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INVITED TECHNICAL REVIEW

Practical application of the linkage disequilibrium method for estimating contemporary effective population size: A review

Robin S. Waples

School of Aquatic and Fishery Sciences, University of Washington, Seattle, Washington, USA

Correspondence
Robin S. Waples, School of Aquatic and Fishery Sciences, University of Washington, Seattle, WA 98195, USA.
Email: robinw3@uw.edu

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Abstract

The method to estimate contemporary effective population size (N_e) based on patterns of linkage disequilibrium (LD) at unlinked loci has been widely applied to natural and managed populations. The underlying model makes many simplifying assumptions, most of which have been evaluated in numerous studies published over the last two decades. Here, these performance evaluations are reviewed and summarized, with a focus on information that facilitates practical application to real populations in nature. Potential sources of bias that are discussed include calculation of r^2 (a measure of LD), adjustments for sampling error, physical linkage, age structure, migration and spatial structure, mutation and selection, mating systems, changes in abundance, rare alleles, missing data, genotyping errors, data filtering choices and methods for combining multiple N_e estimates. Factors that affect precision are reviewed, including pseudoreplication that limits the information gained from large genomics datasets, constraints imposed by small samples of individuals, and the challenges in obtaining robust estimates for large populations. Topics that merit further research include the potential to weight r^2 values by allele frequency, lump samples of individuals, use genotypic likelihoods rather than called genotypes, prune large LD values and apply the method to species practising partial monogamy.

KEYWORDS
computer software, Conservation Genetics, effective population size, LDNe, linkage disequilibrium, population genetics

1 | INTRODUCTION

Effective population size (N_e) is central to evolutionary biology, but collecting the demographic data required to calculate N_e directly can be logistically challenging. As a consequence, considerable interest has focussed on genetic methods to estimate N_e indirectly. As reported by Palstra and Ruzzante (2008), from the 1970s through the mid-2000s most genetic estimates of N_e used the temporal method (Krimbas & Tsakas, 1971; Nei & Tajima, 1981; Waples, 1989), which requires at least two samples separated in time. This changed in the late 2000s when focus shifted to methods that require only a single sample, and within a few years single-sample methods had overtaken the temporal method in popularity (Palstra & Fraser, 2012).

Of the single-sample methods, the most widely used are a bias-adjusted version of Hill's (1981) method based on linkage disequilibrium, LD (Waples, 2006a; Waples & Do, 2008), and the sibship method of Wang (2009). Like all models, these single-sample estimators make numerous simplifying assumptions, sensitivities to which have been evaluated in many cases. However, for the LD method in particular, performance evaluations have been conducted in a wide range of papers published in different journals over ~15 years, making it difficult for practitioners to keep track of all developments. The goal of this review is to summarize the various performance evaluations of the LD method, with focus on information that facilitates practical applications for real populations in nature. Some topics that merit future research are mentioned at the end.

2 | HISTORICAL DEVELOPMENT

2.1 | Definition of linkage disequilibrium

Linkage disequilibrium is a measure of agreement between observed and expected frequencies of gametes that include alleles from more than one gene locus. Let P_A be the frequency of allele A at locus 1 and P_B be the frequency of allele B at locus 2. If these alleles assort randomly, the expected frequency of the gamete that includes both A and B is $E(P_{A,B})=P_AP_B$. A common measure of linkage disequilibrium is then $D_{A,B}=P_{A,B}-P_AP_B$ (Lewontin & Kojima, 1960). A more proper name for $D_{A,B}$ is 'gametic disequilibrium', because physical linkage (occurrence on the same chromosome) is not required for the result that $P_{A,B}\neq P_AP_B$; however, the term 'linkage disequilibrium' and its abbreviation 'LD' are firmly entrenched in the population genetic literature, so they are used here. The brief summary below only highlights some aspects of LD that are most relevant to the goals of this paper. For more comprehensive treatments, see Weir (1979) and Sved and Hill (2018).

A complication for calculation of D is that for most species (and virtually all nonmodel species) what are routinely available are not gametic frequencies but genotypic frequencies. If one assumes random mating, a maximum-likelihood method (Hill, 1974) can be used to estimate gametic frequencies and hence D as above. A simpler approach that does not require the random-mating assumption is the Burrows method, which calculates the composite disequilibrium statistic, Δ (see Weir, 1979, Weir, 1996 for details). Weir (1979) concluded that for genotypic data, Δ generally is preferable to the ML method, even when random mating is a plausible assumption.

D can be positive or negative and is very sensitive to allele frequency (Hedrick, 1987; Lewontin, 1964). Many researchers have found it convenient to work with the square of a standardized version (r^2) that (1) removes most of the dependence on allele frequency, and (2) is always non-negative, with the result that r^2 falls in the range [0,1]. The composite disequilibrium statistic can be standardized to adjust for allele frequency (Weir, 1979, 1996):

$$r_\Delta = \frac{\Delta}{\left[P_A(1-P_A)+(h_1-P_A^2)\right]\left[P_B(1-P_B)+(h_2-P_B^2)\right]} \quad (1)$$

where $h_1-P_A^2$ and $h_2-P_B^2$ are adjustments for Hardy–Weinberg genotypic proportions.

An attractive feature of r^2 or r_Δ^2 is that its expectation can be expressed as a function of two key evolutionary parameters: N_e and recombination fraction (c=probability that two alleles will be shuffled during meiosis). Because r is a ratio, however, an exact expectation has proved elusive, despite many efforts over the years (including Golding, 1984; Hudson, 1985; Ohta & Kimura, 1969; Sved & Feldman, 1973; and Weir & Hill, 1980). For N_e estimation, the most relevant formulation is by Weir and Hill (1980):

$$E(r^2)=\frac{c^2+(1-c)^2}{2N_ec(2-c)}=\frac{\gamma}{N_e}, \quad (2)$$

where

$$\gamma=\frac{c^2+(1-c)^2}{2c(2-c)}. \quad (3)$$

Equation 2 assumes a single, isolated, randomly mating population with discrete generations, within which an equilibrium has been established between generation of LD by random genetic drift and breakdown of existing LD by recombination. Effects of these two processes are captured by the terms for N_e and c, respectively. Equation 2 does not account for sampling and conceptually applies to LD measured in an infinite number of progeny produced by the parents in one generation (Weir & Hill, 1980). When c is very small, Equation 3 can give impossible results [$E(r^2)>1$; Hill, 1981, Sved et al., 2013], but it is generally robust for most practical applications.

2.1.1 | Calculation of r^2

Weir (1979, 1996) showed how to calculate the Burrows Δ from the joint distribution of genotypes at two loci, and the result can then be used to calculate r_Δ using Equation 1. A simpler and exactly equivalent approach is to code genotypes using the 0/1/2 format (Table 1); then, the Pearson product–moment correlation of the genotypic vectors for two loci is identical to Burrows' r_Δ (Gao et al., 2008).

2.2 | Estimation of N_e

2.2.1 | General

Weir and Hill (1980) showed that Equation 2 applies both to monoecious species and dioecious species without permanent pair bonds. They also showed that sampling a finite number (n) of offspring adds approximately $1/n$ to the empirical r^2 value. Subtracting the expected contribution from sampling error and rearranging Equation 2 produces an estimator of effective size as a function of the empirical r^2 and the recombination fraction:

$$\hat{N}_e=\frac{\gamma}{r^2-1/n} \quad (4)$$

The parameter γ is inversely related to c (Table 2), and as $c\rightarrow 0$, $\gamma\rightarrow 1/(4c)$. The coefficient of variation (CV) of $\hat{N}_e$ is a function of γ, the ratio of effective size to sample size (N_e/n), and the number of locus pairs (k) used to compute mean r^2 (Hill, 1981; see Table 2):

$$\mathrm{CV}\left(\hat{N}_e\right)\approx\left(1+\frac{1}{\gamma}\frac{N_e}{n}\right)\sqrt{2/k} \quad (5)$$

Some complications associated with implementing Equation 4 are:

- It requires an estimate of the recombination fraction, c.

- Because c can vary from ~0 to 0.5 depending on which loci are being compared, separate estimates of effective size are associated with each value of c.

Most researchers who have used the LD method to estimate effective population size have adopted one of two strategies.

