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Quantifying genotyping errors in noninvasive population genetics

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Abstract

The use of noninvasively collected samples greatly expands the range of ecological issues that may be investigated through population genetics. Furthermore, the difficulty of obtaining reliable genotypes with samples containing low quantities of amplifiable DNA may be overcome by designing optimal genotyping schemes. Such protocols are mainly determined by the rates of genotyping errors caused by false alleles and allelic dropouts. These errors may not be avoided through laboratory procedure and hence must be quantified. However, the definition of genotyping error rates remains elusive and various estimation methods have been reported in the literature. In this paper we proposed accurate codification for the frequencies of false alleles and allelic dropouts. We then reviewed other estimation methods employed in hair- or faeces-based population genetics studies and modelled the bias associated with erroneous methods. It is emphasized that error rates may be substantially underestimated when using an erroneous approach. Genotyping error rates may be important determinants of the outcome of noninvasive studies and hence should be carefully computed and reported.

Keywords: allelic dropout, amplification success, false allele, genotyping errors, low DNA, noninvasive

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Introduction

Methodological advances in the genotyping of very low DNA quantities, which were first developed in the forensic sciences, have proved increasingly useful in the field of molecular ecology during the last 10 years. One powerful application in animal biology is the ability to sample populations noninvasively, through the collection of animal remnants or droppings such as hair, faeces, feathers, etc. (e.g. see Piggott & Taylor 2003 for a recent discussion). However, the gain in efficiency obtained from such methods is somewhat counteracted by the challenges inherent in low-quality/quantity DNA genotyping (Navidi *et al*. 1992; Taberlet *et al*. 1996, 1999; Gagneux *et al*. 1997). In addition to the general sources of errors encountered in molecular ecology (i.e. mishandled data, variation of temperature during electrophoresis, somatic mutations), noninvasive samples may generate errors specifically associated with the amplification and typing of minute quantities of DNA. First, such analyses are especially sensitive to contamination as the proportion of contaminants may more easily overcome the target DNA in a reaction. However, in most cases both pervasive and sporadic contaminations can be avoided by cautious laboratory work, or at least they can be detected by routinely using negative controls at every stage of the analysis.

More importantly, allelic dropouts [ADO: one allele of a heterozygous individual is not amplified during a positive polymerase chain reaction (PCR)], and false alleles (FA: PCR-generated allele as a result of a slippage artefact during the first cycles of the reaction) constitute two sources of microsatellite genotyping error that cannot be easily monitored (Gerloff *et al*. 1995; Navidi *et al*. 1992; Taberlet *et al*. 1996). The detection of such errors classically relies on the comparison of hair- or faeces-based genotypes with reference genotypes obtained using other sources of DNA (e.g. muscle tissue), or obtained through repeated typing (consensus genotype). Hence, in the context of noninvasive sampling, a genotyping error is defined as an allelic difference

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Table 1 Frequencies of false alleles (FA) and allelic dropouts (ADO) reported in 19 peer-reviewed articles involving genotyping of faeces (F) or hair (H) samples

			FA		ADO	
Reference	Species	Source of DNA	Frequency (%)	Computation method	Rate (%)	Computation method
Bayes *et al*. (2000)	*Papio cynocephalus*	F	—	—	8	ADO_2
Creel *et al*. (2003)	*Canis lupus*	F	5.6	allele-specific*	11.1	allele-specific*
Dallas *et al*. (2003)	*Lutra lutra*	F	—	—	2.1	ADO_2
Ernest *et al*. (2000)	*Puma concolor*	F	—	—	8	ADO_1
Flagstad *et al*. (1999)	*Ovis aries*	F	0	FA_u	2	ADO_1
	Rangifer tarandus	F	0	FA_u	1.5	ADO_1
Frantz *et al*. (2003)	*Meles meles*	F	8	FA_2	*27*	ADO_u
Gagneux *et al*. (1997)	*Pan troglodytes*	H	5.6	FA_2	31.3	ADO_u
Goossens *et al*. (1998)	*Marmota marmota*	H	0	FA_u	0.41	ADO_u
Goossens *et al*. (2000)	*Pongo pygmaeus abelii*	F	2.95	FA_u	4.2	ADO_u
Huber *et al*. (2003)	*Lepus europaeus*	F	13.3	FA_1	2.2	ADO_1
	Cervus elaphus	F	16	FA_1	2.6	ADO_1
Lathuillière *et al*. (2001)	*Macaca sylvanus*	F	15.3	FA_u	3	ADO_u
Launhardt *et al*. (1998)	*Prebytis entellus*	F	0	FA_u	~6.8	ADO_u
Lucchini *et al*. (2002)	*Canis lupus*	F	0.3	FA_1	18	ADO_u
Morin *et al*. (2001)	*Pan troglodytes verus*	F	—	—	24	ADO_u
Parsons (2001)	*Tursiops truncatus*	F	0.97	FA_u	0	ADO_u
Roon *et al*. (2003)	*Ursus arctos*	H	0	FA_u	—	—
Sloane *et al*. (2000)	*Lasiorhinus krefftii*	H	0	FA_u	0	ADO_u
Smith *et al*. (2000)	*Papio cynocephalus*	F	—	—	48	ADO_2
Vege & McCracken (2001)	*Eptesicus fuscus*	F	0	FA_u	0	ADO_u

