Measuring genetic differentiation from Pool-seq data

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Abstract

The recent 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. Last, 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).

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 used 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 costs than sequencing of individuals (Schlötterer et al. 2014). 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). However, it has been shown that, at equal sequencing effort, Pool-seq provides similar, if not more accurate, allele frequency estimates than individual-based (Ind-seq) 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, it is indeed impossible to distinguish pairs of reads that are identical because they were sequenced

from a single gene, from pairs of reads that are identical because they were sequenced from two distinct genes that are identical in state (IIS) (Ferretti et al. 2013). Note that throughout this article, we use the term "gene" to designate a segregating genetic unit (in the sense of the "Mendelian gene" from Orgogozo et al. 2016). A straightforward approach that consists in computing F_{ST} from read counts, as if they were allele counts, ignores the extra variance caused by the sampling of reads from genes during Pool-seq experiments. Furthermore, such computation fails to consider the actual number of lineages in the pool (haploid pool size). A possible alternative amounts to compute F_{ST} from allele counts imputed from read counts using a maximum-likelihood approach conditional on the size of the pools (see, e.g., 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.

Appropriate estimators of diversity and differentiation parameters must therefore be sought, to account for both the sampling of individual genes from the pool and the sampling of reads from these genes. There has been several attempts to define estimators for the parameter F_{ST} for Pool-seq data (Kofler et al. 2011; Ferretti et al. 2013), from ratios of heterozygosities (or from probabilities of genetic identity between pairs of reads) within and between pools. In the following, we will argue that these estimators are biased (i.e., they do not converge towards the expected value of the parameter),

and that some of them have undesired statistical properties (i.e., the bias depends upon sample size and coverage). Here, following Cockerham (1969), Cockerham (1973), Weir and Cockerham (1984), Weir (1996) and Rousset (2007), we define a method-of-moments estimator of the parameter F_{ST} using an analysis-of-variance framework. We then evaluate the accuracy and the precision of this estimator, based on the analysis of simulated datasets, and compare it to estimates from the software package PoPoolation2 (Kofler et al. 2011), and from Ferretti et al. (2013). Furthermore, we test the robustness of our estimators to model misspecifications (including unequal contributions of individuals in pools, and sequencing errors). Last, we reanalyze the prickly sculpin (*Cottus asper*) Pool-seq data (published by Dennenmoser et al. 2017), and show how the use of unbiased F_{ST} estimators may challenge the interpretation of population structure.

MODEL

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 the resulting estimators are combined for several loci. Consider a sample of n_{d} subpopulations, each of which is made of n_{i} genes ($i = 1, \ldots, n_{\mathrm{d}}$) sequenced in pools (hence n_{i} is the haploid sample size of the ith pool). We define c_{ij} as the number of reads sequenced from gene j ($j = 1, \ldots, n_{\mathrm{i}}$) 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 ($r = 1, \ldots, c_{ij}$) from gene j in subpopulation i, such that $X_{ijr:k} = 1$ if the rth read from the jth gene in the ith deme is of type k, and $X_{ijr:k} = 0$ otherwise. In the following, we use standard notations for sample averages, i.e.: $X_{ij\cdot:k} \equiv \sum_r X_{ijr:k}/c_{ij}$, $X_{i\cdot\cdot:k} \equiv \sum_j \sum_r X_{ijr:k}/\sum_j c_{ij}$ and $X_{\cdots:k} \equiv \sum_i \sum_j \sum_r X_{ijr:k}/\sum_i \sum_j c_{ij}$. The analysis of variance is based on the computation of sums of squares, as follows:

$$\begin{aligned}
\sum_i^{n_{\mathrm{d}}} \sum_j^{n_i} \sum_r^{c_{ij}} (X_{ijr:k} - X_{\cdots:k})^2 &= \sum_i^{n_{\mathrm{d}}} \sum_j^{n_i} \sum_r^{c_{ij}} (X_{ijr:k} - X_{ij\cdot:k})^2 \\
&+ \sum_i^{n_{\mathrm{d}}} \sum_j^{n_i} \sum_r^{c_{ij}} (X_{ij\cdot:k} - X_{i\cdot\cdot:k})^2 \\
&+ \sum_i^{n_{\mathrm{d}}} \sum_j^{n_i} \sum_r^{c_{ij}} (X_{i\cdot\cdot:k} - X_{\cdots:k})^2 \\
&\equiv SSR_{:k} + SSI_{:k} + SSP_{:k} \qquad (1)
\end{aligned}$$

As is shown in the Supplemental File S1, the expected sums of squares depend on the expectation of the allele frequency π_k over independent replicates of the evolutionary process, as well as on the IIS probability $Q_{1:k}$ that two genes

in the same pool are both of type k, and the IIS probability $Q_{2:k}$ that two genes from different pools are both of type k. Taking expectations (see the detailed computations in the Supplemental File S1), one has:

$$\mathbb{E}(SSR_{:k}) \quad = \quad 0 \tag{2}$$

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

$$\mathbb{E}(SSI_{:k}) \quad = \quad (C_1 - D_2)\,(\pi_k - Q_{1:k}) \tag{3}$$

where $C_1 \equiv \sum_i \sum_j c_{ij}$ is the total number of observed reads in the full sample, and $D_2 \equiv \mathbb{E}\left(\sum_i \sum_j c_{ij}^2 / C_{1i}\right)$. Because the reads c_{ij} are not observed, D_2 is approximated by assuming a multinomial distribution of the c_{ij}'s across genes: $D_2 \equiv \sum_i (C_{1i} + n_i - 1)\,/n_i$ (see Equation A15 in Supplemental File S1). For reads between genes from different pools, we have:

$$\mathbb{E}(SSP_{:k}) \quad = \quad \left(C_1 - \frac{C_2}{C_1}\right)(Q_{1:k} - Q_{2:k}) + (D_2 - D_2^\star)\,(\pi_k - Q_{1:k}) \tag{4}$$

where $C_2 \equiv \sum_i \left(\sum_j c_{ij}\right)^2$ and $D_2^\star \equiv \mathbb{E}\left(\sum_i \sum_j c_{ij}^2 / C_1\right)$. As for D_2, $D_2^\star$ is also approximated by assuming a multinomial distribution of the reads c_{ij} across genes: $D_2^\star \equiv \left[\sum_i C_{1i}\,(C_{1i} + n_i - 1)\,/n_i\right]/C_1$ (see Equation A16 in Supplemental File S1). Rearranging Equations 3–4, and summing over alleles, we get:

$$Q_1 - Q_2 = \frac{(C_1 - D_2)\,\mathbb{E}(SSP) - (D_2 - D_2^\star)\,\mathbb{E}(SSI)}{(C_1 - D_2)\,(C_1 - C_2/C_1)} \tag{5}$$

and:

$$1 - Q_2 = \frac{(C_1 - D_2)\,\mathbb{E}(SSP) + (n_\mathrm{c} - 1)\,(D_2 - D_2^\star)\,\mathbb{E}(SSI)}{(C_1 - D_2)\,(C_1 - C_2/C_1)} \tag{6}$$

where $n_\mathrm{c} \equiv (C_1 - C_2/C_1)\,/\,(D_2 - D_2^\star)$. Let $MSI \equiv SSI/\,(C_1 - D_2)$ and $MSP \equiv SSP/\,(D_2 - D_2^\star)$. Then:

$$F_\mathrm{ST} \equiv \frac{Q_1 - Q_2}{1 - Q_2} = \frac{\mathbb{E}(MSP) - \mathbb{E}(MSI)}{\mathbb{E}(MSP) + (n_\mathrm{c} - 1)\,\mathbb{E}(MSI)} \tag{7}$$

which yields the method-of-moments estimator:

$$\hat{F}_\mathrm{ST}^\mathrm{pool} = \frac{MSP - MSI}{MSP + (n_\mathrm{c} - 1)\,MSI} \tag{8}$$

Note that Equation A27 in Supplemental File S1 gives an expression of $\hat{F}_\mathrm{ST}^\mathrm{pool}$ in terms of sample frequencies of reads. If we take the limit case where each gene is sequenced exactly once, we recover the Ind-seq model: assuming $c_{ij} = 1$ for all (i, j), then $C_1 = \sum_i^{n_\mathrm{d}} n_i \equiv S_1$, $C_2 = \sum_i^{n_\mathrm{d}} n_i^2 \equiv S_2$, $D_2 = n_\mathrm{d}$ and $D_2^\star = 1$. Therefore, $n_\mathrm{c} = (S_1 - S_2/S_1)\,/\,(n_\mathrm{d} - 1)$, and Equation 8 reduces exactly to the estimator of F_ST for haploids: see Weir (1996), p. 182, and Rousset (2007), p. 977.