The first strategy is inspired by the old adage that where there is danger, opportunity also exists. Although the factors above complicate practical application of Equation 4, especially for large genomics datasets, they also provide an opportunity to detect signatures of past evolutionary events, whose effects are 'frozen' into parts of the genome that rarely experience recombination. Researchers can sort r^2 values for pairs of loci into bins based on their recombination fraction, with each bin representing a relatively small range of c values. With adequately detailed information about genome structure, this approach can lead to a temporal vector of effective size estimates, with $\hat{N}_e$ for pairs of loci with high recombination frequencies applying to more recent time periods and $\hat{N}_e$ for pairs with low c applying to farther in the past. (e.g. Corbin et al., 2012; Hollenbeck et al., 2016; Tenesa et al., 2007). Santiago et al. (2020) described a variation of this approach that uses a different index of LD and, rather than binning, considers the full spectrum of LD present in a dataset. Simulations conducted by Santiago et al. document impressive performance of their software (GONE) to estimate historical N_e within the last ~100 generations.

The second strategy relies on the fact that in general most pairs of loci will be unlinked ($c=0.5$), a result that follows directly from genome structure and Mendel's Second Law of Independent Assortment. If there are C chromosomes of equal size, the probability that a random pair of loci are on different chromosomes (and hence assort independently, with $c=0.5$) is $1-1/C$, which (for example) is 0.95 for $C=20$. Unequal chromosome length can be accounted for by defining an effective number of chromosomes, analogous to the effective number of alleles at a polymorphic gene locus. Given that average chromosome numbers for vertebrates, invertebrates, vascular plants and unicellular eukaryotes are 25, 11, 13 and 17, respectively (data from Li et al., 2011 summarized by Waples et al., 2022), it is easy to see that in most species the preponderance of locus pairs will assort independently. Loci with $c=0.5$ provide information about contemporary N_e (generally speaking, effective size for the time frame encompassed by sampling), and that is of primary interest to applied conservation and management.

The remainder of this paper reviews performance of the LD method to estimate contemporary N_e using unlinked pairs of markers.

TABLE 1 Hypothetical genotypic matrix for 10 individuals scored at 8 loci using the 0/1/2 scoring convention, with the genotypes representing the number of copies of the focal allele each individual carries ('1'=heterozygote; '0' and '2'=alternate homozygotes).

	Locus							
Individual	1	2	3	4	5	6	7	8
1	1	1	0	1	1	1	1	1
2	2	0	0	0	2	1	2	2
3	2	1	1	1	1	2	1	1
4	1	2	1	1	1	1	0	2
5	1	0	0	0	0	1	1	2
6	2	2	1	1	1	2	0	1
7	2	1	0	0	0	2	0	0
8	0	1	1	0	0	1	1	2
9	1	2	2	0	2	1	1	2
10	2	1	0	0	0	2	0	1

Note: For diallelic (SNP) loci, this represents complete information, as data for the other allele are obtained by subtraction. For multiallelic loci, separate columns can be used for each allele. When genotypes are scored this way, the Pearson product–moment correlation of the values in two columns is identical to the Burrows r_Δ calculated in Equation 1, provided that the $n/(n-1)$ adjustment suggested by Weir (1979) is not used in calculating Burrows Δ.

2.2.2 | Unlinked loci

With $c=0.5$, $\gamma = 1/3$ (Table 2), and (after accounting for sampling error) Equations 2 and 4 simplify to

$$E(r^2) \approx \frac{1}{3N_e} + \frac{1}{n}, \tag{6a}$$

TABLE 2 Theoretical expectation of the coefficient of variation (CV) of $\hat{N}_e$ for different combinations of recombination fraction (c), γ (from Equation 3) and the ratio of N_e to number of sampled offspring (n).

		CV ($\hat{N}_e$)					
		$k=10$			$k=10{,}000$		
c	γ	$N_e/n=10$	$N_e/n=5$	$N_e/n=1$	$N_e/n=10$	$N_e/n=5$	$N_e/n=1$
0.001	249.6	0.47	0.46	0.45	0.015	0.014	0.014
0.01	24.6	0.65	0.54	0.47	0.020	0.017	0.015
0.1	2.16	2.52	1.48	0.65	0.080	0.047	0.021
0.3	0.57	8.31	4.38	1.23	0.263	0.138	0.039
0.5	0.33	13.86	7.16	1.79	0.438	0.226	0.057

Note: Results are from Equation 5 assuming $\overline{r^2}$ is averaged across $k=10$ or $k=10{,}000$ independent pairs of loci.

$$\hat{N}_e = \frac{1}{(r^2 - 1/n)} \frac{1}{3} = \frac{1}{3r^{2\prime}}, \quad (6b)$$

where $r^{2\prime}$ is r^2 from Equation 2 adjusted for finite sampling of offspring. The estimate of effective size from the LD method applies primarily to the parental generation of the individuals sampled and hence estimates inbreeding N_e (Laurie-Ahlberg & Weir, 1979; Waples, 2005).

Because precision of $\hat{N}_e$ is an inverse function of recombination fraction and is relatively low for $c = 0.5$ (Table 2), Hill (1981) concluded that unlinked loci have little practical value for estimating effective size. Perhaps largely as a consequence, Hill's method saw few applications for the next quarter century (Bartley et al., 1992 being a notable exception). Two factors eventually caused this to change. First, early developers of genetic methods to estimate N_e generally focussed on potentially very large populations such as *Drosophila* spp. Indeed, Pollak (1983, p. 544) stated that 'there would be no need to estimate [N_e] indirectly by any of the methods discussed in this paper unless it is quite large'. However, for given sample sizes of loci and individuals, precision is higher when N_e is small and the drift signal is stronger (Waples, 1989), and this applies to all genetic methods for estimating contemporary effective size. Beginning in the 1980s, interest rapidly increased in practical application of evolutionary principles to conservation and management of natural populations, including evaluations of how effective sizes measured up to rules of thumb for minimal values consistent with short-term and long-term viability. The second factor is that laboratory techniques eventually advanced to the point where moderately large numbers of alleles were available for analysis, which meant that the number of pairwise comparisons for computing mean r^2 (k in Equation 5) might be large enough to offset at least some of the limitations regarding precision (as indicated on the right side of Table 2).

At this point, a potential problem with the LD method was identified (England et al., 2006): using unlinked loci, $\hat{N}_e$ from Equation 6a was robust only when sample size was comparable to effective size; $\hat{N}_e$ was downwardly biased when $n < N_e$ and dramatically so for $n << N_e$, leading to the result that $\hat{N}_e$ reflected the sample size more than the effective size. Further investigation (Waples, 2006a) showed that the main source of bias was that the $1/n$ term proposed by Weir and Hill (1980) and Hill (1981) was insufficient to account for random contributions to r^2 from sampling offspring. The $1/n$ term was an approximation obtained under the assumption that terms in $1/n^2$ and $1/(nN_e)$ were small enough to ignore (Weir & Hill, 1980). Based on extensive simulations of unlinked loci, Waples (2006a) developed an empirical adjustment to $E(r^2)$ that added terms for $1/n^2$ and $1/N_e^2$. These adjustments were incorporated into the software LDNe (Waples & Do, 2008) and subsequently into NeEstimator (Do et al., 2014). The material below summarizes performance of the version of the LD method that is implemented in LDNe and NeEstimator.

3 | PERFORMANCE OF THE LD METHOD FOR ESTIMATING CONTEMPORARY *Ne*

Many papers have compared results of the LD method with other N_e estimators for empirical datasets, but these are difficult to evaluate because true N_e is generally not known. Material below is drawn from published studies using computer simulations with known parameter values. Evaluations that include comparisons of LDNe with other methods include Waples and Do (2010), Gilbert and Whitlock (2015), and Wang (2016, 2023). What follows here is a detailed review of factors that can bias LD estimates of effective size, followed by a discussion of precision and effects of various sampling strategies and experimental designs.

3.1 | Bias

3.1.1 | Sample size

Adjustments to $E(r^2)$ developed by Waples (2006a) largely eliminated downward bias in $\hat{N}_e$ when $n < N_e$. As discussed in the next section, an interaction between sample size and allele frequency can affect bias to some extent.

Researchers should keep in mind that, assuming other model assumptions are met, two main sources of uncertainty are associated with using genetic methods to estimate effective size: sampling individuals and sampling some of the ~10^9–10^{10} DNA base pairs in a typical genome. For moderate values of these key variables, researchers can often trade off efforts for sampling genes vs individuals, but these experimental-design trade-offs have limitations. In particular, the trend in recent genomics studies to assay larger numbers of SNPs on fewer individuals places important constraints on N_e estimation. In small samples, the amount of LD can be very sensitive to the pedigree of the sampled individuals. As more SNPs are used, the estimate of r^2 will converge on a value that reflects the relationship structure of the sampled individuals and not the population as a whole (the latter generally being what one is interested in). This limitation—which can only be alleviated by taking larger samples of individuals, not by arbitrarily large increases in the number of genetic markers—is particularly acute for analyses (such as estimation of contemporary N_e) that are strongly affected by pedigrees from the most recent generations (King et al., 2018). In coalescent models that describe evolutionary processes over long time scales, individuals become more interchangeable, and just a few individuals might be an adequate sample.