This table includes only those studies that clearly reported the methods used to estimate ADO and/or FA frequencies. Methods for estimating FA and ADO rates are described in the text.
*Allele-specific estimates of FA and ADO frequencies. Method described in Creel *et al*. (2003).

in the genotype determined using a sample collected noninvasively as compared to a reference genotype.

The variability of methods used to estimate genotyping error rates in the literature is noticeable, and often leads to results being incomparable between studies. It also raises the question of the accuracy of the various estimators employed. This is an important issue in the context of noninvasive population genetics, as these estimates are often used to design optimal genotyping protocols, i.e. to reduce the number of repetitions required to obtain reliable genotypes. It has been argued that the cost of the conservative multiple-tubes approach proposed by Taberlet *et al*. (1996) may be reduced by relaxing its inherent assumption of a 100% dropout rate (Miller *et al*. 2002; Valière *et al*. 2002; Paetkau 2003). Taberlet *et al*. (1996) set the ADO rate at maximum to propose a conservative method allowing the avoidance of any risk of error (with a confidence of 99%) in the consensus genotypes obtained after replications, independently of the quantity and quality of DNA present in the PCR templates (note here that the rate of ADO is different from the probability of amplifying only a given allele, which, assuming 100% ADO and equal proportions of the two alleles present in the DNA template for a heterozygous sample, is equal to 0.5). In some cases more efficient PCR strategies may in fact be designed by estimating genotyping error rates during a pilot study, but only if these rates are correctly estimated.

In this paper we review the estimation methods reported in the noninvasive population genetics literature. This review was based on a search in the *Current Contents* database for any population genetics paper involving the sampling of hair or faeces (the two most commonly used noninvasive sources of DNA) published before 2004, and this was completed with published lists of references and our personal literature databases. Out of 145 peer-reviewed articles (complete list of references available on request), we found 19 studies (presented in Table 1) reporting estimates of FA frequency ($n = 14$) and/or ADO rate ($n = 18$), not including articles in which the method used to compute error estimates was not clearly reported.

We first retained accurate definitions for the frequency of PCR-generated false alleles (FA rate) and the rate of allelic dropouts (ADO rate) and then we estimated the bias associated with other methods reported in the literature.

Quantification of error rates

The definition of an ADO is unambiguous: it is 'the possibility of not detecting an allele in heterozygous individuals'

(Taberlet *et al*. 1996). ADO will thus be consistently recognized as such among studies. This is not the case for the detection of FA, which is likely to vary among observers. Any false-positive band may potentially be recorded as an FA, even if this false positive was not generated during the PCR but originated from another source of error, such as contamination or unspecific amplification. The estimations of FA rates we propose are exact for PCR-generated false alleles, assuming that contamination events are detected using negative controls and that primers have been selected to achieve specific amplification of a single target locus.

The definitions proposed below (eqn 1–4) all refer to locus-specific estimates of genotyping errors. These equations are based on PCR counts (number of amplification attempts, either unsuccessful or leading to true or erroneous genotypes). A reliable alternative has been proposed by Creel *et al*. (2003) who reported allele-specific estimates of the dropout rate. It is however, less useful if the aim is to estimate the number of PCR repetitions needed to confirm heterozygous and homozygous genotypes because amplifications are not allele-specific.

