Reliability assessment of null allele detection: inconsistencies between and within different methods

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Running title: Assessment of null allele estimation methods

Abstract

Microsatellite loci are widely used in population genetic studies, but the presence of null alleles may lead to biased results. Here we assessed five methods that indirectly detect null alleles, and found large inconsistencies among them. Our analysis was based on 20 microsatellite loci genotyped in a natural population of *Microtus oeconomus* sampled during 8 years, together with 1200 simulated populations without null alleles, but experiencing bottlenecks of varying duration and intensity, and 120 simulated populations with known null alleles. In the natural population, 29% of positive results were consistent between the methods in pairwise comparisons, and in the simulated dataset this proportion was 14%. The positive results were also inconsistent between different years in the natural population. In the null-allele-free simulated dataset, the number of false positives increased with increased bottleneck intensity and duration. We also found a low concordance in null allele detection between the original simulated populations and their 20% random subsets. In the populations simulated to include null alleles, between 22% and 42% of true null alleles remained undetected, which highlighted that detection errors are not restricted to false positives. None of the evaluated methods clearly outperformed the others when both false positive and false negative rates were considered. Accepting only the positive results consistent between at least two methods should considerably reduce the false positive rate, but this approach may increase the false negative rate. Our study demonstrates the need for novel null allele detection methods that could be reliably applied to natural populations.

Introduction

Highly polymorphic microsatellite markers are widely applied in population genetic studies since their discovery in the late 1980s. The improvement of polymerase chain reaction (PCR) and sequencing technologies allowed the use of these molecular markers to spread fast and wide into many research fields (see Guichoux et al. 2011 for review). However, the potential occurrence of "null alleles", i.e. alleles that fail to amplify during the PCR, creates a disadvantage in using these markers (Oddou-Muratorio et al. 2009). A null allele occurs when an incompatibility between any of the two locus–specific primers and its complementary target region causes the PCR amplification of an allele to fail. Such incompatibilities may be caused by mutations in the primer target region within one species, or between different species (in case of cross-species amplification) (Callen et al. 1993, Primmer et al. 1995, Jarne & Lagoda 1996). In some cases, long alleles may amplify much less efficiently then shorter ones, and therefore may appear as null alleles (Wattier et al. 1998). Low template quality/quantity can also result in the absence of amplification product and may be interpreted as the presence of a null allele (Garcia de Leon et al. 1998).

Null alleles have been reported in many species, e.g. humans (Callen et al. 1993), deers (Pemberton et al. 1995), bears (Paetkau & Strobeck 1995), voles (Ishibashi et al. 1996), fish (McCoy et al. 2001), crayfish (Walker et al. 2002), and oystercatchers (Van Treuren 1998). The detection of null alleles is an important step in population genetic data analysis, as their presence may strongly bias the estimates of population genetics parameters (Pemberton et al. 1995, Chapuis & Estoup 2007). For example, the accuracy of assignment of individuals to populations may be reduced and F_{ST} significantly overestimated (Carlsson 2008). The

presence of null alleles may also lead to an incorrect exclusion of a significant number of true parents in parentage analyses (Dakin & Avise 2004). Despite this, very few studies on population genetic structure and genetic parentage report estimates of null allele frequencies in their data (see Dakin & Avise 2004 for review).

Several methods for null allele estimation are currently available (Dempster et al. 1977, Chakraborty et al. 1992, Brookfield 1996, Summers & Amos 1997, Kalinowski & Taper 2006). They are based on comparing observed and expected heterozygosity for each locus to identify loci with significant heterozygote deficit. This approach is based on the fact that a heterozygous locus with a null allele would be scored as a homozygote, since only the visible allele is detected. Crucially, all these methods assume that a population is in Hardy-Weinberg Equilibrium (HWE), and that all observed deviations towards heterozygote deficit result from the presence of null alleles. The main difference between these methods lies in the way blank results (i.e. individuals without any detectable PCR product at a particular locus) are interpreted. Some methods consider blank results as null allele homozygotes, while others classify them as PCR failures resulting from low DNA quality or human errors; some methods attempt to differentiate between these two cases (see Supplementary Material for details). Another difference lies in the approaches used for null allele frequency estimation. While the estimates of Chakraborty et al. (1992) and Brookfield (1996) are obtained analytically, estimates of Dempster et al. (1977), Summers & Amos (1997), and Kalinowski & Taper (2006) are achieved through iterative optimisation (see Supplementary Material for details).

The above methods showed good to moderate accuracy in estimating frequencies of known null alleles in populations simulated assuming HWE (Kalinowski & Taper 2006,

Chapuis & Estoup 2007). Specifically, Kalinowski & Taper (2006) demonstrated that their method performs better than the methods of Chakraborty et al. (1992) and Summers & Amos (1997), while Chapuis & Estoup (2007) showed that the method of Dempster et al. (1977) performs better than the methods of Chakraborty et al. (1992) and Brookfield (1996). However, Chapuis & Estoup (2007) also showed that the three methods they tested performed worse when applied to two empirical datasets from natural populations, where the presence of null alleles was confirmed by their successful amplification after the primers were re-designed. Moreover, in one of these populations heterozygote deficit remained significant even after the null allele was successfully amplified with the new primers. Although this result was attributed to the presence of additional null alleles (Chapuis & Estoup 2007), the observed heterozygote deficit could have resulted from other factors such as small sample size, high inbreeding levels, or immigration.

The assumption of HWE, common among the methods described above, can be problematic when estimating null alleles in microsatellites scored from natural populations, since natural populations never strictly comply with the assumptions of Hardy-Weinberg law (i.e. infinite size, random mating, lack of mutations, migration and natural selection). Crucially, some of the factors causing deviations from HWE also lead to heterozygote deficit, namely inbreeding, assortative mating, population structure or immigration from a genetically distinct source (Wahlund effect), and disruptive selection (Avise 2004). Heterozygote deficit generated by such population mechanisms may be interpreted as the presence of null alleles, thus leading to false positives. On the other hand, phenomena such as disassortative mating or balancing selection can lead to heterozygote excess, which may result in failure to detect true

null alleles. The effect of other population genetic processes is less obvious. For example, a bottleneck leads to loss of alleles and decline in heterozygosity, but at least under some conditions it may also lead to temporary heterozygote excess (Cornuet & Luikart 1996). Fluctuations in population size, especially if associated with immigration during the growth phase, may lead to temporal fluctuations between heterozygote excess and deficit. In addition, taking a small subsample from a population (which also effectively occurs during founder events) may result in heterozygote deficit in some loci and heterozygote excess in others, due to the stochasticity of the sampling procedure. This may lead to detection of false null alleles in loci with heterozygote deficit.

