase in inbreeding per generation, while for variance effective size, genetic change refers to the variance of the change in allele frequency from one generation to the next (Crow and Denniston 1988; Caballero 1994). At small population sizes the different effective sizes can differ substantially (Crandall et al. 1999). Care must also be taken to specify what generation an estimate of effective size refers to, because a single sampled generation may yield estimates of effective size for its own generation, its parental generation, or its grandparental generation, depending upon which effective size is intended, whether the estimates are genetically based or demographically based, and whether or not the population exhibits selfing (Caballero 1994; Waples 2005).

The linkage disequilibrium method estimates the inbreeding effective size of the parental generation (Waples 2005, 2006). We define the inbreeding effective size of the parental generation to be the number N_e such that its reciprocal, $1/N_e$, is the probability that two gametes, selected at random without replacement from those occurring in the offspring, derive from the same parent. Caballero (1994) shows how this probability is related to the rate of increase in the coefficient of inbreeding per generation in the ideal population. If some parents contribute many more successful gametes than others (non-binomial mating), the probability will be inflated and N_e

will be smaller than the census size. With our definition above, we do not require the randomly selected gametes to be united in a single offspring, so the definition does not require that selfing has taken place. We use this definition to avoid confusion over whether the effective size refers to the parental generation or the grandparental generation in the case of non-selfing populations. For a non-selfing population, two gametes from a single parent cannot unite in an offspring, but two gametes from a single grandparent can, so the generation to which inbreeding N_e applies in a non-selfing population is commonly cited as the grandparental generation (Crow and Denniston 1988; Caballero 1994). However, the linkage disequilibrium method estimates its value for the parental generation (Waples 2005, 2006). We circumvent this confusion by defining N_e via a random selection of two successful gametes that is notional, rather than a selection united in an offspring. Throughout this paper, the inbreeding effective size refers to that of the parental generation.

The reciprocal probability, $1/N_e$, is a more direct driver of evolutionary processes than N_e itself (Wang 2001; Waples 2005). This has led previous authors to report bias in N_e estimators using the harmonic mean of estimated values rather than the arithmetic mean, because this reflects the bias of $1/N_e$. However, this policy has not been made explicit and could lead to confusion because most researchers focus on N_e rather than its reciprocal. In this paper, we will focus on bias in N_e itself, which is assessed by the arithmetic mean of estimated values rather than the harmonic mean.

1.3 Demographic Estimation

Effective population size can be estimated from demographic data on the total number of breeding males and females, and the mean and variance across individuals of their lifetime number of offspring that survive to reproduction (Crow and Denniston 1988, with corrections in Caballero and Hill 1992; Caballero 1994; Rockwell and Barrowclough 1995). However, these parameters are notoriously hard to estimate accurately (Waples 1991; Barrowclough and Rockwell 1993; Schwartz et al. 1998). Additionally, equations used to estimate effective population size from demographic data are not always comprehensive (Frankham 1995), because they do not simultaneously incorporate all three conditions leading to deviation from the ideal population.

1.4 Genetic Estimation

Genetic estimates of effective population size operate by measuring genetic processes that are known to be functions of N_e (Waples 1991). Genetic estimates incorporate all three conditions which lead to deviation from the ideal population (Frankham 1995). The genetic signal from N_e is strongest when the population size is small (Waples 1991), and this is where we have the most potential to estimate N_e accurately (Waples 1991; Wang 2005). For genetic estimation we assume that:

(1) mutation is negligible; (2) the alleles considered are not subject to natural or sexual selection (selectively neutral) and not linked with other loci subject to selection; (3) the samples of individuals for genetic analysis are randomly drawn from a specified population or generation; and (4) there is no immigration from neighbouring populations (Waples 1991).

Genetic estimates of effective population size are most commonly obtained from two or more temporally separated samples from a population (Waples 1989; Williamson and Slatkin 1999; Berthier et al. 2002), and represent an average of N_e over the appropriate time-scale (Waples 2005; Wang 2005). Other methods estimate historical effective population size over longer time periods using coalescent theory and mutation rates (Crandall et al. 1999; Wang 2005), or jointly with other parameters such as migration (Wang and Whitlock 2003) or mutation rate (Garza and Williamson 2001).

By contrast with the temporal and historical methods for estimating N_e, the linkage disequilibrium method gives an estimate of contemporary N_e from just one sampled generation. The single sample exhibits linkage disequilibrium from the two different processes of genetic sampling (selection of parental gametes) and statistical sampling to form the set of individuals for genetic analysis. By quantifying the level of linkage disequilibrium in the sample, an estimate of inbreeding N_e for the parental generation can be obtained (Waples 2006), which we detail below.

1.5 Our Purpose

The effective population size provides a single summary value of the contributions of breeding variability, sex ratio, and fluctuations in population size to the population biology of a species (Wang 2005). Most research in effective population size has focused on rare and endangered species (Nunney and Campbell 1993; Nunney and Elam 1994), where it is considered important to increase effective population size to raise the persistence of a population (Lande and Barrowclough 1987; Lynch and Lande 1998). Our work focuses on invasive species where reducing the census population size is the desired outcome. Invasive species are highly successful colonizers, despite initially small census population sizes and associated limited effective population sizes. This scenario is contrary to what would be expected following experiences with threatened species at small population sizes (Sax and Brown 2000).

Census and effective population sizes are both readily estimated when samples from a population can be taken repeatedly across time and space: for example, mark-recapture methods can be used for census size, and change in heterozygosity over time can be used for effective size. However ecologists commonly operate in the less ideal situation of having only one opportunity to sample a single population. This scenario is particularly the case for pest species where individuals are removed as they are encountered. Where the conservation goal is to remove all individuals as rapidly as possible (eradication) there is very little scope for long-term study of populations. Researchers then have minimal data from which to gain understanding of the population biology.

Our goal is to consider the utility of the linkage disequilibrium method for making inferences on a closed population which can only be sampled once without replacement. We present the theory underlying the linkage disequilibrium method, and use simulation to evaluate its performance. We use selectively neutral and highly variable microsatellite markers to characterise genetic diversity within a population (Selkoe and Toonen 2006). We consider only closed diploid populations with discrete generations. We also examine the performance of the method on real data of approximately known census population size, sex ratio and breeding success. Some previous work has simulated the performance of the linkage disequilibrium method (Waples 2005, 2006; England et al. 2006), and applied it to real datasets (Bartley et al. 1992). We focus on a thorough simulation of the parameters that can affect effective population size, using an ecologically plausible population. We discuss finally the utility of the method, and how this may affect the partitioning of field and laboratory work.

2 Linkage Disequilibrium

Linkage disequilibrium is the non-random association of alleles at different gene loci. Linkage disequilibrium can be produced by a number of factors, including physical linkage (the two loci are on the same chromosome), epistatic selection (alleles interacting to control fitness), genetic hitch-hiking (physical linkage with a selected locus), migration or population admixture, and random drift in finite populations (Hill 1981; Waples 1991). We are concerned only with the effect of genetic drift on linkage disequilibrium, in the absence of the other effects.

Genetic drift linkage disequilibrium is generated from the finite sampling of gametes from the parental generation. Sampling effects in a small sample mean that the sample correlation between the alleles possessed at two loci will not be zero, despite the underlying correlation or physical linkage being zero. The expected squared correlation becomes larger as the population size gets smaller, and can be shown to depend on $1/N_e$ (Sved 1971; Laurie-Ahlberg and Weir 1979; Weir and Hill 1980).

Specifically, consider two alleles A and B at loci 1 and 2 respectively, and suppose for the moment that we have data on individual gametes, as opposed to genotype data (see below). Let g be the total number of gametes whose alleles are known at both loci 1 and 2, g_A be the number of these gametes with allele A at locus 1, g_B be the number with allele B at locus 2, and g_{AB} be the number with both allele A at locus 1 and allele B at locus 2. Under random assortment, the proportion of AB gametes should be approximately the product of the proportion of A gametes and the proportion of B gametes. The linkage disequilibrium measure for alleles A and B at these loci is the difference between the observed and the expected proportions:

$$D_{AB} = \frac{g_{AB}}{g} - \frac{g_A}{g} \times \frac{g_B}{g}$$

A variety of methods can be used for estimating D_{AB} from gametic data (Weir 1996, p. 112). However, it is more common that only genotypic data are available, from which we do not know which gametes the individual's two alleles are located on. When we sample individuals with genotype AA', BB', for example, we do not know whether the alleles are arranged on the individual's two gametes as $(A,B) \mid (A',B')$ or as $(A,B') \mid (A',B)$. Additional to the disequilibrium D_{AB} within the gamete, we can define a second analogous disequilibrium D_B^A referring to opposite gametes within the same individual (Weir and Hill 1980). Neither D_{AB} nor D_B^A is observable from genotypic data, but their sum is. This suggests that we can use a composite disequilibrium measure, attributable to Dr Peter Burrows (see Cockerham and Weir 1977, p. 142, Weir 1979, p. 241, and later unattributed in Weir 1996, p. 126):

$$\Delta_{AB} = D_{AB} + D_B^A$$

The composite Δ_{AB} is known as Burrow's composite D, written as Δ in Weir (1996, p. 126), and D^* in Campton (1987, p. 184). It performs better than an alternative maximum likelihood estimator for linkage disequilibrium (Weir 1979). It is estimated directly from genotype counts as follows:

$$\hat{\Delta}_{AB} = \frac{n_{AB}}{n} - 2\hat{p}_A\hat{q}_B$$

where $n_{AB} = 2n_1 + n_2 + n_4 + n_5/2$, and $n_1, \ldots, n_9$ are genotype counts defined in Table 1, and $n = n_1 + \ldots + n_9$ is the total number of counts (sampled individuals). Here, $\hat{p}_A$ and $\hat{q}_B$ are the sample proportions of alleles A and B in the n individuals typed at both loci: for example $\hat{p}_A = (2n_1 + 2n_2 + 2n_3 + n_4 + n_5 + n_6)/(2n)$. A small-sample correction factor of $n/(n-1)$ should be applied to $\hat{\Delta}_{AB}$ (Weir 1979, p. 241; Campton 1987, p. 185).