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

$$\hat{F}_\mathrm{ST} = \frac{\sum_l MSP_l - MSI_l}{\sum_l MSP_l + (n_\mathrm{c} - 1)\,MSI_l} \tag{9}$$

where MSI and MSP are subscripted with l to denote the lth locus. For Ind-seq data, Bhatia et al. (2013) refer to this multilocus estimate as a "ratio

of averages" by opposition to an "average of ratios", which would consist in averaging single-locus F_{ST} over loci. This approach is justified in the Appendix of Weir and Cockerham (1984) and in Bhatia et al. (2013), who analyzed both estimates by means of coalescent simulations. Note that Equation 9 assumes that the pool size is equal across loci. Also note that the construction of the estimator in Equation 9 is different from Weir and Cockerham's (1984). These authors defined their multilocus estimator as a ratio of sums of components of variance (a, b and c in their notation) over loci, which give the same weight to all loci, whatever the number of sampled genes at each locus. Equation 9 follows Genepop's rationale (Rousset 2008), which gives 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 to the `ms` parameterization $M \equiv 2Nm = 16.569$ for $F_{\mathrm{ST}} = 0.05$ and $M = 3.489$ for $F_{\mathrm{ST}} = 0.20$. The mutation rate was set at $\mu = 10^{-6}$, giving $\theta \equiv 2N\mu = 0.01$. We considered either fixed ($n = 10$ or 100), 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 400,000 sequences for each scenario, in order to retain 50,000 independent single nucleotide polymorphisms (SNPs) from sequences with a single segregating site. Each scenario was replicated 50 times.

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:

$$r_{i:k} \sim \mathrm{Bin}\left(\frac{y_{i:k}}{n_i}, \delta_i\right) \tag{10}$$

where $y_{i:k}$ is the number of genes of type k in the ith 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 $\mu_\text{e} = 0.001$, which is typical of Illumina sequencers (Glenn 2011; Ross et al. 2013). 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 only start this procedure with polymorphic markers, we neglect sequencing errors that would create spurious SNPs. 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 ith pool (C_{1i}) follows a Dirichlet distribution:

$$\{\eta_{ij}\}_{1 \leq j \leq n_i} \sim \text{Dir}\left(\frac{\rho}{n_i}\right) \tag{11}$$

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. (see Gautier et al. 2013): $\epsilon \equiv \sqrt{\mathbb{V}(\eta_{ij})}/\mathbb{E}(\eta_{ij}) = \sqrt{(n_i - 1)/(\rho + 1)}$. 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 $\epsilon = 0.5$, which would correspond to a situation where (for $n_i = 10$) 5 individuals contribute 2.8× 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.

$\mathrm{PP2_d}$: 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:

$$\mathrm{PP2_d} \equiv \frac{\hat{H}_{\mathrm{T}} - \hat{H}_{\mathrm{S}}}{\hat{H}_{\mathrm{T}}} \tag{12}$$

where $\hat{H}_{\mathrm{S}}$ is the average heterozygosity within subpopulations, and $\hat{H}_{\mathrm{T}}$ is the average heterozygosity in the total population (obtained by pooling together all subpopulation to form a single virtual unit). In PoPoolation2, $\hat{H}_{\mathrm{S}}$ is

the unweighted average of within-subpopulation heterozygosities:

$$\hat{H}_{\mathrm{S}} = \frac{1}{n_{\mathrm{d}}} \sum_{i}^{n_{\mathrm{d}}} \left(\frac{n_i}{n_i - 1} \right) \left(\frac{C_{1i}}{C_{1i} - 1} \right) \sum_{k} \left(1 - \hat{\pi}_{i:k}^2 \right) \tag{13}$$

(using the notation from Table 1). Note that in PoPoolation2, $\mathrm{PP2_d}$ is restricted to the case of two subpopulations only ($n_{\mathrm{d}} = 2$). The two ratios in the right-hand side of Equation 13 are presumably borrowed from Nei (1978) to provide an unbiased estimate, although we found no formal justification for this expression. The total heterozygosity is computed as (using the notation from Table 1):

$$\hat{H}_{\mathrm{T}} = \left(\frac{\min_i(n_i)}{\min_i(n_i) - 1} \right) \left(\frac{\min_i(C_{1i})}{\min_i(C_{1i}) - 1} \right) \sum_{k} \left(1 - \hat{\pi}_{k}^2 \right) \tag{14}$$

$\mathrm{PP2_a}$: this is the alternative estimator of F_{ST} provided in the software package PoPoolation2. It is based on an interpretation by Kofler et al. (2011) of Karlsson et al.'s (2007) estimator of F_{ST}, as:

$$\mathrm{PP2_a} \equiv \frac{\hat{Q}_1^{\mathrm{r}} - \hat{Q}_2^{\mathrm{r}}}{1 - \hat{Q}_2^{\mathrm{r}}} \tag{15}$$

where $\hat{Q}_1^{\mathrm{r}}$ and $\hat{Q}_2^{\mathrm{r}}$ are the frequencies of identical pairs of reads within and between pools, respectively, computed by simple counting of IIS pairs. These are estimates of Q_1^{r}, the IIS probability for two reads in the same pool (whether they are sequenced from the same gene or not) and Q_2^{r}, the IIS probability for two reads in different pools. Note that the IIS probabiliy Q_1^{r} is different from Q_1 in Equation 7, which, from our definition, represents the IIS probability between distinct genes in the same pool. This approach 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 IIS genes.

FRP_{13} : 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 12 above, although they estimate heterozygosities within and between pools as "average pairwise nucleotide diversities", which are formally equivalent to IIS probabilities. In particular, they estimate the average heterozygosity within pools as (using the notation from Table 1):

$$\hat{H}_{\mathrm{S}} = \frac{1}{n_{\mathrm{d}}} \sum_{i}^{n_{\mathrm{d}}} \left(\frac{n_i}{n_i - 1} \right) \left(1 - \hat{Q}^{\mathrm{r}}_{1i} \right) \tag{16}$$

and the total heterozygosity among the n_{d} populations as:

$$\hat{H}_{\mathrm{T}} = \frac{1}{n_{\mathrm{d}}^2} \left[\sum_{i}^{n_{\mathrm{d}}} \left(\frac{n_i}{n_i - 1} \right) \left(1 - \hat{Q}^{\mathrm{r}}_{1i} \right) + \sum_{i \neq i'}^{n_{\mathrm{d}}} \left(1 - \hat{Q}^{\mathrm{r}}_{2ii'} \right) \right] \tag{17}$$

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 12 above), hereafter denoted by NC_{83}, or the analysis-of-variance estimator developed by Weir and Cockerham (1984), hereafter denoted by WC_{84}.

All the estimators were computed using custom functions in the R software environment for statistical computing, version 3.3.1 (R Core Team

2017). All these functions were carefully checked against available software packages.

Application example: *Cottus asper*

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

Our aim here was to compare the population structure inferred from pairwise estimates of F_{ST}, using the estimator $\hat{F}_{\mathrm{ST}}^{\mathrm{pool}}$ on the one hand, and $\mathrm{PP2_d}$ on the other hand. Then, to conclude on which of the two estimators per-

forms better, we compared the population structure inferred from $\hat{F}_{\mathrm{ST}}^{\mathrm{pool}}$ and $\mathrm{PP2_d}$ 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.

RESULTS

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

Single-locus estimates $\hat{F}_{\mathrm{ST}}^{\mathrm{pool}}$ 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 $\hat{F}_{\mathrm{ST}}^{\mathrm{pool}}$ across independent replicates decreases as the coverage increases. The correlation between $\hat{F}_{\mathrm{ST}}^{\mathrm{pool}}$ and WC_{84} is stronger for multilocus estimates (see Figure S1A).

Comparing Pool-seq estimators of F_{ST}

We found that our estimator $\hat{F}_{\mathrm{ST}}^{\mathrm{pool}}$ is virtually unbiased: the average across multiple loci and replicates converges towards the simulated value of the 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 $\hat{F}_{\mathrm{ST}}^{\mathrm{pool}}$ over independent replicates are virtually indistinguishable from that of the WC_{84} estimator (see Table S1).

Conversely, we found that $\mathrm{PP2_d}$ (the default estimator of F_{ST} implemented in the software package PoPoolation2) is biased when compared to the expected value of the parameter. We observed that the bias depends on both the sample size, and the coverage (see Figure 2). We note that, as the

coverage and the sample size increase, $PP2_d$ converges to the estimator NC_{83} (Nei and Chesser 1983) computed from individual data (see Figure S1B). This argument was used by Kofler et al. (2011) to validate the approach, even though the estimates $PP2_d$ depart from the true value of the parameter (Figure S1B–C).

