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NEWS AND VIEWS

COMMENT

The number of markers and samples needed for detecting bottlenecks under realistic scenarios, with and without recovery: a simulation-based study

SEAN M. HOBAN,* OSCAR E. GAGGIOTTI†‡ and GIORGIO BERTORELLE*

**Dipartimento di Scienze della Vita e Biotecnologie, Università di Ferrara, Via L Borsari 46, 44100 Ferrara, Italy; †Laboratoire d'Ecologie Alpine, Université Joseph Fourier, 38041 Grenoble, France;*

Abstract

Detecting bottlenecks is a common task in molecular ecology. While several bottleneck detection methods exist, evaluations of their power have focused only on severe bottlenecks (e.g. to *Ne* ~10). As a component of a recent review, Peery *et al.* (2012) analysed the power of two approaches, the M-ratio and heterozygote excess tests, to detect moderate bottlenecks (e.g. to *Ne* ~100), which is realistic for many conservation situations. In this Comment, we address three important points relevant to but not considered in Peery *et al.* Under moderate bottleneck scenarios, we test the (i) relative advantage of sampling more markers vs. more individuals, (ii) potential power to detect the bottleneck when utilizing dozens of microsatellites (a realistic possibility for contemporary studies) and (iii) reduction in power when postbottleneck recovery has occurred. For the realistic situations examined, we show that (i) doubling the number of loci shows equal or better power than tripling the number of individuals, (ii) increasing the number of markers (up to 100) results in continued additive gains in power, and (iii) recovery after a moderate amount of time or gradual change in size reduces power, by up to one-half. Our results provide a practical supplement to Peery *et al.* and encourage the continued use of bottleneck detection methods in the genomic age, but also emphasize that the power under different sampling schemes should be estimated, using simulation modelling, as a routine component of molecular ecology studies.

Keywords: conservation, genomic, heterozygote excess, M-ratio, power, recovery

Correspondence: Sean M. Hoban, Fax: +39 0532 249761;
E-mail: shoban@alumni.nd.edu
‡Present address: Scottish Oceans Institute, School of Biology, University of St Andrews, KY16 8LB, St Andrews, Fife, UK

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Introduction

A recent investigation by Peery *et al.* (2012) makes several important contributions to the analysis of microsatellite data to infer past changes in population size. First, it demonstrates that high error rates can occur for two common bottleneck detection tests (Cornuet & Luikart 1996; Garza & Williamson 2001) if the wrong mutation model is assumed, especially if the proportion of stepwise mutations is greater than the investigator assumes. Second, based on a review of empirical studies of microsatellite mutations, the authors provide guidance on what range of mutation parameter values might be appropriate for these tests, a valuable aid for future investigations. However, regarding the objective, '*to determine how effectively bottleneck tests… can identify populations in need of conservation action*', that is, to evaluate the power of the tests for detecting *moderate* declines (e.g. from *Ne* 2500 to 100), the simulations and conclusions of Peery *et al.* require some additional perspective. We address three points to supplement the utility of their results: (i) determining the relative utility of using more markers vs. more individuals, (ii) determining the power resulting from large, but realistic (for an increasing number of conservation studies), numbers of markers (20–100 microsatellites) and (iii) determining power when population recovery or gradual change in size has occurred.

Point A: higher sampling of markers or individuals

Peery *et al.* test whether 'higher sampling' increases the power of the two tests to detect moderate bottlenecks, and they do in fact find an increase in power (the exact increase they observed depends on the strength of the bottleneck but could reach doubling in power) when doubling the number of loci (from eight to 16) and simultaneously approximately tripling the number of sampled individuals (from 35 to 100). The logical conclusion is that higher sampling should be used than is typical for bottleneck investigations (and the logical corollary that some past studies have had insufficient power). We agree wholeheartedly, but assert that this recommendation is too vague, as it includes both more loci and more individuals. It is of practical interest, when planning a conservation study, whether investment in more markers or more individuals will lead to greater increases in power (Morin *et al.* 2009), but the power of more markers and more individuals was not tested separately. Previous work (Cornuet & Luikart 1996; Luikart & Cornuet 1998) suggests that for the heterozygote excess test, increasing the number of individuals beyond