It can be shown that $\hat{\Delta}_{AB} = \hat{\text{cov}}(K_A, K_B)/2$, where K_A and K_B give respectively the number of A alleles and B alleles possessed by an individual, and each take values 0, 1, or 2. The covariance is taken across individuals. We can then estimate the corresponding correlation coefficient, $r_{AB} = \text{cor}(K_A, K_B)$, as

$$\hat{r}_{AB} = \frac{\hat{\Delta}_{AB}}{\sqrt{\left\{\hat{p}_A(1-\hat{p}_A) + (\hat{h}_{AA} - \hat{p}_A^2)\right\}\left\{\hat{q}_B(1-\hat{q}_B) + (\hat{h}_{BB} - \hat{q}_B^2)\right\}}}$$

Table 1 Possible genotypes and their sample counts for alleles A and B at loci 1 and 2 respectively. A' and B' denote any other alleles

	$B\,B$	$B\,B'$	$B'B'$
$A\,A$	n_1	n_2	n_3
$A\,A'$	n_4	n_5	n_6
$A'A'$	n_7	n_8	n_9

where $\hat{h}_{AA}$ and $\hat{h}_{BB}$ are the observed proportions of AA and BB homozygotes in the sample of size n, for example $\hat{h}_{AA} = (n_1 + n_2 + n_3)/n$. This estimate of the correlation is the ratio of two estimators: the covariance $\hat{\text{cov}}(K_A, K_B) = 2\hat{\Delta}_{AB}$, and the square root of the product of the sample variances for K_A and K_B (Weir 1996, p. 38; equation (2) in Waples 2006).

The correlation coefficient r_{AB} has $E(r_{AB}) = 0$ for unlinked loci, where the expectation is taken over conceptual replicate populations. In finite populations, however, the correlation is likely to take non-zero values, with small populations giving the largest values, so the expectation of its square is non-zero and is a function of the effective population size, N_e. The expression for $E(r_{AB}^2)$ depends upon the mating structure and recombination fraction c in a population (Weir and Hill 1980; Weir et al. 1980), and is also affected by sample size n, because linkage disequilibrium arises from statistical sampling as well as genetic sampling. The distribution of r_{AB}^2 is not known, so the expectation $E(r_{AB}^2)$ is approximated as the ratio of the expectation of the numerator and the denominator (Weir and Hill 1980, p. 484; Laurie-Ahlberg and Weir 1979, p. 1309; Waples 2006, p. 169). For randomly mating populations, this yields

$$E(r_{AB}^2) \approx \frac{c^2 + (1-c)^2}{2N_e^I c(2-c)} + \frac{1}{n}$$

where ${N_e}^I$ is the inbreeding effective size of the parental generation. Thus $E(r_{AB}^2)$ is inversely related to both inbreeding effective population size and sample size. This expression is the same for dioecious species with random pairing and for monoecious species with or without selfing (Weir and Hill 1980). Rearranging the expression and replacing $E(r_{AB}^2)$ with $\hat{r}_{AB}^2$ gives the formula for estimating ${N_e}^I$ in the case of unlinked loci ($c = 0.5$):

$$N_e^{LD} = \frac{1}{3(\hat{r}_{AB}^2 - 1/n)} \tag{1}$$

where ${N_e}^{LD}$ is the linkage disequilibrium estimate of ${N_e}^I$, and n is the number of individuals sampled (Laurie-Ahlberg and Weir 1979; Hill 1981; Waples 1991). Equation 1 incorporates the contribution of both genetic and statistical sampling to the estimate of effective population size (Waples 2006). For species with a mating system of lifetime monogamy, the numerator becomes 2 (Weir and Hill 1980), but we do not consider this case.

The method above shows how ${N_e}^{LD}$ is obtained from data on a single pair of biallelic loci, where there are only two alleles A and A' at locus 1, and B and B' at locus 2. In many applications, there will be several loci which are polymorphic, in other words have more than two alleles. In this case we must derive an estimate $\hat{r}^2$ that combines all possible allele–allele comparisons within a single pair of loci, and additionally all possible pairs of loci. All these comparisons contain information on the underlying parameter r^2.

For a single allele–allele comparison with a single pair of loci, for example A and B above, all other alleles at the loci are binned together as with A' and B' in

Table 1 (Wang 2005). This gives a single allele–allele estimate $\hat{r}^2_{AB}$. Let x_i and x_j be the number of alleles at loci i and j respectively. We obtain $x_i \times x_j$ estimates of r^2, but only $(x_i - 1) \times (x_j - 1)$ of these estimates are independent. This can be seen by noting that (for example) $r_{AB} = \mathrm{cor}(K_A, K_B)$, and the K_A values have to sum to 2 across the x_i different alleles at locus i, and similarly for locus j. The estimate of r^2 within the locus pair is obtained as the arithmetic mean of the $x_i \times x_j$ estimates (England et al. 2006, p. 304). If there are L loci, this produces $L(L-1)/2$ locus-pair estimates of r^2. A single estimate for r^2 across all locus pairs is gained from the weighted arithmetic mean of the estimates for each locus pair, weighted by the number of independent allelic comparisons $(x_i - 1) \times (x_j - 1)$ in each pair. This estimate for r^2 is substituted into equation (1) in place of $\hat{r}^2_{AB}$.

The appropriate sample size n to substitute into equation (1) is complicated by the possibility of missing data for some individuals at some loci, and the different numbers of estimates of r^2 contributed by the different locus pairs. Let n_{ij} be the number of sampled individuals with data available for both loci i and j. There are $(x_i - 1) \times (x_j - 1)$ independent estimates of r^2 available from this locus pair. The final value n in equation (1) is the harmonic mean of the n_{ij} values, where each n_{ij} is included $(x_i - 1) \times (x_j - 1)$ times.

For calculating confidence intervals, the distribution of r^2 is approximated by a chi-square distribution with $M = L(L-1)/2$ degrees of freedom (Hill 1981; Waples 1991). Confidence limits for r^2 are estimated with

$$(1-\alpha)\mathrm{CI} = \left(\hat{r}^2 \times M/\chi^2_{(\alpha/2),M}, \quad \hat{r}^2 \times M/\chi^2_{(1-\alpha/2),M}\right) \tag{2}$$

and confidence intervals for N_e^I are obtained from equation (2) using equation (1).

The method above assumes that loci are neutral (non-selected) and physically unlinked ($c = 0.5$). Microsatellite loci are highly suitable for the linkage disequilibrium method (Schwartz et al. 1998), because they are highly polymorphic and nearly selectively neutral, although this may be compromised by genetic hitchhiking. To avoid physical linkage of microsatellite loci, the loci should be located on different chromosomes where possible. Unfortunately, the greatest information about N_e is provided when loci have tight physical linkage (Hill 1981; Hayes et al. 2003), but this would require knowledge of the recombination fraction c, which is not usually available for natural populations (Waples 1991). As the recombination fraction decreases ($c < 0.5$), the effective population size estimate applies to more distant generations (Hill 1981; Hayes et al. 2003; Waples 2006). For unlinked loci, the linkage disequilibrium signal for N_e is determined by the random reassortment from the breeding process in the parental generation, and is greatest when the population size is small (Waples 1991, 2006).

