A beginner's guide to low-coverage whole genome sequencing for population genomics

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Abstract

Low-coverage whole genome sequencing (lcWGS) has emerged as a powerful and cost-effective approach for population genomic studies in both model and non-model species. However, with read depths too low to confidently call individual genotypes, lcWGS requires specialized analysis tools that explicitly account for genotype uncertainty. A growing number of such tools have become available, but it can be difficult to get an overview of what types of analyses can be performed reliably with lcWGS data, and how the distribution of sequencing effort between the number of samples analyzed and per-sample sequencing depths affects inference accuracy. In this introductory guide to lcWGS, we first illustrate how the per-sample cost for lcWGS is now comparable to RAD-seq and Pool-seq in many systems. We then provide an overview of software packages that explicitly account for genotype uncertainty in different types of population genomic inference. Next, we use both simulated and empirical data to assess the accuracy of allele frequency and genetic diversity estimation, detection of population structure, and selection scans under different sequencing strategies. Our results show that spreading a given amount of sequencing effort across more samples with lower depth per sample consistently improves the accuracy of most types of inference, with a few notable exceptions. Finally, we assess the potential for using imputation to bolster inference from lcWGS data in non-model species, and discuss current limitations and future perspectives for lcWGS-based population genomics research. With this overview, we hope to make lcWGS more approachable and stimulate its broader adoption.

Keywords: genotype likelihoods, bioinformatics, allele frequencies, population structure, selection scan, genotype imputation

1. Introduction

Despite massive reductions in the cost of DNA sequencing over the past decades, researchers remain faced with decisions about how to distribute sequencing effort along three dimensions: 1) how much of the genome to sequence (breath of coverage), 2) how deeply to sequence each sample (depth of coverage), and 3) the total number of samples to sequence. Until recently, reduced-representation sequencing (e.g. RAD-seq), through which a small random portion of the genome can be sequenced deeply in many individuals to allow for simultaneous variant discovery and high-confidence genotyping, has been the most popular approach for population genomics of non-model organisms (Andrews, Good, Miller, Luikart, & Hohenlohe, 2016; Davey et al., 2011; McKinney, Larson, Seeb, & Seeb, 2017). While this approach undoubtedly has led to a breakthrough in our ability to examine genome-wide patterns of variation, an important limitation is that large stretches of the genome between markers remain unsampled (Figure 1A). Accordingly, RAD-seq data may miss signatures of selection and adaptive divergence that are highly localized in the genome (Lowry et al., 2017; Tiffin & Ross-Ibarra, 2014).

In a growing number of cases, whole genome sequencing has identified striking peaks of differentiation or strong associations with phenotypes that went completely undetected with RAD-seq data (see e.g. Aguillon, Walsh, & Lovette, 2020 vs. Aguillon, Campagna, Harrison, & Lovette, 2018; Campagna, Gronau, Silveira, Siepel, & Lovette, 2015 vs. Campagna et al., 2017; Clucas, Lou, Therkildsen, & Kovach, 2019 vs. Clucas et al., 2019; and Szarmach, Brelsford, Witt, & Toews, 2021), suggesting that full genome coverage often is needed to understand mechanisms of adaptation. However, whole genome sequencing at sufficient depths to confidently call individual genotypes is still prohibitively expensive on a population scale for many projects. A popular cost-effective alternative is to sequence pools of individuals (Pool-seq; Schlötterer, Tobler, Kofler, & Nolte, 2014). When the number of individuals pooled and sequencing depth are sufficient, Pool-seq is a powerful approach for obtaining reliable estimates of population-level parameters (Futschik & Schlötterer, 2010; Zhu, Bergland, González, & Petrov, 2012). However, all information about individuals is lost, making it difficult to control for uneven contribution to the pool and precluding individual-level analyses as well as detection of cryptic substructure among sampled individuals (Anderson, Skaug, & Barshis, 2014; Fuentes-Pardo & Ruzzante, 2017).

