Measuring genetic differentiation from Pool-seq data

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Abstract

The advent of high throughput sequencing and genotyping technologies enables the comparison of patterns of polymorphisms at a very large number of markers. While the characterization of genetic structure from individual sequencing data remains expensive for many non-model species, it has been shown that sequencing pools of individual DNAs (Pool-seq) represents an attractive and cost-effective alternative. However, analyzing sequence read counts from a DNA pool instead of individual genotypes raises statistical challenges in deriving correct estimates of genetic differentiation. In this article, we provide a method-of-moments estimator of F_{ST} for Pool-seq data, based on an analysis-of-variance framework. We show, by means of simulations, that this new estimator is unbiased, and outperforms previously proposed estimators. We evaluate the robustness of our estimator to model misspecification, such as sequencing errors and uneven contributions of individual DNAs to the pools. Finally, by reanalyzing published Pool-seq data of different ecotypes of the prickly sculpin *Cottus asper*, we show how the use of an unbiased F_{ST} estimator may question the interpretation of population structure inferred from previous analyses.

INTRODUCTION

It has long been recognized that the subdivision of species into subpopulations, social groups and families fosters genetic differentiation (Wahlund 1928; Wright 1931). Characterizing genetic differentiation as a means to infer unknown population structure is therefore fundamental to population genetics, and finds applications in multiple domains, including conservation biology, invasion biology, association mapping and forensics, among many others. In the late 1940s and early 1950s, Malécot (1948) and Wright (1951) introduced F -statistics to partition genetic variation within and between groups of individuals (Holsinger and Weir 2009; Bhatia et al. 2013). Since then, the estimation of F -statistics has become standard practice (see, e.g., Weir 1996; Weir and Hill 2002; Weir 2012), and the most commonly used estimators of F_{ST} have been developed in an analysis-of-variance framework (Cockerham 1969, 1973; Weir and Cockerham 1984), which can be recast in terms of probabilities of identity of pairs of homologous genes (Cockerham and Weir 1987; Rousset 2007; Weir and Goudet 2017).

Assuming that molecular markers are neutral, estimates of F_{ST} are typically used to quantify genetic structure in natural populations, which is then interpreted as the result of demographic history (Holsinger and Weir 2009): large F_{ST} values are expected for small populations among which dispersal is limited (Wright 1951), or between populations that have long diverged in isolation from each other (Reynolds et al. 1983); when dispersal is spatially restricted, a positive relationship between F_{ST} and the geographical distance for pairs of populations generally holds (Slatkin 1993; Rousset 1997). It has also been proposed to characterize the heterogeneity of F_{ST}

estimates across markers for identifying loci that are targeted by selection (Cavalli-Sforza 1966; Lewontin and Krakauer 1973; Beaumont and Nichols 1996; Vitalis et al. 2001; Akey et al. 2002; Beaumont 2005; Weir et al. 2005; Lotterhos and Whitlock 2014, 2015; Whitlock and Lotterhos 2015).

Next-generation sequencing (NGS) technologies provide unprecedented amounts of polymorphism data in both model and non-model species (Ellegren 2014). Although the sequencing strategy initially involved individually tagged samples in humans (The International HapMap Consortium 2005), whole-genome sequencing of pools of individuals (Pool-seq) is being increasingly popular for population genomic studies (Schlötterer et al. 2014). Because it consists in sequencing libraries of pooled DNA samples and does not require individual tagging of sequences, Pool-seq provides genome-wide polymorphism data at considerably lower cost than sequencing of individuals (Schlötterer et al. 2014). However, non-equimolar amounts of DNA from all individuals in a pool and stochastic variation in the amplification efficiency of individual DNAs have raised concerns with respect to the accuracy of the so-obtained allele frequency estimates, particularly at low sequencing depth and with small pool sizes (Cutler and Jensen 2010; Ellegren 2014; Anderson et al. 2014). Nonetheless, it has been shown that, at equal sequencing effort, Pool-seq provides similar, if not more accurate, allele frequency estimates than individual-based analyses (Futschik and Schlötterer 2010; Gautier et al. 2013). The problem is different for diversity and differentiation parameters, which depend on second moments of allele frequencies or, equivalently, on pairwise measures of genetic identity. With Pool-seq data, however, it is impossible to distinguish pairs of reads that are identical because they were

71 sequenced from a single gene, from pairs of reads that are identical because
 72 they were sequenced from two distinct genes that are identical in state (IIS)
 73 (Ferretti et al. 2013).

74 Appropriate estimators of diversity and differentiation parameters must
 75 therefore be sought, to account for both the sampling of individual genes
 76 from the pool and the sampling of reads from these genes. There has been
 77 several attempts to define estimators for the parameter F_{ST} for Pool-seq data
 78 (Kofler et al. 2011; Ferretti et al. 2013), from ratios of heterozygosities (or
 79 from probabilities of genetic identity between pairs of reads) within and be-
 80 tween pools. In the following, we will argue that these estimators are biased
 81 (i.e., they do not converge towards the expected value of the parameter),
 82 and that some of them have undesired statistical properties (i.e., the bias
 83 depends upon sample size and coverage). Here, following Cockerham (1969),
 84 Cockerham (1973), Weir and Cockerham (1984), Weir (1996), Weir and Hill
 85 (2002) and Rousset (2007), we define a method-of-moments estimator of the
 86 parameter F_{ST} using an analysis-of-variance framework. We then evaluate
 87 the accuracy and the precision of this estimator, based on the analysis of sim-
 88 ulated datasets, and compare it to estimates defined in the software package
 89 PoPoolation2 (Kofler et al. 2011), and in Ferretti et al. (2013). Furthermore,
 90 we test the robustness of our estimators to model misspecifications (including
 91 unequal contributions of individuals in pools, and sequencing errors). Finally,
 92 we reanalyze the prickly sculpin (*Cottus asper*) Pool-seq data (published by
 93 Dennenmoser et al. 2017), and show how the use of biased F_{ST} estimators in
 94 previous analyses may challenge the interpretation of population structure.

95 Note that throughout this article, we use the term “gene” to designate a

96 segregating genetic unit (in the sense of the “Mendelian gene” from Orgogozo
 97 et al. 2016). We further use the term “read” in a narrow sense, as a sequenced
 98 copy of a gene. For the sake of simplicity, we will use the term “Ind-seq” to
 99 refer to analyses based on individual data in which we further assume that
 100 individual genotypes are called without error.

MODEL

F -statistics may be described as intra-class correlations for the probability of identity in state (IIS) of pairs of genes (Cockerham and Weir 1987; Rousset 1996, 2007), and F_{ST} is best defined as:

$$F_{ST} \equiv \frac{Q_1 - Q_2}{1 - Q_2} \quad (1)$$

where Q_1 is the IIS probability for genes sampled within subpopulations, and Q_2 is the IIS probability for genes sampled between subpopulations. In the following, we develop an estimator of F_{ST} for Pool-seq data, by decomposing the total variance of gene frequencies in an analysis-of-variance framework. A complete derivation of the model is provided in the Supplemental File S1.

For the sake of clarity, the notation used throughout this article is given in Table 1. We first derive our model for a single locus, and eventually provide a multilocus estimator of F_{ST} . Consider a sample of n_d subpopulations, each of which is made of n_i genes ($i = 1, \dots, n_d$) sequenced in pools (hence n_i is the haploid sample size of the i th pool). We define c_{ij} as the number of reads sequenced from gene j ($j = 1, \dots, n_i$) in subpopulation i at the locus considered. Note that c_{ij} is a latent variable, that cannot be directly observed from the data. Let $X_{ijr:k}$ be an indicator variable for read r ($r = 1, \dots, c_{ij}$) from gene j in subpopulation i , such that $X_{ijr:k} = 1$ if the r th read from the j th gene in the i th deme is of type k , and $X_{ijr:k} = 0$ otherwise. In the following, we use standard dot notations for sample averages, i.e.: $X_{ij:k} \equiv \sum_r X_{ijr:k} / c_{ij}$, $X_{i:k} \equiv \sum_j \sum_r X_{ijr:k} / \sum_j c_{ij}$ and $X_{:k} \equiv \sum_i \sum_j \sum_r X_{ijr:k} / \sum_i \sum_j c_{ij}$. The analysis of variance is based on the computation of sums of squares, as fol-

123 lows:

$$\begin{aligned}
 \sum_i^{n_d} \sum_j^{n_i} \sum_r^{c_{ij}} (X_{ijr:k} - X_{\dots:k})^2 &= \sum_i^{n_d} \sum_j^{n_i} \sum_r^{c_{ij}} (X_{ijr:k} - X_{ij\cdot:k})^2 \\
 &+ \sum_i^{n_d} \sum_j^{n_i} \sum_r^{c_{ij}} (X_{ij\cdot:k} - X_{i\cdot\cdot:k})^2 \\
 &+ \sum_i^{n_d} \sum_j^{n_i} \sum_r^{c_{ij}} (X_{i\cdot\cdot:k} - X_{\dots:k})^2 \\
 &\equiv SSR_{:k} + SSI_{:k} + SSP_{:k} \quad (2)
 \end{aligned}$$

124 As is shown in the Supplemental File S1, the expected sums of squares depend
 125 on the expectation of the allele frequency π_k over all replicate populations
 126 sharing the same evolutionary history, as well as on the IIS probability $Q_{1:k}$
 127 that two genes in the same pool are both of type k , and the IIS probability
 128 $Q_{2:k}$ that two genes from different pools are both of type k . Taking expecta-
 129 tions (see the detailed computations in the Supplemental File S1), one has:

$$\mathbb{E}(SSR_{:k}) = 0 \quad (3)$$

130 for reads within individual genes, since we assume that there is no sequencing
 131 error, i.e. all the reads sequenced from a single gene are identical and $X_{ijr:k} =$
 132 $X_{ij\cdot:k}$ for all r . For reads between genes within pools, we get:

$$\mathbb{E}(SSI_{:k}) = (C_1 - D_2) (\pi_k - Q_{1:k}) \quad (4)$$

133 where $C_1 \equiv \sum_i \sum_j c_{ij} = \sum_j c_{ij}$ is the total number of reads in the full sample
 134 (total coverage), C_{1i} is the coverage of the i th pool and $D_2 \equiv \sum_i (C_{1i} + n_i - 1) / n_i$.
 135 D_2 arises from the assumption that the distribution of the read counts c_{ij}
 136 is multinomial (i.e., that all genes contribute equally to the pool of reads;

137 see Equation A15 in Supplemental File S1). For reads between genes from
138 different pools, we have:

$$\mathbb{E}(SSP_{:k}) = \left(C_1 - \frac{C_2}{C_1}\right) (Q_{1:k} - Q_{2:k}) + (D_2 - D_2^*) (\pi_k - Q_{1:k}) \quad (5)$$

139 where $C_2 \equiv \sum_i \left(\sum_j c_{ij}\right)^2$ and $D_2^* \equiv [\sum_i C_{1i} (C_{1i} + n_i - 1) / n_i] / C_1$ (see
140 Equation A16 in Supplemental File S1). Rearranging Equations 4–5, and
141 summing over alleles, we get:

$$Q_1 - Q_2 = \frac{(C_1 - D_2) \mathbb{E}(SSP) - (D_2 - D_2^*) \mathbb{E}(SSI)}{(C_1 - D_2) (C_1 - C_2/C_1)} \quad (6)$$

142 and:

$$1 - Q_2 = \frac{(C_1 - D_2) \mathbb{E}(SSP) + (n_c - 1) (D_2 - D_2^*) \mathbb{E}(SSI)}{(C_1 - D_2) (C_1 - C_2/C_1)} \quad (7)$$

143 where $n_c \equiv (C_1 - C_2/C_1) / (D_2 - D_2^*)$. Let $MSI \equiv SSI / (C_1 - D_2)$ and
144 $MSP \equiv SSP / (D_2 - D_2^*)$. Then:

$$F_{ST} \equiv \frac{Q_1 - Q_2}{1 - Q_2} = \frac{\mathbb{E}(MSP) - \mathbb{E}(MSI)}{\mathbb{E}(MSP) + (n_c - 1) \mathbb{E}(MSI)} \quad (8)$$

145 which yields the method-of-moments estimator:

$$\hat{F}_{ST}^{\text{pool}} = \frac{MSP - MSI}{MSP + (n_c - 1) MSI} \quad (9)$$

146 where

$$MSI = \frac{1}{C_1 - D_2} \sum_k \sum_i^{n_d} C_{1i} \hat{\pi}_{i:k} (1 - \hat{\pi}_{i:k}) \quad (10)$$

147 and:

$$MSP = \frac{1}{D_2 - D_2^*} \sum_k \sum_i^{n_d} C_{1i} (\hat{\pi}_{i:k} - \hat{\pi}_k)^2 \quad (11)$$