3.1.2 | Allele frequency

The bias correction developed by Waples (2006a) and incorporated into the LDNe software (Waples & Do, 2008) was based on

simulations of diallelic loci and excluded alleles with frequencies <0.05 or >0.95. In more extensive evaluations that included modelling of highly variable 'microsatellite' loci, Waples and Do (2010) found an upward bias to $\hat{N}_e$ when lower frequency alleles are used, with the bias reduced for larger samples. The most effective strategy for minimizing bias is to screen out all singleton alleles, which only occur in one copy [at frequency $1/(2n)$]. With missing data, usable sample sizes vary among pairs of loci, which means that using a single cut-off for screening out rare alleles (P_{crit}) will not produce uniform results with respect to bias reduction. The software NeEstimator (Do et al., 2014) has an option for screening out only singleton alleles for each pair of loci. Beyond removal of singletons, some modest further reductions in bias can be achieved by removing alleles at frequency up to about 0.05, but generally with some reductions in precision. Figure 1 shows a typical pattern of slightly increasing $\hat{N}_e$ as alleles with lower frequency are allowed into the analysis.

3.1.3 | Linkage

When only a handful of allozymes or microsatellites were available for analysis, it was not unreasonable to assume that the loci were all on separate chromosomes or, if not, far enough apart that they were effectively independent. With genomics-scale datasets, that assumption is no longer tenable, and formally accounting for the effects of physical linkage is essential. This linkage, of course, is vital information for variations of the LD method that aim to estimate historical N_e (see Section 2.2.1), but it will

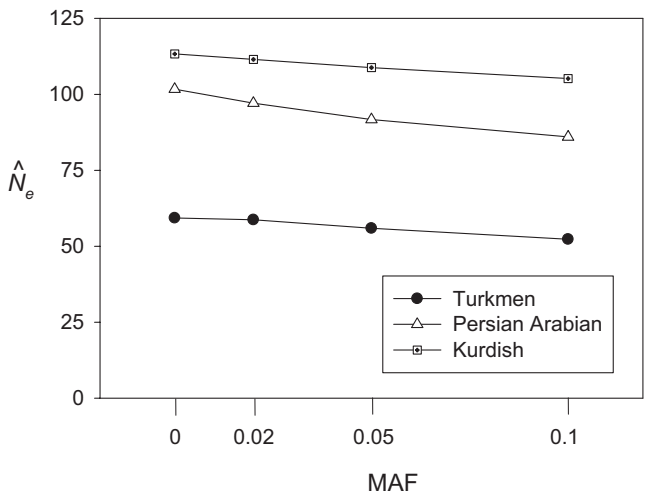

FIGURE 1 Estimates of contemporary N_e for three indigenous Iranian horse breeds, as a function of the criterion for screening out rare alleles (MAF = P_{Crit}). With MAF = 0, all alleles are used. Data are from Mousavi et al. (2023), and results were obtained for the LDNe model in NeEstimator, after restricting comparisons to pairs of loci on different chromosomes. Before screening rare alleles, the dataset included 40,120 SNPs. Sample sizes of individuals were $n = 29$, 52 and 67 for Turkmen, Persian Arabian and Kurdish, respectively.

downwardly bias estimates of contemporary effective size based on theoretically unlinked loci, because LD due to linkage is interpreted as a signal of drift. In stable populations, the magnitude of downward bias does not depend on the number of loci but is inversely correlated with genome size, with a linear regression $\hat{N}_e / \text{true } N_e = .098 + .219 * \ln(C)$, where C is the number of chromosomes of size 100 cM (Waples et al., 2016).

Three general strategies are available for dealing with this bias:

1. Ignore it;
2. adjust for it;
3. restrict comparisons to pairs of loci on different chromosomes.

Whether Option 1 is acceptable depends on one's objectives, tolerance of uncertainty, and study organism. For example, if the focal species has 25 chromosomes (mean number for vertebrates; Li et al., 2011), expected downward bias to $\hat{N}_e$ is only about 20%, but that jumps to 60% for species with only four chromosomes. Option 3 should be optimal for the estimation of current N_e, as all pairs of loci then have $c = 0.5$, and NeEstimator can implement this option provided the required genome-structure information is available. Option 2, which corrects for expected bias using the regression above, is something of a compromise and was developed for a constant-N model, but it could be a Goldilocks 'just right' choice for many species because all it requires besides the raw data is an estimate of C or genome size. If genome-size information is not available for the focal species, it likely is for some closely related ones (Li et al., 2011). Recent studies that have accounted for linkage in estimating contemporary N_e using either Option 2 or 3 include (Bootsma et al., 2021; Nadachowska-Brzyska et al., 2021 and Walsh et al., 2022).

3.1.4 | Changes in abundance

Equations 2, 6a, and similar equations by other authors assume that an equilibrium level of LD has been achieved, such that the amount of LD lost each generation by recombination is offset by new LD generated in all finite populations. Changes in population size can disrupt this process, but for pairs of loci that assort independently the effects are limited by rapid decay of existing LD (50% per generation, measured by D). Furthermore, when D decreases by a factor of 2, D^2 and hence r^2 are reduced by a factor of 4, so for unlinked markers the signature of historical N_e fades quickly and is replaced by a signal related to effective size in the most recent generation(s) (Waples, 2005). This response is particularly rapid following a bottleneck, where new LD from the recent, smaller N_e quickly dominates the signal from higher background N_e. Tallmon et al. (2010), Antao et al. (2011), and Schweizer et al. (2021) all documented the ability of the LD method to detect reduction in effective size within 1–2 generations of a bottleneck, provided a temporal sequence of samples is available. Similarly, the LD method can rapidly detect population fragmentation,

where a single, large population divides into several smaller, isolated ones, each of which has experienced a bottleneck/founder effect (England et al., 2010). Consequences of population size change are not symmetrical with respect to effective size estimation. Following a population expansion, the drift signal for the new N_e is small relative to the large amount of residual LD generated by the formerly smaller effective size, so a few more generations are required for mean r^2 to break down enough to closely approximate the expected value for the new N_e (Waples, 2005).

These results have practical application to computer modelling of evolutionary processes. A common way to initialize an individual-based population genetic model is to draw genotypes in generation 0 randomly and independently for each locus based on specified initial allele frequencies. Conceptually, this is equivalent to modelling reproduction in a population of infinite size, so LD in the offspring (generation 0) is all attributable to sampling error, not genetic drift. By generation 5–6, mean r^2 is essentially indistinguishable from that expected for a stable population with the modelled N_e (Waples, 2005). This allows a very rapid burn-in process with discrete generations, which, however, might have to be extended a bit to deal with age structure (Antao et al., 2020; see Section 3.1.8). In contrast, reaching an equilibrium level of LD can take much longer in models that involve physical linkage and low recombination fractions. Among others, Tallmon et al. (2010), England et al. (2010), Luikart et al. (2021), and Schweizer et al. (2021) have empirically evaluated how changes in abundance affect LD-based estimates of N_e.

3.1.5 | Migration

This section considers the combined effects of migration and subsequent interbreeding on LD in samples taken from a single local population. See Section 3.1.6 for consideration of sampling designs that might incorporate individuals from different populations or demes, with or without actual migration. At equilibrium under migration, $E(r^2)$ has two components, one due to drift and one due to population mixture (Waples & England, 2011):

$$E(r^2) = E(r^2)_{\text{drift}} + E(r^2)_{\text{mixture}} \quad (7)$$

The drift term for unlinked markers (from Equation 6a) is approximately $1/(3N_e)$. The mixture term reflects a kind of two-locus Wahlund effect that occurs in a mixture of genetically divergent individuals (Nei & Li, 1973); its magnitude is a function of the migration fraction (m) and the degree of genetic divergence between the donor and recipient populations, which can be represented by F_{ST} (Waples & England, 2011).

From the point of view of a sample taken from a local population, migration affects both of the terms in Equation 7, but in contrasting ways. The drift term reflects the effective number of parents that produced the sampled offspring. Migration expands the number of potential parents, and (all else being equal) this tends to reduce $(r^2)_{\text{drift}}$. However, genetically divergent migrants introduce mixture LD that increases $(r^2)_{\text{mixture}}$. The net effect of migration on $E(r^2)$ depends on the relative importance of these two factors.