Low-coverage whole genome sequencing (lcWGS) is emerging as a cost-effective alternative that allows population-scale screening of the entire genome while retaining individual information for - in many cases - a comparable cost to RAD-seq and Pool-seq. The underlying strategy is to maximize the information content in the sequence data by spreading it across the entire genomes of many separately barcoded individuals (Figure 1C). This way, we sacrifice depth of coverage (repeated sequencing of the same locus in the same individual), and therefore confidence in individual genotypes, in return for much greater breadth of coverage and potentially also larger sample sizes.

At low depth of coverage, individual genotypes cannot reliably be inferred (Nielsen, Korneliussen, Albrechtsen, Li, & Wang, 2012; Nielsen, Paul, Albrechtsen, & Song, 2011).

However, for most population-level questions, it is not the specific genotype of any particular individual that matters, but rather the overall population characteristics (e.g. allele frequencies, linkage disequilibrium (LD) patterns, etc). Similarly, for questions about genetic relationships between individuals, it is not the genotype at any particular single nucleotide polymorphism (SNP) that matters, but rather patterns of variation across SNPs genome-wide. Accordingly, probabilistic analysis frameworks that account for uncertainty about the true genotype (instead of assuming that any one genotype is correct) can integrate over the uncertainty about individual genotypes for population-level inference of variation at particular SNPs, and integrate over the uncertainty about an individual's genotype at each particular SNP to make inference about that individual's overall genetic signature (e.g. level of inbreeding, admixture proportions) (Buerkle & Gompert, 2013; Nielsen et al., 2012, 2011).

Simulation studies have demonstrated that when sequencing data are analyzed within this type of probabilistic statistical framework that accounts for genotype uncertainty, sampling many individuals each at low read depth actually provides more accurate estimates of many population parameters than higher read depth for fewer individuals (Buerkle & Gompert, 2013; Fumagalli, 2013; Nevado, Ramos-Onsins, & Perez-Enciso, 2014). In fact, these studies have suggested that spreading sequencing depth to 1–2 reads per locus and individual (1–2x coverage or less) - and increasing sample sizes accordingly - maximizes the information gained about a population. Many recent empirical studies have demonstrated the power of this approach (examples are listed in Table S1). Some of the first applications included identification of genomic regions repeatedly associated with marine-freshwater adaptation in stickleback (Jones et al., 2012), adaptation to an Arctic lifestyle in polar bears (Liu et al., 2014), and divergence among killer whale ecotypes (Foote et al., 2016). More recently, lcWGS was used to identify genes involved in rapid adaptation to fisheries-induced size selection in experimental populations of Atlantic silversides (Therkildsen et al., 2019), map hybrid incompatibility genes in swordtail fish (Powell et al., 2020), scan for soft sweeps in response to white-nose syndrome in bats (Gignoux-Wolfsohn et al., 2021), build ultra-dense crossover maps in Arabidopsis (Rowan et al., 2019), and assess admixture patterns and elevated differentiation across massive linkage blocks along environmental gradients in several marine organisms (Clucas et al., 2019; Mérot et al., 2021; Wilder, Palumbi, Conover, & Therkildsen, 2020).

Despite the clear promise, adopting a lcWGS approach can seem daunting because working with genomic data in a probabilistic framework requires both a shift in the way we think about our data and a different toolbox that incorporates genotype uncertainty in downstream analysis. In recent years, there has been a proliferation of programs that can explicitly account for genotype uncertainty in population genomic inference. But for the newcomer, it can be difficult to get an overview of what types of analyses can reliably be performed with this data type and what experimental designs will provide the most robust results for a particular system and question, e.g. how to best divide a given sequencing effort between the number of samples vs. the depth of sequencing per sample.