148 (see Equations A25 and A26 in Supplemental File S1). In Equations 10
149 and 11, $\hat{\pi}_{i:k} \equiv X_{i...k}$ is the average frequency of reads of type k within the i th
150 pool, and $\hat{\pi}_k \equiv X_{...:k}$ is the average frequency of reads of type k in the full sam-
151 ple. Note that from the definition of $X_{...:k}$, $\hat{\pi}_k \equiv \sum_i \sum_j \sum_r X_{ijr:k} / \sum_i \sum_j c_{ij} =$
152 $\sum_i C_{1i} \hat{\pi}_{i:k} / \sum_i C_{1i}$ is the weighted average of the sample frequencies with
153 weights equal to the pool coverage. This is equivalent to the weighted
154 analysis-of-variance in Cockerham (1973) (see also Weir and Cockerham 1984;
155 Weir 1996; Weir and Hill 2002; Rousset 2007; Weir and Goudet 2017). Fi-
156 nally, the full expression of $\hat{F}_{ST}^{\text{pool}}$ in terms of sample frequencies reads:

$$\hat{F}_{ST}^{\text{pool}} = \frac{\sum_k [(C_1 - D_2) \sum_i^{n_d} C_{1i} (\hat{\pi}_{i:k} - \hat{\pi}_k)^2 - (D_2 - D_2^*) \sum_i^{n_d} C_{1i} \hat{\pi}_{i:k} (1 - \hat{\pi}_{i:k})]}{\sum_k [(C_1 - D_2) \sum_i^{n_d} C_{1i} (\hat{\pi}_{i:k} - \hat{\pi}_k)^2 + (n_c - 1) (D_2 - D_2^*) \sum_i^{n_d} C_{1i} \hat{\pi}_{i:k} (1 - \hat{\pi}_{i:k})]} \quad (12)$$

157 If we take the limit case where each gene is sequenced exactly once, we
158 recover the Ind-seq model: assuming $c_{ij} = 1$ for all (i, j) , then $C_1 = \sum_i^{n_d} n_i$,
159 $C_2 = \sum_i^{n_d} n_i^2$, $D_2 = n_d$ and $D_2^* = 1$. Therefore, $n_c = (C_1 - C_2/C_1) / (n_d - 1)$,
160 and Equation 9 reduces exactly to the estimator of F_{ST} for haploids: see Weir
161 (1996), p. 182, and Rousset (2007), p. 977.

162 As in Reynolds et al. (1983), Weir and Cockerham (1984), Weir (1996)
163 and Rousset (2007), a multilocus estimate is derived as the sum of locus
164 specific numerators over the sum of locus-specific denominators:

$$\hat{F}_{ST} = \frac{\sum_l MSP_l - MSI_l}{\sum_l MSP_l + (n_c - 1) MSI_l} \quad (13)$$

165 where MSI and MSP are subscripted with l to denote the l th locus. For
166 Ind-seq data, Bhatia et al. (2013) refer to this multilocus estimate as a “ratio
167 of averages” by opposition to an “average of ratios”, which would consist in av-
168 eraging single-locus F_{ST} over loci. This approach is justified in the Appendix
169 of Weir and Cockerham (1984) and in Bhatia et al. (2013), who analyzed
170 both estimates by means of coalescent simulations. Note that Equation 13
171 assumes that the pool size is equal across loci. Also note that the construc-
172 tion of the estimator in Equation 13 is different from Weir and Cockerham’s
173 (1984). These authors defined their multilocus estimator as a ratio of sums
174 of components of variance (a , b and c in their notation) over loci, which give
175 the same weight to all loci, whatever the number of sampled genes at each
176 locus. Equation 13 follows GENEPOP’s rationale (Rousset 2008), which gives
177 instead more weight to loci that are more intensively covered.

MATERIALS AND METHODS

Simulation study

Generating individual genotypes: we first generated individual genotypes using `ms` (Hudson 2002), assuming an island model of population structure (Wright 1931). For each simulated scenario, we considered 8 demes, each made of $N = 5,000$ haploid individuals. The migration rate (m) was fixed to achieve the desired value of F_{ST} (0.05 or 0.2), using Equation 6 in Rousset (1996) leading, e.g., to $M \equiv 2Nm = 16.569$ for $F_{ST} = 0.05$ and $M = 3.489$ for $F_{ST} = 0.20$. The mutation rate was set at $\mu = 10^{-6}$, giving $\theta \equiv 2N\mu = 0.01$. We considered either fixed, or variable sample sizes across demes. In the latter case, the haploid sample size n was drawn independently for each deme from a Gaussian distribution with mean 100 and standard deviation 30; this number was rounded up to the nearest integer, with min. 20 and max. 300 haploids per deme. We generated a very large number of sequences for each scenario, and sampled independent single nucleotide polymorphisms (SNPs) from sequences with a single segregating site. Each scenario was replicated 50 times (500 times for Figures 3 and S2).

Pool sequencing: for each `ms` simulated dataset, we generated Pool-seq data by drawing reads from a binomial distribution (Gautier et al. 2013). More precisely, we assume that for each SNP, the number $r_{i:k}$ of reads of allelic type k in pool i follows:

$$r_{i:k} \sim \text{Bin}\left(\frac{y_{i:k}}{n_i}, \delta_i\right) \quad (14)$$

where $y_{i:k}$ is the number of genes of type k in the i th pool, n_i is the total number of genes in pool i (haploid pool size), and δ_i is the simulated total coverage for pool i . In the following, we either consider a fixed coverage, with $\delta_i = \Delta$ for all pools and loci, or a varying coverage across pools and loci, with $\delta_i \sim \text{Pois}(\Delta)$.

Sequencing error: we simulated sequencing errors occurring at rate $\mu_e = 0.001$, which is typical of Illumina sequencers (Glenn 2011; Ross et al. 2013). We assumed that each sequencing error modifies the allelic type of a read to one of three other possible states with equal probability (there are therefore four allelic types in total, corresponding to four nucleotides). Note that only biallelic markers are retained in the final datasets. Also note that, since we initiated this procedure with polymorphic markers only, we neglect sequencing errors that would create spurious SNPs from monomorphic sites. However, such SNPs should be rare in real datasets, since markers with a low minimum read count (MRC) are generally filtered out.

Experimental error: non-equimolar amounts of DNA from all individuals in a pool and stochastic variation in the amplification efficiency of individual DNAs are sources of experimental errors in pool sequencing. To simulate experimental errors, we used the model derived by Gautier et al. (2013). In this model, it is assumed that the contribution $\eta_{ij} = c_{ij}/C_{1i}$ of each gene j to the total coverage of the i th pool (C_{1i}) follows a Dirichlet distribution:

$$\{\eta_{ij}\}_{1 \leq j \leq n_i} \sim \text{Dir}\left(\frac{\rho}{n_i}\right) \quad (15)$$

where the parameter ρ controls the dispersion of gene contributions around the value $\eta_{ij} = 1/n_i$, expected if all genes contributed equally to the pool of reads. For convenience, we define the experimental error ϵ as the coefficient of variation of η_{ij} , i.e.: $\epsilon \equiv \sqrt{\mathbb{V}(\eta_{ij})}/\mathbb{E}(\eta_{ij}) = \sqrt{(n_i - 1)/(\rho + 1)}$ (see Gautier et al. 2013). When ϵ tends toward 0 (or equivalently when ρ tends to infinity), all individuals contribute equally to the pool, and there is no experimental error. We tested the robustness of our estimates to values of ϵ comprised between 0.05 and 0.5. The case $\epsilon = 0.5$ could correspond, for example, to a situation where (for $n_i = 10$) 5 individuals contribute $2.8\times$ more reads than the other 5 individuals.

Other estimators

For the sake of clarity, a summary of the notation of the F_{ST} estimators used throughout this article is given in Table 2.

PP2_d : this estimator of F_{ST} is implemented by default in the software package POPOOLATION2 (Kofler et al. 2011). It is based on a definition of the parameter F_{ST} as the overall reduction in average heterozygosity relative to the total combined population (see, e.g., Nei and Chesser 1983):

$$PP2_d \equiv \frac{\hat{H}_T - \hat{H}_S}{\hat{H}_T} \quad (16)$$

where $\hat{H}_S$ is the average heterozygosity within subpopulations, and $\hat{H}_T$ is the average heterozygosity in the total population (obtained by pooling together all subpopulation to form a single virtual unit). In POPOOLATION2, $\hat{H}_S$ is

240 the unweighted average of within-subpopulation heterozygosities:

$$\hat{H}_S = \frac{1}{n_d} \sum_i^{n_d} \left(\frac{n_i}{n_i - 1} \right) \left(\frac{C_{1i}}{C_{1i} - 1} \right) \left(1 - \sum_k \hat{\pi}_{i:k}^2 \right) \quad (17)$$

241 (using the notation from Table 1). Note that in PoPOOLATION2, PP2_d is
 242 restricted to the case of two subpopulations only ($n_d = 2$). The two ratios in
 243 the right-hand side of Equation 17 are presumably borrowed from Nei (1978)
 244 to provide an unbiased estimate, although we found no formal justification
 245 for the expression in Equation 17 for Pool-seq data. The total heterozygosity
 246 is computed as (using the notation from Table 1):

$$\hat{H}_T = \left(\frac{\min_i(n_i)}{\min_i(n_i) - 1} \right) \left(\frac{\min_i(C_{1i})}{\min_i(C_{1i}) - 1} \right) \left(1 - \sum_k \hat{\pi}_k^2 \right) \quad (18)$$

247 PP2_a : this is the alternative estimator of F_{ST} provided in the software
 248 package PoPOOLATION2. It is based on an interpretation by Kofler et al.
 249 (2011) of Karlsson et al.'s (2007) estimator of F_{ST} , as:

$$PP2_a \equiv \frac{\hat{Q}_1^r - \hat{Q}_2^r}{1 - \hat{Q}_2^r} \quad (19)$$

250 where $\hat{Q}_1^r$ and $\hat{Q}_2^r$ are the frequencies of identical pairs of reads within and
 251 between pools, respectively, computed by simple counting of IIS pairs. These
 252 are estimates of Q_1^r , the IIS probability for two reads in the same pool
 253 (whether they are sequenced from the same gene or not) and Q_2^r , the IIS
 254 probability for two reads in different pools. Note that the IIS probability Q_1^r
 255 is different from Q_1 in Equation 1, which, from our definition, represents
 256 the IIS probability between distinct genes in the same pool. This approach
 257 therefore confounds pairs of reads within pools that are identical because

they were sequenced from a single gene, from pairs of reads that are identical because they were sequenced from distinct, yet IIS genes.

FRP₁₃ : this estimator of F_{ST} was developed by Ferretti et al. (2013) (see their Equations 3 and 10–13). Ferretti et al. (2013) use the same definition of F_{ST} as in Equation 16 above, although they estimate heterozygosities within and between pools as “average pairwise nucleotide diversities”, which, from their definitions, are formally equivalent to IIS probabilities. In particular, they estimate the average heterozygosity within pools as (using the notation from Table 1):

$$\hat{H}_S = \frac{1}{n_d} \sum_i^{n_d} \left(\frac{n_i}{n_i - 1} \right) (1 - \hat{Q}_{1i}^r) \quad (20)$$

and the total heterozygosity among the n_d populations as:

$$\hat{H}_T = \frac{1}{n_d^2} \left[\sum_i^{n_d} \left(\frac{n_i}{n_i - 1} \right) (1 - \hat{Q}_{1i}^r) + \sum_{i \neq i'}^{n_d} (1 - \hat{Q}_{2ii'}^r) \right] \quad (21)$$

Analyses of Ind-seq data:

For the comparison of Ind-seq and Pool-seq datasets, we computed F_{ST} on subsamples of 5,000 loci. These subsamples were defined so that only those loci that were polymorphic in all coverage conditions were retained, and the same loci were used for the analysis of the corresponding Ind-seq data. For the latter, we used either the Nei and Chesser’s (1983) estimator based on a ratio of heterozygosity (see Equation 16 above), hereafter denoted by NC₈₃, or the analysis-of-variance estimator developed by Weir and Cockerham (1984), hereafter denoted by WC₈₄.

All the estimators were computed using custom functions in the R soft-

278 were environment for statistical computing, version 3.3.1 (R Core Team
279 2017). All these functions were carefully checked against available software
280 packages, to ensure that they provided strictly identical results.