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~30 may have limited utility, because increasing sample size only increases the confidence in the estimate of heterozygosity, while increasing the number of loci increases the number of replicates for the nonparametric statistical test. We use simulations to separately test the effect of increasing the number of markers and samples. Specifically, we test sampling schemes used in Peery *et al.* (eight markers and 35 samples, which we call 'baseline', and 16 markers and 100 samples), as well as a sampling scheme that only increased the number of markers (doubling the number of markers to 16, with 35 individuals) and one that only increased the number of individuals (tripling the number of samples to 100, with eight markers).

Methods

To test these four sampling schemes, we produced simulated genetic data sets using the coalescent-based simulation software Simcoal2 (Laval and Excoffier 2004). For these simulations, we modelled a bottleneck (as in Peery *et al.*) as an immediate reduction in population size, ten generations before present. The simulations assume an isolated population (no migration) with no age structure. While, in general, few species meet all of these assumptions, many populations of conservation concern tend to be isolated. Furthermore, age structure can be accounted for by measuring time in units of generation. Moreover, the coalescent is a robust approach to modelling genetic diversity change over time (Marjoram & Tavare 2006) and has been used for simulating population size change in a variety of taxa, including fish (Neuenschwander *et al.* 2008), amphibians (Estoup *et al.* 2010), birds, mammals (Alter *et al.* 2012), insects (Mardulyn & Milinkovitch 2005) and plants (Grivet *et al.* 2009). Our model assumes that molecular markers are unlinked and not under selection (an assumption shared by many population genetic analyses, including bottleneck tests). Individuals are assumed to be randomly sampled from the population.

Similar to Peery *et al.*, we simulated a bottleneck from a past effective population size (*Ne*) of either 2500 or 5000 to a current population size of 25, 50 or 100 (six combinations). Many endangered species or populations were recently more abundant but currently persist at small effective sizes (Traill *et al.* 2012), such as Iberian lynx (*Lynx pardinus*), butternut tree (*Juglans cinerea*), Mediterranean monk seal (*Monachus monachus*) and Egyptian vulture (*Neophron percnopterus majorensis*). The *Ne* values that we investigate cover much of the plausible parameter space for *moderate* bottlenecks in a wide range of organisms (many mammals, fish, amphibians, plants and insects have current *Ne* between 50 and 1000). For all simulations, we modelled microsatellite loci under a general stepwise mutation model (mutation rate = 0.0005, proportion of nonstepwise mutations = 0.22, the main parameters used in Peery *et al.*). Peery *et al.* thoroughly examine the consequences of incorrect assumptions about mutation parameters (especially loss of power if the portion of stepwise mutations is much larger than assumed) and generally emphasize that investigations should consider a substantial portion of nonstepwise mutations. To perform the heterozygote excess test, we used the program Bottleneck (Piry *et al.* 1999). We calculated the M-ratio (Garza & Williamson 2001) with Arlequin (Excoffier & Lischer 2010) and used simulations of a constant size population to determine M-critical. Parameter files and processing scripts are in the Appendix S1 (Supporting information).

Results

Our results (Fig. 1) show that doubling the number of loci provides approximately the same (and often slightly higher) power than tripling the number of individuals, for