The second of the two estimators of F_{ST} implemented in PoPoolation2, that we refer to as $PP2_a$, is also biased (see Figure 2). We note that the bias decreases as the sample size increases. However, the bias does not depend on the coverage (only the variance over independent replicates does). The estimator developed by Ferretti et al. (2013), that we refer to as FRP_{13}, is also biased (see Figure 2). However, the bias does neither depend on the pool size, nor on the coverage (only the variance over independent replicates does). FRP_{13} converges to the estimator NC_{83}, 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 the analysis of pairs, 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 3). We found that, at low coverage, unequal sampling

or variable coverage causes a negligible departure from the median of WC_{84} (Weir and Cockerham 1984) 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 3 and Tables S2–S3).

Robustness to sequencing and experimental errors

Figure 4 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 5B). It must be noted, though, that filtering increases the bias in the absence of sequencing error, especially at low coverage (Figure 5A). 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 4). We note that the bias decreases as the size of the pools increases. Filtering (using a minimum read count of 4) does not improve estimation (Figure 5C).

Application example

We measured larger pairwise estimates of multilocus F_{ST} using $PP2_d$ estimator (Kofler et al. 2011), as compared to $\hat{F}_{ST}^{pool}$ (see Figure 6A). Furthermore, $\hat{F}_{ST}^{pool}$ estimates are smaller for within-ecotype pairwise comparisons, as compared to between-ecotype comparisons. Therefore, the inferred relationships between samples based on $\hat{F}_{ST}^{pool}$ estimates show a clear-cut structure separating the two estuarine samples from the freshwater samples (see Figure 6C). We did not recover the same structure using $PP2_d$ estimates (see Figure 6B). Supportingly, the scaled covariance matrix of allele frequencies across samples

is consistent with the structure inferred from $\hat{F}_{\mathrm{ST}}^{\mathrm{pool}}$ estimates (see Figure 6D).

DISCUSSION

Whole-genome sequencing of pools of individuals is being increasingly used 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 sampling error brought by the random sampling of reads from the gene pool during sequencing, which may result in severely biased estimates of differentiation when the pool size is low (see Figure S2). Furthermore, the alternative approach that amounts to impute the allele counts from the read counts, given the haploid size of the pools (e.g., as in Smadja et al. 2012; Leblois et al. 2018) may only be accurate when the coverage is much larger than pool size (see Figure S2).

Here, we developed a new estimator of the parameter F_{ST} for Pool-seq data, in an analysis-of-variance framework (Cockerham 1969, 1973). The precision of this estimator is barely distinguishable from that of the Weir and Cockerham's (1984) estimator for individual data. Furthermore, we have shown that the accuracy of this estimator does neither depend on the pool size nor on the coverage, and is robust to unequal pool sizes and varying coverage across demes and loci.

Analysis of variance and probabilities of identity

In the analysis-of-variance framework, F_{ST} is defined in Equation 7 as an 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 and Cuzick 1979; Mak 1988; Ridout et al. 1999). 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 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 Supplemental File S1, and we provide an unbiased estimator of F_{ST} (Equation A41 in Supplemental File S1). This estimator has 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 A30). 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 provides identical estimates when sample sizes are equal (see also Cockerham and Weir 1987; Karlsson et al. 2007). With unbalanced samples, we found that analysis-of-variance estimates have

lower bias and variance than IIS-based estimates (not shown), the latter being more computationally demanding than the former. Interestingly, we found that IIS-based estimates of F_{ST} for Pool-seq data generally showed lower bias and variance if the overall estimates of IIS probabilities within and between pools were computed as unweighted averages of population-specific or pairwise estimates (see Equations A36 and A40), as compared to weighted averages (as, e.g., in Fleiss and Cuzick 1979).

It follows from the derivations in the Supplemental File S1 that the estimator $\mathrm{PP2_a}$ (Equation 15) is biased, because the IIS probability between pairs of reads within a pool $\left(\hat{Q}_1^{\mathrm{r}}\right)$ 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 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 16 and 17). However, although their Equation 16 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 17), 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 12–14) 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). 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. 2017) 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 $\hat{F}_{\mathrm{ST}}^{\mathrm{pool}}$, we found a clear-cut structure separating the freshwater from the brackish-water ecotypes. Such genetic strucure supports the hypothesis that populations are locally adaptated 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 $\mathrm{PP2_d}$ 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 $\hat{F}_{\mathrm{ST}}^{\mathrm{pool}}$ estimate is unequal contributions of individuals in pools. This is so, because we assume 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 an equal sequencing coverage for all genes (Rode et al. 2017). 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. 2017), and we have shown here that we can achieve very good precision and accuracy of F_{ST} estimates with smaller pool sizes ($n = 10$). 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 the evolutionary process (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 probabilies provided in the Supplemental File S1.

DATA ACCESSIBILITY

A R package, called `poolfstat`, which impletements 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 rth read from the jth individual in the ith 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 ith pool
c_{ij}	Number of reads sequenced from individual j in subpopulation i (unobserved individual coverage)
$C_{1i} \equiv \sum_j c_{ij}$	Total number of reads in the ith 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 ith pool (haploid pool size)
$y_{i:k}$	(Unobserved) number of genes of type k in the ith 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 ith pool
$\hat{\pi}_{i:k} \equiv X_{i\cdot\cdot:k}$	Average frequency of reads of type k in the ith pool
$\hat{\pi}_k \equiv X_{\cdots:k}$	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
$\hat{F}_{\mathrm{ST}}^{\mathrm{pool}}$	Equation 8
FRP_{13}	Ferretti et al. (2013) and Equations 12,16–17
NC_{83}	Nei and Chesser (1983)
$\mathrm{PP2}_{\mathrm{d}}$	Kofler et al. (2011) and Equations 12–14
$\mathrm{PP2}_{\mathrm{a}}$	Kofler et al. (2011) and Equation 15
WC_{84}	Weir and Cockerham (1984)

Table 3 Overall F_{ST} estimates from multiple pools

		Pool-seq		Ind-seq
F_{ST}	n	Cov.	$\hat{F}_{\mathrm{ST}}^{\mathrm{pool}}$	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 $\hat{F}_{\mathrm{ST}}^{\mathrm{pool}}$ (Equation 9). The mean (RMSE) over 50 independent replicates of the `ms` simulations are provided, for all populations ($n_{\mathrm{d}} = 8$). For comparison, we computed WC_{84} from allele count data inferred from individual genotypes (Ind-seq).

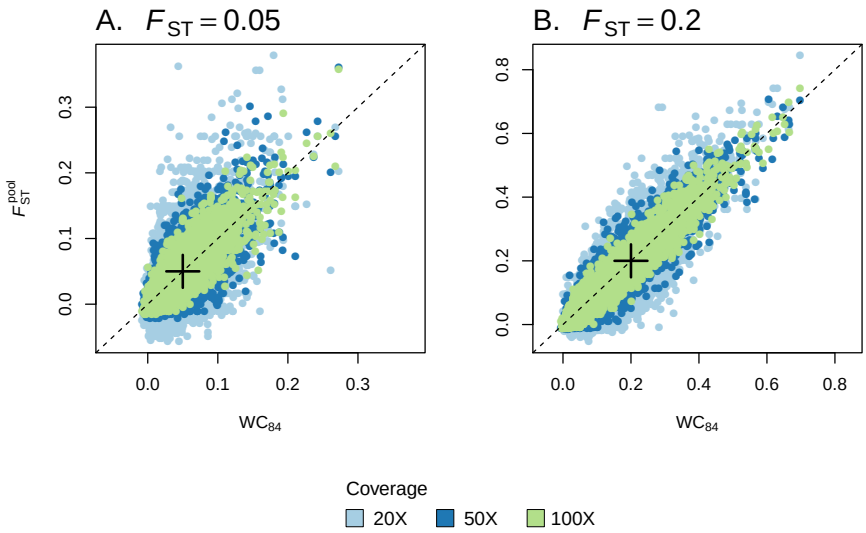

Figure 1 Single-locus estimates of F_{ST}. We compared single-locus estimates of F_{ST} based on allele count data inferred from individual genotypes (Ind-seq), using the WC_{84} estimator, to $\hat{F}_{\mathrm{ST}}^{\mathrm{pool}}$ estimates from pooled data. We simulated 5,000 SNPs using `ms` , in an island model with $n_{\mathrm{d}} = 8$ demes. We used two migration rates corresponding to $F_{\mathrm{ST}} = 0.05$ (A) and $F_{\mathrm{ST}} = 0.20$ (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 true value of F_{ST}.