Many population genetic studies are based on small sample sizes, and in many cases, study populations themselves are small (and therefore subject to strong drift), fluctuate in size, and exhibit considerable deviations from random mating. Such populations do not comply with the assumption that heterozygote deficit results solely from the presence of null alleles. However, the methods assuming HWE are commonly applied to such cases (e.g. see the review by Dakin & Avise 2004). In this study, we address the problem of detecting null alleles in populations that undergo demographic changes and deviate from HWE, and we assess reliability of the five widely used methods (Dempster et al. 1977, Chakraborty et al. 1992, Brookfield 1996, Summers & Amos 1997, Kalinowski & Taper 2006) in such non-equilibrium conditions. For this purpose, we apply these methods to a natural population of root vole, *Microtus oeconomus*, which was sampled over an eight-year period, and underwent substantial density fluctuations during this time. Additionally, in order to test whether population-level factors may lead to the detection of false null alleles, we analysed 1200

simulated populations without null alleles, but affected by a bottleneck with varying levels of intensity and duration.

Materials and Methods

Analysed datasets

We analysed 20 nuclear microsatellite loci in a population of root vole, *Microtus oeconomus*, which was extensively sampled over an eight-year period, and underwent a 7.7-fold change in average density during this time. Detailed information about demography and genetic variability of this population obtained from previous studies (Gliwicz & Jancewicz 2004, Gliwicz & Dąbrowski 2008, Dąbrowski 2010, Pilot et al. 2010) allowed us to follow temporal changes in the estimated null allele frequencies and compare different methods of their detection.

In order to assess the effect of demographic changes and resulting population genetic changes on null allele detection rates under controlled conditions (i.e. with known - rather than estimated – genetic composition and demographic history), we simulated 1200 populations without null alleles, but with varying level and duration of a bottleneck. The simulated data allowed us to explore the effect of demographic changes on inconsistencies in null allele detection that were observed in the natural population. In addition, we created 20% subsets of the simulated populations by random sampling, to assess the effect of population sub-sampling on null allele detection. Finally, we introduced null alleles into the earlier simulated populations in order to (1) assess the performance of each method of null allele estimation in detecting known null alleles, and (2) assess the empirical relationship between

the frequency of null alleles and the frequency of null allele homozygotes in non-equilibrium populations.

We used two general approaches for null allele detection. The first approach was based on methods assessing heterozygote deficit, as described above (Dempster et al. 1977, Chakraborty et al. 1992, Brookfield 1996, Summers & Amos 1997, Kalinowski & Taper 2006). We applied this approach to both the natural population and the simulated populations. The second approach was based on the comparison of genotypes between parent-offspring pairs, and was included here as the method that does not assume HWE. However, it could be only applied to the natural population.

Sample collection from the natural population

The natural population studied was a wild population of the root vole inhabiting a river valley located in a strict reserve of Białowieża National Park in north-eastern Poland. The 1-ha field plot was situated on a vast open sedgeland, and was exposed to seasonal flooding. The root vole is a small rodent with a maximum life span of 18 months (3 months on average). In the studied population, individuals were reproductively active usually only for one breeding season. We used a catch-mark-release (CMR) method, with at least three trapping sessions carried out each year, using 100 live traps placed in a grid of 10 by 10 m. Mean trapping efficiency was over 90% of all individuals present on the plot (Pilot et al. 2010). The population underwent substantial density changes over the study period, with average annual densities ranging from 9 to 69 individuals per hectare as estimated in MARK software, and no individuals trapped in 2007 (Dąbrowski 2010). Such density fluctuations

affected kin structure in the population and could be responsible for deviations from HWE detected in some years (Pilot et al. 2010).

We collected tissue samples for genetic analysis from 94% (739) of the individuals marked from 2000-2008, including 13 recaptures (originally marked in a previous year and re-trapped in the next year; these samples were not duplicated in the genetic analyses). The annual numbers of sampled individuals are presented in Table 1.

Microsatellite genotyping in the wild root vole population

Protocols for DNA extraction and microsatellite genotyping are described in detail in the Supplementary Material. One crucial information to convey here is that there were no blank results in this dataset, i.e. no individuals had missing data at any locus. Tissue samples were obtained as biopsies and immediately stored in ethanol, which allowed us to work only with DNA of good quality. PCR amplification was done using high-quality Taq polymerase (included in QIAGEN Multiplex PCR Kit), and PCR reactions were repeated up to four times for samples that initially failed (see Supplementary Material for details). This allowed us to eliminate any missing data that could have resulted from low quality DNA, human errors and PCR reagent failures. None of these steps would, however, eliminate missing data resulting from the presence of null allele homozygotes. Given that our dataset did not contain any missing data, we can state with a high confidence that no null allele homozygotes existed in our dataset, which implies that null alleles, if present in this dataset, would only occur in low frequencies.

Genetic diversity and null allele detection in the natural population

Genetic diversity estimates for the root vole population, including the number of alleles per locus (N), observed (HO) and expected (HE) heterozygosity, mean polymorphic information content (PIC) and exclusion probability for the first parent (ExP(1)) were calculated in CERVUS 3.0 (Marshall et al. 1998), while departures from HWE were estimated for each locus in GENEPOP v 4.0.10 (Rousset 2008) (Supplementary Table 1).

We tested for the presence of null alleles for each year separately using five different methods: (1) the maximum likelihood (ML) estimator based on observed and expected heterozygosities described by Chakraborty at al. (1992) with the modification of Brookfield (1996) which accounts for the presence of null allele homozygotes, as implemented in MICRO-CHECKER 2.2.1 (van Oosterhout et al. 2004); (2) the ML estimator using chi-square goodness-of-fit, accounting for the presence of null allele homozygotes during optimization rounds (Summers & Amos 1997), as implemented in CERVUS 3.0; (3) the ML estimator accounting for genotyping errors implemented in ML-NullFreq (Kalinowski & Taper 2006); (4) the ML method using iterative EM (expectation and maximization) of Dempster at al. (1977) implemented in GENEPOP v4.0.10.; (5) a method based on the comparison of genotypes of parent-offspring pairs. The algorithms applied in each method (Dempster et al. 1977, Chakraborty et al. 1992, Brookfield 1996, Summers & Amos 1997, Kalinowski & Taper 2006) are described in the Supplementary Material. Hereafter the five methods will be referred to by the names of the software packages that implement them, namely MICRO-CHECKER, CERVUS, ML-NullFreq, GENEPOP, and parent-offspring method. As a result of testing our datasets using these methods, we obtained a binary response variable (presence-

absence of null alleles) for each of the 20 loci and each of the five methods tested. In addition, we tested for the presence of null alleles for the entire dataset with all years pooled (genotypes of recaptured individuals were not duplicated; see Supplementary Material and Supplementary Table 2).