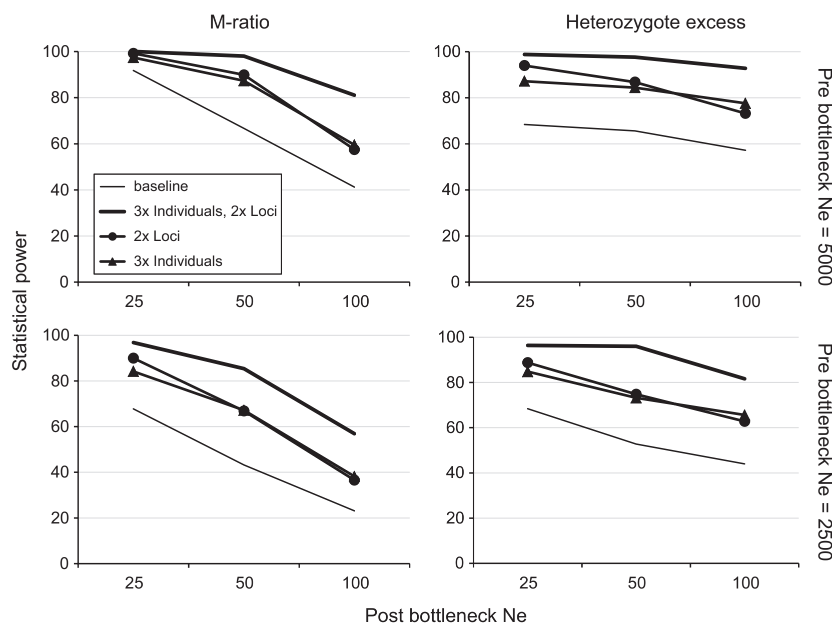

Fig. 1 Power (*y*-axis) to detect bottleneck using two tests (columns), at various postbottleneck *Ne* (*x*-axis) and prebottleneck *Ne* (rows), for four sampling schemes (symbols). In the majority of situations, power is slightly higher for the 16 marker, 35 individual scheme than the eight marker, 100 individual scheme. As in Peery *et al.*, power to detect declines to 500, and from 1000, are very poor and are thus not shown. Results are based on 1000 replicates of each scenario for *M*-ratio and 250 replicates for the heterozygote excess test.

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all six combinations of pre- and postbottleneck sizes, and both statistical tests. Considering that endangered populations are often small (sometimes <100 individuals, for example Hoban *et al.* 2010), and sometimes difficult to access (which can limit the number of individuals that can be sampled), and considering that developing and genotyping more loci is ever more feasible and affordable, future conservation genetic sampling efforts may find it easier to increase the number of loci than number of individuals (Landguth *et al.* 2012). It is encouraging therefore that increasing the number of loci can provide equal or greater power than adding more samples, in the situations explored.

Point B: marker sets in the genomic age

We next consider the number of markers tested by Peery *et al.*, which were based on a review of publications from 2001 to 2010. In this review, the 50th and 90th percentiles were eight and 16 loci. We suggest that this is unrealistically low for some studies in the genomics era, when dozens or hundreds of markers can be developed for even nonmodel organisms (Allendorf *et al.* 2010; Ekblom & Galindo 2011). Particularly in the past 2 years, high throughput sequencing (e.g. 454) has been used to rapidly identify many microsatellites in species having no previous genetic resources. For example, Whitney & Karl (2012) developed 38 loci in hawkfish, and Garibay *et al.* (2012) developed 24 loci in the white-nosed coati. To determine what number of microsatellites will probably be available for conservation genetics studies in the near future, we examined abstracts for three recent issues of Conservation Genetics Resources (March, June and September 2012), for number of novel microsatellite loci (161 articles). The mean number of microsatellite loci was 20 (mode = 14), and the 90th percentile was 27. Fifty-four studies (one-third) developed more than 16 markers, and 39 (one-fifth) developed more than 20. In addition, in the journal Molecular Ecology, the mean number of microsatellites used in population and conservation genetics studies in 2011 was 15–20 (Rieseberg *et al.* 2012). While not all of these studies are testing for bottlenecks, these numbers do suggest that a substantial proportion (perhaps one-third to one-fifth) of near-future conservation genetic studies will be able to utilize substantially more loci than tested by Peery *et al.* (we discuss some caveats to this supposition below). No previous evaluation of bottleneck tests has determined whether dozens of microsatellite markers can detect moderate bottlenecks. We therefore test the power of 8, 16, 20, 25, 30, 40, 50, 75 and 100 loci (and 35 individuals) for detecting bottlenecks from *Ne* 2500 (the median prebottleneck size tested by Peery *et al.*) to postbottleneck *Ne* of 500, 100 and 50, using simulations as explained above. Although 75 and 100 loci are unrealistic for current studies, these values were explored to see whether power reaches an asymptote.