In Wright's (1943) widely used island model, in each generation every local population exchanges a fraction m of individuals with the larger metapopulation as a whole. Under the equilibrium island model, $\hat{N}_e$ based on local samples will provide a largely unbiased estimate of local N_e, provided that m is no higher than about 5%–10% (Waples & England, 2011). The surprising generality of this result across a wide range of plausible migration rates occurs because when migration is common populations are weakly differentiated so little mixture LD is generated, whereas when m is low and F_{ST} is higher, the mixture LD that is generated is uncommon and has relatively little overall effect. When m exceeds about 10%, however, the entire metapopulation behaves more like a single population, and $\hat{N}_e$ based on local samples approaches metapopulation N_e rather than local N_e. Waples and England also modelled episodic migration scenarios, where occasional large pulses of genetically divergent migrants flood into a population. Under those conditions, the mixture term in Equation 7 can be very large, in which case the inflated r^2 translates into a substantial underestimate of local N_e.

Gilbert and Whitlock (2015) examined bias and precision of several single-sample and temporal N_e estimation methods across a wide range of migration scenarios. They found that LDNe performed better than other single-sample methods they considered and was comparable to the best temporal estimators, except when migration rate was high ($m \geq 0.1$) and population size was relatively large ($N_e \geq 500$).

For practical applications, researchers should remember that individual datasets often have idiosyncrasies that cause deviations from 'average' behaviour, which in studies like those cited above typically reflect means across many simulated replicates. Empirical r^2 can depend heavily on which individuals appear in your sample. If the sample by chance includes one or more first-generation migrants, or descendants of recent migrants, effects on r^2 can be substantial, and this is particularly true for smaller samples, where deviant individuals have relatively large influence (Waples & England, 2011). Unless recent immigration can be excluded, it would be prudent for researchers to use PCA, assignment tests, or other methods to try to identify genetic outlier individuals, so their effects on results can be evaluated (see fig. 4 in Waples & England, 2011 for an example illustrating how the criterion for screening out rare alleles (P_{crit}) can be used in this way). Palstra and Ruzzante (2011) and Ruzzante et al. (2019) also illustrate practical application of methods for managing the effects of migration on N_e estimation.

Finally, researchers should be cautious in applying equilibrium models to contemporary samples from species that have been strongly influenced by human-mediated changes to their ecosystems. Although pulse migration—where a large number of genetically divergent individuals appear in a local population—would not be expected under equilibrium conditions, it could occur if anthropogenic

changes have altered natural barriers that previously precluded migration and fostered strong genetic divergence.

3.1.6 | Interactions between sampling and spatial structure

If one expands the geographic scale of sampling beyond a single local deme or population, the sample can include 'pure' individuals from more than one gene pool, even in the absence of recent migration. Effects of these outlier individuals would therefore be similar to first-generation migrants in a metapopulation model.

One spatial structure scenario whose effects on LD have been evaluated is Wright's (1946) isolation-by-distance model for continuously distributed species. Neel et al. (2013) modelled this scenario by creating new individuals each generation for every node in a 2D matrix, with genotypes being drawn randomly from parents within a specified distance of the focal cell; this was referred to as the breeding window, and consequences of natal dispersal were reflected in the emerging genetic structure. Neel et al. (2013) found that when samples were taken within the breeding window, $\widehat{N}_e$ agreed closely with Wright's (1946) neighbourhood size, and other dynamics of the system agreed with predictions of Wright's isolation-by-distance model. As the scale of sampling increased relative to the breeding window, $\widehat{N}_e$ increased more slowly and plateaued at a value substantially below global N_e. This pattern presumably reflects the increasingly strong downward bias from mixture LD as larger sample windows increasingly included more genetically divergent individuals. In support of this interpretation, Neel et al. found that increasingly strong heterozygote deficiencies (positive F_{IS}, consistent with a Wahlund effect) were found as soon as the scale of sampling exceeded the breeding window. Shirk and Cushman (2014) reported results consistent with these patterns in a study of mountain goats (*Oreamnos americanus*) inhabiting a complex landscape. As noted by Battey et al. (2020), departures from many other predictions of standard, discrete-population genetic models can be expected when they are applied to continuously distributed species.

The need for additional research on the effects of combining individuals across more than one partially isolated population is highlighted in Section 5.2.

3.1.7 | Mutation and selection

In contrast to migration and drift, which have profound effects on LD, the other two evolutionary forces (mutation and selection) have little influence on r^2 for unlinked loci. Saura et al. (2015) found that estimating a mutation parameter from empirical genetic data improved an LD-based estimate of N_e, but this adjustment was applied to estimates of historical N_e based on estimates of c for syntenic pairs of loci. Apart from its role in producing new rare alleles (see Section 3.1.2), mutation has no appreciable influence on r^2 for pairs of loci on different chromosomes.

Much of the early research on LD involved searches for evidence of selection, but those were largely unsuccessful. Subsequent research (e.g. Thomson, 1977; Barton, 2000) demonstrated a widespread phenomenon known as genetic hitchhiking, whereby the fate of a neutral allele is affected by selection acting on one or more loci it is linked to. For N_e estimation, however, the key question is not whether selection is associated with LD, but whether selection biases estimates of effective size that assume selective neutrality. Using simulations to address this question, Novo et al. (2022, p. 1) found that estimates of historical N_e from the GONE software developed by Santiago et al. (2020) were 'virtually unaffected by selection'. It is reasonable to conclude that estimates of contemporary N_e also should show little or no bias from selection, given that half of existing LD at unlinked loci breaks down every generation through recombination.

3.1.8 | Age structure

The above analyses all assumed discrete generations. For the large fraction of the world's species that are age-structured and have strongly seasonal reproduction, one needs to be concerned with two effective sizes: for a full generation (N_e), and the effective number of breeders (N_b) in one season (hereafter assumed to be 1 year). N_e is more important for determining the rate of genetic drift, but N_b is generally much easier to estimate and monitor, and it provides key insights into mating systems and reproductive biology. Two general life histories have been studied using the contemporary LD method.

In semelparous species with variable age at maturity (including Pacific salmon, annual plants with seed banks, monocarpic perennials and diverse other taxa), samples from individual cohorts (progeny of adults reproducing in 1 year) that are analysed with the LD method provide an estimate that primarily reflects N_b for that year, with some influence from background LD that is a function of generational N_e. Because each individual reproduces in only 1 year, N_e per generation is an additive function of N_b per year: $N_e \approx gN_b$, where g is generation length. If N_b varies, N_e is approximately g times either the harmonic mean N_b [Pacific salmon (Waples, 2002) and monocarpic perennials (Vitalis et al., 2004)] or the arithmetic mean N_b [annual plants with seed banks (Nunney, 2002)], with the difference determined by the method of population regulation (see Waples, 2006b for details).

It seems intuitive that N_b also should be $<N_e$ in iteroparous species, but it turns out that is not always true: across 63 diverse taxa, Waples et al. (2013) found $N_b < N_e$, $N_b = N_e$, and $N_b > N_e$. The ratio N_b/N_e can be predicted with a reasonable degree of certainty by two widely studied life history traits (age at maturity and adult lifespan), plus a metric that quantifies changes in fecundity with age. To evaluate performance of the LD method to estimate contemporary N_b and N_e under iteroparity, Waples et al. (2014) modelled eco-evolutionary dynamics in 21 of these 63 species having expected N_b/N_e in the range 0.27–1.7, estimated N_e and N_b from genetic samples and compared these with the

pedigree N_e and N_b calculated from the observed mean and variance in offspring number. Results can be summarized as follows:

- For single-cohort samples, after accounting for sampling error, $E(r^{2\prime}) \approx 1/[3 * Hmean(N_b, N_e)]$, where *H*mean is the harmonic mean.
- If $N_e \neq N_b$, the LD-based estimate of N_b ($\widehat{N}_b$) based on single cohorts is biased; this bias can largely be removed by adjusting $\widehat{N}_b$ as follows:

$$\widehat{N}_{b(Adj)} = \frac{\widehat{N}_b}{1.26 - 0.323(N_b / N_e)}, \quad (8)$$

where N_b/N_e can be estimated from life history traits as described above.

- N_e can be estimated indirectly by calculating $\widehat{N}_{b(Adj)}$ from Equation 8 and dividing by the estimated N_b/N_e ratio.
- Waples et al. (2014) found that for all 21 modelled species, direct LD-based genetic estimates based on mixed-age samples of adults underestimated true N_e by ~10%–50%, with less bias generally occurring when the number of age classes in the sample approximated the generation length.
- For three species having a wide range of life history traits and N_b/N_e ratios, Figure 2 shows how raw LD-based estimates compare to true N_b and N_e for experimental designs that randomly sampled all individuals or only adults, or combined samples from 1 to 3 consecutive cohorts. Similar plots for other species are in Waples et al. (2014), and evaluations for additional species are in Robinson and Moyer (2013).