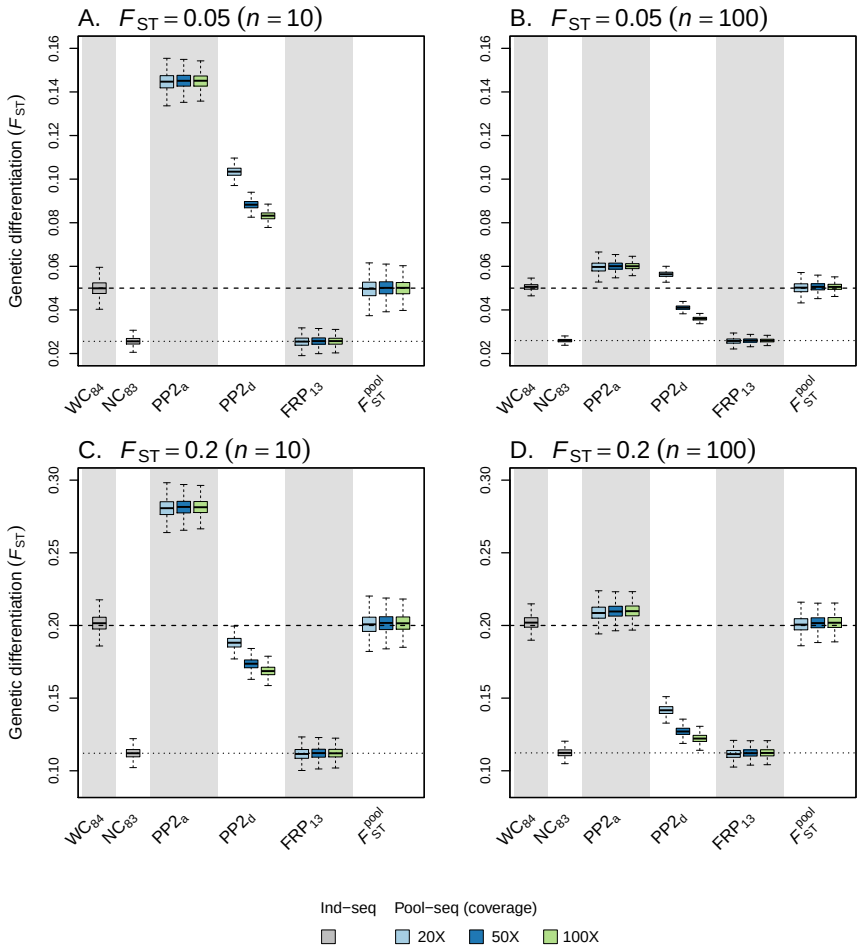

Figure 2 Pairwise estimators of F_{ST}. We considered two estimators based on allele count data inferred from individual genotypes (Ind-seq): WC_{84} and NC_{83}. For pooled data, we computed the two estimators implemented in the software package PoPoolation2, that we refer to as $\mathrm{PP2}_{\mathrm{d}}$ and $\mathrm{PP2}_{\mathrm{a}}$, as well as the FRP_{13} estimator and our estimator $\hat{F}_{\mathrm{ST}}^{\mathrm{pool}}$ (Equation 9). Each boxplot represents the distribution of multilocus F_{ST} estimates across all pairwise comparisons in an island model with $n_{\mathrm{d}} = 8$ demes, and across 50 independent replicates of the `ms` simulations. We used two migration rates, corresponding to $F_{\mathrm{ST}} = 0.05$ (A–B) or $F_{\mathrm{ST}} = 0.20$ (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 true value of F_{ST} and the dotted line indicates the median of the distribution of NC_{83} estimates.

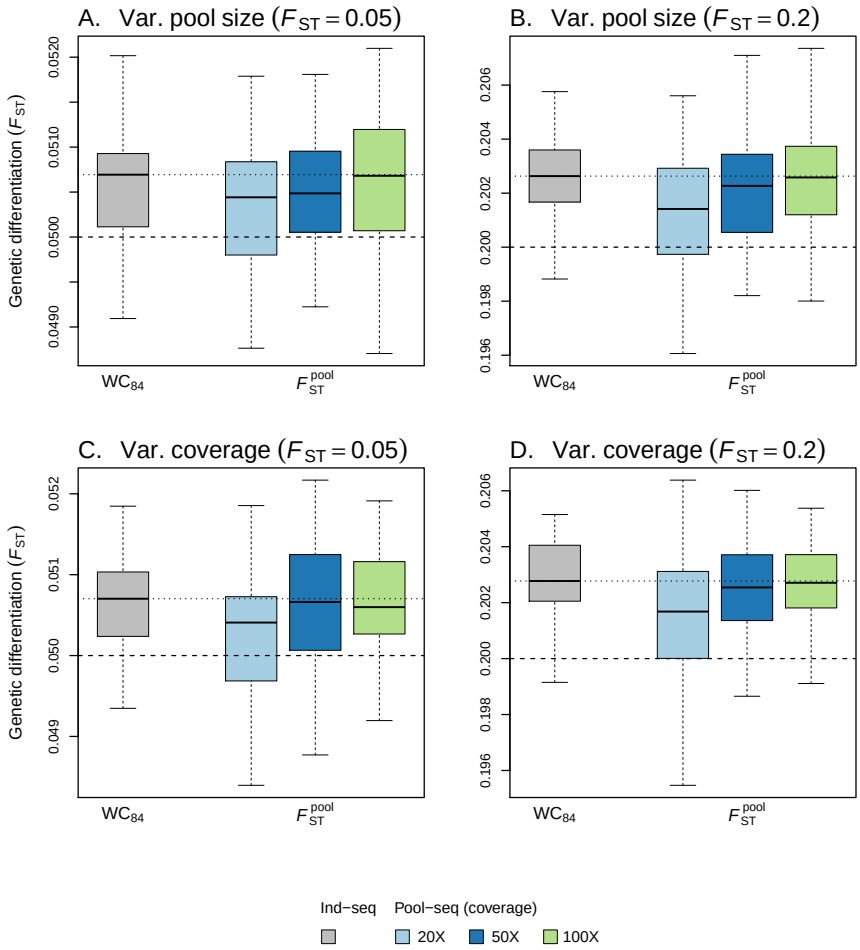

Figure 3 Global estimators of F_{ST}. Our estimator $\hat{F}_{\mathrm{ST}}^{\mathrm{pool}}$ (Equation 9) was calculated from Pool-seq data over all loci and demes and compared to the estimator WC_{84}, computed from allele count data inferred from individual genotypes (Ind-seq). Each boxplot represents the distribution of multilocus F_{ST} estimates across 50 independent replicates of the `ms` simulations. We used two migration rates, corresponding to $F_{\mathrm{ST}} = 0.05$ (A and C) or $F_{\mathrm{ST}} = 0.20$ (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 coverage (δ_i) was varying across demes and loci, with $\delta_i \sim \mathrm{Pois}(\Delta)$ 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 true value of F_{ST} and the dotted line indicates the median of the distribution of WC_{84} estimates.