We observed continued increases in power with added number of markers (Fig. 2). Excepting the M-ratio with the weakest bottleneck, gains in power continue to accumulate somewhat linearly with additional markers, even up to 50 (and 100) loci. For example, when using eight markers for the moderate bottleneck (Ne 2500 to 100), power for the M-ratio and heterozygote excess test was 0.24 and 0.47, while with 30 markers power dramatically increased to 0.55 and 0.84, and with 50 markers it was 0.69 and 0.97. While we only evaluated increasing the number of markers for three bottleneck combinations, it is clear that substantial gain in power to detect bottlenecks in realistic cases can be expected as increasing numbers of markers are used.

Notably, for the case of population size reduction from *Ne* 2500 to 500, there is little power for the M-ratio no matter how many markers, even though, for this same reduction, a linear gain in power is observed for the heterozygote excess test. Clearly, the power of the two tests often differs, and one test may be significant, while the other is not, under the same scenario. While such an observation has been used to make inferences about bottleneck timing or severity (e.g. an 'ancient' bottleneck, Spear *et al.* 2005), our results emphasize that power of the sampling scheme could be an equally valid explanation for nonsignificance of a test.

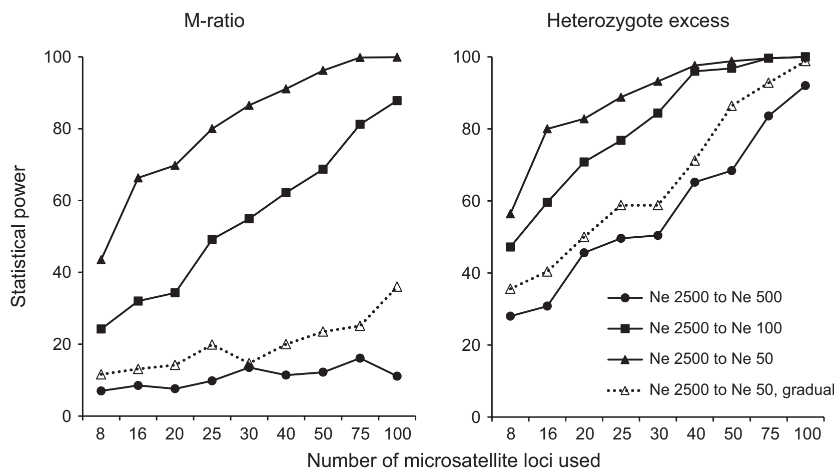

Fig. 2 Power (*y*-axis) to detect bottleneck using two tests (columns), at various postbottleneck *Ne* (symbols) and number of genetic markers employed (*x*-axis).

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There are several caveats to the near-term potential of using dozens of microsatellites in bottleneck studies. First, while development of microsatellites is facilitated by next-generation sequencing, genotyping is usually performed using traditional PCR and capillary-based electrophoresis. Fortunately, increasingly efficient multiplex PCRs can enable amplification of up to 12 loci in a single run (Carvalho *et al.* 2011; Guichoux *et al.* 2011). Such protocols will be necessary for screening large numbers of microsatellite loci. Furthermore, for the M-ratio test, studies typically must discard some (up to one-fourth) loci that do not fit a stepwise model (Peery *et al.* 2012). Therefore, the number developed is higher than the number actually employed. Last, power to detect a bottleneck from 2500 to 500 with the heterozygote excess test is still below 70% with 50 markers, as is the power for a bottleneck from 2500 to 100 with the M-ratio. A power of 80–90% would be most useful to conservation agencies. Despite these caveats, we are optimistic that increasing numbers of markers can increase power in many bottleneck studies (see Conclusion).