Among others, Ruzzante et al. (2016), González-Ferreras et al. (2022) and Brooks et al. (2023) have adjusted raw estimates of N_b or N_e based on a species' life history traits. With long-term datasets, N_b and/or N_e can be monitored over time (Kamath et al., 2015; Waples, Scribner, et al., 2018; Whiteley et al., 2015).

3.1.9 | Mating system

Equations 2 and 6a apply equally to dioecious species with separate sexes and no permanent pair bonds and to monoecious species with random selfing (Weir & Hill, 1980). Under lifetime monogamy, the drift signal for $E(r^2)$ is exactly doubled for $c=0.5$ (being $2/(3N_e)$ rather than the $1/(3N_e)$ shown in Equation 6a; Weir & Hill, 1980). If the selfing rate increases from the random $1/N$ expectation, $\widehat{N}_e$ from the LD method becomes increasingly biased downwards, and the LDNe method can also substantially underestimate N_e in haplodiploid systems (Wang, 2016).

3.1.10 | Combining estimates of effective size

Often researchers want to get an overall $\widehat{N}_e$ by combining two or more individual estimates that in theory are estimating the same

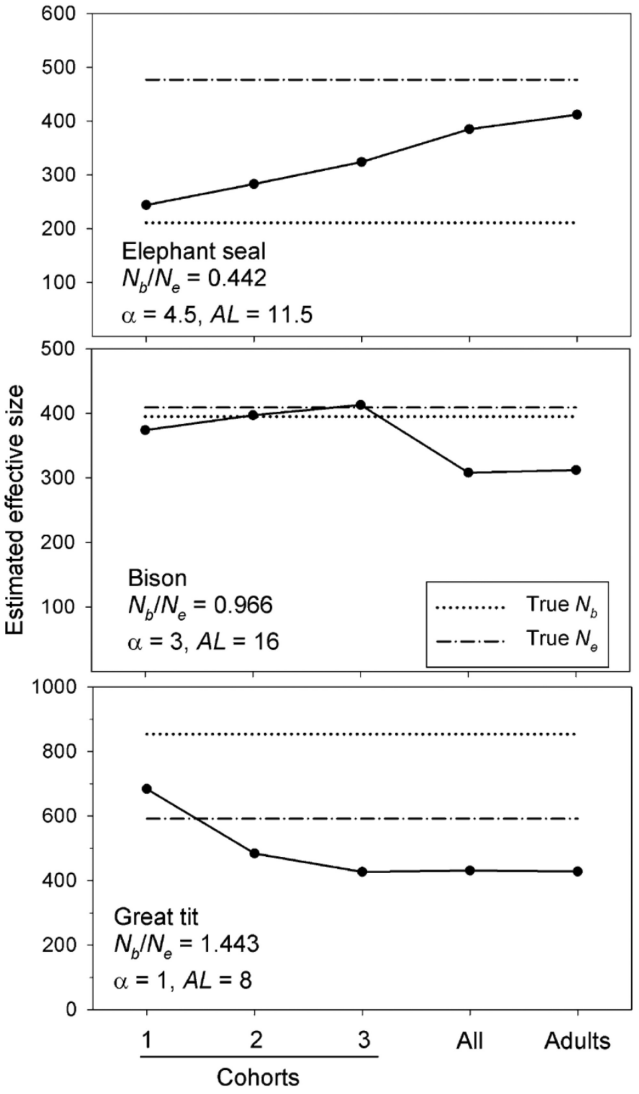

FIGURE 2 Relationship between genetic estimates of contemporary effective size using the LD method (filled circles and solid lines) and true N_e (dashed-dotted lines) and true N_b (dotted lines) in three species with low (male elephant seal), medium (bison) and high (great tit) N_b/N_e ratios. Age-structured simulations modelled each species' vital rates (age-specific survival and fecundity), and results are shown for several sampling strategies (sampling from one to three consecutive cohorts or randomly from all adults or the population as a whole). α = age at maturity and AL = adult lifespan. Reproduced from Waples et al. (2014).

parameter. This might occur, for example, if a researcher had multiple temporal samples from the same population, or a series of replicate estimates from simulated data with a constant N_e. There are two correct (and equivalent) ways of doing this and many incorrect ways that lead to systematic bias (Box 1). The correct ways are to either: (1) compute a grand mean $\overline{r^2}$ across all replicates and use the result to estimate N_e (Method 1); or (2) compute $\widehat{N}_e$ for each replicate and take the harmonic mean of these (Method 2). If the r^2-N_e relationship were as simple as shown in Equation 6a,6b, Methods 1 and 2 should produce identical results, apart from rounding errors. With

the more complicated estimation procedure in LDNe, results should be similar but not necessarily identical.

In my experience, by far the greatest confusion about combining $\hat{N}_e$ values comes when empirical $\overline{r^2}$ is less than the value expected from sampling error alone, with the result that $\overline{r^2}\prime$ is negative and so is the resulting $\hat{N}_e$. Although a negative N_e is conceptually problematical, the estimate still contains valuable information about genetic drift, so the correct approach is to compute the harmonic mean across the entire vector of $\hat{N}_e$ values. Ignoring negative or infinite estimates systematically leads to underestimation of the composite N_e (see Box 1). Some proprietary software might refuse to compute the harmonic mean if any values are negative, but this is simple to accomplish using the definition of a harmonic mean as the reciprocal of the mean of the reciprocals:

$$\hat{N}_{e(\text{composite})} = \frac{1}{\sum_{i=1,k}\left(1/\hat{N}_{e(i)}\right)/k} = \frac{k}{\sum_{i=1,k}\left(\frac{1}{\hat{N}_{e(i)}}\right)},$$

where k is the number of estimates.

If negative or infinite $\hat{N}_e$ values give researchers heartburn, a simple approximation is to replace all such values with a large positive number, like '999999'. These large values contribute little to $\Sigma\left(1/\hat{N}_e\right)$ but they minimize bias associated with omitting them. Alternatively, researchers can compute a composite $\hat{N}_e$ as the median of the individual estimates (including the '999999' values). With an adequate number of replicates, the median will generally be close to the harmonic mean.

3.1.11 | Missing data

Missing data reduce precision but there is no reason it should affect bias of $\hat{N}_e$ from the LD method, provided the missing data points are independent of the true genotype. In LDNe, each pairwise r^2 value is computed across all individuals having data for both loci, and no missing genotypes are imputed. Peel et al. (2013) developed a method for weighting the pairwise r^2 to account for missing data that improved slightly the method used by Waples and Do (2008), and the Peel et al. algorithm is incorporated into the LD module of NeEstimator.

It would be prudent for researchers to evaluate whether missing data are actually independent of genotype, in which case missing data can skew inferred genotypic ratios and bias downstream analyses (Anney et al., 2008), including N_e estimation.

3.1.12 | Genotyping errors

The most common genotyping errors for both microsatellites and SNPs are allelic dropout/null alleles, which lead to apparent heterozygote deficiencies (positive F_{IS}). Null alleles also upwardly bias r^2 and hence downwardly bias $\hat{N}_e$ (Sved et al., 2013; Waples, 2018). Although the increase to raw r^2 might be relatively small [for null alleles at frequency P_n, r^2 is increased by the factor $1/(1-P_n)^2$; Sved et al., 2013], the proportional change to $\overline{r^2}\prime$ (and hence $\hat{N}_e$) can be substantial, especially if $N_e >> n$. Prudent researchers will estimate P_n in their data and evaluate the consequences for $\hat{N}_e$. Bravington et al. (2016) estimated P_n at 25 mSAT loci used in a close-kin mark–recapture study of southern bluefin tuna (*Thunnus maccoyii*); subsequently, Waples, Grewe, et al. (2018) used the same data to estimate N_e, and they evaluated sensitivity to null alleles by repeating the estimates after dropping two loci with estimated $P_n > 0.1$.

3.2 | Precision

In Equation 5, k is the number of locus pairs used to compute $\overline{r^2}$, and these pairs are assumed to be independent. Under that assumption, k is the number of degrees of freedom associated with the estimate, and parametric confidence intervals (CIs) for $\hat{N}_e$ can be calculated using eq. 12 in Waples (2006a). Precision of the LD method has been extensively evaluated for small to moderate numbers of genetic markers, for which the independence assumption is not unreasonable (Waples & Do, 2010; Gilbert & Whitlock, 2015; Luikart et al., 2021; Wang, 2016). These evaluations have shown that the method can provide very robust estimates, provided that true N_e is not too large (e.g. assuming all model assumptions are met, with 50 offspring sampled from a population with $N_e = 100$ and genotyped for 100 SNPs, almost all $\hat{N}_e$ should fall in the range 60–150; Figure 3).