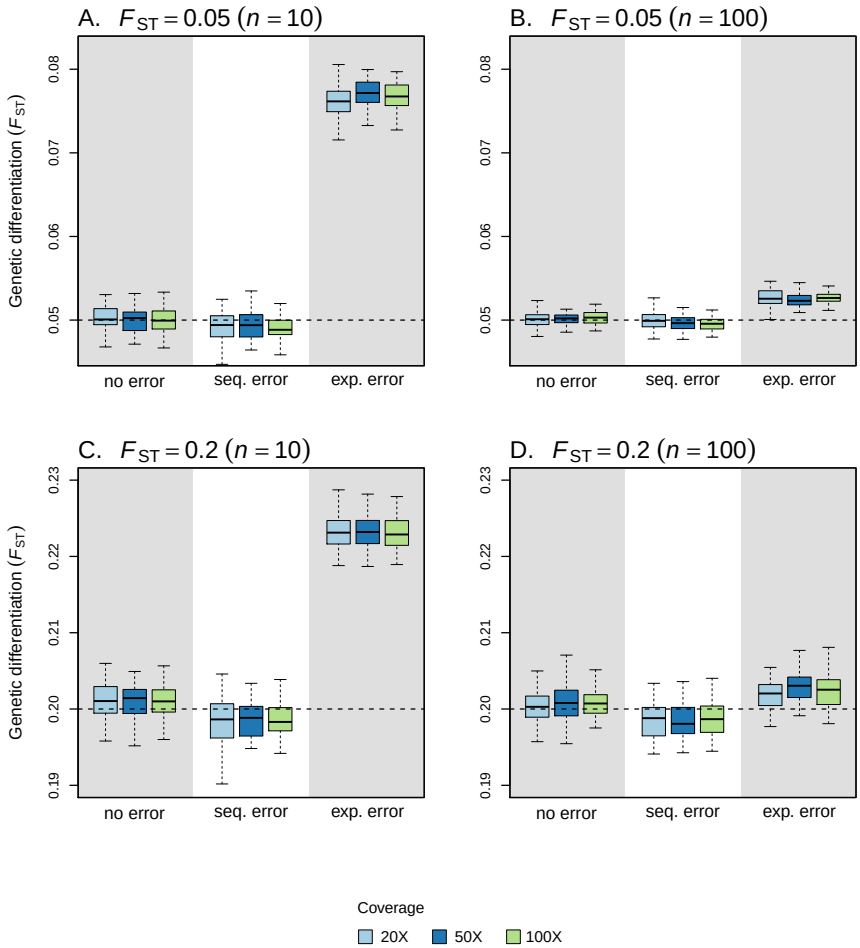

Figure 4 Precision and accuracy of F_{ST} estimates with sequencing and experimental errors. Our estimator $\hat{F}_{\mathrm{ST}}^{\mathrm{pool}}$ (Equation 9) was computed from Pool-seq data over all loci and demes without error, with sequencing error (occurring at rate $\mu_{\mathrm{e}} = 0.001$), 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 $F_{\mathrm{ST}} = 0.05$ (A–B) or $F_{\mathrm{ST}} = 0.20$ (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 true value of F_{ST}.

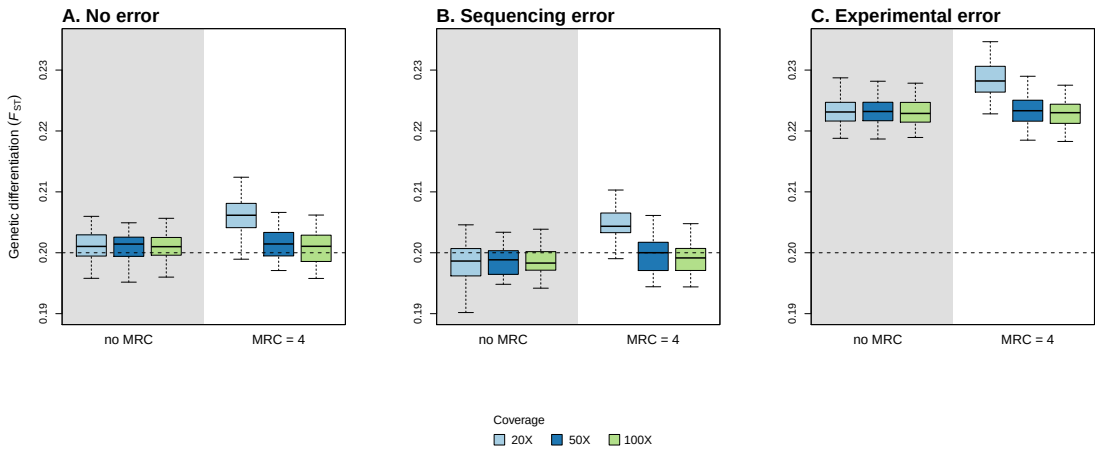

Figure 5 Precision and accuracy of F_{ST} estimates with and without filtering. Our estimator $\hat{F}_{\mathrm{ST}}^{\mathrm{pool}}$ (Equation 9) was computed from Pool-seq data over all loci and demes without error (A), with sequencing error (B) and with experimental error (C) (see the legend of Figure 4 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 $F_{\mathrm{ST}} = 0.20$, and pool size $n = 10$. We show the results for different coverages (20X, 50X and 100X). In each graph, the dashed line indicates the true value of F_{ST}.

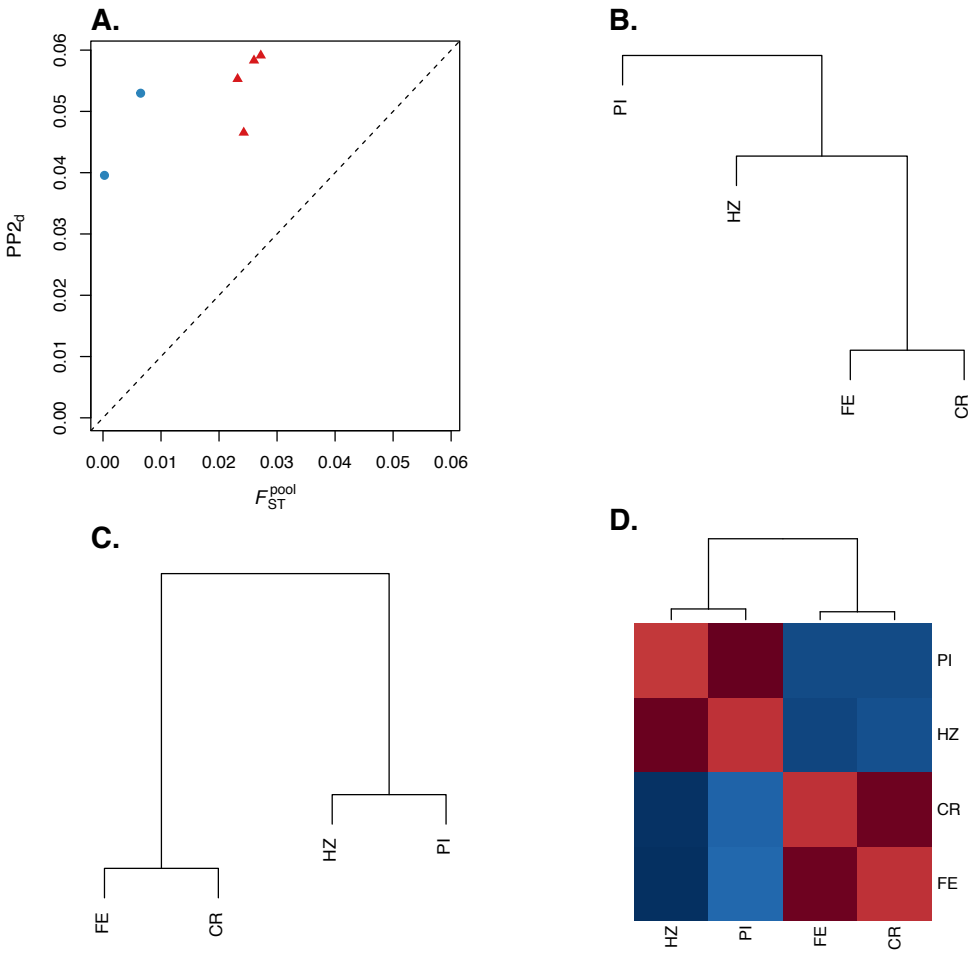

Figure 6 Analysis of the prickly sculpin (*Cottus asper*) Pool-seq data. In (A) we compare the pairwise F_{ST} estimates $\mathrm{PP2_d}$, and $\hat{F}_{\mathrm{ST}}^{\mathrm{pool}}$ (Equation 9) for all pairs of populations from the estuarine (CR and FE) and freshwater samples (PI and HZ). Within-ecotype 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 $\mathrm{PP2_d}$ (B) and $\hat{F}_{\mathrm{ST}}^{\mathrm{pool}}$ (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.

Analysis of variance for Pool-seq data

In the following, we first derive our model for a single locus. Consider a sample of n_{d} subpopulations, each of which is made of n_{i} genes ($i = 1, \ldots, n_{\mathrm{d}}$) sequenced in pools (hence n_{i} is the haploid sample size of the ith pool). We define c_{ij} as the number of reads sequenced from gene j ($j = 1, \ldots, n_{\mathrm{i}}$) 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 ($r = 1, \ldots, c_{ij}$) from gene j in subpopulation i, such that $X_{ijr:k} = 1$ if the rth read from the jth gene in the ith deme is of type k, and $X_{ijr:k} = 0$ otherwise. In the following, we use standard notations for sample averages, i.e.: $X_{ij\cdot:k} \equiv \sum_r X_{ijr:k}/c_{ij}$, $X_{i\cdot\cdot:k} \equiv \sum_j \sum_r X_{ijr:k}/\sum_j c_{ij}$ and $X_{\cdots:k} \equiv \sum_i \sum_j \sum_r X_{ijr:k}/\sum_i \sum_j c_{ij}$. The analysis of variance is based on the computation of sums of squares, as follows:

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

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

$$\begin{aligned} SSR_{:k} &= \sum_{i}^{n_{\mathrm{d}}} \sum_{j}^{n_i} \sum_{r}^{c_{ij}} \left(X_{ijr:k} - X_{ij\cdot:k}\right)^2 \\ &= 0 \end{aligned} \tag{A2}$$