281 **Application example: *Cottus asper***

282 Dennenmoser et al. (2017) investigated the genomic basis of adaption to
283 osmotic conditions in the prickly sculpin (*Cottus asper*), an abundant eury-
284 haline fish in northwestern North America. To do so, they sequenced the
285 whole-genome of pools of individuals from two estuarine populations (CR,
286 Capilano River Estuary; FE, Fraser River Estuary) and two freshwater pop-
287 ulations (PI, Pitt Lake and HZ, Hatzic Lake) in southern British Columbia
288 (Canada). We downloaded the four corresponding BAM files from the Dryad
289 Digital Repository (doi: 10.5061/dryad.2qg01) and combined them into a sin-
290 gle mpileup file using **SAMtools** version 0.1.19 (Li et al. 2009) with default
291 options, except the maximum depth per BAM that was set to 5,000 reads.
292 The resulting file was further processed using a custom **awk** script, to call
293 SNPs and compute read counts, after discarding bases with a Base Align-
294 ment Quality (BAQ) score lower than 25. A position was then considered
295 as a SNP if: (i) only two different nucleotides with a read count > 1 were
296 observed (nucleotides with ≤ 1 read being considered as a sequencing error);
297 (ii) the coverage was comprised between 10 and 300 in each of the four align-
298 ment files; (iii) the minor allele frequency, as computed from read counts,
299 was ≥ 0.01 in the four populations. The final data set consisted of 608,879
300 SNPs.

301 Our aim here was to compare the population structure inferred from pair-
302 wise estimates of F_{ST} , using the estimator $\hat{F}_{ST}^{pool}$ on the one hand, and PP2_d

on the other hand. Then, to conclude on which of the two estimators performs better, we compared the population structure inferred from $\hat{F}_{ST}^{pool}$ and PP2_d to that inferred from the Bayesian hierarchical model implemented in the software package BAYPASS (Gautier 2015). BAYPASS allows indeed the robust estimation of the scaled covariance matrix of allele frequencies across populations for Pool-seq data, which is known to be informative about population history (Pickrell and Pritchard 2012). The elements of the estimated matrix can be interpreted as pairwise and population-specific estimates of differentiation (Coop et al. 2010), and therefore provide a comprehensive description of population structure that makes full use of the available data.

Data availability

The authors state that all data necessary for confirming the conclusions presented in this article are fully represented within the article, figures, and tables. Supplemental Tables S1–S3 and Figures S1–S4 are available at FigShare, along with a complete derivation of the model in the Supplemental File S1 at FigShare.

RESULTS

Comparing Ind-seq and Pool-seq estimates of F_{ST}

Single-locus estimates $\hat{F}_{ST}^{pool}$ are highly correlated with the classical estimates WC_{84} (Weir and Cockerham 1984) computed on the individual data that were used to generate the pools in our simulations (see Figure 1). The variance of $\hat{F}_{ST}^{pool}$ across independent replicates decreases as the coverage increases. The correlation between $\hat{F}_{ST}^{pool}$ and WC_{84} is stronger for multilocus estimates (see Figure S1A).

Comparing Pool-seq estimators of F_{ST}

We found that our estimator $\hat{F}_{ST}^{pool}$ has extremely low bias ($< 0.5\%$ over all scenarios tested: see Tables 3 and S1-S3). In other words, the average estimates across multiple loci and replicates closely equals the expected value of the F_{ST} parameter, as given by Equation 6 in Rousset (1996), which is based on the computation of IIS probabilities in an island model of population structure. In all the situations examined, the bias did neither depend on the sample size (i.e., the size of each pool) nor on the coverage (see Figure 2). Only the variance of the estimator across independent replicates decreases as the sample size increases and/or as the coverage increases. At high coverage, the mean and root mean squared error (RMSE) of $\hat{F}_{ST}^{pool}$ over independent replicates are virtually indistinguishable from that of the WC_{84} estimator (see Table S1).

Figure 3 shows the RMSE of F_{ST} estimates for a wide range of pool sizes and coverage. The RMSE decreases as the pool size and/or the coverage increases. The F_{ST} estimates are more precise and accurate when differen-

343 tiation is low. Figure 3 provides some clues to evaluate the pool size and
 344 the coverage that is necessary to achieve the same RMSE than for Ind-seq
 345 data. Consider, for example, the case of samples of $n = 20$ haploids. For
 346 $F_{ST} \leq 0.05$ (in the conditions of our simulations), the RMSE of F_{ST} estimates
 347 based on Pool-seq data tends to the RMSE of F_{ST} estimates based on Ind-seq
 348 data either by sequencing pools of ca. 200 haploids at 20X, or by sequencing
 349 pools of 20 haploids at ca. 200X. However, the same precision and accuracy
 350 are achieved by sequencing ca. 50 haploids at ca. 50X.

351 Conversely, we found that PP2_d (the default estimator of F_{ST} imple-
 352 mented in the software package POPOOLATION2) is biased when compared
 353 to the expected value of the parameter. We observed that the bias depends
 354 on both the sample size, and the coverage (see Figure 2). We note that, as the
 355 coverage and the sample size increase, PP2_d converges to the estimator NC₈₃
 356 (Nei and Chesser 1983) computed from individual data (see Figure S1B).
 357 This argument was used by Kofler et al. (2011) to validate the approach,
 358 even though the estimates PP2_d depart from the true value of the parameter
 359 (Figure S1B–C).

360 The second of the two estimators of F_{ST} implemented in POPOOLATION2,
 361 that we refer to as PP2_a, is also biased (see Figure 2). We note that the bias
 362 decreases as the sample size increases. However, the bias does not depend
 363 on the coverage (only the variance over independent replicates does). The
 364 estimator developed by Ferretti et al. (2013), that we refer to as FRP₁₃, is
 365 also biased (see Figure 2). However, the bias does neither depend on the pool
 366 size, nor on the coverage (only the variance over independent replicates does).
 367 FRP₁₃ converges to the estimator NC₈₃, computed from individual data (see

Figure 2). At high coverage, the mean and RMSE over independent replicates are virtually indistinguishable from that of the NC_{83} estimator.

Last, we stress out that our estimator $\hat{F}_{ST}^{pool}$ provides estimates for multiple populations, and is therefore not restricted to pairwise analyses, contrary to PoPOOLATION2's estimators. We show that, even at low sample size and low coverage, Pool-seq estimates of differentiation are virtually indistinguishable from classical estimates for Ind-seq data (see Table 3).

Robustness to unbalanced pool sizes and variable sequencing coverage

We evaluated the accuracy and the precision of the estimator $\hat{F}_{ST}^{pool}$ when sample sizes differ across pools, and when the coverage varies across pools and loci (see Figure 4). We found that, at low coverage, unequal sampling or variable coverage causes a negligible departure from the median of WC_{84} estimates computed on individual data, which vanishes as the coverage increases. At 100X coverage, the distribution of $\hat{F}_{ST}^{pool}$ estimates is almost indistinguishable from that of WC_{84} (see Figure 4 and Tables S2–S3).

Robustness to sequencing and experimental errors

Figure 5 shows that sequencing errors cause a negligible negative bias for $\hat{F}_{ST}^{pool}$ estimates. Filtering (using a minimum read count of 4) improves estimation slightly, but only at high coverage (Figure 6B). It must be noted, though, that filtering increases the bias in the absence of sequencing error, especially at low coverage (Figure 6A). With experimental error, i.e., when individuals do not contribute evenly to the final set of reads, we observed a positive bias for $\hat{F}_{ST}^{pool}$ estimates (Figure 5). We note that the bias decreases

as the size of the pools increases. Figure S2 shows the RMSE of F_{ST} estimates for a wider range of pool sizes, coverage and experimental error rate. For $\epsilon \geq 0.25$, increasing the coverage cannot improve the quality of the inference, if the pool size is too small. When Pool-seq experiments are prone to large experimental error rates, increasing the size of pools is the only way to improve the estimation of F_{ST} . Filtering (using a minimum read count of 4) does not improve estimation (Figure 6C).

Application example

The reanalysis of the prickly sculpin data revealed larger pairwise estimates of multilocus F_{ST} using PP2_d estimator, as compared to $\hat{F}_{ST}^{pool}$ (see Figure 7A). Furthermore, we found that $\hat{F}_{ST}^{pool}$ estimates are smaller for within-ecotype pairwise comparisons as compared to between-ecotype comparisons. Therefore, the inferred relationships between samples based on pairwise $\hat{F}_{ST}^{pool}$ estimates show a clear-cut structure, separating the two estuarine samples from the freshwater ones (see Figure 7C). We did not recover the same structure using PP2_d estimates (see Figure 7B). Supportingly, the scaled covariance matrix of allele frequencies across samples is consistent with the structure inferred from $\hat{F}_{ST}^{pool}$ estimates (see Figure 7D).

DISCUSSION

Whole-genome sequencing of pools of individuals is being increasingly popular for population genomic research on both model and non-model species (Schlötterer et al. 2014). The development of dedicated software packages (reviewed in Schlötterer et al. 2014) has undoubtedly something to do with the breadth of research questions that have been tackled using pool-sequencing. Yet, the analysis of population structure from Pool-seq data is complicated by the double sampling process of genes from the pool and sequence reads from those genes (Ferretti et al. 2013).

The naive approach that consists in computing F_{ST} from read counts, as if they were allele counts (e.g., as in Chen et al. 2016), ignores the extra variance brought by the random sampling of reads from the gene pool during Pool-seq experiments. Furthermore, such computation fails to consider the actual number of lineages in the pool (haploid pool size). Altogether, these limits may result in severely biased estimates of differentiation when the pool size is low (see Figure S3). A possible alternative is to compute F_{ST} from allele counts imputed from read counts using a maximum-likelihood approach conditional on the haploid size of the pools (e.g., as in Smadja et al. 2012; Leblois et al. 2018), or from allele frequencies estimated using a model-based method that accounts for the sampling effects and the sequencing error probabilities inherent to pooled NGS experiments (see Fariello et al. 2017). However, these latter approaches may only be accurate in situations where the coverage is much larger than pool size, allowing to reduce sampling variance of reads (see Figure S3).

Here, we therefore developed a new estimator of the parameter F_{ST} for

435 Pool-seq data, in an analysis-of-variance framework (Cockerham 1969, 1973).
 436 The accuracy of this estimator is barely distinguishable from that of the
 437 Weir and Cockerham's (1984) estimator for individual data. Furthermore,
 438 does neither depend on the pool size nor on the coverage, and is robust
 439 to unequal pool sizes and varying coverage across demes and loci. In our
 440 analysis the frequency of reads within pools is a weighted average of the
 441 sample frequencies with weights equal to the pool coverage. Therefore, our
 442 approach follows Cockerham's (1973) one, which he referred to as a weighted
 443 analysis-of-variance (see also Weir and Cockerham 1984; Weir 1996; Weir and
 444 Hill 2002; Weir and Goudet 2017).

445 With unequal pool sizes, weighted and unweighted analyses differ. As dis-
 446 cussed recently in Weir and Goudet (2017), the unweighted approach seems
 447 appropriate when the between component exceed the within component, i.e.
 448 when F_{ST} is large (Tukey 1957). It turns out that optimal weighting depends
 449 upon the parameter to be estimated (Cockerham 1973) and is only efficient
 450 at lower levels of differentiation (Robertson 1962). In a likelihood analysis
 451 of the island model, Rousset (2007) derived asymptotically efficient weights
 452 that are proportional to n_i^2 for the sum of squares of different samples (i.e.,
 453 as in Robertson 1962). To the best of our knowledge, such optimal weighting
 454 has never been considered in the literature. Nevertheless, if these arguments
 455 are true for estimators of variance components, they do not necessarily apply
 456 to estimates of intra-class correlations (Cockerham 1973).

457 **Analysis of variance and probabilities of identity**

458 In the analysis-of-variance framework, F_{ST} is defined in Equation 1 as an
 459 intraclass correlation for the probability of identity in state (Cockerham and

Weir 1987; Rousset 1996). Extensive statistical literature is available on estimators of intraclass correlations. Beside analysis-of-variance estimators, introduced in population genetics by Cockerham (1969, 1973), estimators based on the computation of probabilities of identical response within and between groups have been proposed (see, e.g., Fleiss 1971; Fleiss and Cuzick 1979; Mak 1988; Ridout et al. 1999; Wu et al. 2012), which were originally referred to as kappa-type statistics (Fleiss 1971; Landis and Koch 1977). These estimators have later been endorsed in population genetics, where the “probability of identical response” was then interpreted as the frequency with which the genes are alike (Cockerham 1973; Cockerham and Weir 1987; Weir 1996; Rousset 2007; Weir and Goudet 2017).

This suggests that, with Pool-seq data, another strategy could consist in computing F_{ST} from IIS probabilities between (unobserved) pairs of genes, which requires that unbiased estimates of such quantities are derived from read count data. We have done so in the second section of the Supplemental File S1, and we provide alternative estimators of F_{ST} for Pool-seq data (see Equations A44 and A48 in Supplemental File S1). These estimators (denoted by $\hat{F}_{ST}^{\text{pool-PID}}$ and $\tilde{F}_{ST}^{\text{pool-PID}}$) have exactly the same form as the analysis-of-variance estimator if the pools have all the same size and if the number of reads per pool is constant (Equation A33). This echoes the derivations by Rousset (2007) for Ind-seq data, who showed that the analysis-of-variance approach (Weir and Cockerham 1984) and the simple strategy of estimating IIS probabilities by counting identical pairs of genes provide identical estimates when sample sizes are equal (see Equation A28 and also Cockerham and Weir 1987; Weir 1996; Karlsson et al. 2007). With unbalanced samples, we

found that analysis-of-variance estimates have better precision and accuracy than IIS-based estimates, particularly for low levels of differentiation (see Figure S4). Interestingly, we found that IIS-based estimates of F_{ST} for Pool-seq data have generally lower bias and variance if the overall estimates of IIS probabilities within and between pools are computed as unweighted averages of population-specific or pairwise estimates (see Equations A39 and A43), as compared to weighted averages. Equation A28 further shows that our estimator may be rewritten as a function close to $(\hat{Q}_1 - \hat{Q}_2) / (1 - \hat{Q}_2)$, except that it also depends on the sums $\sum_i (\hat{Q}_{1i} - \hat{Q}_1)$ in both the numerator and the denominator. This suggests that if the Q_{1i} 's differ among subpopulations, then our estimator provides an estimate of an average of population-specific F_{ST} (Weir and Hill 2002; Weir and Goudet 2017).