Modern genomics datasets can have thousands to millions of SNPs. Because the number of locus pairs increases with the square of the number of diallelic loci, this suggests that the LD method might have essentially unlimited precision to estimate contemporary N_e, even when effective size is very large. In real-world applications, however, a large number of locus pairs can never be independent, for two reasons. First, with L loci there are $L(L-1)/2$ pairs but only $L/2$ nonoverlapping pairs, so the full matrix of r^2 values contains many pairs that share one locus with another pair, and this creates positive correlations among r^2 values. Second, many pairs of loci will

BOX 1 Combining multiple estimates of N_e

Two essentially equivalent methods are optimal for combining multiple estimates of effective size: compute a grand mean $\overline{r^2}$ across all replicates and use that to compute the composite $\hat{N}_e$ (Method 1); or compute $\hat{N}_e$ for each replicate and take the harmonic mean of these (Method 2). To illustrate the underlying issues involved, Monte Carlo methods were used to simulate LD in 1000 replicate populations. For each population, $\overline{r^2}$ was calculated across all pairs of loci, and this was used to estimate N_e using the LDNe method (details and code to replicate the simulations are provided in Data S1). True N_e and sample size of individuals (n) were fixed at 200 and 50, respectively.

BOX 1 (Continued)

Mean $\overline{r^2}$ is a random variable and its distribution converges on the normal as the number of pairs of loci increases. In Scenario A, with 100 SNPs and 4950 locus pairs, the distribution of $\overline{r^2}$ was ~normal, with an overall mean of 0.02291 (Figure B1a, top). With $n=50$, in the LDNe model this translates to an overall estimate of $\hat{N}_{e(Method1)} = 202$. Because of the inverse relationship between r^2 and N_e (Equation 6a,6b), the distribution of $\hat{N}_e$ is not normal; instead, it is skewed towards high values (Figure B1a, bottom). Consequently, the arithmetic mean is not a good measure of central tendency, and the harmonic mean is used instead. Harmonic mean $\hat{N}_e$ across all replicates is $\hat{N}_{e(Method2)} = 202$, the same as obtained under Method 1. In contrast, arithmetic mean $\hat{N}_e$ (243) is biased high by the right tail of large values.

Scenario B considers a harder estimation problem, using only 30 loci. Still, with 435 locus pairs to average over, the distribution of $\overline{r^2}$ is ~normal and symmetrical around the overall mean 0.02286; however, with less data the spread of $\overline{r^2}$ values is considerably increased (Figure B1b, top). Using LDNe, the overall $\overline{r^2}$ produces $\hat{N}_{e(Method1)} = 209$. The wider range of $\overline{r^2}$ values leads to a wider range and increased skew of $\hat{N}_e$ (Figure B1b, bottom). Furthermore, in 16% of the replicates, empirical $\overline{r^2}$ was less than that expected from sampling error alone $[E(r^2_{sample}) = 1/n + 3.19/n^2 = 0.021276]$, in which case both $\overline{r^2}' = \overline{r^2} - E\left(r^2_{sample}\right)$ and $\hat{N}_e$ are negative. These replicates provide no evidence for genetic drift, so the interpretation is that $\hat{N}_e = \infty$. Despite the presence of negative $\hat{N}_e$ values, the correct way to implement Method 2 is to take the harmonic mean of all the estimates, which here leads to $\hat{N}_{e(Method2)} = 206$, nearly the same as obtained under Method 1. Confused by how to interpret negative or infinite $\hat{N}_e$ values, many researchers simply ignore them. This is a serious error; these estimates are consistent with a large N_e and trimming them systematically leads to downward bias. In this example, computing harmonic mean $\hat{N}_e$ only for the positive estimates leads to $\hat{N}_{e(Trim)} = 161$, which is biased downwards by 20%.

These examples have balanced designs and used unweighted means and harmonic means. If numbers of loci and/or individuals used differ among replicates, individual estimates can be weighted as described in Waples and Do (2010).

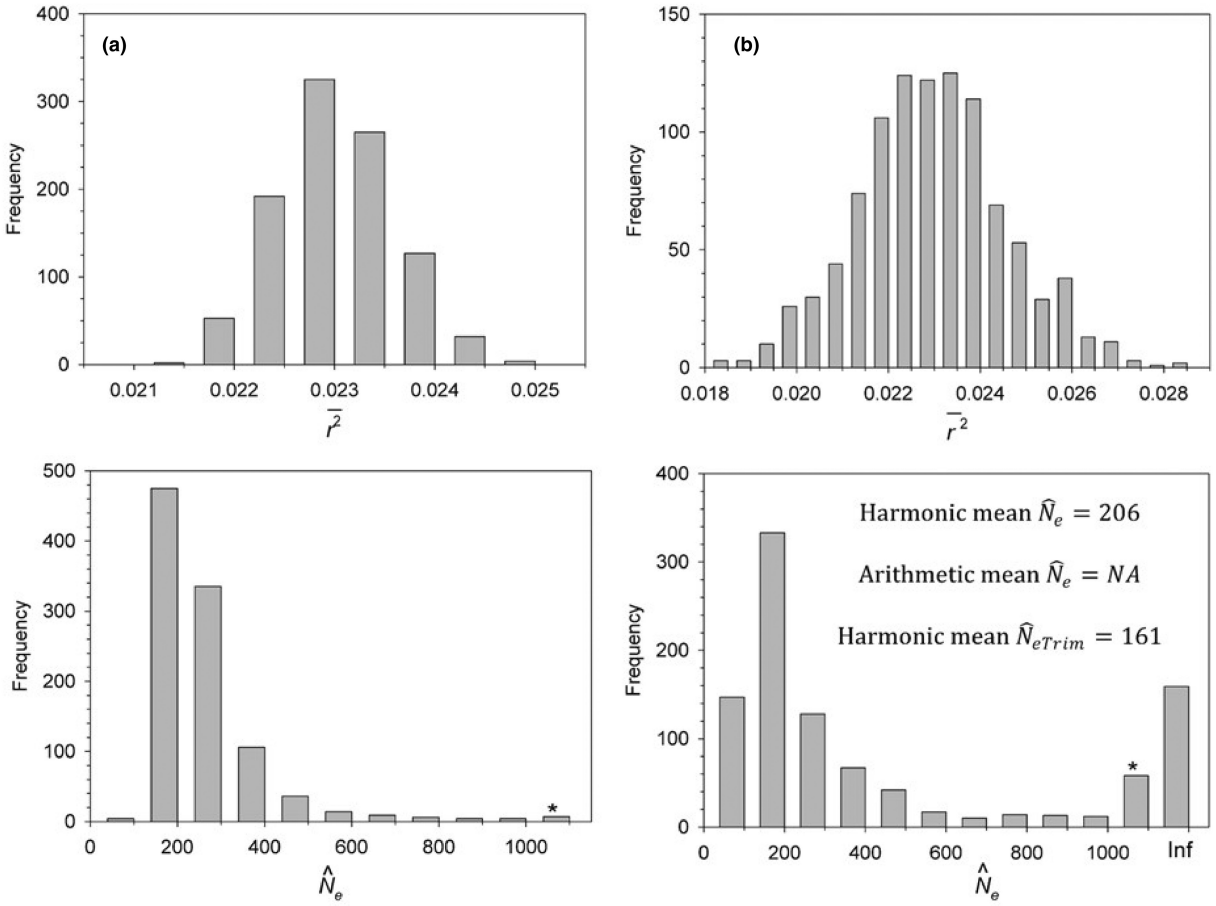

FIGURE B1 Distribution of $\overline{r^2}$ (top panels) and $\hat{N}_e$ (bottom panels) in simulated populations with true Ne = 200, sample size of n = 50 individuals and $\overline{r^2}$ averaged over pairwise comparisons of 100 (Panels a) or 30 (Panels b) diallelic loci. In the bottom panels, the bar with the '*' represents all estimates that were finite but >1000; in panel b, bottom, the bar labelled 'Inf' represents all negative estimates, which imply $\hat{N}_e = \infty$. Arithmetic mean $\hat{N}_e$ is not meaningful when some of the estimates are negative (Panel b, bottom).