The relationship between the estimated r^2 and ${N_e}^{LD}$ takes the form of a hyperbolic curve (Fig. 1). When $\hat{r}^2$ is less than $1/n$, negative estimates of ${N_e}^{LD}$ are possible. In these cases, which are most likely to arise when the sample size is small, the contribution of genetic drift to linkage disequilibrium is swamped by the contribution from statistical sampling. Because it is not possible for N_e to be negative, the conventional way of interpreting a negative ${N_e}^{LD}$ is to replace it with an

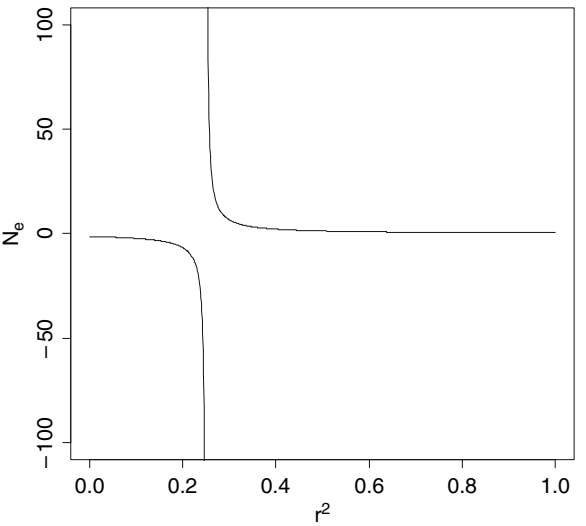

Fig. 1 Relationship between $N_e{}^{LD}$ and $\hat{r}^2$ ($n = 4$ for illustration)

estimate of infinity (Waples 1991), meaning that the observed linkage disequilibrium is estimated to be entirely due to sampling and with zero contribution due to drift, as would occur in an infinite population. This scenario is a considerable disadvantage to the linkage disequilibrium method. There is also a singularity (undefined value) associated with $N_e{}^{LD}$ (equation 1) when $\hat{r}^2 = 1/n$.

The linkage disequilibrium in a sample is also affected by residual disequilibria from previous generations. If a population of effective size N_e is initially drawn from an infinite population at time 0, and then remains at constant size N_e for subsequent generations, the expected value of r^2 takes a few generations to reach its equilibrium value. The rate of convergence is given by Sved (1971), and is $1-(1/4)^t$ for unlinked loci, where t is the number of generations (Waples 2005). It follows that r^2 reaches its equilibrium value after about four generations. Waples (2005) performed preliminary simulations for populations with recent increases and decreases in N_e, and confirmed that accumulated disequilibria over multiple generations can affect $N_e{}^{LD}$. Populations suffering recent declines were only affected for about one generation until N_e stabilized. For populations that had undergone recent increases, residual effects could persist for a few generations, and caused negative bias in estimates of N_e, depending upon the severity of the bottleneck and the magnitude of the subsequent increase in N_e.

England et al. (2006) simulated ideal populations and found that the $N_e{}^{LD}$ estimator was robust to different distributions of allele frequencies with up to five alleles per locus. However, for small sample sizes (less than 100), they found serious negative bias in $N_e{}^{LD}$ when the sample size was smaller than the true value of N_e. Waples (2006) additionally noted a less serious positive bias in $N_e{}^{LD}$ when the sample size was larger than the true N_e. The conclusion that the sample size should be

approximately equal to the quantity that we are trying to estimate, in order for the estimates to be unbiased, is clearly problematic. To improve the method, Waples (2006) used simulated data to derive an empirical correction factor to adjust the estimated r^2 to its correct value. This was to take account of the second order terms in $(1/N_e^I)$ and $(1/n)$ that were omitted in the original derivation of the approximation $E(r^2) \approx 1/(3N_e^I) + 1/n$. He derived two separate modified equations for ${N_e}^{LD}$, one for $n < 30$ and one for $n \geq 30$. Using biallelic simulations, he showed that the harmonic mean of the corrected N_e estimates compared well with the true values (Fig. 4 of Waples 2006; Waples personal communication). The harmonic mean was used because the method adjusted bias in r^2, which is related to the reciprocal $(1/N_e^I)$. He also found that dependencies in r^2 effectively lower the degrees of freedom for estimating var(${N_e}^{LD}$). Both England et al. (2006) and Waples (2006) stated that their simulations were only exploratory and that a thorough evaluation of the linkage disequilibrium method was still required.

The linkage disequilibrium method has seen limited application to real datasets. Bartley et al. (1992) applied the method to natural populations, though obvious errors in their equations (2) and (3), and incorrect sample variances for allele frequencies in estimating r, should all be noted. Recent studies have estimated ${N_e}^{LD}$ using the software package NEESTIMATOR (Peel et al. 2004), following Bartley et al. (1992), but do not provide confidence intervals for their estimates (Lippé et al. 2006), or compare it to inappropriate demographic estimates (Schmeller and Merilä 2007). A new software package LDNE estimates ${N_e}^{LD}$ using the bias-corrected method, removing alleles below a specified proportion, and implementing bootstrap confidence intervals (Waples and Do in press).

3 Simulation

We are concerned with populations consisting of two sexes (male and female; i.e. dioecious and diploid), two distinct generations (adults and juveniles), and a small number of offspring annually from each adult (number of offspring ≤ 15), as is common for terrestrial vertebrate species. We consider only populations with discrete breeding generations where breeding occurs once within a generation. We simulate small populations ($N < 200$) of individuals for four discrete generations after initially drawing alleles from an effectively infinite pool. This allows us to estimate N_e from genetic data on a generation once r^2 has reached its equilibrium value.

We first generate a population of individuals with L loci and A_i alleles in locus i, where $A_1, ..., A_L$ can be set *a priori* as constant, or drawn from a Normal distribution (rounded to integer values) to create polymorphism. Initial allele frequencies for the simulation are drawn from a Dirichlet distribution with a specified shape parameter which controls allele rarity. We use relatively common allele frequencies ($p > 0.1$) to reduce the chance of alleles being lost from the population through genetic drift over the generations.

The first generation of N_1 individuals is given alleles drawn from an infinite sampling distribution with the selected frequencies, which effectively means that

$N_0 = \infty$. Generations 1–4 have finite sizes $N_1, ..., N_4$ determined by the chosen value of N_c for the simulations. Over these four generations genetic drift occurs, which creates the desired linkage disequilibrium but can alter the number of alleles and their frequencies from those specified for the simulation.

In each generation, individuals are assigned male or female sex so that the generation's sex ratio is exactly equal to that specified by the simulation parameters. The ideal population has a 1:1 sex ratio. Males and females in the population contribute k gametes (or, equivalently, k offspring when gametes are united) to the next generation, where the value of k for any individual is drawn from a negative binomial distribution with mean μ_k and variance σ_k^2, and μ_k and σ_k^2 are specified separately for males and females. For a population of constant size and equal sex ratio, the mean must be $\mu_k = 2$ for each sex.

The ratio of offspring variance to mean is called the index of variability in breeding success: $IV = \sigma_k^2/\mu_k$ (Barrowclough and Rockwell 1993; Waples 2006). It is a key parameter for controlling departures from the ideal population. If some individuals have much greater breeding success (k) than others, the index of variability is high, and the inbreeding N_e is lowered due to an increased probability of two randomly selected gametes being derived from the same, successful, parent. We control the index of variability separately for each sex. In an infinite ideal population, $IV = 1$ and k follows a Poisson distribution. This specifies a randomly mating (promiscuous) population. For $IV > 1$, the negative binomial distribution for k has greater than Poisson variance, which is characteristic of polygamous populations where a few individuals have the most successful matings. We do not consider the unusual case where $IV < 1$.

For creating simulated populations with IV exactly equal to the value specified by the simulation parameters, we require vectors of gametes $(k_1, ..., k_M)$ for the M male adults such that the mean and variance of $(k_1, ..., k_M)$ are exactly equal to the specified values for μ_k and σ_k^2, and similarly for the female adults. We achieve this by reformulating the vectors as $(n_0, n_1, ..., n_K)$, where n_k is the number of males contributing k gametes, to a maximum allowed number of gametes $K = 15$. We have three equations for the $K + 1$ unknown integers $n_0, n_1, ..., n_K$, where these are $\sum_{k=0}^{K} n_k = M$, $\sum_{k=0}^{K} k n_k = M\mu_k$, and $\sum_{k=0}^{K} (k - \mu_k)^2 n_k = M\sigma_k^2$. We select guesses for all but three of the $n_0, n_1, ..., n_K$, and solve a matrix equation to find the remaining three values, which leads to some initial guesses being discarded because there are no solutions in integers ≥ 0. Only certain combinations of μ_k and σ_k^2 yield exact solutions in non-negative integers for M individuals. We restrict our simulations to these combinations where possible, or use closely approximate solutions otherwise when the sex ratio and index of variability are both non-ideal.

Once the gamete vectors have been generated for both sexes, gametes from each sex are united with those from the other sex at random without replacement, to create offspring from breeding pairs. Because each offspring is formed from the union of a male and female gamete, the two sexes contribute the same number of gametes, even when sex ratios differ. For unequal sex ratios, μ_k and σ_k^2 are determined separately for each sex to achieve the target population size and index of variability.