It follows from the derivations in the Supplemental File S1 that the estimator PP2_a (Equation 19) is biased, because the IIS probability between pairs of reads within a pool $(\hat{Q}_1^r)$ is a biased estimator of the IIS probability between pairs of distinct genes in that pool (see Equation A34 in Supplemental File S1). This is so, because the former confounds pairs of reads that are identical because they were sequenced from a single gene copy, from pairs of reads that are identical because they were sequenced from distinct, yet IIS genes.

A more justified estimator of F_{ST} has been proposed by Ferretti et al. (2013), based on previous developments by Futschik and Schlötterer (2010). Note that, although they defined F_{ST} as a ratio of functions of heterozygosities, they actually worked with IIS probabilities (see Equations 20 and 21). However, although their Equation 20 is strictly identical to our Equation A34

in Supplemental File S1, we note that they computed the total heterozygosity by integrating over pairs of genes sampled both within and between populations (see Equation 21), which may explain the observed bias (see Figure 2).

Comparison with alternative estimators

An alternative framework to Weir and Cockerham’s (1984) analysis-of-variance has been developed by Masatoshi Nei and coworkers to estimate F_{ST} from gene diversities (Nei 1973, 1977; Nei and Chesser 1983; Nei 1986). The estimator $PP2_d$ (see Equations 16–18) implemented in the software package **POPOOLATION2** (Kofler et al. 2011) follows this logic. However, it has long been recognized that both frameworks are fundamentally different in that the analysis-of-variance approach considers both statistical and genetic (or evolutionary) sampling, whereas Nei and coworkers’ approach do not (Weir and Cockerham 1984; Excoffier 2007; Holsinger and Weir 2009). Furthermore, the expectation of Nei and coworkers’ estimators depend upon the number of sampled populations, with a larger bias for lower numbers of sampled populations (Goudet 1993; Excoffier 2007; Weir and Goudet 2017). This is so, because the computation of the total diversity in Equations 18 and 21 includes the comparison of pairs of genes from the same subpopulation, whereas the computation of IIS probabilities between subpopulations do not (see, e.g., Excoffier 2007). Therefore, we do not recommend using the estimator $PP2_d$ implemented in the software package **POPOOLATION2** (Kofler et al. 2011).

Applications in evolutionary ecology studies

Pool-seq is being increasingly used in many application domains (Schlötterer et al. 2014), such as conservation genetics (see, e.g., Fuentes-Pardo 2017),

invasion biology (see, e.g., Dexter et al. 2018) and evolutionary biology in a broader sense (see, e.g., Collet et al. 2016). These studies use a large range of methods, which aim at characterizing fine-scaled population structure (see, e.g., Fischer et al. 2017), reconstructing past demography (see, e.g., Chen et al. 2016; Leblois et al. 2018), or identifying footprints of natural or artificial selection (see, e.g., Chen et al. 2016; Fariello et al. 2017; Leblois et al. 2018).

Here, we reanalyzed the Pool-seq data produced by Dennenmoser et al. (2017), who investigated the adaptive genomic divergence between freshwater and brackish-water ecotypes of the prickly sculpin *C. asper*, an abundant euryhaline fish in northwestern North America. Measuring pairwise genetic differentiation between samples using $\hat{F}_{ST}^{pool}$, we found a clear-cut structure separating the freshwater from the brackish-water ecotypes. Such genetic structure supports the hypothesis that populations are locally adapted to osmotic conditions in these two contrasted habitats, as discussed in Dennenmoser et al. (2017). This structure, which is at odds with that inferred from PP2_d estimates, is not only supported by the scaled covariance matrix of allele frequencies, but also by previous microsatellite-based studies, who showed that populations were genetically more differentiated between ecotypes than within ecotypes (Dennenmoser et al. 2014, 2015).

Limits of the model and perspectives

We have shown that the stronger source of bias for the $\hat{F}_{ST}^{pool}$ estimate is unequal contributions of individuals in pools. This is so, because we assume in our model that the read counts are multinomially distributed, which supposes that all genes contribute equally to the pool of reads (Gautier et al. 2013), i.e. that there is no variation in DNA yield across individuals and that all

genes have equal sequencing coverage (Rode et al. 2018). Because the effect of unequal contribution is expected to be stronger with small pool sizes, it has been recommended to use pool-seq with at least 50 diploid individuals per pool (Lynch et al. 2014; Schlötterer et al. 2014). However, this limit may be overly conservative for allele frequency estimates (Rode et al. 2018), and we have shown here that we can achieve very good precision and accuracy of F_{ST} estimates with smaller pool sizes. Furthermore, because genotypic information is lost during Pool-seq experiments, we assume in our derivations that pools are haploid (and therefore that F_{IS} is nil). Analyzing non-random mating populations (e.g., in selfing species) is therefore problematic.

Finally, our model, as in Weir and Cockerham (1984), formally assumes that all populations provide independent replicates of some evolutionary process (Excoffier 2007; Holsinger and Weir 2009). This may be unrealistic in many natural populations, which motivated Weir and Hill (2002) to derive a population-specific estimator of F_{ST} for Ind-seq data (see also Vitalis et al. 2001). Even though the use of Weir and Hill’s (2002) estimator is still scarce in the literature (but see Weir et al. 2005; Vitalis 2012), Weir and Goudet (2017) recently proposed a re-interpretation of population-specific estimates of F_{ST} in terms of allelic matching proportions, which are strictly equivalent to IIS probabilities between pairs of genes. It would therefore be straightforward to extend Weir and Goudet’s (2017) estimator of population-specific F_{ST} for the analysis of Pool-seq data, using the unbiased estimates of IIS probabilities provided in the Supplemental File S1.

DATA ACCESSIBILITY

A R package, called `poolfstat`, which implements F_{ST} estimates for Pool-seq data, is available at the Comprehensive R Archive Network (CRAN): <https://cran.r-project.org/web/packages/poolfstat/index.html>.

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Table 1 Summary of main notations

Notation	Parameter definition
$X_{ijr:k}$	Indicator variable: $X_{ijr:k} = 1$ if the r th read from the j th individual in the i th pool is of type k , and $X_{ijr:k} = 0$ otherwise
$r_{i:k} = \sum_j \sum_r X_{ijr:k}$	Number of reads of type k in the i th pool
c_{ij}	Number of reads sequenced from individual j in sub-population i (unobserved individual coverage)
$C_{1i} \equiv \sum_j c_{ij}$	Total number of reads in the i th pool (pool coverage)
$C_1 \equiv \sum_i C_{1i}$	Total number of reads in the full sample (total coverage)
$C_2 \equiv \sum_i C_{1i}^2$	Squared number of reads in the full sample
n_i	Total number of genes the i th pool (haploid pool size)
$y_{i:k}$	(Unobserved) number of genes of type k in the i th pool
$\pi_k \equiv \mathbb{E}(X_{ijr:k})$	Expected frequency of reads of type k in the full sample
$\hat{\pi}_{ij:k} \equiv X_{ij\cdot:k}$	(Unobserved) average frequency of reads of type k for individual j in the i th pool
$\hat{\pi}_{i:k} \equiv X_{i\cdot\cdot:k}$	Average frequency of reads of type k in the i th pool
$\hat{\pi}_k \equiv X_{\dots:k}$	Average frequency of reads of type k in the full sample
Q_1 (resp. Q_2)	IIS probability for two genes sampled within (resp. between) pools
Q_1^r (resp. Q_2^r)	IIS probability for two reads sampled within (resp. between) pools
$\hat{Q}_1^{\text{pool}}$ (resp. $\hat{Q}_2^{\text{pool}}$)	Unbiased estimator of the IIS probability for genes sampled within (resp. between) populations

Table 2 Definition of the F_{ST} estimators used in the text

Notation	Definition
$\hat{F}_{ST}^{pool}$	Equation 9
FRP ₁₃	Ferretti et al. (2013) and Equations 16,20–21
NC ₈₃	Nei and Chesser (1983)
PP2 _d	Kofler et al. (2011) and Equations 16–18
PP2 _a	Kofler et al. (2011) and Equation 19
WC ₈₄	Weir and Cockerham (1984)

Table 3 Overall F_{ST} estimates from multiple pools

F_{ST}	n	Pool-seq		Ind-seq
		Cov.	$\hat{F}_{ST}^{pool}$	WC ₈₄
0.05	10	20×	0.050 (0.002)	
0.05	10	50×	0.051 (0.002)	0.050 (0.002)
0.05	10	100×	0.050 (0.002)	
0.05	100	20×	0.050 (0.001)	
0.05	100	50×	0.050 (0.001)	0.051 (0.001)
0.05	100	100×	0.050 (0.001)	
0.20	10	20×	0.200 (0.002)	
0.20	10	50×	0.201 (0.002)	0.201 (0.002)
0.20	10	100×	0.201 (0.002)	
0.20	100	20×	0.201 (0.003)	
0.20	100	50×	0.202 (0.003)	0.203 (0.003)
0.20	100	100×	0.203 (0.003)	

Overall F_{ST} was estimated for various conditions of expected F_{ST} , pool size (n) and coverage (Cov.). For Pool-seq data, we computed our estimator $\hat{F}_{ST}^{pool}$ (Equation 13). The mean (RMSE) over 50 independent replicates of the **ms** simulations are provided, for all populations ($n_d = 8$). For comparison, we computed WC₈₄ from allele count data inferred from individual genotypes (Ind-seq).

Figure 1 Single-locus estimates of F_{ST} . We compared single-locus estimates of F_{ST} based on allele count data inferred from individual genotypes (Ind-seq), using the WC_{84} estimator, to $\hat{F}_{ST}^{pool}$ estimates from Pool-seq data. We simulated 5,000 SNPs using *ms* in an island model with $n_d = 8$ demes. We used two migration rates corresponding to $F_{ST} = 0.05$ (A) and $F_{ST} = 0.20$ (B). The size of each pool was fixed to 100. We show the results for different coverages (20X, 50X and 100X). In each graph, the cross indicates the simulated value of F_{ST} .

Figure 2 Precision and accuracy of pairwise estimators of F_{ST} . We considered two estimators based on allele count data inferred from individual genotypes (Ind-seq): WC_{84} and NC_{83} . For pooled data, we computed the two estimators implemented in the software package POPOOLATION2, that we refer to as $PP2_d$ and $PP2_a$, as well as the FRP_{13} estimator and our estimator $\hat{F}_{ST}^{pool}$ (Equation 13). Each boxplot represents the distribution of multilocus F_{ST} estimates across all pairwise comparisons in an island model with $n_d = 8$ demes, and across 50 independent replicates of the `ms` simulations. We used two migration rates, corresponding to $F_{ST} = 0.05$ (A–B) or $F_{ST} = 0.20$ (C–D). The size of each pool was either fixed to 10 (A and C) or to 100 (B and D). For Pool-seq data, we show the results for different coverages (20X, 50X and 100X). In each graph, the dashed line indicates the simulated value of F_{ST} and the dotted line indicates the median of the distribution of NC_{83} estimates.

Figure 3 Root mean squared error (RMSE) of F_{ST} estimates for a wide range of pool sizes and coverage, with F_{ST} varying from 0.005 to 0.2 (A–F). Each density plot gives the RMSE of our estimator $\hat{F}_{ST}^{pool}$, using simple linear interpolation from a set of 44×44 pairs of pool size and coverage values. For each pool size and coverage, 500 replicates of 5,000 markers were simulated. Plain white isolines represent the RMSE of the WC_{84} estimator computed from Ind-seq data, for various sample sizes ($n = 5, 10, 20, \text{ and } 50$). Each isline was fitted using a thin plate spline regression with smoothing parameter $\lambda = 0.005$, implemented in the `fields` package for R (Nychka et al. 2017).