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

$$\begin{aligned} SSI_{:k} &= \sum_{i}^{n_{\mathrm{d}}} \sum_{j}^{n_i} \sum_{r}^{c_{ij}} \left(X_{ij\cdot:k} - X_{i\cdot\cdot:k}\right)^2 \\ &= \sum_{i}^{n_{\mathrm{d}}} \sum_{j}^{n_i} \sum_{r}^{c_{ij}} \left(X_{ij\cdot:k} - \pi_k\right)^2 - \sum_{i}^{n_{\mathrm{d}}} \sum_{j}^{n_i} \sum_{r}^{c_{ij}} \left(X_{i\cdot\cdot:k} - \pi_k\right)^2 \\ &= \sum_{i}^{n_{\mathrm{d}}} \sum_{j}^{n_i} c_{ij} \left(X_{ij\cdot:k} - \pi_k\right)^2 - \sum_{i}^{n_{\mathrm{d}}} C_{1i} \left(X_{i\cdot\cdot:k} - \pi_k\right)^2 \end{aligned} \tag{A3}$$

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

$$\begin{aligned} SSP_{:k} &= \sum_{i}^{n_{\mathrm{d}}} \sum_{j}^{n_i} \sum_{r}^{c_{ij}} \left(X_{i\cdot\cdot:k} - X_{\cdot\cdot\cdot:k}\right)^2 \\ &= \sum_{i}^{n_{\mathrm{d}}} C_{1i} \left(X_{i\cdot\cdot:k} - \pi_k\right)^2 - C_1 \left(X_{\cdot\cdot\cdot:k} - \pi_k\right)^2 \end{aligned} \tag{A4}$$

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

type k within the ith pool: $\hat{\pi}_{i:k} \equiv X_{i\cdot\cdot:k}$, and of the average allele frequency of reads of type k in the full sample: $\hat{\pi}_k \equiv X_{\cdots:k}$. Then, developing the square in the first term in the right-hand side of Equation A3, we get:

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

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

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

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

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

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

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

Hence, developing the first term in the right-hand side of Equation A3 using Equation A5, we have:

$$
\sum_i^{n_\mathrm{d}} \sum_j^{n_i} c_{ij} \left(X_{ij\cdot:k} - \pi_k \right)^2 = C_1 \left(\hat{\pi}_k - 2\pi_k \hat{\pi}_k + \pi_k^2 \right) \qquad \text{(A8)}
$$

Likewise, developing the second term in the right-hand side of Equation A3 using Equation A6, we get:

$$\begin{aligned}\sum_{i}^{n_{\mathrm{d}}} C_{1i}\left(X_{i\cdot:k}-\pi_k\right)^2 &= C_1\left(\hat{\pi}_k-2\pi_k\hat{\pi}_k+\pi_k^2\right)+\sum_{i}^{n_{\mathrm{d}}}\sum_{j}^{n_i}\frac{c_{ij}^2}{C_{1i}}\left(\hat{\pi}_{ij:k}-\hat{\pi}_{i:k}\right)\\ &+ \sum_{i}^{n_{\mathrm{d}}}\left(C_{1i}-\sum_{j}^{n_i}\frac{c_{ij}^2}{C_{1i}}\right)\left(\hat{Q}_{1i:k}-\hat{\pi}_{i:k}\right) \qquad \text{(A9)}\end{aligned}$$

Last, developing the second term in the right-hand side of Equation A4 using Equation A7, we get:

$$\begin{aligned}C_1\left(X_{\cdots:k}-\pi_k\right)^2 &= C_1\left(\hat{\pi}_k-2\pi_k\hat{\pi}_k+\pi_k^2\right)+\sum_{i}^{n_{\mathrm{d}}}\sum_{j}^{n_i}\frac{c_{ij}^2}{C_1}\left(\hat{\pi}_{ij:k}-\hat{\pi}_k\right)\\ &+ \left(\frac{C_2}{C_1}-\sum_{i}^{n_{\mathrm{d}}}\sum_{j}^{n_i}\frac{c_{ij}^2}{C_1}\right)\left(\hat{Q}_{1:k}-\hat{\pi}_k\right)\\ &+ \left(C_1-\frac{C_2}{C_1}\right)\left(\hat{Q}_{2:k}-\hat{\pi}_k\right) \qquad \text{(A10)}\end{aligned}$$

Then, from Equations A3, A8 and A9:

$$\begin{aligned}SSI_{:k} &= \sum_{i}^{n_{\mathrm{d}}}\sum_{j}^{n_i}\frac{c_{ij}^2}{C_{1i}}\left(\hat{\pi}_{i:k}-\hat{\pi}_{ij:k}\right)\\ &+ \sum_{i}^{n_{\mathrm{d}}}\left(C_{1i}-\sum_{j}^{n_i}\frac{c_{ij}^2}{C_{1i}}\right)\left(\hat{\pi}_{i:k}-\hat{Q}_{1i:k}\right) \qquad \text{(A11)}\end{aligned}$$

and from Equations A4, A9 and A10:

$$\begin{aligned}SSP_{:k} &= \sum_{i}^{n_{\mathrm{d}}}\sum_{j}^{n_i}\frac{c_{ij}^2}{C_{1i}}\left(\hat{\pi}_{ij:k}-\hat{\pi}_{i:k}\right)-\sum_{i}^{n_{\mathrm{d}}}\left(C_{1i}-\sum_{j}^{n_i}\frac{c_{ij}^2}{C_{1i}}\right)\left(\hat{\pi}_{i:k}-\hat{Q}_{1i:k}\right)\\ &+ \sum_{i}^{n_{\mathrm{d}}}\sum_{j}^{n_i}\frac{c_{ij}^2}{C_1}\left(\hat{\pi}_k-\hat{\pi}_{ij:k}\right)+\left(\frac{C_2}{C_1}-\sum_{i}^{n_{\mathrm{d}}}\sum_{j}^{n_i}\frac{c_{ij}^2}{C_1}\right)\left(\hat{\pi}_k-\hat{Q}_{1:k}\right)\\ &+ \left(C_1-\frac{C_2}{C_1}\right)\left(\hat{\pi}_k-\hat{Q}_{2:k}\right) \qquad \text{(A12)}\end{aligned}$$

Taking expectation over independent replicates of the evolutionary process, we get, from Equation A3:

$$\begin{aligned}
\mathbb{E}(SSI_{:k}) &= \mathbb{E}\left(\hat{\pi}_{i:k} - \hat{\pi}_{ij:k}\right)\mathbb{E}\left(\sum_{i}^{n_\mathrm{d}}\sum_{j}^{n_i}\frac{c_{ij}^2}{C_{1i}}\right) \\
&+ \mathbb{E}\left(\hat{\pi}_{i:k} - \hat{Q}_{1i:k}\right)\mathbb{E}\left(\sum_{i}^{n_\mathrm{d}}\left(C_{1i} - \sum_{j}^{n_i}\frac{c_{ij}^2}{C_{1i}}\right)\right) \\
&= (\pi_k - Q_{1:k})\left(C_1 - \mathbb{E}\left(\sum_{i}^{n_\mathrm{d}}\sum_{j}^{n_i}\frac{c_{ij}^2}{C_{1i}}\right)\right) \qquad \text{(A13)}
\end{aligned}$$

where $Q_{1:k}$ is the expected IIS probability that two genes in the same pool are both of type k. Likewise, from Equation A4:

$$\begin{aligned}
\mathbb{E}(SSP_{:k}) &= \mathbb{E}\left(\hat{\pi}_{i:k} - \hat{\pi}_{ij:k}\right)\mathbb{E}\left(\sum_{i}^{n_\mathrm{d}}\sum_{j}^{n_i}\frac{c_{ij}^2}{C_{1i}}\right) + \mathbb{E}\left(\hat{\pi}_{k} - \hat{\pi}_{ij:k}\right)\mathbb{E}\left(\sum_{i}^{n_\mathrm{d}}\sum_{j}^{n_i}\frac{c_{ij}^2}{C_{1}}\right) \\
&- \mathbb{E}\left(\hat{\pi}_{i:k} - \hat{Q}_{1i:k}\right)\mathbb{E}\left(\sum_{i}^{n_\mathrm{d}}\left(C_{1i} - \sum_{j}^{n_i}\frac{c_{ij}^2}{C_{1i}}\right)\right) \\
&+ \mathbb{E}\left(\hat{\pi}_{k} - \hat{Q}_{1:k}\right)\mathbb{E}\left(\frac{C_2}{C_1} - \sum_{i}^{n_\mathrm{d}}\sum_{j}^{n_i}\frac{c_{ij}^2}{C_{1}}\right) \\
&+ \left(C_1 - \frac{C_2}{C_1}\right)\mathbb{E}\left(\hat{\pi}_{k} - \hat{Q}_{2:k}\right) \\
&= (\pi_k - Q_{1:k})\left(\frac{C_2}{C_1} - \mathbb{E}\left(\sum_{i}^{n_\mathrm{d}}\sum_{j}^{n_i}\frac{c_{ij}^2}{C_{1}}\right)\right) \\
&- (\pi_k - Q_{1:k})\left(C_1 - \mathbb{E}\left(\sum_{i}^{n_\mathrm{d}}\sum_{j}^{n_i}\frac{c_{ij}^2}{C_{1i}}\right)\right) \\
&+ \left(C_1 - \frac{C_2}{C_1}\right)(\pi_k - Q_{2:k}) \qquad \text{(A14)}
\end{aligned}$$