Finally, we simulate statistical sampling from the generated population by drawing a sample of n individuals at random without replacement from the final

generation (N_4). The genotypes of these n individuals are inspected, and genotyping errors may be added with a specified probability to mimic real laboratory conditions (van Oosterhout et al. 2004; Hoffman and Amos 2005). We simulated two types of errors. The first is allelic drop-out, where one of two alleles for an individual is not typed. This causes the individual to appear homozygous when in fact it is heterozygous but one allele was not typed. We simulate allelic dropout for an individual by replacing one of its two alleles with the other one. The second type of error is missing data, where the individual fails to type for both alleles at a locus. This reduces the sample size for that locus across the population. All error rates are assigned across individuals × loci. For a single individual at a single locus, allelic dropout is assigned first (yes or no), then missing data, which will override allelic dropout if both are selected. We do not consider other microsatellite typing errors which change the length of the microsatellite allele due to either contamination (allele is drawn from the population frequencies) or stutter error (allele is altered by a multiple of the repeat unit). We assume that the error rates are independent and multiplicative. Reported error rates from studies may be conservative, since missing data errors can mask allelic dropout, and both can mask typing errors.

The inbreeding effective population size at time $t-1$ for the non-selfing populations such as in our simulation is approximated from demographic parameters as:

$$\frac{1}{N_e^I} = \frac{\mu_k - 1 + \sigma_k^2/\mu_k}{N_{t-1}\mu_k - 2} \tag{3}$$

(equations 2, 2′ and 2″ in Crow and Denniston 1988; equation (23) in Caballero 1994). Here, the overall μ_k and σ_k^2 for both sexes combined are given by $\mu_k = 2m\mu_m = 2f\mu_f$ and $\sigma_k^2 = m\sigma_m^2 + f\sigma_f^2 + mf(\mu_m - \mu_f)^2$, where m and f are the proportions of adult males and females, μ_m and μ_f are the mean number of progeny of adult males and females, and σ_m^2 and σ_f^2 are the variances in the number of progeny of adult males and females. N_{t-1} is the total number of adults in the parental generation (time $t-1$), given by $N_m + N_f$.

Because inbreeding is slightly retarded in non-selfing populations, $N_e{}^I$ is slightly less than N even in an ideal population without selfing. Our estimate of $N_e{}^{LD}$ is for a non-selfing population, and so we do not adjust $N_e{}^I$ so that our effective population size is exactly equal to our census population size under ideal conditions (Caballero and Hill 1992; Waples 2006). Equation 3 is thus an appropriate true value for comparison to our simulation $N_e{}^{LD}$ estimates when linkage disequilibrium is generated from the non-selfing parental population.

Using this model, we simulate across a range of ecologically realistic parameter values (Table 2), and consider how each parameter influences our estimates of the mean $N_e{}^{LD}$ and 95% confidence intervals, compared to the true comprehensive demographic $N_e{}^I$ calculated from equation (3).

The default simulation values involve eight loci with five alleles per locus at approximately equal allele frequencies, and the entire generation is sampled to estimate effective population size ($n = N$). First we consider ideal populations of different census population sizes. We then determine how the linkage disequilibrium estimate is affected by deviation from each of the three ideal population conditions:

Table 2 Simulation parameters and values. Bold indicates default values that are used unless the simulation specifies that the associated parameter is to be varied. The number of alleles per locus is an integer generated by rounding a Normal(s,v) variate with mean s and variance v given in the table

	Parameter	Values
Population properties	Census population size	10, **50**, 100 and 200
	Index of variability	1, 2, **3**, 4 and 5
	Sex ratio	**1:1**, 1:1.5
Sample properties	Proportion sampled	**1**, 0.5, 0.2
	Number of loci	**8**, 16, 24
	Allele numbers	**N(5,0)**, N(2,0), N(10,0), N(5,1)
	Sequencing errors	Dropout (1%)
		Missing (5%)

constant population size; equal sex ratio; and random mating with $IV = 1$. To investigate the effect of non-constant population size, we run the simulation for a further four generations after r^2 has stabilized in generation 4, and allow N_e to change over generations 5–8 before using the generation 8 data to calculate the estimate $N_e{}^{LD}$. Table 3 shows the patterns of population change that we simulate, which we label increasing, decreasing, fluctuating up (where 'up' refers to the last change from generation 7 to 8), and fluctuating down. The populations are ideal in all characteristics except for the changes in population size.

The sequences of population sizes chosen in Table 3 are dictated by our requirement that the mean number of gametes per individual is exactly equal to the variance, to ensure that $IV = 1$, and that the new population size (half the mean number of gametes, times the old population size) is an integer. Sequences of population sizes meeting these requirements are unusual and hard to generate. We restrict our simulations to these exact sequences so that the performance of the $N_e{}^{LD}$ estimator is not confounded by the unknown effect of using an approximate *IV*.

After investigating the impact of the three departures from the ideal population, we then test how $N_e{}^{LD}$ is affected by sampling properties. We select ecologically realistic population parameters, for which $N_e^I = N_{t-1}/2$, and vary the proportion of the population sampled; the number of loci typed; the number of alleles occurring per locus (distributed Normally and rounded to 0 d.p.); and the presence of genotyping errors.

Multiple paternity (offspring within a litter sired by multiple fathers) can impact on the effective population size (Sugg and Chesser 1994), but we do not model this

Table 3 Simulation parameters for changing population sizes in an otherwise ideal population

Population change	$N_1, \ldots N_4$	N_5	N_6	N_7	N_8
Increasing	12	24	36	48	60
Decreasing	64	48	36	24	12
Fluctuating up	36	48	36	24	36
Fluctuating down	36	24	36	48	36

separately because its only effect would be to alter σ_k^2 (and hence IV) contingent on the model for multiple paternity. Dominance multiple paternity where the most successful male breeders also sire other litters increases σ_k^2, while sneaky multiple paternity where unsuccessful male breeders sire other litters reduces σ_k^2, and random multiple paternity should not alter σ_k^2.

4 Results

We simulated over a range of N_e values from 6 to 199, where $N_e = 6$ occurs with census size $N = 10$, sex ratio 1.5:1, and $IV = 2$; and $N_e = 199$ occurs with $N = 200$, sex ratio 1:1, and $IV = 1$. For most values of census size N, we allowed IV to vary from 1 to 5, but for $N = 10$ we only used IV of 1 and 2, because exact solutions for larger IV were not possible. Substantial loss of alleles due to genetic drift over the four generations of equilibration was only problematic for populations with $N = 10$. We refer to the original linkage disequilibrium method as the standard method (SM), and the small sample bias correction introduced by Waples (2006) as the Waples adjustment (WA). WA results are discussed but not shown.

4.1 Population Properties

For both methods, ${N_e}^{LD}$ has a right-skewed distribution (Fig. 2; only showing SM). The reciprocal $1/{N_e}^{LD}$, which estimates the probability that two randomly selected successful gametes derive from the same parent, is distributed approximately Normally. For the ideal population ($IV = 1$), both the arithmetic mean and the harmonic mean of ${N_e}^{LD}$ are almost equal to the true value of N_e, for both the SM and WA methods. Because most researchers currently focus on estimators for N_e rather than its reciprocal, we focus on the bias of ${N_e}^{LD}$ rather than the bias of $1/{N_e}^{LD}$ and therefore report arithmetic means of our results rather than harmonic means. Estimates of infinity are omitted when calculating the arithmetic mean for each simulation, which is justifiable in the sense that these estimates would be discarded by practitioners when they occur in practice. Omitting these poor results will enhance the apparent performance of the ${N_e}^{LD}$ estimator to some extent, but in practice they only occurred in one of our simulations (the biallelic plot A2 in Fig. 6 below). Infinite values are recorded on the boxplots when they occur within the central 95% of the distribution of simulated ${N_e}^{LD}$ estimates, which again only occurred once.

In the ideal populations, where sample size $n \approx N_e$, a positive bias in ${N_e}^{LD}$ is present which increases with census population size (Fig. 3; upper left plot). This is consistent with the suggestion that the ${N_e}^{LD}$ method should work best in small populations, where the genetic signal is strongest (Waples 1991, 2006). The SM performs better than the WA (graphs not shown). For each of the 10,000 simulations in every boxplot, 95% confidence intervals are calculated using the chi-squared approximation in equations (2) and (1). Except for very small populations, uninformative upper