Genotyping errors are defined as differences between noninvasive and reference genotypes; in the equations below, observed ADO and FA refer to errors obtained using noninvasive samples and detected by comparison with reference genotypes of the same individuals. Hence every individual included in the sample used to quantify error rates has to be genotyped at least twice (typing of noninvasive vs. invasive samples, or repeated typing of noninvasive samples). It is also worth noting that the samples used to determine reference genotypes must be highly reliable (high quality/quantity of DNA). Blood, for instance, should be used carefully as some genotyping errors have been observed using this tissue as a source of DNA (e.g. Jeffery *et al*. 2001; Fernando *et al*. 2003).

Allelic dropouts

As correctly pointed out by Creel *et al*. (2003), a rate of genotyping error should be computed as the number of errors detected, divided by the total number of cases in which an error can be detected (which includes cases in which an error has indeed been detected). ADO can be detected in heterozygous genotypes only, and hence the rate of ADO should be estimated considering only such genotypes. Note that undetected ADO in homozygous genotypes are not a problem since they do not lead to erroneous data. Hence, if we call 'positive amplification' a successful PCR leading to the determination of a genotype (even if this genotype is erroneous), the rate of ADO can be estimated as the ratio of the number of observed ADO at locus j (D_j; number of amplifications involving the loss of one allele) on the number of positive amplifications of individuals determined as heterozygous according to their reference genotype at locus j (A_{het_j}). If we call p the frequency of ADO at locus j (as in Taberlet *et al.* 1996 and later publications, but different from the parameter 'p' reported in Navidi *et al*. 1992), we have:

$$p_j = \frac{D_j}{A_{het_j}} \tag{1}$$

In the noninvasive population genetics literature, most authors reported the ratio of observed ADO over L loci on the total number of heterozygous genotypes, which is the weighted average of p_j for L loci (see Appendix for details of formulae):

$$p = \bar{p}_w = \frac{\sum_{j=1}^{L} D_j}{\sum_{j=1}^{L} A_{het_j}} \tag{2}$$

False alleles

FA generated during amplification reactions lead to erroneous genotypes in both homozygous and heterozygous genotypes. Hence the probability of occurrence of FA (here noted f) at locus j can be defined as:

$$f_j = \frac{F_j}{A_j} \tag{3}$$

where F_j is the number of amplifications leading to the creation of one or more false alleles at locus j and A_j is the number of amplifications (positive amplification of one sample at locus j, either homozygous or heterozygous). Note here that several false alleles may be generated during a single PCR, which would not be recorded using our definition. f_j is a measure of the frequency of FA leading to an erroneous genotype, because only this number is directly relevant to the determination of the number of repetitions needed to achieve reliable genotypes. The overall FA rate over L loci is then:

$$f = \bar{f}_w = \frac{\sum_{j=1}^{L} F_j}{\sum_{j=1}^{L} A_j} \tag{4}$$

The definitions proposed above for p and f lead to unbiased estimates of genotyping error frequencies (hereafter referred to as ADO_u and FA_u, respectively), and they appeared to be the most commonly employed in the literature (respectively in 11 of 18 and 9 of 14 studies). However, unless $A_{het_j} = A_j$, the two terms p_j and f_j cannot be summed to give an overall genotyping error rate.

Other computation methods reported in the literature

Allelic dropouts

Two different approaches that generally lead to inaccurate results have been reported in the literature (Table 1).

Method ADO$_1$. The ADO rate is calculated considering all PCR attempts (P_j), either successful or not:

$$p_j^1 = \frac{D_j}{P_j} \tag{5}$$

This method, employed in three papers (Table 1), generally underestimated p_j because negative amplification reactions and positive amplification of homozygous genotypes cannot lead to the detection of allelic dropouts.

Method ADO$_2$. The ADO rate is calculated considering all successful amplifications (A_j):

$$p_j^2 = \frac{D_j}{A_j} \tag{6}$$

This method, also reported in three papers (Table 1), may underestimate p_j because homozygous genotypes cannot lead to the detection of ADO.

False alleles

As already highlighted, the detection and quantification of FA is highly dependent on the observer, introducing some variance in parameter F_j. In addition to this source of variation that we were unable to quantify, two different approaches to the calculation of FA frequency have been reported in the literature.

Method FA$_1$. FA frequency is calculated considering all PCR attempts (P_j), whether successful or not:

$$f_j^1 = \frac{F_j}{P_j} \tag{7}$$

This method, used in two papers (Table 1), generally underestimated f_j, because negative amplification reactions cannot lead to the detection of false alleles.