The application of the parent-offspring method to the root vole population was possible since a careful reconstruction of its kin structure was available from earlier studies (Dąbrowski 2010, Pilot et al. 2010, see Supplementary Material). The program CERVUS allows for a small number of mismatches between parent and offspring genotypes, if the probability of the estimated relationship is high based on the conformity of the remaining loci. Therefore, we could use mismatching loci to detect putative null alleles. We created a list of parent-offspring pairs based on the results of the previous studies on this population (Dąbrowski 2010, Pilot et al. 2010). The average rate of mismatches between parental and offspring genotypes estimated using *error rate analysis* implemented in CERVUS was 0.08 (SD = 0.097). Presence of a null allele in a locus was reported only if the observed mismatch in a parent-offspring pair fitted the pattern expected by the presence of a null allele. For example, if a female with genotype AB at a particular locus mates with a male with genotype CN (where N is a null allele), 50% of their offspring are expected to have genotypes with this null allele (either AN or BN). Visible genotypes of the father (CC) and the offspring (AA, BB) will be inconsistent with the father-offspring relationship, therefore creating a mismatch at this locus. In contrast to the methods based on the heterozygote deficit, the parent-offspring method does not require the assumption of HWE.

The parent-offspring method could not be applied to the entire dataset, but only to

closely related individuals, which reduced the sample size to 511 parent-offspring pairs. In contrast, the other four methods were tested using all sampled individuals. Due to smaller sample size, the parent-offspring method may detect fewer null alleles compared with the other methods. However, if each method detects null alleles correctly, the null alleles detected by the parent-offspring method should be confirmed by the other methods.

Generation and analysis of simulated datasets

Using SPAms (Parreira et al. 2009), we simulated 1200 populations, each comprised of 100 individuals with 20 loci. The reason for generating this data was to test the way bottlenecks affect the detection of putative null alleles. In order to do this, the *one population size change model* with *instantaneous size change* was applied with the following options: *ancestral pop effective diploid size*: 100,000; *present pop diploid size* (six variants): (1) 99,999, (2) 50,000, (3) 25,000, (4) 10,000, (5) 5,000, (6) 2,500; *duration of event* (four variants): 3, 30, 300, 3000 generations; and *mutation rate*: 0.0001. The first size change variant (from 100,000 to 99,999 individuals) was used as a control, where bottleneck effect was not present. Each combination of the population size change (from ancestral to present number of individuals) and time of this event was simulated in 50 replicates. In all 1200 simulated populations the presence of putative null alleles was tested using four programs: CERVUS, GENEPOP, MICRO-CHECKER and ML-NullFreq. Genetic diversity in the simulated populations was estimated using the same methods as for the natural population of root voles (see above).

To test the effect of random sampling on null allele detection, we randomly selected

10% (n = 120) of the simulated populations, and from each of them we randomly sampled 20% of individuals. This simulated the effects of three different real life scenarios: (A) estimation of null allele frequencies based on a subset of individuals sampled from a population, (B) founder effect, or (C) sudden change in number of individuals within one breeding season. We tested for the presence of null alleles in each original population (n = 100 individuals) and its random subset (n = 20 individuals, i.e. a 20% subset) separately. Then we considered only the loci with null alleles detected in at least one of the original populations or their subsets. For these populations, we calculated the Kendall's coefficient of concordance as a measure of similarity of null allele detection between the original and the subset populations.

For the next analysis, we selected 120 out of 1200 populations simulated in SPAmp, in which no null alleles (false positives) were detected by any method. This set included populations that underwent all levels of the simulated bottleneck. Then, we simulated the presence of two null alleles in each of these 120 populations using NullAlleleGenerator (http://www.lcb.uu.se/papers/dabrowski/NullAlleleGenerator.zip). NullAlleleGenerator randomly selected a locus (out of the 20 loci simulated) and changed one random allele into a null allele. This procedure was repeated for two loci, thus simulating two null alleles per population. Whenever genotyped in heterozygous form, the allele selected as the null allele was replaced by the other allele from that locus. Whenever occurring in a homozygous state, the simulated null allele was marked as a blank result (missing data). This way we obtained populations with true known null alleles, for which we assessed the performance of CERVUS, GENEPOP, MICRO-CHECKER and ML-NullFreq in detecting null alleles.

Finally, we repeated the previously described procedure of simulating two null alleles per population for the second time, but unlike in the first case, this time the 120 populations were selected randomly. In this analysis, we checked the relationship between the frequency of simulated null alleles and the frequency of null allele homozygotes. In a population under HWE, the expected frequency of a null allele homozygote is p^2, where p is the frequency of the respective null allele. However, here we simulated populations that underwent a bottleneck, and thus many of them deviated from HWE. Therefore, we checked empirically how the frequency of null allele homozygotes depended on the frequency of null alleles. This was needed for the interpretation of the lack of null allele homozygotes in the natural population we studied (which also deviated from HWE and underwent substantial demographic fluctuations).

Statistical analysis

In order to investigate the presence/absence of null alleles in a particular locus in subsequent years, we applied a generalized linear mixed model (GLMM) with binomial error distribution, and logit link function. We used this approach because several parameters can potentially affect null allele presence and these parameters need to be included within one statistical design. Moreover, as the study covers different years and different loci, both year effect and loci effect have to be included as random variables to avoid pseudoreplications. We thus used the occurrence pattern of null alleles (present vs. absent) as a dependent variable, while the method of null allele estimation (marked as 1-5) was used as a fixed categorical factor. Difference between observed (H_O) and expected (H_E) heterozygosity (hereafter H_OH_E),

number of individuals trapped in a given year and number of alleles at a particular locus in a given year were used as three separate covariates in the model, whereas year and locus were included as random categorical factors. We implemented the GLMM using the “lmer” package (Bates et al. 2011) in R (R Development Core Team 2011).

The expected number of loci with null alleles was estimated as a function of the number of years analysed. For this purpose we used rarefaction curves implemented in EstimateS 800 (Colwell 2005). The curves were based on the years resampled in a random order. We constructed the curves independently for each method of null allele estimation.

In order to assess how similar the five methods were in their estimates of null alleles for a given locus in a particular year, we calculated a similarity index as the probability that a null allele detected by one method will be confirmed by another. This index was calculated pairwise between the methods, and visualized with 2x2 contingency tables.

We also conducted pairwise comparisons between individual null allele estimations, independent of year and method (8 years * 5 methods = 40 estimations; year 2007 was removed due to lack of voles) using EstimateS 800 (Colwell 2005). In order to understand what drives similarity among randomly selected null allele estimations, we carried out the GLM analysis as follows: For each possible pair of estimations (n = 780 pairs) we computed: (1) temporal distance (ranged from 0 to 8 years), (2) pooled number of null alleles indicated by two estimations and (3) logical statement (yes or no) indicating whether both estimations for a given pair were obtained with the same method (e.g. CERVUS vs. CERVUS) or different method (CERVUS vs. GENEPOP). These three variables were used as explanatory variables, whereas the number of shared null alleles in a given pair (indicating similarity level between

two estimations compared, which ranged from 0 to 6, mean = 0.77, SD = 0.95) was used as response variable in GLM with Poisson error distribution and log link in R package (R Development Core Team 2011).