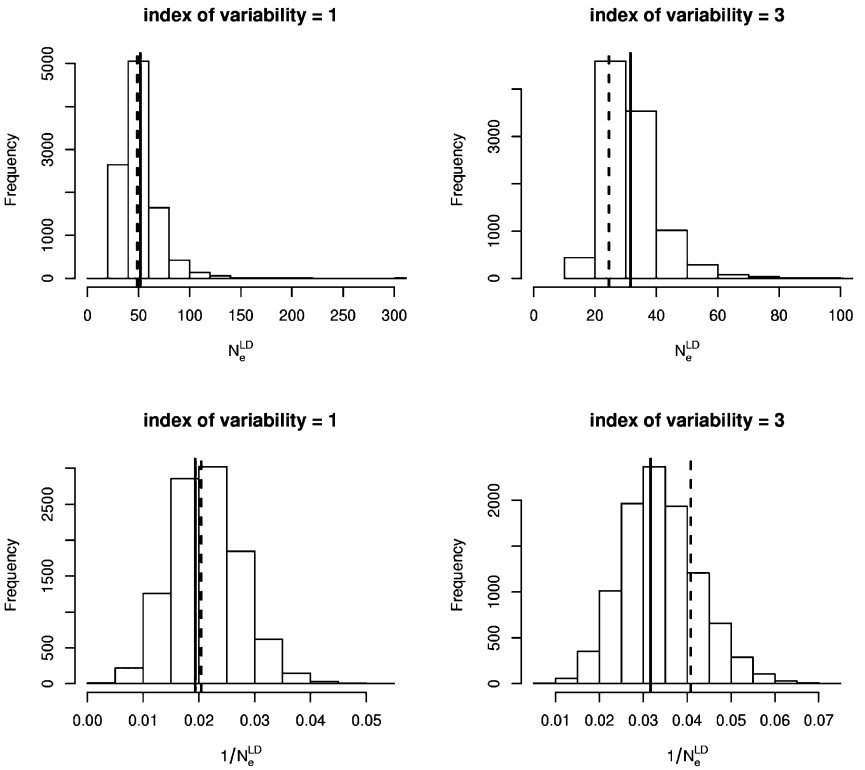

Fig. 2 Distribution of linkage disequilibrium SM estimates from 10,000 simulations with $N = 50$, equal sex ratio and index of variability 1 (an ideal population), and index of variability 3. Bold lines are means, dotted lines are true values. The entire generation is sampled

95% confidence interval estimates of infinity are ubiquitous when the SM is applied to ideal populations. Precision is constant in ratio to the mean for increasing census population sizes. Changing the sex ratio from the ideal 1:1 to the non-ideal 1.5:1 has very little effect on the calculation of N_e by equation (3), and also has very little effect on the bias or precision of either method (Fig. 3).

Figure 4 shows the impact of increasing the index of variability, simultaneously for both sexes, for different population sizes. The bias of both methods increases with increasing *IV* (Fig. 4), in the sense of the true value lying below the 25% quantile of the estimator distribution, and the bias of the WA becomes more similar to that of the SM. For populations with identical N_e the bias is greater at higher indices of variability, but precision remains similar. Both biases are considerable at high indices of variability, with the true value lying at about or below the 25% quantile of the estimator distribution, but the SM still has less bias than the WA at our maximum index of variability (5). High indices of variability did substantially decrease the number of upper 95% confidence interval estimates of infinity, beyond that expected from a decrease in N_e alone.

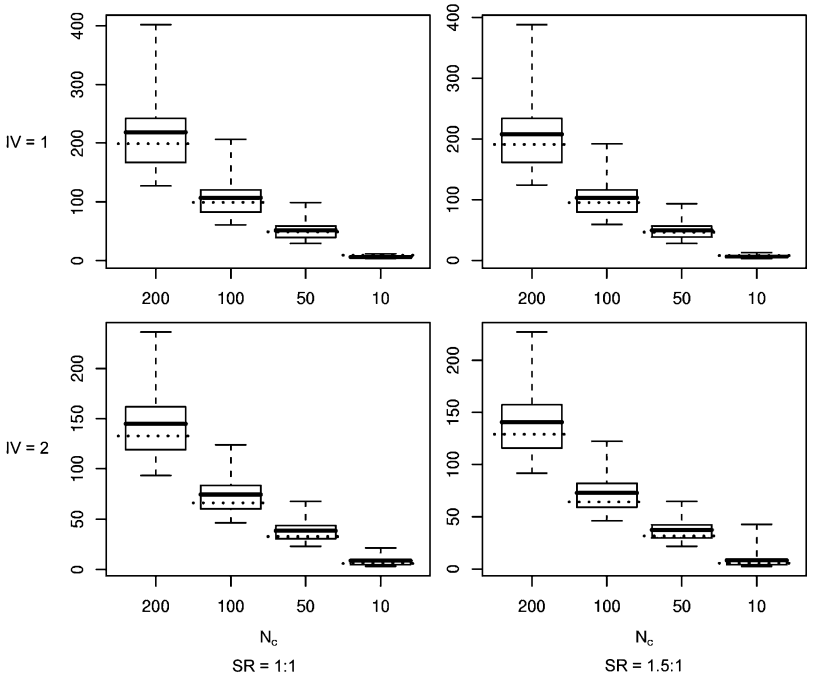

Fig. 3 Boxplots of the SM N_e^{LD} estimator for sex ratios 1:1 and 1.5:1, and indices of variability 1 and 2 for populations $N = 200$, 100, 50 and 10 from 10,000 simulations. Boxplots show 2.5, 25%, mean, 75 and 97.5% quantiles of the estimator distribution. Dotted lines are true N_e values

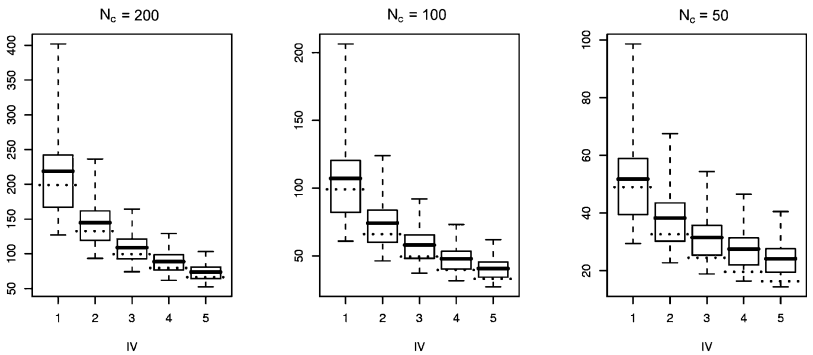

Fig. 4 Boxplots of the SM N_e^{LD} estimator for sex ratio 1:1 and indices of variability 1–5 for populations $N = 200$, 100 and 50 from 10,000 simulations. Boxplots show 2.5, 25%, mean, 75 and 97.5% quantiles of the estimator distribution. Dotted lines are true N_e values

If males and females are given different values of *IV*, the resulting bias is greater than the bias obtained if the same overall average *IV* is used and is equal for both sexes (results not shown). If the *IV* differs between sexes and the sex ratio is also

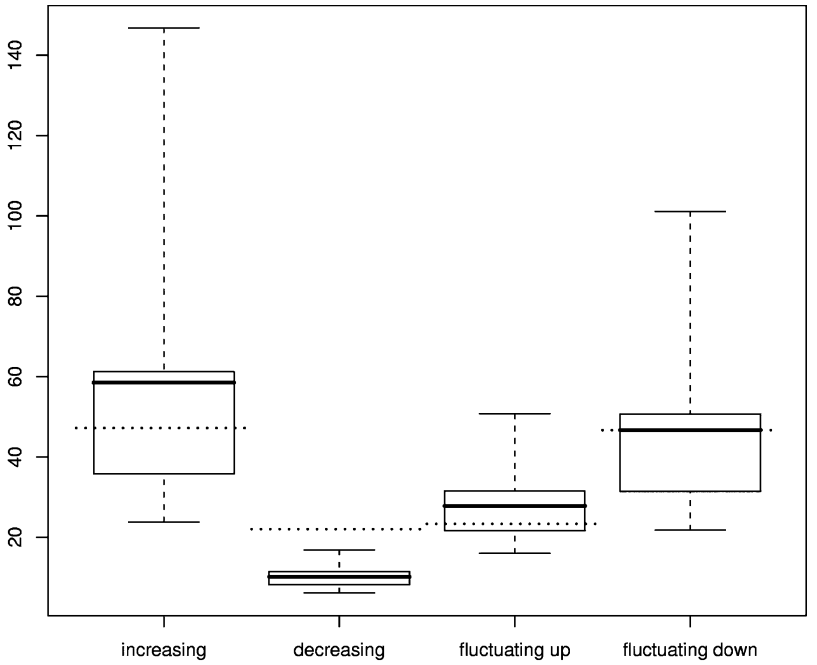

Fig. 5 Boxplots of the SM $N_e{}^{LD}$ estimator when generations $N_5, \ldots, N_8$ are steadily increasing, steadily decreasing, and fluctuating with a final increase and final decrease, from 10,000 simulations. Boxplots show 2.5, 25%, mean, 75 and 97.5% quantiles of the estimator distribution. Dotted lines are true N_e values

deviated from 1:1, we see an additional, but small, positive bias again. Precision is similar to that in Fig. 4 when both sexes are given the average of the two *IV* values.

Figure 5 shows the results of changing the census population size over generations 5–8 in populations that are otherwise ideal, using the population size sequences in Table 3. For both SM and WA methods, the $N_e{}^{LD}$ estimate is affected differently depending on the form of the population change. The $N_e{}^{LD}$ estimate from generation 8 will be distorted by residual linkage disequilibrium from roughly the previous four generations (Sved 1971). For a systematically increasing population $N_e{}^{LD}$ was positively biased, and imprecise (Fig. 5). For a systematically decreasing population $N_e{}^{LD}$ was negatively biased but very precise. For the 'fluctuating up' population where the final fluctuation was upwards, $N_e{}^{LD}$ was also slightly positively biased, but not to the extent of the 'increasing' population. For the 'fluctuating down' population where the final fluctuation was downwards, $N_e{}^{LD}$ showed no bias. Waples (2005) also found that decreases in population size had less severe effects than increases. Bias reflected the persistence of directional population change, while precision reflected the final census population size as expected from previous results. In all cases the SM performed better than the WA, which was highly positively biased for both cases of decreasing populations. The $N_e{}^{LD}$ for systematically

increasing and decreasing populations was in fact more closely aligned with the census population size of the offspring generation 8 rather than that of the parental generation 7, which would correspond to the variance effective population size rather than the inbreeding effective size. Further investigation beyond that of our limited population sequences is warranted for this ecologically important scenario.