Method FA$_2$. FA frequency is calculated on a subsample comprising only heterozygous genotypes. This approach was adopted in two papers where both FA and ADO frequencies were estimated on the same sample (Gagneux *et al*. 1997; Frantz *et al*. 2003). It gives unbiased estimates of f_j if the frequency of FA does not differ in homozygote and heterozygote genotypes. This seems to be a robust assumption although, to our knowledge, it has not been tested yet. This approach enables one to add FA and ADO frequencies to give an overall genotyping error estimate. However, such an estimate is only accurate for the focal sample of heterozygous genotypes, and is not representative of any other sample including homozygous genotypes (the rate of genotyping error would in that case be over-estimated).

Bias associated with erroneous methods of computation

Definition of bias

We codified the level of error introduced in the computation of p_j and f_j by the three most commonly used approaches leading to biased estimates (p_j^1, p_j^2 and f_j^1). We note ε and δ, respectively the relative divergence from the real ADO and FA rates p_j and f_j, such that:

$$\varepsilon = \frac{p_j - \hat{p}_j}{p_j} \tag{8}$$

and

$$\delta = \frac{f_j - \hat{f}_j}{f_j} \tag{9}$$

where $\hat{p}_j$ and $\hat{f}_j$ are the estimates obtained using methods 1 or 2, and p_j and f_j are real values of genotyping error rates at locus j.

These relative levels of error may be expressed as functions of the heterozygosity at locus j (H_j), the success of amplification reactions (s), and the ratio of numbers of repetitions of positive amplifications of homozygous and heterozygous individuals (r). The value of r directly derives from the number of repetition experiments involved when following a multiple-tubes based approach, which generally differs between homozygous and heterozygous individuals (for instance $r = 7/3$ in Taberlet's multiple-tubes approach). The relative error obtained when estimating the rate of ADO using method 1 is then (see Appendix for details):

$$\varepsilon_1 = \frac{p_j - p_j^1}{p_j} = \frac{H_j(1-s) + r(1-H_j)}{H_j + r(1-H_j)} \tag{10}$$

and for method 2 it is:

$$\varepsilon_2 = \frac{p_j - p_j^2}{p_j} = \frac{r(1-H_j)}{H_j + r(1-H_j)} \tag{11}$$

The effects of H_j and r on the level of error made when estimating ADO rates using methods 1 and 2 are presented in Fig. 1. The relative error made when using method 1 or 2 rapidly increases when H_j decreases and r increases. The level of amplification success (s) also influences the bias introduced by method ADO$_1$. Decreasing amplification success increases the relative error ε_1, which is in turn generally higher than the bias ε_2 introduced by method