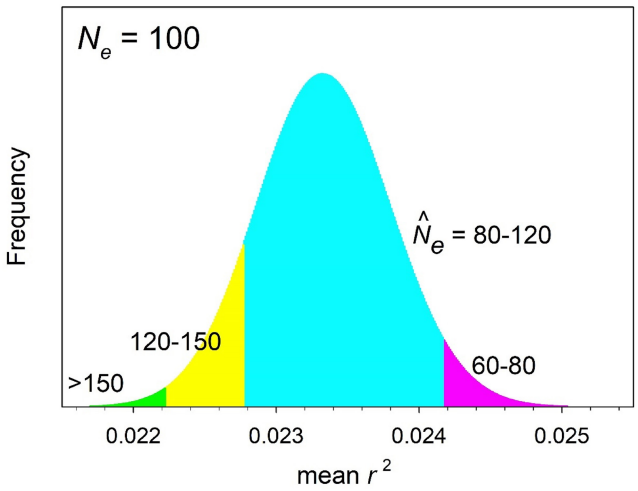

FIGURE 3 Expected distribution of $\overline{r^2}$ (coloured graph segments) and resulting $\hat{N}_e$ estimates (numbers) for scenarios where true $N_e = 100$, $n = 50$ offspring were randomly sampled, and $\overline{r^2}$ was averaged across all $k = 100 \times (100 - 1)/2 = 4950$ pairs of 100 diallelic (SNP) loci. Expected distribution of $\overline{r^2}$ reflects the expectation that $\overline{r^2} / E(\overline{r^2})$ is distributed as chi-square with k degrees of freedom (Hill, 1981). The $\overline{r^2}$ values were converted into $\hat{N}_e$ using Equation 6b. These results apply to the inference model of Weir and Hill (1980) and Hill (1981), which assumed discrete generations, constant N_e, and no selection or migration.

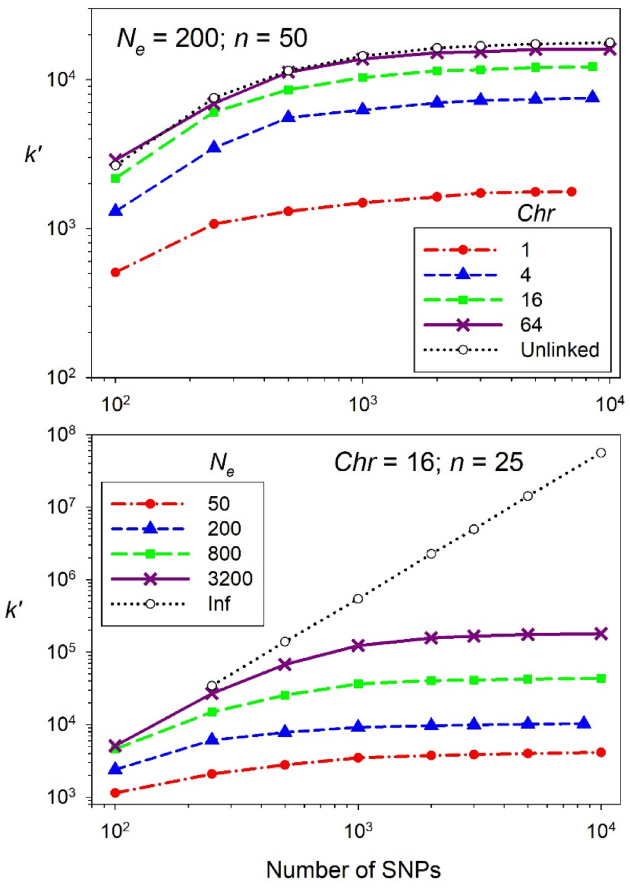

FIGURE 4 Effective degrees of freedom = effective number of locus pairs (k') for $\overline{r^2}$ as a function of the number (L) of diallelic (SNP) loci used to calculate $\overline{r^2}$. k' was calculated from simulated data based on the rate of decline in var($\overline{r^2}$) as more loci were used. Top: Effect of number of chromosomes (Chr), with $N_e = 200$ and $n = 50$. Bottom: effect of N_e, with Chr = 16 and $n = 25$. Modified from Waples et al. (2022).

be linked and hence provide correlated information. Both of these factors reduce the information content of pairwise LD data, with the result that the effective degrees of freedom (k') is less than the number of locus pairs (k). A key question thus becomes, how much is k' reduced compared with k? Unfortunately, a general analytical answer to this question does not seem to be feasible.

In the original implementation of LDNe, Waples and Do (2008) estimated k' by jackknifing over loci, but this proved to be inadequate. Jones et al. (2016) showed that a better approach is to jackknife over individuals, and their improved method for generating CIs for $\overline{r^2}$ and $\hat{N}_e$ was incorporated into NeEstimator V2. The new jackknife approach is very useful for application to specific datasets but less useful for understanding the factors that influence the ratio k'/k, and this information is crucial for researchers in planning experimental designs. Furthermore, previous evaluations of precision had primarily considered datasets with no more than a few dozen microsatellites (or their equivalent in SNPs), so little information was available regarding precision to be expected in large genomics-scale datasets, where L often is 10^3–10^7, even for nonmodel species.

To address these data gaps, Waples et al. (2022) explored a 4-dimensional parameter space (n, N_e, L and C) and across many replicate simulations estimated k' by measuring how quickly the variance of $\overline{r^2}$ declined as L increased. The main themes of their results are shown in Figure 4. Genome size has a predictable effect: as the number of loci increases compared with the number of chromosomes, lack of independence becomes more severe and k'/k declines. With 64 chromosomes, k'/k was largely indistinguishable from the value expected when all loci are unlinked. However, even for large genomes k' is greatly reduced compared with k when the number of loci is large, and this effect is due to the finite size of all real populations. As evident from Equation 2, LD disappears entirely and $E(r^2)$ = sampling error alone if $N_e = \infty$. As N_e becomes smaller, effects of linkage are seen at greater distances along a chromosome. Finite N_e also strongly enhances the effects of overlapping pairs of loci in making pairwise r^2 values nonindependent, and these effects are seen even for unlinked loci. The net result is that precision does not increase much after a few thousand SNPs (or their equivalent in multiallelic loci) are used.

Why is large N_e so difficult to estimate reliably? From Equation 5, for a given amount of data (numbers of individuals and loci), CV($\hat{N}_e$) increases almost linearly with N_e. When the ratio N_e/n is very large, uncertainty associated with the estimate generally also will be very large (Marandel et al., 2019; Wang, 2023; Waples, 2016). The underlying problem is that large N_e produces a very weak drift signal, so most empirical $\overline{r^2}$ arises from random sampling error. In that scenario, $\overline{r^2}'$ will generally be close to 0, and tiny changes in r^2 have large effects on $\hat{N}_e$. For example, with the same amount of data that provides high power to estimate N_e when true $N_e = 100$ (Figure 3), if N_e is 10^5, about half the estimates will be ∞, about half will fall in the range 100–10,000, and only a tiny fraction will be finite and $>10^4$ (Figure 5A). With large N_e and only a moderate sample of individuals, one typically obtains a bimodal distribution of $\hat{N}_e$: many estimates that are much

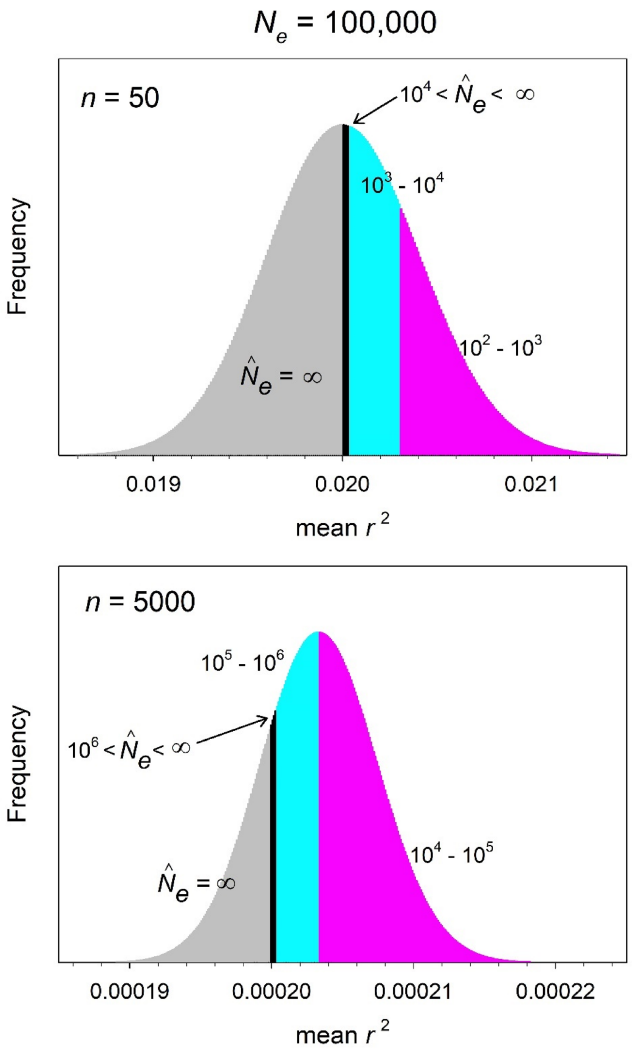

FIGURE 5 As in Figure 3, using 100 'SNP' loci, but for true $N_e = 100{,}000$. Sample size (n) was 50 individuals in the top panel and 5000 in the bottom panel. After accounting for sampling error by subtracting $1/n = 0.02$ (top panel) or 0.0002 (bottom panel) from the raw $\overline{r^2}$ values, adjusted $\overline{r^2}'$ that were ≤0 were treated as $\hat{N}_e = \infty$. The thin black vertical bar is the range of $\overline{r^2}$ values that lead to finite estimates that are larger than 10^4 (top) or 10^6 (bottom).