$N_e{}^{LD}$ estimates of infinity are a concern for inference from the linkage disequilibrium method, but in our simulations they only occurred once (biallelic case: plot A2, Fig. 6 below). They occur because the $N_e{}^{LD}$ estimator is sensitive to variation in r^2 (Fig. 1). The estimator is constructed by replacing the expectation $E(r^2)$ by the sample value $\hat{r}^2$ in equation (1), so a large variance in $\hat{r}^2$ could draw the estimator into the part of the parameter space left of the singularity in Fig. 1, which would be impossible for the true expectation $E(r^2)$. The result is a negative estimate of $N_e{}^{LD}$,

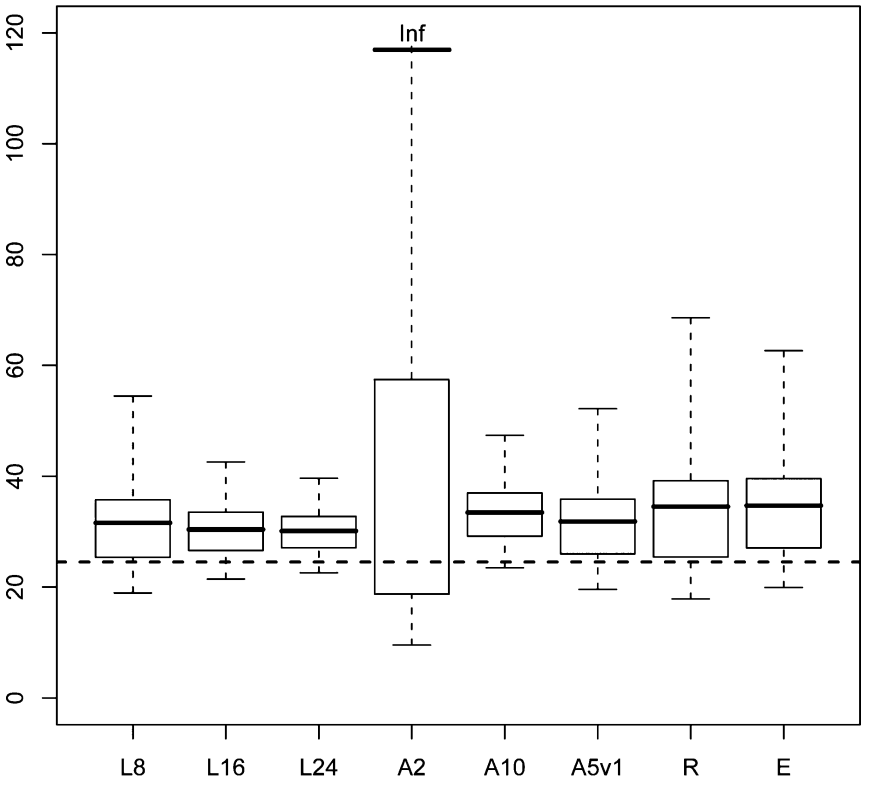

Fig. 6 Boxplot of the SM $N_e{}^{LD}$ estimator for N = 50, sex ratio 1:1 and index of variability 3 from 10,000 simulations (except A2 = 20,000 simulations). Boxplots show 2.5, 25%, mean, 75 and 97.5% quantiles of the estimator distribution. The dotted line is the true value N_e = 25. L8 = standard reference sample with 8 loci and 5 alleles; L16 = 16 loci; L24 = 24 loci; A2 = biallelic loci, i.e. number of alleles per locus is Normal(2,0); A10 = polymorphic loci with N(10,0) alleles per locus; A5v1 = N(5,1) alleles per locus; R = rare alleles in initial population ($p < 0.1$); E = errors in genetic sequencing at an individual rate of allelic dropout = 0.01 and missing data = 0.05

which has to be interpreted as infinity. A greater problem in our simulations is upper confidence limits of infinity, which are gained using the chi-squared approximation in the SM and are very common. The SM consistently overestimates the variance of $N_e{}^{LD}$. Except for very small census population sizes of $N = 10$, the 95% confidence intervals contained the true value 100% of the time, usually with uninformative upper confidence intervals of infinity. The chi-square approximation for the variance of r^2 does not appear to be appropriate for estimating suitable confidence intervals.

By contrast, coverage of the 95% confidence intervals using the WA method was usually below 95%, sometimes as low as 40% for small census population sizes. Only for large census population sizes with significant deviation from the ideal population did the WA coverage approach 100%. Waples (2006) did some investigations of confidence interval coverage and also found that for $N_e < 100$ his bias-corrected confidence intervals would have coverage well below 95% (Figs. 5 and 6 in Waples 2006). The poor coverage of the WA confidence intervals was attributable to a decrease in the estimator variance, coupled with an increase to the positive bias already present when sample size was greater than N_e. This often led to the lower confidence interval being above the true value. The WA 95% confidence interval was additionally problematic when the square root component of his adjusted N_e equations (Waples 2006) was positive for the corrected $\hat{r}^2$, but not for the lower 95% confidence interval of $\hat{r}^2$. This meant a second approximate equation was necessary to estimate the lower 95% confidence interval for $N_e{}^{LD}$ in the WA method.

4.2 Sample Properties

Effects of number of loci sampled, allele numbers and rarity, and genotyping errors were investigated with the ecologically reasonable value $IV = 3$ (e.g. Heiberg et al. 2006). Increasing the number of loci sampled substantially improves the precision of the $N_e{}^{LD}$ estimate for both methods (Fig. 6, plots L8, L16, L24), and reduces the number of upper confidence interval estimates of infinity in the SM. However, increasing the number of loci sampled has little effect on the bias, and confidence interval coverage remains at 100% for the SM. For the WA, confidence interval coverage decreased markedly to 40% with 24 loci. For strictly biallelic loci, 20,000 simulations were required to attain stability. Biallelic loci generated a substantial positive bias in both methods (Fig. 6, plot A2), due to the presence of many large estimate values, including infinity. The biallelic loci case was the only case where the WA performed better than the SM, which is notable because the WA method was derived using biallelic loci (Waples 2006, p. 182). In the biallelic loci case for the SM, confidence interval coverage was reduced slightly less than 95%, and the precision of the estimator was reduced to the extent that it included infinity in the central 95% of its distribution. However, the harmonic mean of $N_e{}^{LD}$ was almost exactly correct in the biallelic case for the SM.

With an increased polymorphism of 10 alleles per locus (plot A10), precision was improved slightly but bias was also increased slightly over the 5 allele setting (plot L8). Normally distributed variation in the number of alleles had no effect on the

estimates from either method (plot A5v1). The presence of rare alleles meant some were lost during genetic drift, creating a positive bias and decrease in precision as found for the extreme biallelic case (plot R). The presence of errors in the data at our specified rates also created a positive bias and decrease in precision (plot E).

The important effect of sample size n on the $N_e{}^{LD}$ estimator was emphasised by both Waples (2006) and England et al. (2006). For the SM we found a similar pattern to that reported in Fig. 1 of Waples (2006). When $n > N_e$ (so sampling fraction $S = n/N_c$ satisfies $S > N_e/N_c$), positive bias occurs (Fig. 7). When $n < N_e$ ($S < N_e/N_c$), we obtain severe negative bias. The least bias occurs when $n \approx N_e$ (Fig. 7: $S = 1$ with $IV = 1$; and $S = 0.5$ with $IV = 3$). In our simulations, the WA