The goal of this paper is to provide a practical "field guide" for researchers considering a lcWGS approach. We first illustrate that lcWGS is now a feasible option for many research projects by

comparing the current costs and requirements of lcWGS to alternative sequencing strategies (Section 2). Next, we introduce the basic statistical framework used to account for genotype uncertainty inherent to lcWGS data, and provide an overview of current analytical tools built under a probabilistic framework to help readers identify software that can robustly perform common types of population genomics inference with lcWGS data (Section 3). To guide experimental design, we then use both genetic simulations (Section 4) and downsampling of empirical data (Section 5) to assess the accuracy of population genomic inference under different sequencing strategies. We evaluate trade-offs between sample size and depth of coverage, compare the power of lcWGS to RAD-seq and Pool-seq, and explore the potential of genotype imputation for bolstering inference with lcWGS data. Finally, in Sections 6 and 7, we review challenges and limitations associated with lcWGS data and discuss future perspectives. With this practitioner-centered overview, we hope to make lcWGS seem more approachable and stimulate broader adoption of this powerful approach, while inspiring future development of population genomic inference methods for lcWGS data.

2. Feasibility: What does lcWGS cost and what resources are required?

2.1 Current sequencing costs

It is a widespread assumption that whole genome sequencing approaches are still too expensive for researchers working on modest budgets. Yet, due to spectacular drops in sequencing costs over the past decades (the cost per Mb of sequencing is today >600,000 times cheaper than in 2000; (Wetterstrand, 2021), lcWGS can now - in many cases - be performed at similar per-sample costs as reduced-representation techniques. Table 1 provides estimates of the total per-sample cost for both library preparation and sequencing (based on November 2020 pricing) for organisms of different genome sizes. The cost of lcWGS inevitably scales with genome size (because more sequence data are needed to provide a target coverage level of a large vs. a small genome), and this approach therefore may remain impractical for organisms with extremely large genome sizes. However, even for organisms with sizeable genomes around 1 Gb (e.g. many birds, fish, invertebrates, and plants), the per-sample cost with 1-2x sequencing coverage (20-32 USD) is now on par with the 30 USD recently reported for genotyping 20,000 variable RAD-seq loci,15 USD for a custom sequence capture approach for 500 - 10,000 loci (Meek & Larson, 2019) and 48 USD for custom exome capture (Puritz & Lotterhos, 2018). For organisms with smaller genome sizes, lcWGS can be cheaper than reduced-representation approaches, and prices are likely to drop further as sequencing costs continue to decrease.

2.2. Library preparation

Depending on target coverage levels, Pool-seq approaches remain the most cost-effective way to obtain genome-wide population-level data because it only requires preparation of a single sequencing library per population. The obvious downside is that all individual-level information is

lost, precluding many types of analysis. Despite this limitation, Pool-seq has gained popularity because preparation of separate indexed libraries for hundreds of individuals used to be labor-intensive and costly (the costs for preparing hundreds of libraries could easily outweigh the cost of sequencing). LcWGS has now become a viable alternative because of the development of cheap library preparation methods with efficient workflows that make it both practical and affordable to process hundreds of samples. (Nina Overgaard Therkildsen & Palumbi, 2017) for example, describe a robust easy-to-implement protocol based on reduced reaction volumes of Illumina's Nextera kit, which brings per-sample reagent costs down to ~8 USD (based on current reagent pricing). Several other protocols that stretch reagents in commercial kits reach similar price points (e.g. Gaio et al., 2019; Li et al., 2019). An advantage of commercial kit-based protocols is that they often work "straight out of the box" or require only limited optimization. Substantial further cost savings can be achieved with protocols based on in-house expression and purification of tn5 transposase (the enzyme used in Illumina's Nextera tagmentation approach), such as described by Hennig et al., (2018) and Picelli et al., (2014). With those protocols, per-sample library costs can be brought to <<1 USD, substantially reducing overall project costs when analyzing hundreds of samples and essentially eliminating the added cost of individually indexed libraries, making total costs for lcWGS equivalent to Pool-seq for similar total sequencing effort per population.