Point C: the effect of recovery

A final point regards when a threatened species has exhibited full or partial recovery since the bottleneck, thus showing a reasonably high current census (see Fig. 3). A bottleneck test can reveal whether a decline occurred in the past with subsequent recovery (e.g. a cryptic decline, Hailer *et al.* 2006), but Peery *et al.* and much past work (though see Williamson-Natesan 2005) has focused on power to detect only permanent bottlenecks (no recovery). As yet, no studies have tested the power to detect *moderate bottlenecks followed by recovery*. A situation of recovery for endangered species is not uncommon; well-known examples of recovery include the alpine ibex, grey whale, hihi, El Hierro giant lizard and peregrine falcon (Brown *et al.* 2007; Biebach & Keller 2009; Miras *et al.* 2009; Brekke *et al.* 2011; Alter *et al.* 2012). A recovery might be expected to decrease the power of a test, if enough time passes (Brown *et al.* 2007; Hundertmark & Van Daele 2010), but the effect of recovery is rarely considered. To investigate a situation of recovery, we simulated (with Simcoal2) a reduction from 2500 to 100 and from 2500 to 50, followed by full recovery. The bottleneck began either 50, 40, 30, 20 or 15 generations before present, with recovery 10 generations later, so the bottleneck was the same length as in the previous simulations (e.g. a bottleneck lasted from 50 to 40 generations before present time, when samples are taken).

We chose to model a ten-generation bottleneck followed by recovery for two reasons. First, this allows comparison to results from above in which no recovery was modelled. Second, ten generations is plausible in many threatened species whose size was reduced and then increased following successful conservation efforts or end of exploitation (grey whale, hihi, El Hierro giant lizard). Of course, some species experienced longer bottlenecks (ibex or bison, several dozen generations), while others experienced shorter (peregrine falcon, two generations). The recovery times simulated cover a range of plausible values (for ibex, for example, it has been ~20–25 generations since antihunting laws enabled recovery, Maudet *et al.* 2002). As with simulations from Point A, population size reduction and recovery are assumed to be instantaneous (see below for discussion, and Fig. 3).

Our results show that for recovery taking place most recently (five or 10 generations ago), power is similar to when no recovery occurs. However, when recovery occurred more than ~10 or 20 generations ago, power decreased substantially (Table 1, results shown are for 16 markers but results for eight markers show similar decrease). The degree to which power is reduced depends on the degree of bottleneck, and test employed. In an

Table 1 Power to detect bottleneck if a recovery occurs, depending on the number of generations before present that the recovery occurs

	16 markers			
	Bottleneck from 2500 to 100		Bottleneck from 2500 to 50	
Time before present of start and end of the bottleneck	Het exc test	M-ratio test	Het exc test	M-ratio test
50–40	44	23.2	48	48.2
40–30	50.4	30.7	45.6	56.1
30–20	55.2	27.2	61.6	55.7
20–10	57.2	34.4	63.6	60.9
15–5	62	41.1	73.6	69.9

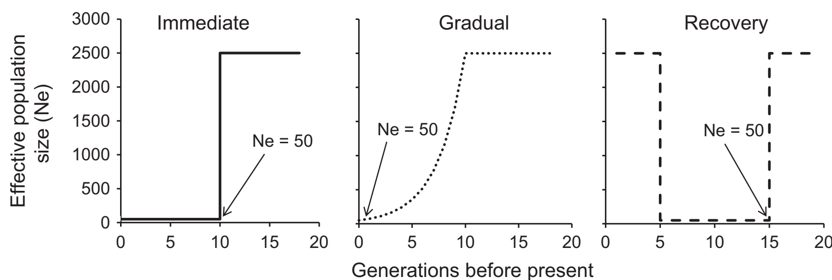

Fig. 3 Illustration of population size over time for immediate compared to exponential/gradual declines, and recovery.

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extreme case, if recovery from a moderate bottleneck occurred 40 generations ago, power with the M-ratio and 16 markers is reduced by nearly half compared to power if recovery occurred five generations ago, and power with the heterozygote excess test is reduced by about one-third. This differs from previous investigations of simulated extreme bottlenecks (e.g. to *Ne* ~10), which showed long-lasting (>100 generations) signals of bottlenecks (see Garza & Williamson 2001, Figs 5 and 6).

These simulations (and most previous investigations) assume that bottlenecks occur instantaneously. As a final evaluation of bottleneck detection methods in realistic conservation situations, we simulated an exponential (gradual) decline (Fig. 3), which may better represent some species (e.g. grey whale, lynx). For these simulations, negative growth rate is calculated from current (*N*) and past population size (N_0), and time (*t*), as $N = N_0 * e^{-rt}$. We model exponential population decline beginning ten generations ago, from *Ne* 2500 to 50, and evaluated up to 100 markers. Results show that power is lower, for both tests, under exponential decline (Fig. 2).