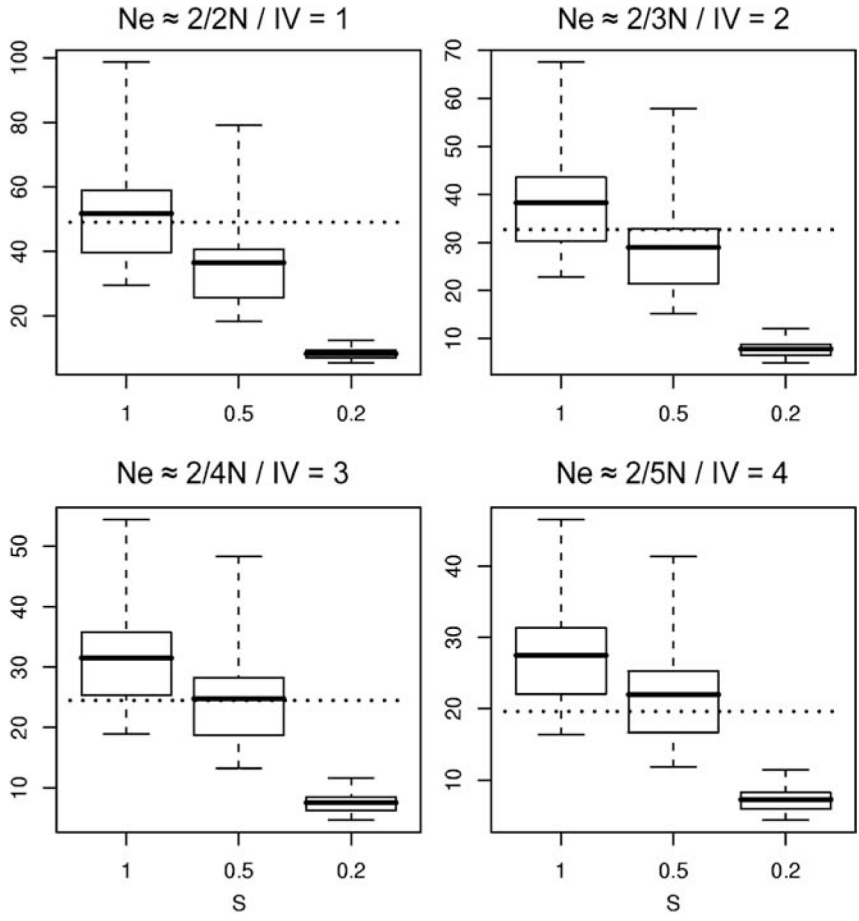

Fig. 7 Boxplots of the SM $N_e{}^{LD}$ estimator for $N = 50$, sex ratio 1:1 and indices of variability 1–4 from 10,000 simulations. Boxplots show 2.5, 25%, mean, 75 and 97.5% quantiles of the estimator distribution. The dotted line is the true value N_e. $S = n/N_c$ is the proportion of the census population size sampled

removed the negative bias but created a positive bias of at least similar magnitude. The WA did however reduce the bias of the reciprocal estimator when $n < N_e$. As the sampling fraction decreased, the coverage of the WA 95% confidence interval for N_e decreased to below 50%, because the positive bias lifted the lower confidence interval above the true value. The SM became more precise at smaller sampling fractions, but the estimator distribution did not include the true value. By contrast, the WA estimator became less precise at lower sampling fractions. Increasing the number of loci typed when the sample size was very small led to the very poor interaction of large bias and high precision, because the estimator became more precise with more loci, but had confidence interval coverage below 50% due to the negative bias. Increasing the number of loci did however reduce the bias of the WA.

5 Rat Populations

We now compare demographic and linkage disequilibrium estimates of inbreeding effective population size using data collected from closed rat populations on two small (<10 ha) islands in New Zealand, with negligible migration. Random mating can be achieved as all rats can physically find each other on the islands. We assume that rats follow a breeding model with discrete generations. In the wild, rats rarely survive longer than one year (Innes 2005a, b), usually only breeding in one generation (season). Hence we assume that any adults caught can not be offspring of other adults in our sample from a previous generation. As many rats as possible were sampled prior to eradication from the islands. Rats were classified by sex and assigned as adult or juvenile based on an arbitrary weight value and breeding condition. On each island we therefore have a total population of size N, comprising two generations of rats (N_t and N_{t-1}), from which we will use the linkage disequilibrium method with a sample of size n_t juveniles to estimate the effective population size of generation $t-1$. In each case, N was approximately known due to exhaustive eradication sampling and follow-up observation.

We estimate the mean and variance of the number of progeny of adults, separately for males and females, by applying the two-stage parentage assignment software CERVUS (Marshall et al. 1998) to the juvenile genotypes. Missing data rates were estimated from the real populations, and a default mis-typing rate of 1% was assumed. We then use equation 3 to estimate an approximate demographic N_e^I for the parental generation. We assume equal catchability between age classes, and therefore estimate the total number of adults (N_{t-1}) by dividing the number of adults caught by the approximate sampling proportion of the population. Our demographic estimates are only approximate due to possible incorrect assignment of parentage and missing individuals, which will influence our estimates of mean and variance in breeding success. We have essentially ignored the statistical issues in estimating these demographic parameters (Crow and Denniston 1988), and do so merely for comparative purposes with the linkage disequilibrium estimates. We assume individuals were sampled at random so that our estimates are representative of the entire population.

5.1 Ship Rats (*Rattus rattus*) – Hawere (9.3 ha)

A total of $n = 29$ ship rats were genotyped from a known population of $N = 31$ individuals, giving a sampling proportion of $S = 0.94$ for the population. An arbitrary weight of 120 g was used to distinguish between adults and juveniles, giving 7 male and 11 female adults ($n_{t-1} = 18$), and 6 male and 5 female juveniles ($n_t = 11$). Parentage was assigned with 80% confidence for the fathers of 8/11 juveniles, and mothers of 10/11 juveniles. We estimated $\mu_m = 1.14$, $\sigma_m^2 = 0.48$, $\mu_f = 0.91$, and $\sigma_f^2 = 1.69$. Using the expressions below equation (3) (but using $\mu_k = m\mu_m + f\mu_f$ to allow for unequal parentage assignment for male and female adults), the overall estimates were $\mu_k = 1.00$ and $\sigma_k^2 = 1.23$. By equation (3):

$$\frac{1}{N_e^I} \approx \frac{1.00 - 1 + 1.23/1.00}{18/0.94 \times 1.00 - 2}$$

This gives our demographic estimate $N_e{}^I = 13.93$ (73% of N_{t-1}). The $N_e{}^{LD}$ estimates from the linkage disequilibrium method were: SM = 7.52, 95%CI = [2.35, ∞]; and WA = 30.68 [17.37, 47.34].

5.2 Norway Rats (*Rattus norvegicus*) – Moturemu (5.0 ha)

A total of $n = 27$ Norway rats were genotyped from a population of approximately $N \approx 39$ individuals, giving a sampling proportion of $S = 0.69$ for the population. An arbitrary weight of 220 g was used to distinguish between adults and juveniles, giving 11 male and 5 female adults ($n_{t-1} = 16$), and 4 male and 7 female juveniles ($n_t = 11$). Most likely parentage was assigned for the fathers and mothers of all 11 offspring (ties in parentage were assigned 0.5 to each parent), giving estimates $\mu_m = 1.00$, $\sigma_m^2 = 2.40$, $\mu_f = 2.20$, and $\sigma_f^2 = 11.08$. The overall estimates were therefore $\mu_k = 1.38$ and $\sigma_k^2 = 5.42$, so by equation (3):

$$\frac{1}{N_e^I} \approx \frac{1.38 - 1 + 5.42/1.38}{16/0.69 \times 1.38 - 2}$$

therefore $N_e{}^I = 6.92$ (30% of N_{t-1}), while the $N_e{}^{LD}$ estimates were SM = 1.92, 95%CI = [0.85, 4.39], and WA = 1.16 [0.63, 1.84].

6 Discussion

6.1 Simulations

Data on linkage disequilibrium in a population provide a method of estimating inbreeding effective population size in the parental generation, and its reciprocal, the probability that two randomly chosen successful gametes derive from the same parent. The concepts underlying the method are intuitively challenging, and this has led to some confusion in the literature regarding gametic and genotypic

estimators (Waples 1991; Bartley et al. 1992), and variance and inbreeding effective population sizes (Leberg 2005). The method can be used when just one sample is available from a population, complementing alternative methods for estimating effective population size when multiple samples are available (Wang 2005).

Throughout our study, we evaluated the bias of $N_e{}^{LD}$ as an estimator of N_e, rather than using the harmonic mean of $N_e{}^{LD}$ estimates. The harmonic mean would be appropriate if the parameter of interest were the reciprocal, $1/N_e$, rather than N_e itself, and has been used by previous authors in assessing bias of $N_e{}^{LD}$ (Waples 2006). We intentionally conducted our investigations on $N_e{}^{LD}$ rather than the reciprocal estimator, because this is the parameter that researchers focus on and set out to estimate in conservation biology. The bias correction method (WA) of Waples (2006) was developed in such a way that it improves the bias of $1/N_e{}^{LD}$ for biallelic loci, especially when the sample size is much less than N_e, but this has the effect of exacerbating upwards bias in N_e itself in most other situations. For the standard method (SM), the bias in the arithmetic mean and harmonic mean of $N_e{}^{LD}$ estimates were similar in direction and magnitude. For the Waples (2006) adjusted method (WA), the bias in the harmonic mean was generally reduced slightly below that of the SM, but the bias in the arithmetic mean was increased substantially above that of the SM. Using the arithmetic mean had the disadvantage that infinite values had to be discarded before assessing bias, but this happened only in the biallelic simulation A2 (Fig. 6).