LcWGS library preparation methods also tend to be very efficient and scalable. For example, tagmentation-based protocols (like the one used by Therkildsen & Palumbi 2017) make it possible to prepare 96 libraries in <5 hours (with <3 hours hands-on time) - substantially less time than needed for most RAD-seq protocols (Meek & Larson, 2019). The Therkildsen and Palumbi (2017) protocol also works well for relatively degraded DNA and requires only very small amounts of input DNA (~2.5 ng). For highly degraded DNA, we have had great success with the Carøe et al. (Carøe et al., 2018) single-tube method. Other cost-effective protocols produce successful lcWGS libraries even from picogram-levels of input DNA (Hennig et al., 2018; Meier, Salazar, Kučka, Davies, & Dréau, 2020; Picelli et al., 2014), for example enabling high throughput production of libraries from individual zooplankters (Beninde, Möst, & Meyer, 2020). Methods that sidestep DNA extraction with tagmentation directly on cells or tissue may lead to additional efficiencies for lcWGS library preparation in the future (Vonesch et al., 2020).

2.3. The need for a reference genome

For non-model organisms, a key constraint associated with lcWGS is the need for a reference genome to map the short-read sequence data generated from each individual. If a reference genome is not already available for the species of interest, a common solution is to map to a reference genome of a related species. While this can work well in some contexts, increasing phylogenetic divergence between the re-sequenced species and the reference genome can restrict mapping to the genomic regions that are most conserved between the two taxa and bias estimates of population genomic parameters (Bohling, 2020; Nevado et al., 2014). Major differences in genome organization (e.g. structural and copy number variants) can also exist even between closely related species (Ekblom & Wolf, 2014). For these reasons, a species-specific reference sequence is preferable where it can be obtained.

As a shortcut to obtaining species-specific reference sequence without de novo assembling a full genome, Therkildsen and Palumbi (2017) mapped lcWGS reads to a reference transcriptome, in practice performing 'in-silico' exome capture. However, major advances in affordable long-read sequencing, powerful genome scaffolding techniques, and improved assembly algorithms now enable chromosome-scale assemblies at a much lower cost and faster speed than earlier approaches (reviewed by Rice & Green, 2019), facilitating high-quality assemblies of mammalian-sized genomes (several Gb) with chromosome-length scaffolds for as little as 1,000 USD (Dudchenko et al., 2018; Gatter, von Löhneysen, Drozdova, Hartmann, & Stadler, 2020). Therefore, at this point, it probably makes sense to start most new lcWGS studies with a de novo genome assembly or upgrade, if a reference sequence of sufficient quality is not available.

BOX 1: Glossary

Bayesian inference: a statistical inference strategy that estimates model parameters by characterizing its posterior probability distribution (i.e. P(parameter | data)). By the Bayes theorem, the posterior probability is formulated as a product of the likelihood function and the prior probability distribution (probability distribution of model parameters before considering the data) divided by the marginal probability of the data (which is a constant), i.e. P(parameter | data) = P(data | parameter) * P(parameter) / P(data)

Genotype dosage: the expected genotypic count. For diploid individuals, genotype dosage = P(AA | data)*0 + P(AB | data)*1 + P(BB | data)*2, where A and B represent the two alleles at the site, and e.g. P(AB | data) represents the posterior probability of the heterozygous genotype.

Genotype imputation: A method to infer missing genotypes and bolster genotype likelihood estimation by identifying stretches of haplotypes shared between individuals.

Genotype likelihoods (GLs): the probability of observing the sequencing data at a certain site in an individual given that the individual has each of the possible genotypes at this site (e.g. for diploids there are 10 possible genotypes, which can be reduced to 3 if the major and minor alleles are known), i.e. P(data | genotype), or L(genotype).

Genotype likelihood model: the mathematical model used to estimate GLs. Different GL models are built under different assumptions about the data, in particular about the sequencing error profile. For example, the GATK model assumes that the sequencing quality scores accurately capture the probability of sequencing error, and that all errors are independent. In comparison, the Samtools model assumes that once a first error occurs at a certain site in an individual, subsequent errors are more likely.