where $Q_{2:k}$ is the expected IIS probability that two genes from different pools are both of type k. Note that the terms $\sum_i \sum_j c_{ij}^2/C_{1i}$ and $\sum_i \sum_j c_{ij}^2/C_1$ in Equations A13 and A14 depend on the latent variable c_{ij}, that cannot be

directly observed from the data. Therefore, we must make an assumption on the distribution of the c_{ij}'s to proceed. In the following, we assume that for each pool i, c_{ij} follows a multinomial distribution with parameter C_{1i} (the number of trials, i.e. the total number of reads in the ith pool) and probabilities $(1/n_i, \ldots, 1/n_i)$ for the n_i individuals in the pool. Then:

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

and:

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

Hence, from Equations A13 and A15, we have:

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

and from Equations A14 and A16:

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

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

$$\mathbb{E}(SSI) = \sum_k \mathbb{E}(SSI_{:k}) = (C_1 - D_2)(\pi - Q_1) \qquad \text{(A19)}$$

and for genes between individuals from different pools:

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

Rearranging Equations A19–A20, we get:

$$Q_1 - Q_2 = \frac{(C_1 - D_2)\,\mathbb{E}(SSP) - (D_2 - D_2^\star)\,\mathbb{E}(SSI)}{(C_1 - D_2)(C_1 - C_2/C_1)} \qquad \text{(A21)}$$

and:

$$1 - Q_2 = \frac{(C_1 - D_2)\,\mathbb{E}(SSP) + (n_\mathrm{c} - 1)(D_2 - D_2^\star)\,\mathbb{E}(SSI)}{(C_1 - D_2)(C_1 - C_2/C_1)} \qquad \text{(A22)}$$

where $n_{\mathrm{c}} \equiv (C_1 - C_2/C_1) / (D_2 - D_2^{\star})$. Let $MSI \equiv SSI/(C_1 - D_2)$ and $MSP \equiv SSP/(D_2 - D_2^{\star})$. Then, rearranging Equations A21–A22, we get:

$$F_{\mathrm{ST}} \equiv \frac{Q_1 - Q_2}{1 - Q_2} = \frac{\mathbb{E}(MSP) - \mathbb{E}(MSI)}{\mathbb{E}(MSP) + (n_{\mathrm{c}} - 1)\,\mathbb{E}(MSI)} \tag{A23}$$

which yields the method-of-moments estimator:

$$\hat{F}_{\mathrm{ST}}^{\mathrm{pool}} = \frac{MSP - MSI}{MSP + (n_{\mathrm{c}} - 1)\,MSI} \tag{A24}$$

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

$$\begin{aligned} SSI &= \sum_k SSI_{:k} = \sum_k \sum_i^{n_{\mathrm{d}}} \sum_j^{n_i} \sum_r^{c_{ij}} \left(X_{ij\cdot:k} - X_{i\cdot\cdot:k}\right)^2 \\ &= \sum_k \sum_i^{n_{\mathrm{d}}} C_{1i} \hat{\pi}_{i:k} \left(1 - \hat{\pi}_{i:k}\right) \end{aligned} \tag{A25}$$

and:

$$\begin{aligned} SSP &= \sum_k SSP_k = \sum_k \sum_i^{n_{\mathrm{d}}} \sum_j^{n_i} \sum_r^{c_{ij}} \left(X_{i\cdot\cdot:k} - X_{\cdots:k}\right)^2 \\ &= \sum_k \sum_i^{n_{\mathrm{d}}} C_{1i} \left(\hat{\pi}_{i:k} - \hat{\pi}_k\right)^2 \end{aligned} \tag{A26}$$

our estimator then takes the form:

$$\hat{F}_{\mathrm{ST}}^{\mathrm{pool}} = \frac{\frac{\sum_k \sum_i^{n_{\mathrm{d}}} C_{1i} (\hat{\pi}_{i:k} - \hat{\pi}_k)^2}{D_2 - D_2^{\star}} - \frac{\sum_k \sum_i^{n_{\mathrm{d}}} C_{1i} \hat{\pi}_{i:k} (1 - \hat{\pi}_{i:k})}{C_1 - D_2}}{\frac{\sum_k \sum_i^{n_{\mathrm{d}}} C_{1i} (\hat{\pi}_{i:k} - \hat{\pi}_k)^2}{D_2 - D_2^{\star}} + (n_{\mathrm{c}} - 1)\, \frac{\sum_k \sum_i^{n_{\mathrm{d}}} C_{1i} \hat{\pi}_{i:k} (1 - \hat{\pi}_{i:k})}{C_1 - D_2}} \tag{A27}$$

If the pools have all the same size and if the number of reads per pool is constant, i.e. if $n_i = n$ and $C_{1i} = C$, for all $i \in (1, \ldots, n_{\mathrm{d}})$ then one can show

that Equations A25–A26 reduce to:

$$SSI = n_{\mathrm{d}}(C-1)\left(1-\hat{Q}_1^{\mathrm{r}}\right) \tag{A28}$$

and:

$$SSP = C(n_{\mathrm{d}}-1)\left(1-\hat{Q}_2^{\mathrm{r}}\right) - (n_{\mathrm{d}}-1)(C-1)\left(1-\hat{Q}_1^{\mathrm{r}}\right) \tag{A29}$$

where $\hat{Q}_1^{\mathrm{r}}$ and $\hat{Q}_2^{\mathrm{r}}$ are the frequencies of identical pairs of reads within and between pools, respectively, computed by simple counting of IIS pairs. These are (unweighted) averages of the population-specific estimates $\hat{Q}_{1i}^{\mathrm{r}}$ (Equation A31) and the pairwise estimates $\hat{Q}_{2ii'}^{\mathrm{r}}$ (Equation A37), respectively. Then, from Equation A24, we get:

$$\hat{F}_{\mathrm{ST}}^{\mathrm{pool}} = \frac{\left(1-\hat{Q}_2^{\mathrm{r}}\right) - \left(1-\hat{Q}_1^{\mathrm{r}}\right)\left(\frac{n}{n-1}\right)}{\left(1-\hat{Q}_2^{\mathrm{r}}\right)} \tag{A30}$$

IIS probabilities for Pool-seq data

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

$$\hat{Q}^{\mathrm{r}}_{1i} \equiv \frac{\sum_k r_{i:k}\left(r_{i:k}-1\right)}{C_{1i}\left(C_{1i}-1\right)} \tag{A31}$$

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

$$\mathbb{E}\left(\hat{Q}^{\mathrm{r}}_{1i} \mid y_{i:k}\right) = \frac{\sum_k \mathbb{E}\left(r^2_{i:k} - r_{i:k}\right)}{C_{1i}\left(C_{1i}-1\right)} = \sum_k \left(\frac{y_{i:k}}{n_i}\right)^2 \tag{A32}$$

Since

$$\hat{Q}_{1i} \equiv \frac{\sum_k y_{i:k}\left(y_{i:k}-1\right)}{n_i\left(n_i-1\right)} \tag{A33}$$

is an unbiased estimate of the IIS probability between pairs of distinct genes in the ith pool, Equation A32 implies that $\hat{Q}^{\mathrm{r}}_{1i}$ (Equation A31) is a biased estimate of that quantity (i.e., the IIS probability between pairs of reads 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 A32 suggests that an unbiased estimate of $\hat{Q}_{1i}$ may be given by:

$$\hat{Q}_{1i}^{\text{pool}} \equiv 1 - \frac{n_i}{n_i - 1}\left(1 - \hat{Q}_{1i}^{\text{r}}\right) \tag{A34}$$