To assess whether a bottleneck influences the number of null alleles detected by each method tested, we compared the number of detected null alleles between the no-bottleneck variant with the five bottleneck variants using a χ^2 test with Bonferroni correction. In order to test whether the duration of the bottleneck affects the number of null alleles detected, we used a Kruskal-Wallis test. To assess the level of consistency in null allele detection between the tests applied to the entire population and to the corresponding randomly selected 20% subset, we used Kendall's coefficient of concordance Wt implemented in R (R Development Core Team 2011). All these calculations were carried out for each method of null allele detection.

Finally, using Wilcoxon Signed-Rank Test in R (R Development Core Team 2011), we assessed whether the observed frequency of null allele homozygotes in populations with simulated null alleles (see Generation and analysis of simulated datasets), was significantly different from the frequency expected under HWE.

Results

Null allele detection in the root vole population

The number of alleles per locus in the root vole population ranged from 4 to 25 (mean = 14; SD = 5.6). Expected heterozygosity (H_E) ranged from 0.107 to 0.925 (mean = 0.780; SD = 0.218), and observed heterozygosity (H_O) from 0.080 to 0.926 (mean = 0.762; SD = 0.215). The analysed loci had high mean polymorphic information content (mean = 0.760, SD =

0.220) and high exclusion probability for the first parent (mean = 0.50, SD = 0.207), which allowed us to use them successfully in parentage and kinship analyses (see Pilot et al. 2010). In all but one locus, significant deviations from HWE were detected in different years, with both heterozygosity deficit (59%) and excess (41%) being observed (Supplementary Table 1). In 60% of the loci (n = 12) the allele distribution had no missing alleles of any length within the expected range. In 15% of the loci (n = 3), the allele distribution had one missing allele length, other 15% (n = 3) had two missing allele lengths, and in the remaining 10% of the loci (n = 2), more than two allele lengths were missing. At the same time, we did not detect any null allele homozygotes, which would be indicated as a locus with no detectable product (blank result).

The number of loci in which putative null alleles were detected varied among years and depended on the estimation method (Table 1). The set of 20 loci was analysed for each study year separately, which resulted in 8 replicates and a total of 160 loci*replicates (number of loci multiplied by number of years). In total, CERVUS detected the lowest number of putative null alleles at 14 loci*replicates, while MICRO-CHECKER detected putative null alleles in 15 loci*replicates, ML-NullFreq in 36 loci*replicates and GenePop in 46 loci*replicates. Altogether, using these four different methods we recorded 67 loci*replicates with putative null alleles out of the total number of 160 loci*replicates. Among them, 68.5% (n = 46) were detected only by one out of 4 methods, 12% (n = 8) by 2 methods, 4.5% (n = 3) by 3 methods, and 15% (n = 10) by 4 methods (Table 1). Only two loci had no null alleles detected in any year. There were no loci where null alleles were detected in each year (Table 1). Moreover, the number of loci with detected putative null alleles estimated for the entire

dataset with all years pooled also depended on the estimation method (see Supplementary Table 2). The frequency of detected putative null alleles (Table 1) was lower than 10% in 73% of cases (Figure 1). We found no correlation between the number of discontinuities in allele distribution and the number of detected null alleles within a locus. Finally, the frequency of null allele detection at loci originally developed for *M. oeconomus* did not differ from the frequency at loci originally developed for other species.

Null allele detection based on parent-offspring genotype comparison in the root vole population

We investigated 270 father-offspring pairs and 241 mother-offspring pairs, resulting in the detection of 18 parent-offspring pairs carrying putative null alleles. As shown in Table 1, the parent-offspring method detected null alleles in 11 loci*replicates. Seven loci were indicated as having null alleles once (in one out of 8 years), and 2 loci were indicated twice (in two out of 8 years). Five loci*replicates with putative null alleles detected using the parent-offspring analysis were also detected by all four ML programs tested (Table 1). Three other loci*replicates indicated by the parent-offspring analysis were not confirmed by any of these four programs. At the same time, four other loci*replicates with null alleles detected by all the other four programs, were not confirmed by the parent-offspring analysis.

Null allele detection in the simulated populations

In the 1200 simulated populations the number of alleles per locus ranged from 2 to 34 (mean = 14; SD = 4.2). Expected heterozygosity (H_E) ranged from 0.068 to 0.957 (mean =

0.861; SD = 0.081), and observed heterozygosity (H_O) from 0.07 to 1.0 (mean = 0.861; SD = 0.087). The analysed loci had high mean polymorphic information content (mean = 0.860; SD = 0.091) and high exclusion probability for the first parent (mean = 0.420, SD = 0.129) (for detailed information see Supplementary Table 3). In some loci, significant deviations from HWE were detected (Supplementary Table 4).

Among 24,000 loci*replicates, the number of loci with putative null alleles detected was highest for ML-NullFreq (n = 1255 loci*replicates; 5.2% of the total number) and GENEPOP (n = 1123 loci*replicates; 4.7%), followed by MICRO-CHECKER (n = 500 loci*replicates; 2.1%), and it was lowest for CERVUS (n = 327 loci*replicates; 1.4%) (Supplementary Table 5). Altogether, using the four different methods we detected 2532 loci*replicates (10.5% out of 24,000 analysed) with putative null alleles. Among them, 81% (n = 2056) were detected only by one of the 4 methods, 12% (n = 296) by 2 methods, 6% (n = 163) by 3 methods, and 1% (n = 17) by all 4 methods (Supplementary Table 4).

All the null alleles detected in these 1200 simulated populations were false positives, as the program SPAms used for their generation does not simulate null alleles. Therefore, we selected 120 populations where no null alleles were detected, introduced simulated null alleles by using NullAllelesGenerator, and repeated the analysis with the same four methods. In this case, MICRO-CHECKER, CERVUS and ML-NullFreq detected either none or very low frequencies of false positives (0.1% of loci that were actually free of null alleles and 1.6-1.9% of all loci with null alleles detected). In contrast, GENEPOP detected a considerable number of false positives: they were found in 9% of loci that were actually free of null alleles and constituted 55% of null alleles detected by this method (Table 2). Each of the four programs

produced a considerable number of false negatives (i.e. true null alleles that remained undetected) in proportions that ranged between 22% (in ML-NullFreq) and 42% (in CERVUS) of all loci with known true null alleles.