too high, many that are much too low, and very few that are close to the true value (Waples, 2016). Using more loci increases precision, but those benefits decline rapidly after a few thousand SNPs are used (Figure 4). As a consequence, when true effective size is very large, robust estimates of N_e cannot be expected from the LD method with small samples of individuals, regardless of how many loci are used. In that scenario, the only way to meaningfully increase precision is to increase the sample size and hence reduce the N_e/n ratio. This can be effective even with relatively modest numbers of genetic markers: When 5000 rather than 50 progeny were sampled from the large population shown in Figure 5A, the majority of the $\hat{N}_e$ were within an order of magnitude of the true effective size (Figure 5B). In a real-world application, Bravington et al. (2016) used genetic data (26 highly variable microsatellite loci) from over 13,000 juvenile and adult southern bluefin tuna to obtain a large abundance estimate (~2 million adults) using close-kin mark–recapture. Subsequently, the same data were used in the LD method to obtain a robust estimate of the N_e/N ratio of ~0.1–0.5 (Waples, Grewe, et al., 2018).

3.3 | Sampling issues

All of the above assumes random sampling, which implies both equiprobability and independence: Each individual has an equal chance of being sampled, and whether individual A is sampled has no effect on whether individual B, individual C, or any other individual is also sampled. Perfectly random sampling is generally difficult or impossible to achieve in nature, so the goal should be to avoid major departures from randomness that can substantially bias results. For N_e estimation, the most common (and often the most serious) sampling bias occurs when close relatives are sampled at higher rates than they occur in the population as a whole. In population genetics analyses, researchers often prune samples by removing all but one member of a putative family group (Goldberg & Waits, 2010; Hess et al., 2015; Peterman et al., 2016). However, this is dangerous for N_e estimation because the incidence of relatives is a core part of the genetic-drift signal. Removing putative siblings predictably makes N_e appear larger. This is counterproductive if the sample is truly random; if close relatives are over-represented in the sample, without additional information it is impossible to know how many to remove to approximate a random sample (see Waples & Anderson, 2017 for details).

4 | SUMMARY

For practical applications, the most important considerations regarding the LD method are the following:

- Researchers should endeavour to approximate random sampling as closely as possible. If progeny are not well mixed, local sampling will provide a drift signal from local parents rather than the population as a whole. Purging some putative siblings is not a reliable way of removing this bias.
- Little extra precision is gained by using more than a few thousand SNPs (or their equivalent in multiallelic markers)—but the benefits of using more loci are greater when true N_e is large.
- If $N_e >> 1000$, robust estimates cannot be expected from small samples of offspring, regardless of how many loci are used.
- The most effective way to minimize upward bias from rare alleles is to exclude singletons [alleles present in one heterozygote, at frequency $1/(2n)$].
- Physical linkage is inevitable in all but the smallest datasets and will downwardly bias $\hat{N}_e$ unless it is accounted for, by either excluding comparisons of loci on the same chromosome, or applying a bias correction.
- Equilibrium migration has relatively little effect on the estimation of local N_e unless it is high in genetic terms (>5%–10%). Pulse migration of genetically divergent individuals can lead to

substantial bias, so researchers should examine evidence for genetic outlier individuals.

- The LD method is well suited for rapid bottleneck detection within one to a few generations, if a series of samples is available; it is a little slower to detect expansion, as residual LD from the smaller size in the recent past can depress estimates N_e for several generations.
- In age-structured populations, the most robust estimates are of N_b for one year/season, based on sampling a single cohort of offspring. Mixed-age samples generally relate to N_e, but with more variation across species. $\hat{N}_b$ can be converted to $\hat{N}_e$ based on the species-specific N_b/N_e ratio, which can be estimated from life history data.
- $\hat{N}_e$ is skewed high so the arithmetic mean is not a reliable indicator of central tendency; the harmonic mean or median $\hat{N}_e$ should be used in computing composite estimates.
- Do not exclude negative estimates; they suggest that genetic drift is negligible and hence N_e is large, so ignoring them leads to downward bias in composite $\hat{N}_e$. Even if the point estimate is infinity, the lower bound to $\hat{N}_e$ can be informative for conservation and management.

It is important here to reflect on why one wants to estimate N_e, because that affects how one should interpret and use the estimates. The LD method primarily provides information about local effective size, as equilibrium migration has relatively little influence unless it is high. But even very low migration rates (~1 individual per generation) are sufficient to spread alleles widely, so the amount of genetic diversity in a local population reflects local N_e only under strong and long-term isolation. For similar reasons, as noted by Ryman et al. (2019), in local populations the rates of inbreeding and the amount of additive genetic variance (factors that led to the '50–500' rule of thumb for how large N_e should be to promote short-term and long-term persistence, respectively) can depend heavily on metapopulation dynamics. But estimates of local N_e are still valuable, for several reasons. First, they provide useful information about reproductive biology and mating system dynamics. And local N_e influences the effectiveness of natural and sexual selection, which also primarily occur locally. Second, the distribution and variability of local N_e are key elements in metapopulation models that more comprehensively predict dynamics of interacting groups of individuals. Finally, local N_e is a good predictor of future levels of genetic diversity and genetic variance in populations that become fragmented and isolated, which is an increasingly common occurrence in the Anthropocene.

5 | TOPICS THAT MERIT FURTHER STUDY

5.1 | Weighting alleles

Sved et al. (2013) suggested weighting r^2 for different pairs of alleles by allele frequency (P), because alleles at higher frequency occur in more copies and hence contain more information. A weighting scheme also could potentially alleviate bias associated with null alleles. This idea merits further exploration for effects on bias and precision, including different weighting schemes (e.g. perhaps weighting by variance in allele frequency [$P(1-P)$] rather than linearly by P).

5.2 | Combining samples

Given that small n limits precision, under what conditions is it reasonable to lump samples from different geographic areas? This makes sense if the area encompassed by samples represents a single, random-mating population: In that case, the separate samples are estimating the same parameter. When spatial and genetic structure exists, however, the merits of lumping samples (or not) depend on the relative strengths of the drift and mixture contributions to LD (Equation 7). More extensive evaluations might uncover general rules to guide practical applications.

5.3 | LD pruning

Pruning pairwise r^2 values based on magnitude or statistical significance does not remove all effects of physical linkage (see Section 3.1.3 and Waples et al., 2016), so it is not a substitute for restricting comparisons to loci on different chromosomes. Furthermore, the bias adjustment for linkage based on genome size (Waples et al., 2016) assumes that loci are randomly sprinkled across the genome, including some that are by chance tightly linked. Pruning those largest r^2 values would therefore affect performance of the bias correction. Nevertheless, judicious pruning might be warranted in some circumstances. Restriction-site-associated DNA sequencing (RAD-seq) and related methods start with restriction sites widely spread across the genome and then generate short sequence reads from each site. Limiting analysis to one SNP per RAD site or contig therefore could be consistent with the simulation model used by Waples et al. (2016) to develop the adjustment for linkage.

5.4 | Genotype likelihoods

All of the above evaluations assume that discrete genotypes are called for every individual at every locus. Some methods (e.g. ANGSD; Korneliussen et al., 2014) instead use the distribution of genotypic likelihoods in downstream analyses, and one (ngsLD by Fox et al., 2019) is designed to estimate LD from genotypic likelihoods. Evaluations are needed for potential biases to r^2 (and hence $\hat{N}_e$) for pairs of loci that are unlinked.

5.5 | Partial monogamy

Weir and Hill (1980) derived $E(r^2)$ for random mating and permanent pair bonds and showed that the drift component to r^2 is twice as

large under monogamy. Those two models are available in LDNe and NeEstimator, but many species practise only seasonal monogamy or find new partners if theirs dies. Empirical evaluations might uncover useful rules for these common scenarios. Santiago et al.. (unpublished) carried out some limited evaluations of effects of partial monogamy on LD.

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CONFLICT OF INTEREST STATEMENT

The author declares no conflict of interest.

DATA AVAILABILITY STATEMENT

No new data were generated for this study except the simulation in Box 1, code for which is in Data S1.

ORCID

Robin S. Waples https://orcid.org/0000-0003-3362-7590

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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