Figure 4 Precision and accuracy of F_{ST} estimates with varying pool size or varying coverage. Our estimator $\hat{F}_{ST}^{pool}$ (Equation 13) was calculated from Pool-seq data over all loci and demes and compared to the estimator WC_{84} , computed from allele count data inferred from individual genotypes (Ind-seq). Each boxplot represents the distribution of multilocus F_{ST} estimates across 50 independent replicates of the **ms** simulations. We used two migration rates, corresponding to $F_{ST} = 0.05$ (A and C) or $F_{ST} = 0.20$ (B and D). In A–B the pool size was variable across demes, with haploid sample size n drawn independently for each deme from a Gaussian distribution with mean 100 and standard deviation 30; n was rounded up to the nearest integer, with min. 20 and max. 300 haploids per deme. In C–D, the pool size was fixed ($n = 100$), and the coverage (δ_i) was varying across demes and loci, with $\delta_i \sim \text{Pois}(\Delta)$ where $\Delta \in \{20, 50, 100\}$. For Pool-seq data, we show the results for different coverages (20X, 50X and 100X). In each graph, the dashed line indicates the simulated value of F_{ST} and the dotted line indicates the median of the distribution of WC_{84} estimates.

Figure 5 Precision and accuracy of F_{ST} estimates with sequencing and experimental errors. Our estimator $\hat{F}_{ST}^{pool}$ (Equation 13) was computed from Pool-seq data over all loci and demes without error, with sequencing error (occurring at rate $\mu_e = 0.001$), and with experimental error ($\epsilon = 0.5$). Each boxplot represents the distribution of multilocus F_{ST} estimates across 50 independent replicates of the **ms** simulations. We used two migration rates, corresponding to $F_{ST} = 0.05$ (A–B) or $F_{ST} = 0.20$ (C–D). The size of each pool was either fixed to 10 (A and C) or to 100 (B and D). For Pool-seq data, we show the results for different coverages (20X, 50X and 100X). In each graph, the dashed line indicates the simulated value of F_{ST} .

Figure 6 Precision and accuracy of F_{ST} estimates with and without filtering. Our estimator $\hat{F}_{ST}^{\text{pool}}$ (Equation 13) was computed from Pool-seq data over all loci and demes without error (A), with sequencing error (B) and with experimental error (C) (see the legend of Figure 5 for further details). For each case, we computed F_{ST} without filtering (no MRC) and with filtering (using a minimum read count $MRC = 4$). Each boxplot represents the distribution of multilocus F_{ST} estimates across 50 independent replicates of the **ms** simulations. We used a migration rate corresponding to $F_{ST} = 0.20$, and pool size $n = 10$. We show the results for different coverages (20X, 50X and 100X). In each graph, the dashed line indicates the simulated value of F_{ST} .

Figure 7 Analysis of the prickly sculpin (*Cottus asper*) Pool-seq data. In (A) we compare the pairwise F_{ST} estimates $PP2_d$, and $\hat{F}_{ST}^{pool}$ (Equation 13) for all pairs of populations from the estuarine (CR and FE) and freshwater samples (PI and HZ). Within-ecotype comparisons are depicted as blue dots, and between-ecotype comparisons as red triangles. In (B–C) we show a UPGMA hierarchical cluster analyses based on $PP2_d$ (B) and $\hat{F}_{ST}^{pool}$ (C) pairwise estimates. In (D), we show a heatmap representation of the scaled covariance matrix among the four *C. asper* populations, inferred from the Bayesian hierarchical model implemented in the software package BAYPASS.

811 SUPPLEMENTAL FILE S1: DETAILED MATHEMATICAL DERIVATIONS

812 Analysis of variance for Pool-seq data

813 In the following, we first derive our model for a single locus. Consider a
814 sample of n_d subpopulations, each of which is made of n_i genes ($i = 1, \dots, n_d$)
815 sequenced in pools (hence n_i is the haploid sample size of the i th pool). We
816 define c_{ij} as the number of reads sequenced from gene j ($j = 1, \dots, n_i$) in
817 subpopulation i at the locus considered. Note that c_{ij} is a latent variable,
818 that cannot be directly observed from the data. Let $X_{ijr:k}$ be an indicator
819 variable for read r ($r = 1, \dots, c_{ij}$) from gene j in subpopulation i , such that
820 $X_{ijr:k} = 1$ if the r th read from the j th gene in the i th deme is of type k ,
821 and $X_{ijr:k} = 0$ otherwise. In the following, we use standard dot notations
822 for sample averages, i.e.: $X_{ij\cdot:k} \equiv \sum_r X_{ijr:k}/c_{ij}$, $X_{i\cdot\cdot:k} \equiv \sum_j \sum_r X_{ijr:k}/\sum_j c_{ij}$
823 and $X_{\cdot\cdot\cdot:k} \equiv \sum_i \sum_j \sum_r X_{ijr:k}/\sum_i \sum_j c_{ij}$. The analysis of variance is based
824 on the computation of sums of squares, as follows:

$$\begin{aligned}
 \sum_i^{n_d} \sum_j^{n_i} \sum_r^{c_{ij}} (X_{ijr:k} - X_{\cdot\cdot\cdot:k})^2 &= \sum_i^{n_d} \sum_j^{n_i} \sum_r^{c_{ij}} (X_{ijr:k} - X_{ij\cdot:k})^2 \\
 &+ \sum_i^{n_d} \sum_j^{n_i} \sum_r^{c_{ij}} (X_{ij\cdot:k} - X_{i\cdot\cdot:k})^2 \\
 &+ \sum_i^{n_d} \sum_j^{n_i} \sum_r^{c_{ij}} (X_{i\cdot\cdot:k} - X_{\cdot\cdot\cdot:k})^2 \\
 &\equiv SSR_{\cdot:k} + SSI_{\cdot:k} + SSP_{\cdot:k} \quad (A1)
 \end{aligned}$$

825 We express the sum of squares for reads within individuals as:

$$\begin{aligned} SSR_{:k} &= \sum_i^{n_d} \sum_j^{n_i} \sum_r^{c_{ij}} (X_{ijr:k} - X_{ij:k})^2 \\ &= 0 \end{aligned} \quad (A2)$$

826 since we assume that there is no sequencing error, i.e. all the reads sequenced
827 from a single gene are identical (therefore $X_{ijr:k} = X_{ij:k}$, for all r). The sum
828 of squares for genes within pools reads:

$$\begin{aligned} SSI_{:k} &= \sum_i^{n_d} \sum_j^{n_i} \sum_r^{c_{ij}} (X_{ij:k} - X_{i:k})^2 \\ &= \sum_i^{n_d} \sum_j^{n_i} \sum_r^{c_{ij}} (X_{ij:k} - \pi_k)^2 - \sum_i^{n_d} \sum_j^{n_i} \sum_r^{c_{ij}} (X_{i:k} - \pi_k)^2 \\ &= \sum_i^{n_d} \sum_j^{n_i} c_{ij} (X_{ij:k} - \pi_k)^2 - \sum_i^{n_d} C_{1i} (X_{i:k} - \pi_k)^2 \end{aligned} \quad (A3)$$

829 where π_k is the expectation of the frequency of allele k over independent
830 replicates of the evolutionary process, and $C_{1i} \equiv \sum_j c_{ij}$ is the total number
831 of observed reads in the i th pool. Likewise, the sum of squares for genes
832 between pools reads:

$$\begin{aligned} SSP_{:k} &= \sum_i^{n_d} \sum_j^{n_i} \sum_r^{c_{ij}} (X_{i:k} - X_{...:k})^2 \\ &= \sum_i^{n_d} C_{1i} (X_{i:k} - \pi_k)^2 - C_1 (X_{...:k} - \pi_k)^2 \end{aligned} \quad (A4)$$

833 where $C_1 \equiv \sum_i \sum_j c_{ij}$ is the total number of observed reads in the full sample.
834 These sums can be expressed as functions of the average frequency of reads
835 of type k for individual j : $\hat{\pi}_{ij:k} \equiv X_{ij:k}$, of the average frequency of reads of

836 type k within the i th pool: $\hat{\pi}_{i:k} \equiv X_{i\ldots:k}$, and of the average frequency of reads
837 of type k in the full sample: $\hat{\pi}_k \equiv X_{\ldots:k}$. Note that from the definition of
838 $X_{\ldots:k}$, $\hat{\pi}_k \equiv \sum_i \sum_j \sum_r X_{ijr:k} / \sum_i \sum_j c_{ij} = \sum_i C_{1i} \hat{\pi}_{i:k} / \sum_i C_{1i}$ is the weighted
839 average of the sample frequencies with weights equal to the pool coverage.
840 Our approach is therefore equivalent to the weighted analysis-of-variance in
841 Cockerham (1973) (see also Weir and Cockerham 1984; Weir 1996; Weir and
842 Hill 2002; Rousset 2007; Weir and Goudet 2017). Then, developing the square
843 in the first term in the right-hand side of Equation A3, we get:

$$\begin{aligned}
 (X_{ij:k} - \pi_k)^2 &= \left(\frac{\sum_r^{c_{ij}} (X_{ijr:k} - \pi_k)}{c_{ij}} \right)^2 \\
 &= \frac{1}{c_{ij}^2} \left(\sum_r^{c_{ij}} X_{ijr:k} - c_{ij} \pi_k \right)^2 \\
 &= \frac{1}{c_{ij}^2} \left(\sum_r^{c_{ij}} X_{ijr:k}^2 + \sum_{r \neq r'}^{c_{ij}} X_{ijr:k} X_{ijr':k} - 2c_{ij}^2 X_{ij:k} \pi_k + c_{ij}^2 \pi_k^2 \right) \\
 &= \frac{1}{c_{ij}^2} (c_{ij} X_{ij:k} + c_{ij} (c_{ij} - 1) X_{ij:k} \\
 &\quad - 2c_{ij}^2 X_{ij:k} \pi_k + c_{ij}^2 \pi_k^2) \\
 &= \hat{\pi}_{ij:k} - 2\pi_k \hat{\pi}_{ij:k} + \pi_k^2
 \end{aligned} \tag{A5}$$

844 The sums of squares also depend on the unobserved frequency of pairs of
845 genes sampled in the i th pool that are both of type k , i.e. the probability
846 of identity in state (IIS) for allele k , for two distinct genes in the i th pool:
847 $\hat{Q}_{1i:k} \equiv \left(\sum_{j \neq j'} \sum_{r, r'} X_{ijr:k} X_{ij'r':k} \right) / \left(C_{1i}^2 - \sum_j c_{ij}^2 \right)$. Then, developing the

848 square in the second term in the right-hand side of Equation A3, we get:

$$\begin{aligned}
 (X_{i\cdots k} - \pi_k)^2 &= \left(\frac{\sum_j^{n_i} \sum_r^{c_{ij}} (X_{ijr:k} - \pi_k)}{C_{1i}} \right)^2 \\
 &= \frac{1}{C_{1i}^2} \left(\sum_j^{n_i} \sum_r^{c_{ij}} X_{ijr:k} - C_{1i} \pi_k \right)^2 \\
 &= \frac{1}{C_{1i}^2} \left(\sum_j^{n_i} \sum_r^{c_{ij}} X_{ijr:k}^2 + \sum_j^{n_i} \sum_{r \neq r'}^{c_{ij}} X_{ijr:k} X_{ijr':k} \right. \\
 &\quad \left. + \sum_{j \neq j'}^{n_i} \sum_{r, r'}^{c_{ij}} X_{ijr:k} X_{ij'r':k} - 2C_{1i}^2 X_{i\cdots k} \pi_k + C_{1i}^2 \pi_k^2 \right) \\
 &= \frac{1}{C_{1i}^2} \left(\sum_j^{n_i} c_{ij} X_{ij\cdots k} + \sum_j^{n_i} c_{ij} (c_{ij} - 1) X_{ij\cdots k} \right. \\
 &\quad \left. + \left(C_{1i}^2 - \sum_j^{n_i} c_{ij}^2 \right) \hat{Q}_{1i:k} - 2C_{1i}^2 X_{i\cdots k} \pi_k + C_{1i}^2 \pi_k^2 \right) \\
 &= \frac{1}{C_{1i}^2} \left(\sum_j^{n_i} c_{ij}^2 (X_{ij\cdots k} - X_{i\cdots k}) + \left(C_{1i}^2 - \sum_j^{n_i} c_{ij}^2 \right) (\hat{Q}_{1i:k} - X_{i\cdots k}) \right. \\
 &\quad \left. + C_{1i}^2 X_{i\cdots k} - 2C_{1i}^2 X_{i\cdots k} \pi_k + C_{1i}^2 \pi_k^2 \right) \\
 &= \hat{\pi}_{i:k} - 2\pi_k \hat{\pi}_{i:k} + \pi_k^2 + \sum_j^{n_i} \frac{c_{ij}^2}{C_{1i}^2} (\hat{\pi}_{ij:k} - \hat{\pi}_{i:k}) \\
 &\quad + \left(1 - \sum_j^{n_i} \frac{c_{ij}^2}{C_{1i}^2} \right) (\hat{Q}_{1i:k} - \hat{\pi}_{i:k}) \tag{A6}
 \end{aligned}$$

849 Last, the sums of squares depend on the unobserved frequency of pairs
 850 of genes sampled in the same pool that are both of type k , i.e. the IIS
 851 probability for allele k for two distinct genes in the same pool: $\hat{Q}_{1:k} \equiv$
 852 $\left(\sum_i \sum_{j \neq j'} \sum_{r, r'} X_{ijr:k} X_{ij'r':k} \right) / \left(C_2 - \sum_i \sum_j c_{ij}^2 \right)$, and of the unobserved
 853 frequency of pairs of genes sampled in different pools that are both of type
 854 k : $\hat{Q}_{2:k} \equiv \left(\sum_{i \neq i'} \sum_{j, j'} \sum_{r, r'} X_{ijr:k} X_{i'j'r':k} \right) / (C_1^2 - C_2)$, where $C_2 \equiv \sum_i C_{1i}^2$.