Statistical analysis

The GLMM revealed that the probability of null allele detection in the root vole population depended on the method applied. It was lowest for the parent-offspring method (as expected due to smaller sample size used for this analysis – see Materials and Methods) and highest for the GENEPOP method (Table 3). The number of null alleles detected using the parent-offspring method was about 16 times lower as compared to GENEPOP and nearly 10 times lower as compared to ML-NullFreq method. Within the remaining methods, the differences were also significant: the frequency of null allele detection was higher in ML-NullFreq than in CERVUS (P <0.0001), and higher in GENEPOP than in CERVUS (P < 0.0001) but no differences were found between CERVUS and MICRO-CHECKER (P = 0.673).

The GLMM also revealed that differences between observed and expected heterozygosity ($H_O H_E$) in the root vole population had a significant influence on the detection probability of putative null alleles (Table 3). The effects of number of individuals trapped in a particular year, and the number of alleles in a given locus in a particular year were non-significant (Table 3).

Although the expected cumulative number of loci where putative null alleles were detected increased asymptotically with increasing sample size, the rate of increase differed between the five methods applied (Figure 2). Depending on the method, null alleles occurred

in 25% to 75% of loci for the all eight years cumulatively.

Inspection of the similarity patterns shows that a null allele detected in the root vole population by a given method is usually a very weak predictor of it being detected by another method. As a consequence, consistent estimates of null alleles by two methods were rare and ranged from 12% to 58% of method-pairs (mean = 29.05%, Figure 3). In the simulated populations, the observed similarity pattern was even lower and ranged from 1.8% to 35.4% (mean = 13.6%, Figure 3).

GLMs showed that the pooled number of null alleles estimated in the root vole population by two randomly selected methods explained the number of null alleles shared by these two methods (GLM, B = 0.19, SE = 0.01, z = 18.06, P <0.001). Contrary to expectations, the number of shared putative null alleles was similar in the "between-methods" and the "within-method" pairs of estimates (B = 0.14, SE = 0.10, z = 1.36, P = 0.174). The effect of temporal distance between samples from different years was insignificant for the similarity among estimates (B = -0.04, SE = 0.02, z = 1.85, P = 0.064).

In the simulated populations (n = 100 individuals each), a change in the population size (bottleneck effect) significantly affected the number of loci with detected null alleles (Table 4). GENEPOP detected significantly higher number of loci with null alleles in four out of five comparisons, CERVUS in three, and the remaining two programs in one (Table 4). We also found that the cumulative number of loci with detected null alleles increased with the increased bottleneck duration (Figure 4). Bottleneck duration also significantly affected the number of null alleles detected by GENEPOP (Kruskal-Wallis Hc = 11.9; P < 0.05), although no significant correlation was detected in other programs.

In the “sub-sampled” simulated populations (n = 20 individuals), we observed very low concordance of null alleles detected (using each of the four methods) as compared with the original population (n = 100 individuals) (mean Kendall's coefficient of concordance for four programs Wt = 0.11; SD = 0.06) (Table 5).

Finally, in populations with simulated null alleles, the observed frequency of null allele homozygotes differed significantly from the expected frequency (V = 10070; P < 0.001; Supplementary Figure 1). We observed deviations toward both null allele homozygote deficiency and excess (Supplementary Figure 1). For null allele frequencies below 0.17 we observed cases were no null allele homozygotes occurred, but there were no such cases for null allele frequencies higher than 0.17.

Discussion

Detection of null alleles using indirect methods is susceptible to errors, given that these methods are based on assumptions that are commonly violated in natural populations. Methods based on comparing observed and expected heterozygosity (Dempster et al. 1977, Chakraborty et al. 1992, Brookfield 1996, Summers & Amos 1997, Kalinowski & Taper 2006) assume that null alleles can be detected based on observed deviations from HWE towards heterozygote deficit. However, natural populations may deviate from HWE because they do not meet the assumptions of the Hardy-Weinberg law, and/or because they are often studied based on a small number of samples, which may lead to random deviations from the equilibrium at different loci. The parentage method does not assume HWE, but may be prone to other types of errors, e.g. human errors with microsatellite scoring. The error rate in null

allele detection in natural populations is difficult to estimate, because the actual null allele frequencies are usually unknown. Our study was based on a natural population that was sampled for several consecutive years, and so the same null alleles were expected to occur throughout the entire study period. This allowed us to test the reliability of several methods of null allele estimation through the comparison of results between different years. Application of the same methods to 1200 simulated populations that underwent bottlenecks of different intensity and duration allowed us to further examine the effect of strong genetic drift on null allele detection.

Accuracy of null allele detection in the root vole population

We found inconsistencies in null allele estimation both across years for each method and among different methods within each year. We also failed to find any statistically significant temporal repeatability in null allele detection at any locus. Each method detected a considerable number of null alleles in the 1200 simulated datasets, and there were significant differences in null allele estimates among the methods. However, the number of null alleles detected was positively correlated with the bottleneck size in each of the methods tested. Crucially, the simulated populations did not originally include any null alleles (the program SPAms used for their generation does not simulate null alleles), so all the detected null alleles were false positives.

We thus conclude that all putative null alleles detected in the root vole population are likely to be false positives. Our conclusion is supported by the following evidence: First, given that real null alleles are derived from primer compatibility problems during PCR

amplification and PCR protocols did not change between years, we would expect the same null alleles to be present in each year of the study, or at least in most years (accounting for the sampling effect – see below). Yet, none of the methods tested detected such continuous presence for any of the loci. One explanation for this could be that individuals having null alleles in their genotypes were not sampled every year. In that case the number of individuals sampled in a particular year should have a significant influence on null allele detection probability. The GLMM analysis did not find such a correlation, which allows us to reject this explanation. Additionally, according to MARK estimate, over 90% of all individuals present in the study population were genotyped, and therefore the probability of omitting all individuals with a given null allele is negligible, unless this allele has a very low frequency in the population. However, the impact of null alleles with such low frequencies on results of population-level genetic analyses would be negligible.

Second, lack of missing data in the root vole genotype dataset is also consistent with low frequency or lack of null alleles in this population. Although the relationship between null allele frequency and the frequency of null allele homozygotes based on the Hardy-Weinberg law does not necessarily hold in non-equilibrium populations, these two parameters are always dependent as demonstrated for the simulated populations. Therefore, it is expected that in a locus with high null allele frequency, some blank results should occur. There are some cases in the root vole population where the estimated null allele frequency was above 20% (Figure 1). Under the Hardy-Weinberg law, such loci should contain over 4% of null allele homozygotes. However, we detected none, despite a large number of genotyped individuals.

Third, kin clustering and non-random mating have been earlier demonstrated in this

population (Dąbrowski 2010). We also found that significant multi-annual changes in density and random environmental events (e.g. seasonal floods) have a strong impact on rates of seasonal migration, male dispersal, and female philopatry (Pilot et al. 2010, Dąbrowski 2010). Heterozygosity could thus have been lost (and regained) from year to year due to both genetic drift and migration (Pilot et al. 2010, Dąbrowski 2010). We also found genetic signatures of bottleneck in this population (Pilot et al. 2010), and we show in this study, based on simulated data, that bottlenecks may significantly increase the frequency of false null alleles detected by each of the methods tested. Therefore, we conclude that the pattern of the putative null allele occurrence observed in the study population, is more likely to result from population genetic processes like density fluctuations, migration and non-random mating, than from factors associated with PCR amplification outcomes.