Low-coverage whole genome re-sequencing (lcWGS): We use this term to refer to whole genome re-sequencing of individuals (i.e. labeled with unique barcodes) with depth too low to

reliably call genotypes without imputation (<5x). Note, however, that even for medium sequencing depth (5-20x), inference accuracy may improve under a probabilistic analysis framework based on GLs, rather than working with called genotypes (Nielsen et al., 2011).

Maximum likelihood inference: a statistical inference strategy that estimates model parameters by choosing the parameters that maximize the likelihood of the data. In other words, the maximum likelihood estimators of model parameters = argmax(L(parameter))

Posterior genotype probability: the probability of an individual having one of the possible genotypes at a certain site given the sequencing data, i.e. P(genotype | data).

Prior genotype probability: the probability of an individual having one of the possible genotypes at a certain site before considering the sequencing data for this individual at this site, i.e. P(genotype). The prior genotype probability can be uniform (i.e. all genotypes are equally likely to occur), or can be informed by the allele frequency or the site frequency spectrum (SFS) at this site for all individual samples. It is often used for the estimation of posterior genotype probability in Bayesian inference.

Restriction site-associated DNA sequencing (RAD-seq): a group of techniques for sequencing short flanking regions around restriction enzyme cut sites to obtain random samples of genetic markers across the entire genome. These markers are typically sequenced at high depth (e.g. >20x) for each individual so that individual genotypes can be confidently determined.

Sample allele frequency (SAF) likelihood: the probability of observing sequencing data at a certain site across all individual samples given each possible sample allele frequency at this site (e.g. for diploids, the possible sample allele frequencies range from 0 to 2n; n=sample size), i.e. P(data | sample allele frequency).

Whole genome sequencing of pools of individuals (Pool-seq): a whole genome sequencing strategy in which unlabeled DNA from multiple individuals is pooled before sequencing. The sequencing depth is typically low on a per-individual level but high for each pool (e.g. >50x). Due to the absence of individual barcodes, all individual-level information is lost in the sequencing data.

3. The toolbox: What types of analysis can we do with low-coverage data?

The major challenge in working with lcWGS data is that individual genotypes cannot be accurately inferred (Li, Sidore, Kang, Boehnke, & Abecasis, 2011; Nielsen et al., 2012, 2011). Many analytical tools that incorporate the uncertainty about individuals have therefore been developed in recent years, covering the most common types of population genomic inference. We briefly introduce the most widely used applications (see Table 2 for a more comprehensive

list) and also provide a tutorial with example data as a starting point for exploration: https://github.com/nt246/lcwgs-guide-tutorial.

Currently, the most widely used program for lcWGS analysis is ANGSD (Korneliussen, Albrechtsen, & Nielsen, 2014), a comprehensive package that implements an extensive variety of analysis options. Because of its broad use and versatility, ANGSD will feature prominently in this section's overview of available tools. However, we also seek to highlight that a variety of alternative programs are available for most types of analysis (Table 2).

3.1. Accounting for genotype uncertainty

The most common way to incorporate uncertainty about true genotypes is to use genotype likelihoods (GLs) rather than genotype calls in downstream analyses. A GL reflects the probability of observing the sequencing reads that cover a specific site in an individual if said individual has a particular genotype at this site. GLs refer to the set of likelihoods computed for each of all possible genotypes that individual could hold at that site (e.g. for diploids there are ten possible genotypes: AA, AC, AG, AT, CC, CG, CT, GG, GT, and TT, which can be reduced to three possible genotypes if the major and minor allele at a site is known, i.e. major-major, major-minor, minor-minor.