855 Developing the second term in the right-hand side of Equation A4, we get:

$$\begin{aligned}
 (X_{\dots:k} - \pi_k)^2 &= \left(\frac{\sum_i^{n_d} \sum_j^{n_i} \sum_r^{c_{ij}} (X_{ijr:k} - \pi_k)}{C_1} \right)^2 \\
 &= \frac{1}{C_1^2} \left(\sum_i^{n_d} \sum_j^{n_i} \sum_r^{c_{ij}} X_{ijr:k} - C_1 \pi_k \right)^2 \\
 &= \frac{1}{C_1^2} \left(\sum_i^{n_d} \sum_j^{n_i} \sum_r^{c_{ij}} X_{ijr:k}^2 + \sum_i^{n_d} \sum_j^{n_i} \sum_{r \neq r'}^{c_{ij}} X_{ijr:k} X_{ijr':k} \right. \\
 &\quad + \sum_i^{n_d} \sum_{j \neq j'}^{n_i} \sum_{r, r'}^{c_{ij}} X_{ijr:k} X_{i'j'r':k} + \sum_{i \neq i'}^{n_d} \sum_{j, j'}^{n_i} \sum_{r, r'}^{c_{ij}} X_{ijr:k} X_{i'j'r':k} \\
 &\quad \left. - 2C_1^2 X_{\dots:k} \pi_k + C_1^2 \pi_k^2 \right) \\
 &= \frac{1}{C_1^2} \left(\sum_i^{n_d} \sum_j^{n_i} c_{ij} X_{ij:k} + \sum_i^{n_d} \sum_j^{n_i} c_{ij} (c_{ij} - 1) X_{ij:k} \right. \\
 &\quad + \left(C_2 - \sum_i^{n_d} \sum_j^{n_i} c_{ij}^2 \right) \hat{Q}_{1:k} + (C_1^2 - C_2) \hat{Q}_{2:k} - 2C_1^2 X_{\dots:k} \pi_k + C_1^2 \pi_k^2 \Big) \\
 &= \frac{1}{C_1^2} \left(\sum_i^{n_d} \sum_j^{n_i} c_{ij}^2 (X_{ij:k} - X_{\dots:k}) + \left(C_2 - \sum_i^{n_d} \sum_j^{n_i} c_{ij}^2 \right) (\hat{Q}_{1:k} - X_{\dots:k}) \right. \\
 &\quad + \left. (C_1^2 - C_2) (\hat{Q}_{2:k} - X_{\dots:k}) + C_1^2 X_{\dots:k} - 2C_1^2 X_{\dots:k} \pi_k + C_1^2 \pi_k^2 \right) \\
 &= \hat{\pi}_k - 2\pi_k \hat{\pi}_k + \pi_k^2 + \sum_i^{n_d} \sum_j^{n_i} \frac{c_{ij}^2}{C_1^2} (\hat{\pi}_{ij:k} - \hat{\pi}_k) \\
 &\quad + \left(\frac{C_2}{C_1^2} - \sum_i^{n_d} \sum_j^{n_i} \frac{c_{ij}^2}{C_1^2} \right) (\hat{Q}_{1:k} - \hat{\pi}_k) + \left(1 - \frac{C_2}{C_1^2} \right) (\hat{Q}_{2:k} - \hat{\pi}_k) \quad (A7)
 \end{aligned}$$

856 Hence, developing the first term in the right-hand side of Equation A3 using

857 Equation A5, we have:

$$\sum_i^{n_d} \sum_j^{n_i} c_{ij} (X_{ij:k} - \pi_k)^2 = C_1 (\hat{\pi}_k - 2\pi_k \hat{\pi}_k + \pi_k^2) \quad (A8)$$

858 Likewise, developing the second term in the right-hand side of Equation A3

859 using Equation A6, we get:

$$\begin{aligned} \sum_i^{n_d} C_{1i} (X_{i:k} - \pi_k)^2 &= C_1 (\hat{\pi}_k - 2\pi_k \hat{\pi}_k + \pi_k^2) + \sum_i^{n_d} \sum_j^{n_i} \frac{c_{ij}^2}{C_{1i}} (\hat{\pi}_{ij:k} - \hat{\pi}_{i:k}) \\ &+ \sum_i^{n_d} \left(C_{1i} - \sum_j^{n_i} \frac{c_{ij}^2}{C_{1i}} \right) (\hat{Q}_{1i:k} - \hat{\pi}_{i:k}) \end{aligned} \quad (A9)$$

860 Last, developing the second term in the right-hand side of Equation A4 using

861 Equation A7, we get:

$$\begin{aligned} C_1 (X_{\dots:k} - \pi_k)^2 &= C_1 (\hat{\pi}_k - 2\pi_k \hat{\pi}_k + \pi_k^2) + \sum_i^{n_d} \sum_j^{n_i} \frac{c_{ij}^2}{C_1} (\hat{\pi}_{ij:k} - \hat{\pi}_k) \\ &+ \left(\frac{C_2}{C_1} - \sum_i^{n_d} \sum_j^{n_i} \frac{c_{ij}^2}{C_1} \right) (\hat{Q}_{1:k} - \hat{\pi}_k) \\ &+ \left(C_1 - \frac{C_2}{C_1} \right) (\hat{Q}_{2:k} - \hat{\pi}_k) \end{aligned} \quad (A10)$$

862 Then, from Equations A3, A8 and A9:

$$\begin{aligned} SSI_{:k} &= \sum_i^{n_d} \sum_j^{n_i} \frac{c_{ij}^2}{C_{1i}} (\hat{\pi}_{i:k} - \hat{\pi}_{ij:k}) \\ &+ \sum_i^{n_d} \left(C_{1i} - \sum_j^{n_i} \frac{c_{ij}^2}{C_{1i}} \right) (\hat{\pi}_{i:k} - \hat{Q}_{1i:k}) \end{aligned} \quad (A11)$$

863 and from Equations A4, A9 and A10:

$$\begin{aligned} SSP_{:k} &= \sum_i^{n_d} \sum_j^{n_i} \frac{c_{ij}^2}{C_{1i}} (\hat{\pi}_{ij:k} - \hat{\pi}_{i:k}) - \sum_i^{n_d} \left(C_{1i} - \sum_j^{n_i} \frac{c_{ij}^2}{C_{1i}} \right) (\hat{\pi}_{i:k} - \hat{Q}_{1i:k}) \\ &+ \sum_i^{n_d} \sum_j^{n_i} \frac{c_{ij}^2}{C_1} (\hat{\pi}_k - \hat{\pi}_{ij:k}) + \left(\frac{C_2}{C_1} - \sum_i^{n_d} \sum_j^{n_i} \frac{c_{ij}^2}{C_1} \right) (\hat{\pi}_k - \hat{Q}_{1:k}) \\ &+ \left(C_1 - \frac{C_2}{C_1} \right) (\hat{\pi}_k - \hat{Q}_{2:k}) \end{aligned} \quad (A12)$$

864 Taking expectation over all possible samples from all replicate populations
865 sharing the same evolutionary history, we get from Equation A11:

$$\begin{aligned}\mathbb{E}(SSI_{:k}) &= \sum_i^{n_d} \sum_j^{n_i} \mathbb{E}(\hat{\pi}_{i:k} - \hat{\pi}_{ij:k}) \mathbb{E}\left(\frac{c_{ij}^2}{C_{1i}}\right) \\ &+ \sum_i^{n_d} \mathbb{E}\left(\hat{\pi}_{i:k} - \hat{Q}_{1i:k}\right) \mathbb{E}\left(C_{1i} - \sum_j^{n_i} \frac{c_{ij}^2}{C_{1i}}\right) \\ &= (\pi_k - Q_{1:k}) \left(C_1 - \mathbb{E}\left(\sum_i^{n_d} \sum_j^{n_i} \frac{c_{ij}^2}{C_{1i}}\right)\right)\end{aligned}\quad (\text{A13})$$

866 where $Q_{1:k}$ is the expected IIS probability that two genes in the same pool
867 are both of type k . Likewise, from Equation A12:

$$\begin{aligned}\mathbb{E}(SSP_{:k}) &= \sum_i^{n_d} \sum_j^{n_i} \mathbb{E}(\hat{\pi}_{i:k} - \hat{\pi}_{ij:k}) \mathbb{E}\left(\frac{c_{ij}^2}{C_{1i}}\right) + \sum_i^{n_d} \sum_j^{n_i} \mathbb{E}(\hat{\pi}_k - \hat{\pi}_{ij:k}) \mathbb{E}\left(\frac{c_{ij}^2}{C_1}\right) \\ &- \sum_i^{n_d} \mathbb{E}\left(\hat{\pi}_{i:k} - \hat{Q}_{1i:k}\right) \mathbb{E}\left(C_{1i} - \sum_j^{n_i} \frac{c_{ij}^2}{C_{1i}}\right) \\ &+ \mathbb{E}\left(\hat{\pi}_k - \hat{Q}_{1:k}\right) \mathbb{E}\left(\frac{C_2}{C_1} - \sum_i^{n_d} \sum_j^{n_i} \frac{c_{ij}^2}{C_1}\right) \\ &+ \left(C_1 - \frac{C_2}{C_1}\right) \mathbb{E}\left(\hat{\pi}_k - \hat{Q}_{2:k}\right) \\ &= (\pi_k - Q_{1:k}) \left(\frac{C_2}{C_1} - \mathbb{E}\left(\sum_i^{n_d} \sum_j^{n_i} \frac{c_{ij}^2}{C_1}\right)\right) \\ &- (\pi_k - Q_{1:k}) \left(C_1 - \mathbb{E}\left(\sum_i^{n_d} \sum_j^{n_i} \frac{c_{ij}^2}{C_{1i}}\right)\right) \\ &+ \left(C_1 - \frac{C_2}{C_1}\right) (\pi_k - Q_{2:k})\end{aligned}\quad (\text{A14})$$

868 where $Q_{2:k}$ is the expected IIS probability that two genes from different pools
869 are both of type k . Note that the expected sums $\mathbb{E}\left(\sum_i \sum_j c_{ij}^2\right)/C_{1i}$ and
870 $\mathbb{E}\left(\sum_i \sum_j c_{ij}^2\right)/C_1$ in Equations A13 and A14 depend on the latent variable

871 c_{ij} , that cannot be directly observed from the data. Therefore, we must make
 872 an assumption on the distribution of the c_{ij} 's to proceed. In the following,
 873 we assume that for each pool i , c_{ij} follows a multinomial distribution with
 874 parameter C_{1i} (the number of trials, i.e. the total number of reads in the
 875 i th pool) and probabilities $(1/n_i, \dots, 1/n_i)$ for the n_i individuals in the pool.
 876 Then:

$$\begin{aligned} \mathbb{E} \left(\sum_i^{n_d} \sum_j^{n_i} \frac{c_{ij}^2}{C_{1i}} \right) &= \sum_i^{n_d} \frac{1}{C_{1i}} \sum_j^{n_i} \mathbb{E} (c_{ij}^2) \\ &= \sum_i^{n_d} \frac{1}{C_{1i}} \sum_j^{n_i} \left(\mathbb{E} (c_{ij})^2 + \mathbb{V} (c_{ij}) \right) \\ &= \sum_i^{n_d} \frac{1}{C_{1i}} \sum_j^{n_i} \left(\left(\frac{C_{1i}}{n_i} \right)^2 + \frac{C_{1i}}{n_i} \left(\frac{n_i - 1}{n_i} \right) \right) \\ &= \sum_i^{n_d} \left(\frac{C_{1i}}{n_i} + \left(\frac{n_i - 1}{n_i} \right) \right) \equiv D_2 \end{aligned} \quad (\text{A15})$$

877 and:

$$\begin{aligned} \mathbb{E} \left(\sum_i^{n_d} \sum_j^{n_i} \frac{c_{ij}^2}{C_1} \right) &= \frac{1}{C_1} \sum_i^{n_d} \sum_j^{n_i} \mathbb{E} (c_{ij}^2) \\ &= \frac{1}{C_1} \sum_i^{n_d} C_{1i} \left[\frac{C_{1i}}{n_i} + \left(\frac{n_i - 1}{n_i} \right) \right] \equiv D_2^* \end{aligned} \quad (\text{A16})$$