Inconsistencies among different methods of null allele detection

Our study revealed large inconsistencies among the compared methods of null allele detection in both the natural root vole population and the simulated datasets. The average similarity among the methods used to detect null alleles was 29.05% for the root vole population and only 13.6% for the simulated populations. While detection of false null alleles may be explained by population genetic processes leading to deviations from HWE, inconsistencies among the methods cannot be accounted for solely by this explanation. The method based on parentage analysis relied on different assumptions and smaller pool of individuals than the heterozygosity-based methods, and the resulting differences were consistent with expectations. However, the four heterozygosity-based methods applied the

same general assumptions (see Supplementary Material). They differed in the way missing data was interpreted, but neither the root vole population nor the original SPAms-generated populations included any missing data. Therefore, we conclude that the discrepancies among these methods do not result from differences in the theoretical assumptions, but rather from differences in the particular optimisation algorithms applied.

How to combine different methods to minimise errors in null allele detection?

Our study raises a question regarding whether estimates of null alleles reported in the literature, which are usually inferred using indirect methods, are always reliable. The number of null allele occurrences within different allele frequency classes calculated in this study for the root vole population (Figure 1) has a similar distribution to the one shown in Dakin & Avise (2004), based on an extensive literature review. Given that our results show that most null alleles detected in the root vole population are likely false positives (see above), this similarity raises a further question of whether the recommendation for discarding loci showing null alleles from analysed datasets (De Sousa et al. 2005) should be followed unconditionally.

In the case of the root vole population, several loci with putative null alleles would have to be excluded following this recommendation, with different number of loci excluded depending on the year and the detection method used. Moreover, if sampling was carried out for a longer period, we may expect that the number of loci with putative null alleles would increase with the number of study years (see Figure 2), because we observed no consistent detection pattern among years for any locus.

It may be thus useful to devise strategies that combine different methods to minimise errors in null allele detection based on the results from our study. We found that in the simulated populations without null alleles, 81% of false positives were detected by only one out of the four heterozygosity-based methods, while only 1% of false positives were detected by all the four methods. At the same time, in the simulated populations where null alleles were included, 58% of true null alleles were detected by all the four methods. Therefore, combining two or more methods and considering only the consistent putative null alleles should considerably reduce the detection of false positives. However, it may also result in non-detection of some true null alleles, especially if more than two methods are applied.

Therefore, it may be useful to assess which of the four methods tested are less error-prone. In the simulated populations without null alleles, CERVUS and MICRO-CHECKER detected less false positives (1.4% and 2.1%, respectively) as compared with the two other methods. On the other hand, in the simulated populations with null alleles, ML-NullFreq had the lowest proportion of false negatives (22%), while for CERVUS and MICRO-CHECKER this proportion was 42% and 33%, respectively. GENEPOP was the only method that still detected a considerable number of false positives in the simulated datasets (prior to the simulation of true null alleles) that were pre-selected specifically as having no false positives detected by any of the four programs. Therefore, this program seems to be particularly error-prone in terms of the detection of false null alleles. We thus suggest that the best strategy to minimise the errors in null allele detection would be the combined use of two or three of the remaining methods (ML-NullFreq, CERVUS and MICRO-CHECKER). The combination of CERVUS and MICRO-CHECKER is best for minimising the false positives' rate, while the

combination of ML-NullFreq and MICRO-CHECKER is best for minimising the false negatives' rate.

However, before applying these methods, it is important to account for the occurrence of other types of genotyping errors like allelic dropouts or false alleles (e.g. resulting from stuttering), which can be detected e.g. using MICRO-CHECKER and/or by replicating the genotyping for a number of individuals. It is also important to minimise the occurrence of missing data due to reasons other than null allele homozygotes by repeating failed PCRs at least once.

Because heterozygosity-based methods assume HWE, it is important to minimise errors that may result from violations of the assumptions of Hardy-Weinberg law. For example, if population genetic structure is detected, the presence of null alleles should be assessed for each sub-population separately. The parentage-based method does not assume HWE, so it may help minimising the detection of false null alleles if used in addition to the heterozygosity-based methods; however, we recognise it won't always be possible or practical to use this method, due to its reliance on a detailed reconstruction of parent-offspring relationships within the study population. Finally, our study showed that material collected from the same population during several seasons (if there is sufficient generational turnover) may help interpreting the results of null allele detection and prevent their overestimation of their numbers. Alternatively, if sample size is sufficiently large, the accuracy of null allele detection may be improved by comparing the results obtained from different random sub-sets of the entire dataset analysed.

Conclusions

Our study shows that many commonly used null allele detection methods exhibit low reliability and consistency when applied to non-equilibrium populations. When we account for both false null allele detection rate and non-detection rate of the true null alleles, no method can be considered as clearly superior over the others. We thus suggest the combined use of at least two methods and considering only putative null alleles detected consistently by different methods. This should considerably reduce the detection of false positives. However, this approach is compromised by an increased rate of false negatives (non-detected real null alleles), and thus provides only a sub-optimal solution. Our study demonstrates the need to develop null allele detection methods that could be applied to non-equilibrium populations without violating the model assumptions.

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MJD and MP designed the project. MP and JG supervised the project. MJD and MP wrote the paper. MJD performed laboratory work, generated simulated populations and carried out null allele detection analysis. MK designed and implemented the algorithm for simulating null alleles, contributed to the statistical analyses and editing of the manuscript. MZ designed and conducted most of the statistical analyses and contributed to writing the manuscript. HMU participated in programming and automating of the process of comparing null allele detection results. This project used data collected as a result of a long-term research project on rodents in Białowieża National Park supervised by JG.

Data Accessibility

Data underlying this manuscript are available in the online supplemental information. *Microtus oeconomus* genotypes, simulated populations genotypes and subpopulations genotypes are available on Dryad, doi: 10.5061/dryad.4p41m

NullAlleleGenerator, along with its full documentation and example data is available from http://www.lcb.uu.se/papers/dabrowski/NullAlleleGenerator.zip

Figure legends

Figure 1. Histogram of frequencies of putative null alleles detected using different algorithms implemented in the evaluated programs: CERVUS (algorithm of Summers & Amos); MICRO-CHECKER (algorithms: Oosterhout, Chakraborty, Brookfield 1, Brookfield 2), and GENEPOP (EM algorithm of Dempster 1977).

Figure 2. Expected cumulative number (left axis) and percentage (right axis) of loci where putative null alleles were detected using five different methods, are presented as a function of the number of years studied. C – CERVUS; M – MICRO-CHECKER; N – ML-NullFreq; G – GENEPOP.