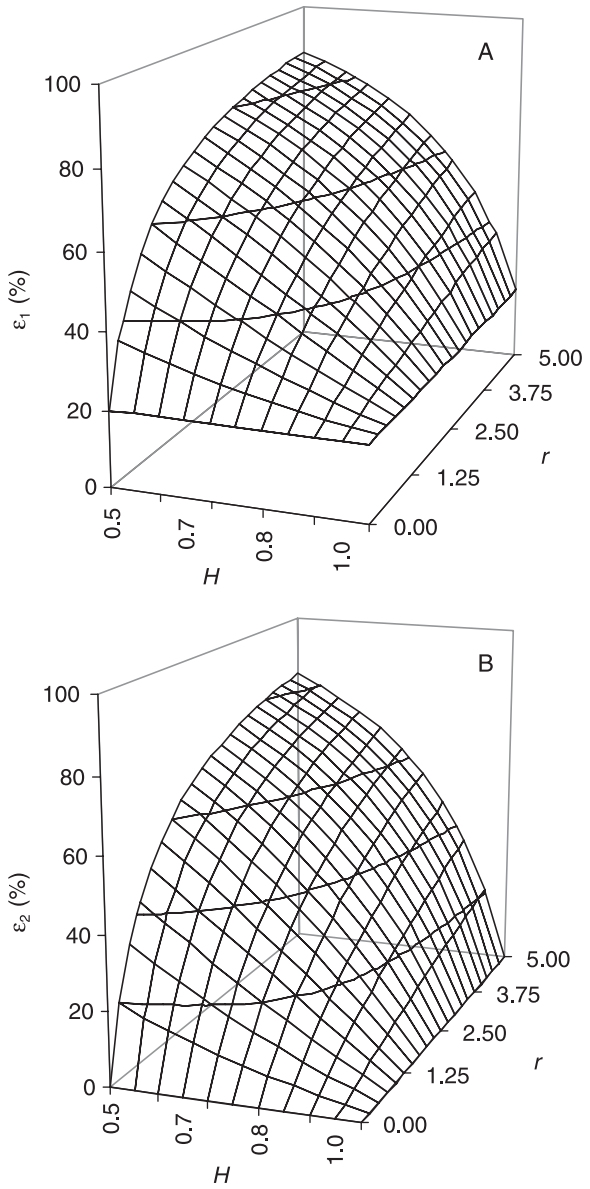

Fig. 1 Effect of heterozygosity (*H*) and of the ratio of repetitions of homozygous and heterozygous genotypes (*r*) on the relative error made when calculating the rate of allelic dropout with method 1 (A) and 2 (B). In (A), amplification success (*s*) was arbitrarily set to 0.8 as an example.

ADO_2. In Fig. 1(A), amplification success was arbitrarily set to 80% as an example. With this value the minimum relative error made when estimating p_j is 20%. Using methods 1 or 2, common values for *H* and *r* ($H = 0.7$ and $r = 1$), respectively, lead to a relative error of 44 or 30% on p_j estimate, whereas these values can reach 60 or 50% when $r = 7/3$. It is worth noting that repeating homozygous individuals to obtain reliable genotypes (multiple-tubes approach) also rapidly increases the underestimation of p_j when using methods 1 or 2.

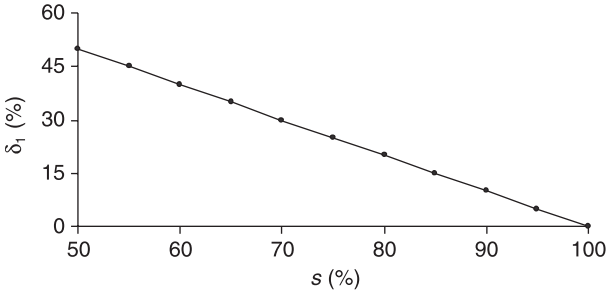

Fig. 2 Effect of amplification success (*s*) on the relative error made when calculating the frequency of false alleles with method 1.

The relative error obtained using method 1 for estimating f_j reduces to (see Appendix for details):

$$\delta_1 = \frac{f_j - f_j^1}{f_j} = 1 - s \qquad (12)$$

This bias depends solely on the level of amplification success (*s*) (Fig. 2), the underestimation of f_j being inversely proportional to *s*.

An example: re-analysis of ADO estimates reported by Smith et al. *(2000)*

Smith *et al*. thoroughly addressed the reliability of using human microsatellites in savannah baboons. They assessed the reliability of their genotypes using four different methods. In particular, to avoid any mistyping caused by ADO and FA, genotyping of heterozygous individuals was repeated 8–12 times, whereas homozygous genotypes were amplified 16 times. However, ADO frequencies were calculated following method ADO_2 (eqn 6; Susan Alberts & Kerri Smith, personal communication). As the authors reported the number of repetitions and locus-specific estimates of heterozygosity, we were able to quantify the bias associated with their estimates of ADO frequencies. Although limited by high levels of heterozygosity and by a low ratio of homozygous vs. heterozygous repetitions ($r = 1.6$), the bias in their estimates ranged from 5 to 30% (Table 2).

Conclusion

One may be interested in quantifying genotyping errors for several reasons. Strictly speaking, any approach presented here may be used to compare the efficiencies of given types of samples (e.g. Gagneux *et al.* 1997; Goossens *et al.* 1998), of strategies for the conservation of samples (e.g. Roon *et al.* 2003), or of extraction methods (e.g. Flagstad *et al.* 1999; Huber *et al.* 2003), as long as any potential bias is similar in every term in the comparison. For obvious reasons however, we recommend the use of unbiased estimates, either locus-specific (eqn 1 and 3) or allele-specific (Creel *et al.* 2003). Such methods become

Table 2 Re-analysis of error rates reported by Smith *et al.* (2000)