878 Hence, from Equations A13 and A15, we have:

$$\mathbb{E}(SSI_{:k}) = (C_1 - D_2) (\pi_k - Q_{1:k}) \quad (\text{A17})$$

879 and from Equations A14 and A16:

$$\begin{aligned}
 \mathbb{E}(SSP_{:k}) &= \left(\frac{C_2}{C_1} - D_2^* \right) (\pi_k - Q_{1:k}) - (C_1 - D_2) (\pi_k - Q_{1:k}) \\
 &+ \left(C_1 - \frac{C_2}{C_1} \right) (\pi_k - Q_{2:k}) \\
 &= \left(C_1 - \frac{C_2}{C_1} \right) (Q_{1:k} - Q_{2:k}) \\
 &+ (D_2 - D_2^*) (\pi_k - Q_{1:k})
 \end{aligned} \tag{A18}$$

880 Summing over alleles, we get the following expressions for the expected sums
881 of squares for genes between individuals within pools:

$$\mathbb{E}(SSI) = \sum_k \mathbb{E}(SSI_{:k}) = (C_1 - D_2) (1 - Q_1) \tag{A19}$$

882 and for genes between individuals from different pools:

$$\begin{aligned}
 \mathbb{E}(SSP) &= \sum_k \mathbb{E}(SSP_{:k}) \\
 &= \left(C_1 - \frac{C_2}{C_1} \right) (Q_1 - Q_2) + (D_2 - D_2^*) (1 - Q_1)
 \end{aligned} \tag{A20}$$

883 Rearranging Equations A19–A20, we get:

$$Q_1 - Q_2 = \frac{(C_1 - D_2) \mathbb{E}(SSP) - (D_2 - D_2^*) \mathbb{E}(SSI)}{(C_1 - D_2) (C_1 - C_2/C_1)} \tag{A21}$$

884 and:

$$1 - Q_2 = \frac{(C_1 - D_2) \mathbb{E}(SSP) + (n_c - 1) (D_2 - D_2^*) \mathbb{E}(SSI)}{(C_1 - D_2) (C_1 - C_2/C_1)} \tag{A22}$$

885 where $n_c \equiv (C_1 - C_2/C_1) / (D_2 - D_2^*)$. Let $MSI \equiv SSI / (C_1 - D_2)$ and
886 $MSP \equiv SSP / (D_2 - D_2^*)$. Then, rearranging Equations A21–A22, we get:

$$F_{ST} \equiv \frac{Q_1 - Q_2}{1 - Q_2} = \frac{\mathbb{E}(MSP) - \mathbb{E}(MSI)}{\mathbb{E}(MSP) + (n_c - 1) \mathbb{E}(MSI)} \quad (\text{A23})$$

887 which yields the method-of-moments estimator:

$$\hat{F}_{ST}^{\text{pool}} = \frac{MSP - MSI}{MSP + (n_c - 1) MSI} \quad (\text{A24})$$

888 Since SSI (Equation A3) and SSP (Equation A4) may be rewritten in terms
889 of sample frequencies as:

$$\begin{aligned} SSI &= \sum_k SSI_{:k} = \sum_k \sum_i^{n_d} \sum_j^{n_i} \sum_r^{c_{ij}} (X_{ij:k} - X_{i:k})^2 \\ &= \sum_k \sum_i^{n_d} C_{1i} \hat{\pi}_{i:k} (1 - \hat{\pi}_{i:k}) \end{aligned} \quad (\text{A25})$$

890 and:

$$\begin{aligned} SSP &= \sum_k SSP_k = \sum_k \sum_i^{n_d} \sum_j^{n_i} \sum_r^{c_{ij}} (X_{i:k} - X_{j:k})^2 \\ &= \sum_k \sum_i^{n_d} C_{1i} (\hat{\pi}_{i:k} - \hat{\pi}_k)^2 \end{aligned} \quad (\text{A26})$$

891 our estimator then takes the form:

$$\hat{F}_{ST}^{\text{pool}} = \frac{\sum_k [(C_1 - D_2) \sum_i^{n_d} C_{1i} (\hat{\pi}_{i:k} - \hat{\pi}_k)^2 - (D_2 - D_2^*) \sum_i^{n_d} C_{1i} \hat{\pi}_{i:k} (1 - \hat{\pi}_{i:k})]}{\sum_k [(C_1 - D_2) \sum_i^{n_d} C_{1i} (\hat{\pi}_{i:k} - \hat{\pi}_k)^2 + (n_c - 1) (D_2 - D_2^*) \sum_i^{n_d} C_{1i} \hat{\pi}_{i:k} (1 - \hat{\pi}_{i:k})]} \quad (\text{A27})$$

892 The estimator in Equation A24 can also be expressed as a function of the

893 frequencies of identical pairs of genes $\hat{Q}_1 = \sum_k \hat{Q}_{1:k}$ and $\hat{Q}_2 = \sum_k \hat{Q}_{2:k}$, as:

$$\hat{F}_{ST}^{pool} = \frac{(\hat{Q}_1 - \hat{Q}_2) \alpha + \left(C_1 - \sum_i \sum_j \frac{c_{ij}^2}{C_1}\right) \beta}{(1 - \hat{Q}_2) \alpha + \left(C_2/C_1 - \sum_i \sum_j \frac{c_{ij}^2}{C_1}\right) \beta} \quad (A28)$$

894 where:

$$\alpha \equiv \left(C_1 - \sum_i \sum_j \frac{c_{ij}^2}{C_{1i}}\right) \left(C_1 - \frac{C_2}{C_1}\right) \quad (A29)$$

895 and:

$$\beta \equiv \sum_i \left(C_{1i} - \sum_j \frac{c_{ij}^2}{C_{1i}}\right) (\hat{Q}_{1i} - \hat{Q}_1) \quad (A30)$$

896 If we take the limit case where the number of sequenced reads per gene is
 897 constant, i.e. if $C_{1i} = C$, for all $i \in (1, \dots, n_d)$, then it can be shown that
 898 Equation A28 reduces exactly to Equations 28A29–28A30 in Rousset (2007),
 899 p. 977. Furthermore, if the pools have all the same size, i.e. if $n_i = n$ for all
 900 $i \in (1, \dots, n_d)$, then $\hat{F}_{ST}^{pool} = (\hat{Q}_1 - \hat{Q}_2) / (1 - \hat{Q}_2)$.

901 If the pools have all the same size and if the number of reads per pool is
 902 constant, then one can also show that Equations A25–A26 reduce to:

$$SSI = n_d(C - 1) (1 - \hat{Q}_1^r) \quad (A31)$$

903 and:

$$SSP = C(n_d - 1) (1 - \hat{Q}_2^r) - (n_d - 1)(C - 1) (1 - \hat{Q}_1^r) \quad (A32)$$

904 where $\hat{Q}_1^r$ and $\hat{Q}_2^r$ are the frequencies of identical pairs of reads within and be-
 905 tween pools, respectively, computed by simple counting of IIS pairs. These

are (unweighted) averages of the population-specific estimates $\hat{Q}_{1i}^r$ (Equation A34) and the pairwise estimates $\hat{Q}_{2ii'}^r$ (Equation A40), respectively. Then, from Equation A24, we get:

$$\hat{F}_{ST}^{\text{pool}} = 1 - \left(\frac{1 - \hat{Q}_1^r}{1 - \hat{Q}_2^r} \right) \left(\frac{n}{n-1} \right) \quad (\text{A33})$$

909 IIS probabilities for Pool-seq data

910 In this Appendix, we provide unbiased estimates of IIS probabilities between
 911 pairs of genes, computed from read count data. Let $r_{i:k} = \sum_j \sum_r X_{ijr:k}$ be
 912 the number of reads of type k in the i th pool. A straightforward estimate of
 913 the IIS probability between pairs of reads in the i th pool is given by:

$$\hat{Q}_{1i}^r \equiv \frac{\sum_k r_{i:k} (r_{i:k} - 1)}{C_{1i} (C_{1i} - 1)} \quad (\text{A34})$$

914 where $C_{1i} = \sum_k r_{i:k}$. As above (see Equations A15 and A16), we assume that
 915 in each pool, the conditional distribution of the read counts $r_{i:k}$, given the
 916 (unobserved) allele counts $y_{i:k}$, is binomial, i.e.: $r_{i:k} \mid y_{i:k} \sim \text{Bin}(y_{i:k}/n_i, C_{1i})$.
 917 The conditional expectation of the number of reads is therefore given by:
 918 $\mathbb{E}(r_{i:k} \mid y_{i:k}) = C_{1i} (y_{i:k}/n_i)$, and the conditional expectation of the squared
 919 number of reads by: $\mathbb{E}(r_{i:k}^2 \mid y_{i:k}) = C_{1i} (C_{1i} - 1) (y_{i:k}/n_i)^2 + C_{1i} (y_{i:k}/n_i)$.
 920 Therefore, the conditional expectation of the IIS probability between pairs
 921 of reads in the i th pool reads:

$$\mathbb{E}(\hat{Q}_{1i}^r \mid y_{i:k}) = \frac{\sum_k \mathbb{E}(r_{i:k}^2 - r_{i:k})}{C_{1i} (C_{1i} - 1)} = \sum_k \left(\frac{y_{i:k}}{n_i} \right)^2 \quad (\text{A35})$$

922 Since

$$\hat{Q}_{1i} \equiv \frac{\sum_k y_{i:k} (y_{i:k} - 1)}{n_i (n_i - 1)} \quad (\text{A36})$$

923 is an unbiased estimate of the IIS probability between pairs of distinct genes
 924 in the i th pool, Equation A35 implies that $\hat{Q}_{1i}^r$ (Equation A34) is a biased
 925 estimate of that quantity (i.e., the IIS probability between pairs of reads
 926 within a pool is a biased estimate of the IIS probability between pairs of

distinct genes in that pool). This is so, because the former confounds pairs of reads that are identical because they were sequenced from a single gene copy, from pairs of reads (from distinct gene copies) that are identical because they share a common ancestor. However, inspection of Equation A35 suggests that an unbiased estimate of $\hat{Q}_{1i}$ may be given by:

$$\hat{Q}_{1i}^{\text{pool}} \equiv 1 - \frac{n_i}{n_i - 1} \left(1 - \hat{Q}_{1i}^r\right) \quad (\text{A37})$$

Taking expectation of Equation A37, we get indeed:

$$\begin{aligned} \mathbb{E} \left(\hat{Q}_{1i}^{\text{pool}} \mid y_{i:k} \right) &= \frac{n_i}{n_i - 1} \mathbb{E} \left(\hat{Q}_{1i}^r \right) - \frac{1}{n_i - 1} \\ &= \frac{n_i}{n_i - 1} \sum_k \left(\frac{y_{i:k}}{n_i} \right)^2 - \frac{n_i}{n_i(n_i - 1)} \\ &= \frac{\sum_k y_{i:k}^2}{n_i(n_i - 1)} - \frac{\sum_k y_{i:k}}{n_i(n_i - 1)} \\ &= \frac{\sum_k y_{i:k}(y_{i:k} - 1)}{n_i(n_i - 1)} \equiv \hat{Q}_{1i} \end{aligned} \quad (\text{A38})$$

Following Weir and Goudet (2017), we define the overall IIS probability between pairs of genes within pools as the unweighted average of population-specific estimates, leading to:

$$\hat{Q}_1^{\text{pool}} \equiv \frac{\sum_i \hat{Q}_{1i}^{\text{pool}}}{n_d} \quad (\text{A39})$$

A straightforward estimate of the IIS probability between pairs of reads taken in different pools i and i' is given by:

$$\hat{Q}_{2ii'}^r \equiv \frac{\sum_k r_{i:k} r_{i':k}}{C_{1i} C_{1i'}} \quad (\text{A40})$$

Since we assume that pools are conditionally independent, taking expectation

939 gives:

$$\begin{aligned}\mathbb{E}\left(\hat{Q}_{2ii'}^r \mid y_{i:k}, y_{i':k}\right) &= \frac{\sum_k \mathbb{E}(r_{i:k}) \mathbb{E}(r_{i':k})}{C_{1i} C_{1i'}} \\ &= \sum_k \left(\frac{y_{i:k} y_{i':k}}{n_i n_{i'}} \right) \equiv \hat{Q}_{2ii'}\end{aligned}\quad (\text{A41})$$

940 Therefore, the IIS probability between pairs of reads sampled in different
941 pools is an unbiased estimate of the IIS probability between pairs of genes in
942 these pools, and an unbiased estimate of the IIS probability of genes sampled
943 from different pools is given by:

$$\hat{Q}_{2ii'}^{\text{pool}} \equiv \hat{Q}_{2ii'}^r \quad (\text{A42})$$

944 As above, we define the overall IIS probability between pairs of genes sampled
945 from different pools as the unweighted average of pairwise estimates, i.e.:

$$\hat{Q}_2^{\text{pool}} \equiv \frac{\sum_{i \neq i'} \hat{Q}_{2ii'}^{\text{pool}}}{n_d(n_d - 1)} \quad (\text{A43})$$