Figure 3. Similarity of null allele estimates between the methods applied for the natural root vole population and the simulated populations. The plot gives an average expectation that a null allele detected by one method will also be detected by the other method (gray – the simulated data; white – the root vole data).

Figure 4. Cumulative number of loci with putative null alleles within the simulated populations (n = 100 individuals each) with different bottleneck scenarios. “Time” denotes the bottleneck duration in generations. Cumulative number of loci is the sum of loci where null alleles were detected using any of the four methods.

Table 1. The presence of putative null alleles in the root vole population in each locus per year, estimated using five different methods.

Study year	2000	2001	2002	2003	2004	2005	2006	2007	2008
Sample size	150	116	130	39	70	147	84	0	16
Locus									
AV12			N	N	G	P			G
AV13	CMNG		CMNGP	CMNG	NG				
AV14				NG		N	G		
AV15	N								
Moe1		G	M	G		G	N		G
Moe2				G		G			
Moe3	G	NG	G			MNG	G		
Moe4	CMNGP	NG		CMN	N		CMNGP		N
Moe5		P			N				
Moe6	MN	G	N	G	P		N		CNG
Moe7		CMNGP	MG	G		CMNG	CMNGP		G
MSCRB4			C	C					
MSCRB6		G		G					
MSMM2									
MSMM3	N		P		N	N			
MSMM4	N			N		NG	G		
MSMM5	NG	G	G	G	G	G			
MSMM6		CMNGP		G		CMNG			G
MSMM7	NP			G					
MSMM8									

The presence of a putative null allele is marked by the symbol of the program (or multiple programs) that detected it: C – CERVUS; M – MICRO-CHECKER; N – ML-NullFreq; G – GENEPOP, and P – comparison of mismatching loci in parent-offspring genotypes. In the second row, the number of individuals sampled in each year is shown (Sample size).

Table 2. Null alleles detected using MICRO-CHECKER, CERVUS, ML-NullFreq and GENEPOP for 120 simulated populations containing two null alleles each. Loci with known null alleles were compared with loci detected using different programs (0- loci without null alleles; 1- loci with null alleles). Black background: true positives, grey background: true negatives, white background: false negatives, underline value: false positives.

		Null Alleles Generator	
		0	1
MICRO-CHECKER	0	2164	78
	1	2	156
CERVUS	0	2166	99
	1	0	135
ML-NullFreq	0	2163	52
	1	3	182
GENEPOP	0	1967	72
	1	199	162

Table 3. Summary results of a generalized linear mixed model with binomial error distribution and logit link, explaining the presence of the null alleles as a function of the four predictors: (1) difference between observed and expected heterozygosity ($H_O H_E$), (2) number of individuals trapped in a given year, (3) number of alleles in a particular locus in a given year and (4-8) the method of null allele detection. Symbols of different methods are explained in Table 1. Year and locus were included as random categorical factors in the model. For every level of each predictor the following parameters are given: estimate (B), with standard errors (SE), exponentiated estimate (Exp(B)), tests statistic (z-value), and significance (*P* -value).

Effect	B	SE	Exp(B)	z-value	*P* -value
Intercept	-3.507	0.593	0.030	-5.918	0.000
(1) $H_O H_E$	-28.760	3.070	0.000	-9.368	< 0.001
(2) N of individuals	0.000	0.003	1.000	-0.013	0.990
(3) N of alleles	-0.037	0.031	0.964	-1.175	0.240
(4) Method = G	0.579	0.554	1.785	1.045	0.296
(5) Method = M	2.782	0.495	16.154	5.623	< 0.001
(6) Method = N	0.811	0.541	2.249	1.498	0.134
(7) Method = P	2.283	0.498	9.806	4.586	< 0.001
(8) Method = C	0.000		1.000		

Table 4. Pairwise comparisons of the number of loci with putative null alleles between variant 1 of the simulation (no bottleneck) with the other five variants with different level of bottleneck. Comparisons were made for each program separately. Values presented in the table are results of χ^2 test. Statistically significant results are marked with (*).

Bottleneck pair variants	CERVUS	GENEPOP	MICRO-CHECKER	ML-NullFreq
1-2	0.272	2.010	0.286	< 0.001
1-3	2.502	70.040**	3.704	4.560
1-4	9.318*	148.700**	0.008	0.011
1-5	42.760**	206.500**	1.347	1.612
1-6	114.700**	176.500**	24.940**	16.870*

(*) $P < 0.01$; (**) $P < 0.001$; Bottleneck pair variants: 1 - 99,999; 2 - 50,000; 3 - 25,000; 4 - 10,000; 5 – 5,000; 6 – 2,500. The ancestral number of individuals for all bottleneck variants was set to 100,000 individuals.

Table 5. Pairwise comparison of loci with detected null alleles in two data sets: simulated original populations (n = 100 individuals per each population) and sub-sampled populations (n = 20 individuals randomly selected from original population).

Variable	CERVUS	GENEPOP	MICRO-CHECKER	ML-NullFreq
Wt	0.182	0.040	0.093	0.123
N	229	223	59	214
CP	13	9	6	30
new-P	204	129	24	96
not-CP	12	85	29	88

Wt –Kendall's coefficient of concordance corrected for ties; N – number of loci with null alleles detected in at least one dataset (either original or under-sampled); CP – conserved positives: loci with detected null alleles in both sets of populations; new-P – new positives: loci with null alleles detected only in under-sampled data set; not-CP – not conserved positives: loci with null alleles detected in the original data set which were not confirmed within under-sampled data set.

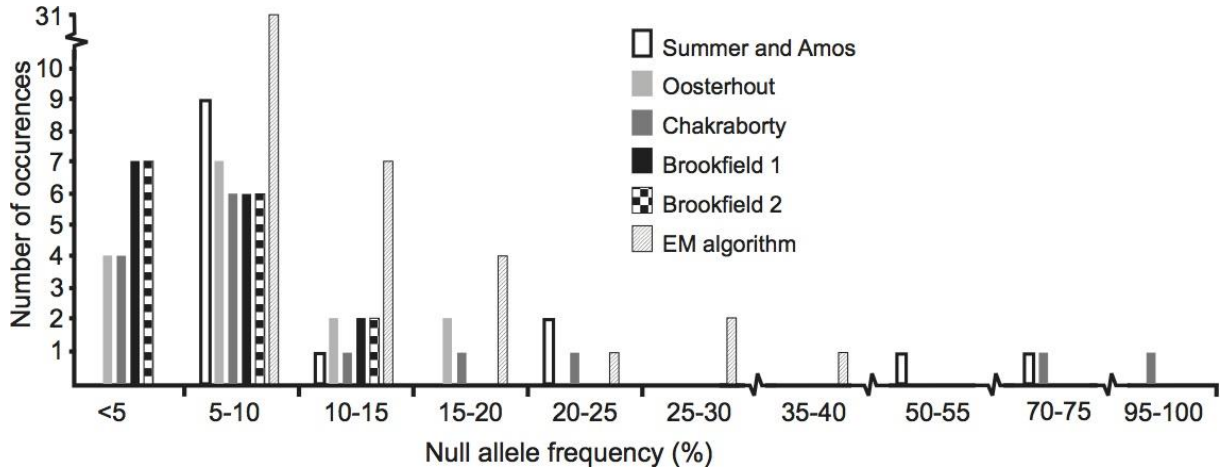
Number of occurences
31
10
9
8
7
6
5
4
3
2
1
Summer and Amos
Oosterhout
Chakraborty
Brookfield 1
Brookfield 2
EM algorithm
<5
5-10
10-15
15-20
20-25
25-30
35-40
50-55
70-75
95-100
Null allele frequency (%)