Our simulation results suggest that the $N_e{}^{LD}$ estimator performs poorly for non-ideal populations and when the sample size is either substantially greater than or less than the true value N_e, which we wish to estimate. Deviation from ideal sex ratios has little effect, but deviation from random (binomial) breeding by using index of variability $IV > 1$ leads to bias in the method. Of some concern is that the size of the census population, and recent fluctuations in it, have considerable effects on $N_e{}^{LD}$ (Waples 2005). This is a problem as much of the interest in $N_e{}^{LD}$ is in its application to natural populations, where usually N is unknown and changing. Non-constant population size can cause considerable bias, because $N_e{}^{LD}$ is affected by residual disequilibria from previous generations. Waples (2005) simulated changes in population size and the subsequent rate of recovery in $N_e{}^{LD}$ for the population at its final, stable, size. For a population that had gone through a bottleneck and then increased, the bias was considerable for several generations because the small population size during the bottleneck generated strong levels of linkage disequilibrium which took several generations to decay. In our limited simulations of non-constant population size, we found bias in the direction of the most recent population change. The bias became greater as population change persisted in a single direction.

The bias in $N_e{}^{LD}$ changes direction according to whether the sample size is larger or smaller than the true value N_e, and this is a considerable drawback of the method (Hill 1981; England et al. 2006; Waples 2006). The bias correction method proposed by Waples (2006) only improves properties of the reciprocal estimator $1/N_e{}^{LD}$ when n is much less than N_e, but in our simulations did not perform as well as the standard method when considering bias, precision and confidence interval coverage for estimating N_e itself. England et al. (2006) suggested a way of addressing the problem

that the optimal sample size is the same as the value of the unknown parameter we wish to estimate. They recommended sub-sampling the available sample to create several N_e^{LD} estimates from different sample sizes and plotting the results against sample size to investigate whether it stabilizes. Stability suggests that the correct value has been reached, because the bias from $n > N_e$ is much less than that from $n < N_e$. The sample size effect has been observed in every evaluation of the N_e^{LD} undertaken to date, and it would be wise for researchers applying the method to real data to routinely investigate results from this sub-sampling procedure.

The index of breeding variability exerts a strong influence on N_e, and another tactic for overcoming the problem of requiring $n \approx N_e$ is to produce a rudimentary estimate of the index of variability within a population. With this, it may be possible to gain a crude estimate of N_e from demographic data using equation (3), and hence an estimate of N_e/N_c and the proportion of the population that should be sampled. For example, assuming $IV = 3$, $N_e \approx N/2$, so $n > N/2$ is appropriate. Estimates of the proportion of the population sampled can be obtained from removal data and catch-effort modeling methods (Seber 1982). However, these approaches are themselves subject to considerable statistical error.

Waples (2006, p. 180) remarked that simulating ideal populations of $N = N_e$, and assuming that estimators will behave similarly if N_e is of an equivalent value under non-ideal conditions, may be a reasonable approximation; however, this was not always the case with our simulations. Different forms of deviation from the ideal population led to substantially different biases in the linkage disequilibrium estimate. Waples (2006) simulated a standard population of sample size 50, which we also used as our default value and which performs very well in most simulations. As census (and effective) population size increase, the magnitude of the positive bias, and lack of precision, in the method increases. This is consistent with the point made by Waples (1991, 2006) that the linkage disequilibrium method is most useful for small populations in which the genetic signal from linkage disequilibrium is strongest.

Although genetic drift over the four generations of burn-in for our simulations did reduce allelic diversity for small census population sizes or for populations with rare alleles ($p < 0.1$), no loci ever became monomorphic. Monomorphic loci positively bias the linkage disequilibrium method because all their pair-wise loci combinations have $r = 0$, which will lead to infinite estimates of N_e^{LD}. In general as the number of alleles decreases for a locus (reduced polymorphism, or apparent fixation), estimates of N_e^{LD} will become increasingly positively biased due to a poor ability to detect linkage disequilibrium, causing r^2 to be underestimated in some samples. With a high level of polymorphism, precision is improved and there appears to be little if any effect on bias. Researchers should be aware of the effect of polymorphisms and select appropriate loci for estimating N_e^{LD}. The presence of occasional genotyping errors had little effect on N_e^{LD} in our simulations.

Increasing the number of loci sampled improves precision substantially, but this is only desirable when bias is relatively small compared to precision, otherwise

misleading estimates which are precise but highly biased are possible. Practitioners should therefore focus primarily on increasing their sample size of individuals. Once they are confident that their sample size exceeds the effective population size, increasing the number of loci sampled is a useful secondary consideration in order to give precise estimates which will be useful for inference. Increasing the number of loci sampled with an inadequate sample size can lead to highly misleading results. This recommendation is similar to advice given when estimating census population size through mark-recapture studies, where captures of new individuals provide more information than recaptures of previously caught individuals (Seber 1982; Borchers et al. 2002).

6.2 Rats

With a removal sample from a population, an estimate of census population size is possible, but this is prone to poor precision and accuracy (Borchers et al. 2002). By estimating the index of variability for all adults from trapping data and parentage assignment, and with a reliable estimate of N_e (and knowledge of any recent fluctuations in N) it may be possible to improve or corroborate estimates of N. As others have previously noted this relies heavily on the accurate estimation of the index of variability (Barrowclough and Rockwell 1993), which is problematic.

The sample sizes (parents and offspring) for our rat datasets were 29 and 27, and the total population sizes were 31 and approximately 39 respectively. Despite small sample sizes, n was very likely to be greater than N_e for both populations. Both populations had a mean number of offspring less than two, suggesting declining populations, which our simulations suggested would lead to negative bias in ${N_e}^{LD}$. Consistent with this, the standard method estimates were less than the demographic estimates in each case. The index of variability of the Norway rats was three times that of the ship rats, for which the index of variability was only a little greater than the ideal value of 1. The ship rat confidence interval was far too wide, and included infinity as expected from our simulations for populations close to $IV = 1$, while the Norway rat confidence interval was unexpectedly narrow and did not include our demographic estimate. As N_e approaches N, we expect the WA to have some positive bias with a wide confidence interval approaching 95% coverage, and this is somewhat consistent with the ship rat results. For $N_e < N$ we expect the WA confidence interval to have very poor coverage as seems likely for the Norway rat results.

For ship rats, the demographic, SM and WA methods give substantially different answers. It is not possible to make a judgement as to which is correct, as all three methods have associated problems. The ${N_e}^{LD}$ estimates between 1 and 2 for Norway rats are likely to be underestimates for a population of this size. The comparison of estimates is encouraging in appearing to corroborate some of our simulation results, but also illustrates the poor results that may be obtained from the ${N_e}^{LD}$ method with real data, even though the sampling fractions are large and N_e is small enough for a strong genetic signal to be expected.

6.3 Application

The effective population size provides a single statistic which simultaneously adjusts for the effects of fluctuations in population size, deviations from random mating, and unequal sex ratios when measuring genetic change in a population. As such, it can be a useful summary value in population dynamics, particularly for conservation managers (Wang 2005). It does not directly quantify the genetic diversity in a population, however, for example the number of different alleles per locus. Populations can display local adaptation and persistence with low genetic diversity and effective population size (McKay et al. 2001). The linkage disequilibrium method for estimating effective population size is particularly useful for providing information about populations that can only be sampled once. However, the methodology is still at a stage where estimates must be treated with caution.

Threatened species at small population sizes are routinely found to have low ratios of effective to census population sizes (Frankham 1995), which are implicated in their bottleneck. This creates something of a paradox, however, as introduced invading populations created from small numbers of founders also undergo a severe bottleneck and might be expected to be poorly adapted to successful establishment (Sax and Brown 2000). We would expect this to be reflected in invasive species also having a low ratio of effective to census population size. Effective population size can therefore play an important comparative role in understanding the persistence of not just threatened but also invading species (Holland 2000). Particularly, it is of interest to study whether there are certain mechanisms that help invasive species to overcome the long-term effects of severe bottlenecks and low effective population sizes.

We have performed a reasonably extensive simulation on the effect of multiple and simultaneous deviations from ideal conditions, as well as sampling properties, on the linkage disequilibrium estimate of effective population size. From this and other work (England et al. 2006; Waples 2005, 2006) we now have a reasonable understanding of the effect of multiple loci and alleles on the method, and sensitivity to allele frequencies and genotyping errors. Violation of critical assumptions needed for genetic estimation of effective population size remain to be investigated, including selected or linked markers; mutation; population admixture, migration, and sub-division; non-random sampling; and overlapping generations, which will all affect the method (Vitalis and Couvet 2001; Waples 2006). Our simulation had no spatial component, and so was biased towards ‘random mating’, in that it implicitly assumed all individuals could access all other individuals, which would only be possible in small closed populations. In other situations, a ‘Wahlund effect’ might occur where genetically similar family clusters are created. The presence of such population subdivision will affect the linkage disequilibrium estimate (Wang and Caballero 1999). More importantly, a thorough treatment of r^2 is required, considering both its accurate estimation with respect to population and sample size, and the impact of dependencies among pairwise loci comparisons. From this it may be possible to propose more robust methods of estimating N_e from data on linkage disequilibrium.

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