946 We can then derive an IIS-based estimator of F_{ST} , as:

$$\begin{aligned}\hat{F}_{\text{ST}}^{\text{pool-PID}} &\equiv \frac{\hat{Q}_1^{\text{pool}} - \hat{Q}_2^{\text{pool}}}{1 - \hat{Q}_2^{\text{pool}}} = 1 - \frac{1 - \hat{Q}_1^{\text{pool}}}{1 - \hat{Q}_2^{\text{pool}}} \\ &= 1 - \frac{\sum_i \left[\left(1 - \hat{Q}_{1i}^r\right) n_i / (n_i - 1) \right]}{\sum_{i \neq i'} \left(1 - \hat{Q}_{2ii'}^r\right) / (n_d - 1)}\end{aligned}\quad (\text{A44})$$

947 which, to the extent that we may take the expectation of a ratio to be the
948 ratio of expectations, is unbiased. If the pools have all the same size (i.e., if

949 $n_i = n$ for all i), then Equation A44 reduces to:

$$\hat{F}_{ST}^{\text{pool-PID}} = 1 - \left(\frac{1 - \hat{Q}_1^r}{1 - \hat{Q}_2^r} \right) \left(\frac{n}{n-1} \right) \quad (\text{A45})$$

950 where $\hat{Q}_1^r \equiv \sum_i \hat{Q}_{1i}^r / n_d$ and $\hat{Q}_2^r \equiv \sum_{i \neq i'} \hat{Q}_{2ii'}^r / [n_d(n_d - 1)]$. Note that Equa-
 951 tion A45 is strictly identical to Equation A33. Therefore, if the pools have all
 952 the same size and if the number of reads per pool is constant, the analysis-
 953 of-variance estimator $\hat{F}_{ST}^{\text{pool}}$ is strictly equivalent to the estimator $\hat{F}_{ST}^{\text{pool-PID}}$
 954 based on the computation of IIS probabilities between pairs of reads, with
 955 appropriate bias correction (see Equation A37). This echoes the derivations
 956 by Rousset (2007) for Ind-seq data, who showed that the analysis-of-variance
 957 approach (Weir and Cockerham 1984) and the simple strategy of estimat-
 958 ing IIS probabilities by counting identical pairs of genes provides identical
 959 estimates when sample sizes are equal (see also Cockerham and Weir 1987;
 960 Karlsson et al. 2007).

961 Alternatively, the overall IIS probability between pairs of genes within
 962 pools may be defined as the weighted average of population-specific estimates,
 963 with weights equal to the number of pairs of genes in each pool (see Rousset
 964 2007), i.e.:

$$\tilde{Q}_1^{\text{pool}} \equiv \frac{\sum_i n_i(n_i - 1) \hat{Q}_{1i}^{\text{pool}}}{\sum_i n_i(n_i - 1)} \quad (\text{A46})$$

965 Likewise, the overall IIS probability between pairs of genes sampled from
 966 different pools may be defined as the weighted average of pairwise estimates,
 967 with weights equal to the number of pairs of genes sampled between pools,

968 i.e.:

$$\tilde{Q}_2^{\text{pool}} \equiv \frac{\sum_{i \neq i'} n_i n'_i \hat{Q}_{2ii'}^{\text{pool}}}{\sum_{i \neq i'} n_i n'_i} \quad (\text{A47})$$

969 We can then derive an IIS-based estimator of F_{ST} , using weighted IIS prob-
970 abilities, as:

$$\begin{aligned} \tilde{F}_{\text{ST}}^{\text{pool-PID}} &\equiv \frac{\tilde{Q}_1^{\text{pool}} - \tilde{Q}_2^{\text{pool}}}{1 - \tilde{Q}_2^{\text{pool}}} = 1 - \frac{1 - \tilde{Q}_1^{\text{pool}}}{1 - \tilde{Q}_2^{\text{pool}}} \\ &= 1 - \frac{\sum_i \left[n_i^2 \left(1 - \hat{Q}_{1i}^{\text{r}} \right) \right] / \sum_i n_i (n_i - 1)}{\sum_{i \neq i'} n_i n'_i \left(1 - \hat{Q}_{2ii'}^{\text{r}} \right) / \sum_{i \neq i'} n_i n'_i} \end{aligned} \quad (\text{A48})$$

971 If the pools have all the same size (i.e., if $n_i = n$ for all i), then Equation A48
972 reduces to Equation A45, and $\tilde{F}_{\text{ST}}^{\text{pool-PID}} = \hat{F}_{\text{ST}}^{\text{pool-PID}}$. With unbalanced sam-
973 ples, simulation analyses show that $\tilde{F}_{\text{ST}}^{\text{pool-PID}}$ has larger bias and variance
974 than $\hat{F}_{\text{ST}}^{\text{pool-PID}}$, in particular for low levels of differentiation (see Figure S4).

Table S1 Comparison of pairwise F_{ST} estimates

F_{ST}	n	Pool-seq		Ind-seq
		Cov.	$\hat{F}_{ST}^{pool}$	WC ₈₄
0.05	10	20×	0.051 (0.004)	
0.05	10	50×	0.051 (0.004)	0.051 (0.003)
0.05	10	100×	0.051 (0.003)	
0.05	100	20×	0.051 (0.003)	
0.05	100	50×	0.051 (0.003)	0.051 (0.002)
0.05	100	100×	0.051 (0.002)	
0.20	10	20×	0.203 (0.007)	
0.20	10	50×	0.202 (0.006)	0.202 (0.007)
0.20	10	100×	0.201 (0.006)	
0.20	100	20×	0.201 (0.006)	
0.20	100	50×	0.201 (0.006)	0.201 (0.005)
0.20	100	100×	0.202 (0.005)	

F_{ST} was estimated for various conditions of expected F_{ST} , pool size (n) and coverage (Cov.). For Pool-seq data, we computed our estimator $\hat{F}_{ST}^{pool}$ (Equation 13). The mean (RMSE) over 50 independent replicates of the **ms** simulations are provided for a single pair of populations. For comparison, we computed WC₈₄ from allele count data inferred from individual genotypes (Ind-seq).

Table S2 Effect of unequal sampling on pairwise F_{ST} estimates

F_{ST}	n	Pool-seq		Ind-seq
		Cov.	$\hat{F}_{ST}^{pool}$	WC ₈₄
0.05	$\mathcal{N}(100, 30)$	20×	0.051 (0.003)	
0.05	$\mathcal{N}(100, 30)$	50×	0.052 (0.003)	0.051 (0.002)
0.05	$\mathcal{N}(100, 30)$	100×	0.051 (0.002)	
0.20	$\mathcal{N}(100, 30)$	20×	0.202 (0.007)	
0.20	$\mathcal{N}(100, 30)$	50×	0.202 (0.006)	0.202 (0.006)
0.20	$\mathcal{N}(100, 30)$	100×	0.202 (0.006)	

Pairwise F_{ST} was estimated for various conditions of expected F_{ST} and coverage (Cov.). The pool size (n) was variable across demes, with haploid sample size n drawn independently for each deme from a Gaussian distribution with mean 100 and standard deviation 30; n was rounded up to the nearest integer, with min. 20 and max. 300 haploids per deme. For Pool-seq data, we computed our estimator $\hat{F}_{ST}^{pool}$ (Equation 13). The mean (RMSE) over 50 independent replicates of the **ms** simulations are provided, for a single pair of populations. For comparison, we computed WC₈₄ (Weir and Cockerham 1984) from allele count data inferred from individual genotypes (Ind-seq).

Table S3 Effect of variable coverage on pairwise F_{ST} estimates

F_{ST}	n	Pool-seq		Ind-seq
		Δ	$\hat{F}_{ST}^{pool}$	WC ₈₄
0.05	10	20	0.050 (0.006)	
0.05	10	50	0.050 (0.004)	0.050 (0.004)
0.05	10	100	0.050 (0.004)	
0.05	100	20	0.051 (0.003)	
0.05	100	50	0.051 (0.002)	0.051 (0.002)
0.05	100	100	0.051 (0.002)	
0.20	10	20	0.200 (0.007)	
0.20	10	50	0.200 (0.007)	0.200 (0.007)
0.20	10	100	0.200 (0.007)	
0.20	100	20	0.202 (0.006)	
0.20	100	50	0.203 (0.006)	0.203 (0.005)
0.20	100	100	0.203 (0.005)	

Pairwise F_{ST} was estimated for various conditions of expected F_{ST} and pool size (n). The coverage (δ_i) was varying across demes and loci, with $\delta_i \sim \text{Pois}(\Delta)$. For Pool-seq data, we computed our estimator $\hat{F}_{ST}^{pool}$ (Equation 13). The mean (RMSE) over 50 independent replicates of the **ms** simulations are provided, for a single pair of populations. For comparison, we computed WC₈₄ from allele count data inferred from individual genotypes (Ind-seq).

Figure S1 Pairwise estimators of F_{ST} . A. Multilocus estimates $\hat{F}_{ST}^{pool}$ (computed using Equation 13) as a function of WC_{84} estimates computed from allele count data inferred from individual genotypes. B. Multilocus estimates $PP2_d$, as a function of NC_{83} estimates computed from allele count data inferred from individual genotypes. C. Multilocus estimates $\hat{F}_{ST}^{pool}$ as a function of $PP2_d$ estimates. In each graph, the dots represent multilocus estimates of F_{ST} across all pairs of subpopulations from an 8-island model, and across 50 replicate *ms* simulations. We specified the migration rate corresponding to $F_{ST} = 0.20$. The size of each pool was fixed to 100. The results are shown for different coverages (20X, 50X and 100X). The cross indicates the simulated value of the parameter F_{ST} .

Figure S2 Root mean squared error (RMSE) of F_{ST} estimates for a wide range of pool sizes and coverage, with experimental error rate ϵ varying from 0 to 0.5 (A–E). Each density plot gives the RMSE of our estimator $\hat{F}_{ST}^{\text{pool}}$, using simple linear interpolation from a set of 44×44 pairs of pool size and coverage values. For each pool size and coverage, 500 replicates of 5,000 markers were simulated. Plain white isolines represent the RMSE of the WC_{84} estimator computed from Ind-seq data, for various sample sizes ($n = 5, 10, 20, \text{ and } 50$). Each isoline was fitted using a thin plate spline regression with smoothing parameter $\lambda = 0.005$, implemented in the `fields` package for R (Nychka et al. 2017).

Figure S3 Global estimators of F_{ST} . We considered one estimator based on allele count data inferred from individual genotypes (Ind-seq): WC₈₄. For pooled data, we computed F_{ST} using the WC₈₄ estimator: (i) directly from read counts, as if they were allele counts (“reads”); (ii) from allele counts imputed by maximum-likelihood (“imput”), as in Leblois et al. (2018). Each boxplot represents the distribution of multilocus F_{ST} estimates across all demes comparisons in an 8-island model, and across 50 independent replicates of the **ms** simulations. We used two migration rates, corresponding to $F_{ST} = 0.05$ (A) or $F_{ST} = 0.20$ (B). The size of each pool was fixed to 10. For Pool-seq data, we show the results for different coverages (20X, 50X and 100X). In each graph, the dashed line indicates the simulated value of F_{ST} .

Figure S4 Precision and accuracy of alternative estimators of F_{ST} with varying pool size, for various levels of differentiation (A–D). The haploid pool size n drawn independently for each deme from a Gaussian distribution with mean 100 and standard deviation 30; n was rounded up to the nearest integer, with min. 20 and max. 300 haploids per deme. We considered three estimators based on allele count data inferred from individual genotypes (Ind-seq): WC_{84} , $\hat{F}_{ST} \equiv (\hat{Q}_1 - \hat{Q}_2) / (1 - \hat{Q}_2)$ (where $\hat{Q}_1$ and $\hat{Q}_2$ are the weighted frequencies of identical pairs of genes within and between subpopulations, respectively, with weights equal to the number of pairs of genes) and $\tilde{F}_{ST} \equiv (\tilde{Q}_1 - \tilde{Q}_2) / (1 - \tilde{Q}_2)$ (where $\tilde{Q}_1$ and $\tilde{Q}_2$ are the unweighted frequencies of identical pairs of genes within and between subpopulations, respectively). For Pool-seq data, we considered the estimators $\hat{F}_{ST}^{pool}$ (Equation 12), $\hat{F}_{ST}^{pool-PID}$ (Equation A44) and $\tilde{F}_{ST}^{pool-PID}$ (Equation A45). Each boxplot represents the distribution of multilocus F_{ST} across 50 independent replicates of the *ms* simulations. For Pool-seq data, we show the results for different coverages (20X, 50X and 100X). In each graph, the dashed line indicates the simulated value of F_{ST} and the dotted line indicates the median of the distribution of WC_{84} estimates.