Our results suggest that simulations of permanent reduction with no recovery, and/or simulations of immediate reduction, as in Peery *et al.* and other works, may overestimate power to detect bottlenecks in situations in which the population or species recovered some time ago and/or experienced gradual decline.

Conclusions

We concur with the suggestion of Peery *et al.* that some (maybe many) past bottleneck studies may have had insufficient power and warrant reassessment (perhaps reanalysing samples with more markers). Our simulation results suggest the following general conclusions, for the bottleneck situations considered (which are realistic scenarios for species of conservation concern): (i) sampling twice the number of loci usually shows equal or better power for both tests than sampling three times the number of individuals, (ii) increasing the number of markers (up to at least 50 markers) results in continued additive gains in power, and (iii) recovery after a moderate amount of time, or gradual size change, reduces power, by up to one-half, depending on the test.

A specific practical note for conservation planning is that we observe 80% power to detect declines from *Ne* 2500 to 100 (a realistic conservation situation) with the heterozygosity excess test using 30 microsatellite loci. This is balanced by the observation that a decline from 2500 to 500 was never detectable with the M-ratio and was only detectable two-thirds of the time even with 50 loci with the heterozygote excess test. Therefore, it is likely that some ecologically relevant bottlenecks (e.g. reduction of 50–80%) will remain undetectable by these tests even with large numbers of markers. Clearly, a failure of these tests (e.g. Lindsay *et al.* 2008) does not mean that populations have not experienced large drops in size. To illustrate this, we carried out a final simulation whose parameters are similar to the endangered tree, butternut (*Juglans cinerea*), which underwent a bottleneck of 90–95% in many populations, due to an introduced fungal disease. We simulated a population of *Ne* 1000 reduced to 50, instantaneously, seven generations ago. In this case, power was only 26.4% with 10 markers and the heterozygote excess test, and 15.9% with the M-ratio. Therefore, one likely explanation of nonsignificant bottleneck tests (Hoban *et al.* 2010) is low statistical power. This example highlights the point that some previous studies lacked power, and that prestudy simulations could help determine the number of markers needed.

We conclude by recommending (i) that the number of markers developed for and used in conservation studies be increased as much as possible up to at least several dozen and (ii) that realistic simulations (including recovery, if appropriate) should be used (*sensu* Swatdipong *et al.* 2010) to determine the expected power of the sampling employed to detect a range of potential bottleneck sizes, to ensure that the study has sufficient power. Many simulation software are now available (Segelbacher *et al.* 2010; Hoban *et al.* 2012), and thus we suggest that simulation of power to detect a bottleneck for particular investigations should become a routine aspect of planning a study and poststudy interpretation of results (Williamson-Natesan 2005; Peery *et al.* 2012), as has been suggested for studies of genetic connectivity (Ryman *et al.* 2006; Morin *et al.* 2009). User-friendly software is available for such power estimation (Hoban *et al.* (2013); http://www.congressgenetics.eu/page.aspx?SP=Simulator_Start).

Based on the results of Peery *et al.* and our simulations, it is clear that further work is needed to test the utility of bottleneck detection methods in realistic situations. Future investigations should test the effects of overlapping generations (Hailer *et al.* 2006) and gene flow (Swatdipong *et al.* 2010). Continued evaluation of bottleneck detection and other methods will help ensure their proper application and interpretation in conservation management.

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S.H. planned the primary set of simulations, performed simulations and analysis, and drafted the manuscript. O.G. and G.B. planned additional simulations, discussed concepts and results, and assisted writing the manuscript.

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Data accessibility

Representative genetic data sets are archived in Dryad (doi:10.5061/dryad.f9j15).

Supporting information

Additional supporting information may be found in the online version of this article.

Appendix S1 Contains example parameter files for Simcoal2, Arlequin data files, analysis scripts and output. These files, and analysis instructions, are described in the readme text file.

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