Locus	H (%)	$\hat{p}$ (%)	p (%)	ε_2 (%)
GATA164D10	97	69	72.42	4.72
GATA124D04	79	33	47.04	29.84
AFM240WA9	87	23	28.50	19.29
GATA144D04	87	37	45.85	19.29
GATA69D06	83	47	62.40	24.68

H and $\hat{p}$ are expected heterozygosity and ADO rate reported in Table 3 of Smith *et al.* who computed ADO following our eqn 6. Unbiased estimates, p, of ADO rates and bias ε_2 associated with the estimate of Smith *et al*. were computed following eqn 11, and assuming $r = 1.6$.

necessary if one wants to assess whether a single-tube approach is reliable (e.g. Goossens *et al*. 1998; Flagstad *et al.* 1999; Sloane *et al*. 2000; Huber *et al.* 2003) or to determine the number of repetitions needed in the multiple-tubes framework (e.g. Bayes *et al.* 2000; Ernest *et al.* 2000; Parsons 2001). Finally, the estimation of population size based on noninvasive sampling also requires the computation of accurate frequencies of genotyping error (Kohn *et al*. 1999; Creel *et al.* 2003; Frantz *et al*. 2003). We suggest that weighted averages of error rates (eqn 2 and 4) may be reported as a general trend to characterize the overall quality of data in a study, but that locus-specific estimates (eqn 1 and 3) must be chosen if one wants to use error rate estimates analytically, for instance to determine the number of repetitions required to achieve reliable genotypes.

Whatever computation method is chosen for the estimation of error rates, it should be clearly reported. Moreover, as the detection of FA is not straightforward, the definition adopted in papers should be reported (term F_j in eqn 3 and 4). Finally, an overall rate of genotyping error including both ADO and FA may only be computed over a limited sample (heterozygous genotypes only), and may not be representative of all genotypes, unless one wants to give an inaccurate but conservative rate of error.

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Appendix

Following eqn 1, the overall allelic dropout rate for L loci is the overall number of observed dropouts divided by the overall number of amplifications of heterozygous samples. Rearranging the equation shows that it corresponds to the weighted average $\bar{p}_w$ of p_j .

$$\frac{\sum_{j=1}^{L} D_j}{\sum_{j=1}^{L} A_{het_j}} = \frac{\sum_{j=1}^{L} p_j \cdot A_{het_j}}{\sum_{j=1}^{L} A_{het_j}} = \sum_{j=1}^{L} p_j \cdot \frac{A_{het_j}}{\sum_{j=1}^{L} A_{het_j}} = \bar{p}_w$$

The relative error obtained when estimating p_j using methods 1 or 2 is shown below.

Using method 1:

$$\varepsilon_1 = \frac{p_j - p_j^1}{p_j} = \frac{\frac{D_j}{A_{het_j}} - \frac{D_j}{P_j}}{\frac{D_j}{A_{het_j}}} = \frac{P_j - A_{het_j}}{P_j} = \frac{A_j - sA_{het_j}}{A_j}$$

With I_j = number of individuals genotyped at locus j, n = number of repetitions of positive amplifications of heterozygous individuals, and m = number of repetitions of positive amplifications of homozygous individuals, we have:

$$\varepsilon_1 = \frac{I_j(nH_j + m(1-H_j)) - snH_jI_j}{I_j(nH_j + m(1-H_j))} = \frac{nH_j(1-s) + m(1-H_j)}{nH_j + m(1-H_j)}$$

and if $r = \frac{m}{n}$

$$\text{then } \varepsilon_1 = \frac{H_j(1-s) + r(1-H_j)}{H_j + r(1-H_j)}$$

Similarly, for method 2 we have:

$$\varepsilon_2 = \frac{p_j - p_j^2}{p_j} = \frac{A_j - A_{het_j}}{A_j} = \frac{I_j(nH_j + m(1-H_j)) - nH_jI_j}{I_j(nH_j + m(1-H_j))}$$
$$= \frac{m(1-H_j)}{nH_j + m(1-H_j)} = \frac{r(1-H_j)}{H_j + r(1-H_j)}$$

The relative error obtained when estimating f_j using method 1 is given by:

$$\delta_1 = \frac{f_j - f_j^1}{f_j} = \frac{\frac{F_j}{A_j} - \frac{F_j}{P_j}}{\frac{F_j}{A_j}} = \frac{P_j - A_j}{P_j} = \frac{A_j - sA_j}{A_j} = 1 - s$$