Taking expectation of Equation A34, we get indeed:

$$\begin{aligned}
\mathbb{E}\left(\hat{Q}_{1i}^{\text{pool}} \mid y_{i:k}\right) &= \frac{n_i}{n_i - 1}\mathbb{E}\left(\hat{Q}_{1i}^{\text{r}}\right) - \frac{1}{n_i - 1} \\
&= \frac{n_i}{n_i - 1}\sum_k \left(\frac{y_{i:k}}{n_i}\right)^2 - \frac{n_i}{n_i(n_i - 1)} \\
&= \frac{\sum_k y_{i:k}^2}{n_i(n_i - 1)} - \frac{\sum_k y_{i:k}}{n_i(n_i - 1)} \\
&= \frac{\sum_k y_{i:k}(y_{i:k} - 1)}{n_i(n_i - 1)} \equiv \hat{Q}_{1i}
\end{aligned} \tag{A35}$$

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:

$$\hat{Q}_1^{\text{pool}} \equiv \frac{\sum_i \hat{Q}_{1i}^{\text{pool}}}{n_{\text{d}}} \tag{A36}$$

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

$$\hat{Q}_{2ii'}^{\text{r}} \equiv \frac{\sum_k r_{i:k} r_{i':k}}{C_{1i} C_{1i'}} \tag{A37}$$

Since we assume that pools are conditionally independent, taking expectation gives:

$$\begin{aligned}\mathbb{E}\left(\hat{Q}^{\mathrm{r}}_{2ii'} \mid y_{i:k}, y_{i':k}\right) &= \frac{\sum_k \mathbb{E}(r_{i:k})\mathbb{E}(r_{i':k})}{C_{1i}C_{1i'}} \\ &= \sum_k \left(\frac{y_{i:k}y_{i':k}}{n_i n'_i}\right) \equiv \hat{Q}_{2ii'} \end{aligned} \tag{A38}$$

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

$$\hat{Q}^{\mathrm{pool}}_{2ii'} \equiv \hat{Q}^{\mathrm{r}}_{2ii'} \tag{A39}$$

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

$$\hat{Q}^{\mathrm{pool}}_{2} \equiv \frac{\sum_{i\neq i'} \hat{Q}^{\mathrm{pool}}_{2ii'}}{n_{\mathrm{d}}(n_{\mathrm{d}}-1)} \tag{A40}$$

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

$$\hat{F}^{\mathrm{pool}}_{\mathrm{ST}} \equiv \frac{\hat{Q}^{\mathrm{pool}}_{1} - \hat{Q}^{\mathrm{pool}}_{2}}{1-\hat{Q}^{\mathrm{pool}}_{2}} \tag{A41}$$

which, to the extent that we may take the expectation of a ratio to be the ratio of expectations, is unbiased. Note that Equation A41 has exactly the same form as Equation A30. Therefore, if the pools have all the same size and if the number of reads per pool is constant, the analysis-of-variance estimator is strictly equivalent to the estimator based on the computation of IIS probabilities between pairs of reads with appropriate bias correction.

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 provides identical estimates when sample sizes are equal.

Table S1 Comparison of pairwise F_{ST} estimates

		Pool-seq		Ind-seq
F_{ST}	n	Cov.	$\hat{F}_{\mathrm{ST}}^{\mathrm{pool}}$	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 $\hat{F}_{\mathrm{ST}}^{\mathrm{pool}}$ (Equation 9). The mean (RMSE) over 50 independent replicates of the `ms` simulations are provided for a single pair of populations. For comparison, we computed WC_{84} from allele count data inferred from individual genotypes (Ind-seq).

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

		Pool-seq		Ind-seq
F_{ST}	n	Cov.	$\hat{F}_{\mathrm{ST}}^{\mathrm{pool}}$	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 $\hat{F}_{\mathrm{ST}}^{\mathrm{pool}}$ (Equation 9). The mean (RMSE) over 50 independent replicates of the `ms` simulations are provided, for a single pair of populations. For comparison, we computed WC_{84} (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

		Pool-seq		Ind-seq
F_{ST}	n	Δ	$\hat{F}_{\mathrm{ST}}^{\mathrm{pool}}$	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 $\delta_i \sim \mathrm{Pois}(\Delta)$. For Pool-seq data, we computed our estimator $\hat{F}_{\mathrm{ST}}^{\mathrm{pool}}$ (Equation 9). The mean (RMSE) over 50 independent replicates of the `ms` simulations are provided, for a single pair of populations. For comparison, we computed WC_{84} from allele count data inferred from individual genotypes (Ind-seq).

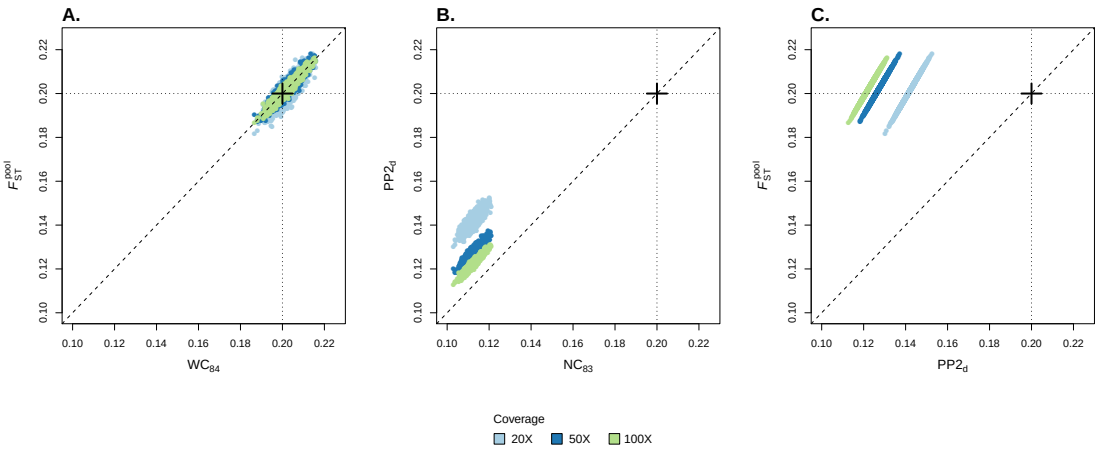

Figure S1 Pairwise estimators of F_{ST}. A. Multilocus estimates $\hat{F}_{\mathrm{ST}}^{\mathrm{pool}}$ (computed using Equation 9) as a function of WC_{84} estimates computed from allele count data inferred from individual genotypes. B. Multilocus estimates $\mathrm{PP2_d}$, as a function of NC_{83} estimates computed from allele count data inferred from individual genotypes. C. Multilocus estimates $\hat{F}_{\mathrm{ST}}^{\mathrm{pool}}$ as a function of $\mathrm{PP2_d}$ 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 $F_{\mathrm{ST}} = 0.20$. The size of each pool was fixed to 100. The results are shown for different coverages (20X, 50X and 100X). The cross indicates the true (simulated) value of the parameter F_{ST}.

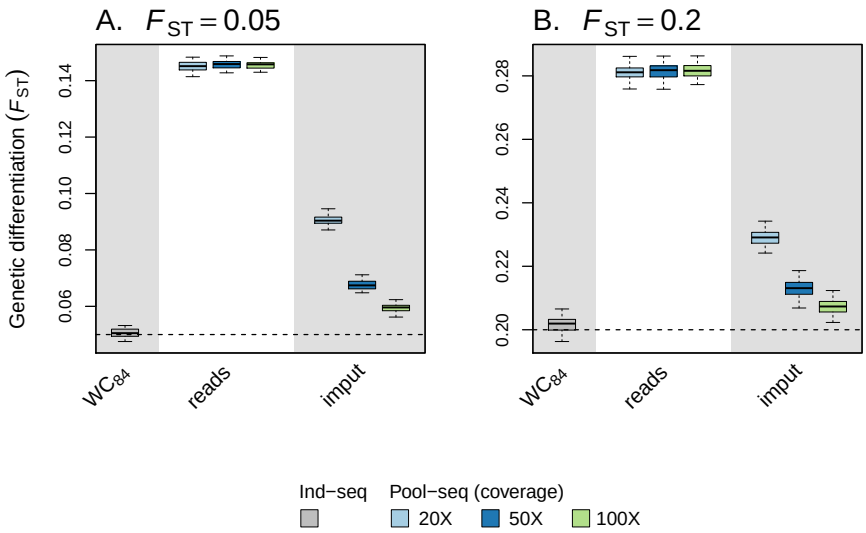

Figure S2 Global estimators of F_{ST}. We considered one estimator based on allele count data inferred from individual genotypes (Ind-seq): WC_{84}. For pooled data, we computed F_{ST} using the WC_{84} estimator: (i) directly from read counts, as if they were allele counts ("reads"); (ii) from allele 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 $F_{\mathrm{ST}} = 0.05$ (A) or $F_{\mathrm{ST}} = 0.20$ (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 true value of F_{ST}.