Figure 1

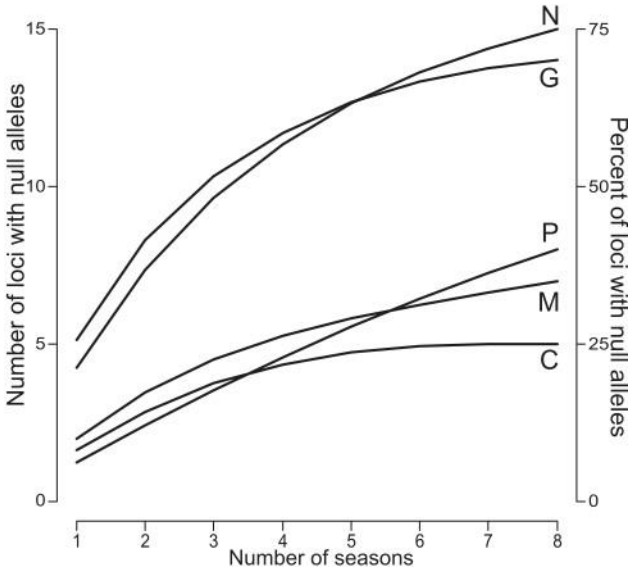
N
G
P
M
C
15
10
5
0
75
50
25
0
Number of loci with null alleles
Percent of loci with null alleles
1
2
3
4
5
6
7
8
Number of seasons

Figure 2

CERVUS	22.4%	57.9%	31.6%	33.3%
2.3%	GENEPOP	24.0%	28.1%	12.0%
35.4%	1.8%	MICRO-CHECKER	33.3%	30.0%
13.6%	2.9%	25.3%	ML-NullFreq	17.9%
-	-	-	-	Parent-offspring

Figure 3

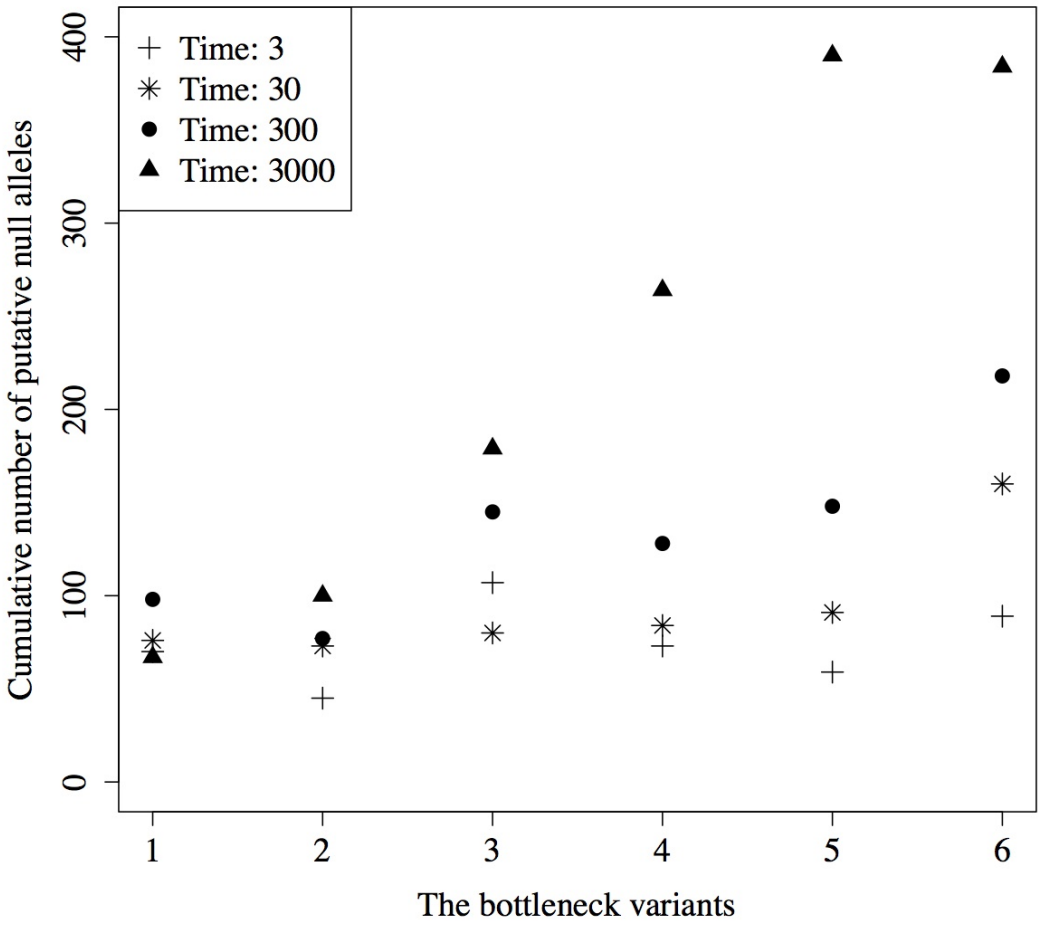
Time: 3
Time: 30
Time: 300
Time: 3000
Cumulative number of putative null alleles
0
100
200
300
400
1
2
3
4
5
6
The bottleneck variants

Figure 4

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Characteristics of 20 microsatellite loci analysed in the root vole population, organized in four multiplex PCR reactions.

Table S2 Loci with putative null alleles confirmed for the entire dataset (root vole population, all years pooled) using all four methods.

Table S3 Characteristics of the simulated populations. Range, mean values and SDs were computed separately for 50 replicates representing each scenario of the bottleneck size and duration (in generations).

Table S5 Number of loci with null alleles detected using four programs in populations simulated with various levels and duration of a bottleneck.

Fig. S1 The observed and expected frequency of homozygotes vs. null allele frequency in the loci where null alleles were simulated using NullAlleleGenerator in 120 randomly selected populations.

Table S4 Genetic estimates and null allele detection results computed for simulated populations affected by bottleneck scenarios.