The key factors that prevent us from confidently identifying the true genotype with lcWGS data is uncertainty about 1) whether both alleles of a diploid individual have been sampled in the stochastic sequencing process, 2) whether the base call (A, C, G, T) at each position of a sequencing read is correct, and 3) whether sequencing reads have been mapped to the correct position in the genome. Several models for how we should account for these uncertainties in computing GLs from sequencing data have been proposed, with the main difference between current models is their assumptions about how base quality scores relate to the true probabilities of sequencing error (i.e. issue 2 above; see Supplementary text for more detail; Blischak, Kubatko, & Wolfe, 2018; Korneliussen et al., 2014; Kousathanas et al., 2017). Unfortunately, the effects of GL model choice on downstream analyses are still incompletely understood. While GL model choice has been suggested to make little difference for most downstream analyses, inference that depends on accurate detection of rare alleles can be more sensitive (Korneliussen et al., 2014). In general, the sensitivity to GL model choice may depend on the accuracy of base calling, read coverage distribution and filtering, sample size, and particular individuals included in the sample (see Box 4 in Fuentes-Pardo & Ruzzante, 2017). In Section 4.1, we report one example where the choice of GL model can strongly influence the number of low frequency SNPs estimated from simulated low coverage (≤2x) data but more research is needed to compare the performance of these different models. In the meantime, it may be prudent to compare inference with several different models with a subset of the data for each new dataset, particularly for analyses that rely on rare alleles.

3.2. From raw reads to SNP identification

The initial steps in processing lcWGS data are similar to those used in many other NGS approaches, such as high-coverage whole genome sequencing and Pool-Seq (Figure 2). These include trimming adapter sequence and bases with low quality scores, mapping (aligning) reads to a suitable reference genome, removing poorly mapped and duplicated reads, and optionally realigning reads that span indels (see e.g. Therkildsen & Palumbi 2017). It is in the downstream processing of the resulting filtered bam files that high-coverage and low-coverage workflows diverge and where a probabilistic framework based on GLs becomes central for low-coverage data.

The optimal approach in a GL-based framework would arguably be to compute GLs for every site in the genome for all analysis, including sites that appear to be invariant in a sample (because with lcWGS data we cannot be completely confident that we have not missed an alternative allele in one or more of our samples). While this approach is required for some analysis (e.g. for estimation of the site frequency spectrum and related estimates of genetic diversity), other types of analysis are more tractable and computationally efficient if only polymorphic sites are considered. Thus, a more practical solution is to initially identify likely polymorphic sites and restrict most GL-based analyses to those sites.

Although many types of genetic variants exist, lcWGS analysis is typically restricted to bi-allelic single-nucleotide polymorphisms (SNPs). A range of programs can identify SNPs from lcWGS data (Table 2). Because of built-in integration of a broad variety of downstream analysis tools, ANGSD is often a convenient option. ANGSD identifies SNPs as sites with minor allele frequencies significantly larger than zero. In this case, the number of alleles at each site is restricted to two (major and minor allele), with the identities of these alleles either determined through a maximum likelihood approach, setting the more common allele as the major allele (Jørsboe & Albrechtsen, 2019; Skotte, Korneliussen, & Albrechtsen, 2012) or by user specification (e.g. setting the reference or ancestral allele as the major allele). ANGSD currently does not allow for identification of indels or multi-nucleotide polymorphisms, but users could potentially identify biallelic indels with a different tool, such as Freebayes (Garrison & Marth, 2012) or GATK (McKenna et al., 2010), and import estimated GLs into ANGSD for use in downstream analysis. Regardless of the program used, quality control filters can be crucial to ensure data reliability. Table 3 provides an overview of the key filters that should be considered for different types of analysis with lcWGS data.

3.3. Individual-level analyses

Despite the lack of called genotypes, lcWGS data can be used for a wide range of individual-level analyses, which we define as those that do not require a priori grouping individual samples. It should be noted that the input formats for the different approaches differ between programs and that in some cases the SNP identification can be performed as part of the analyses (see specific manuals). Note also that none of the analyses listed in this subsection are possible with Pool-seq data.

Population structure: A key component of many population genomic studies is to characterize population structure, using dimensionality reduction (e.g. PCA and PCoA) and/or model-based clustering (e.g. admixture analysis). Dimensionality reduction methods are based on a covariance matrix (PCA) or distance matrix (PCoA). Several methods for computing these matrices while accounting for genotype uncertainty have been implemented. ANGSD, for example, can either randomly sample one read per individual per site or use the most common allele to represent the individual's allele frequency at this site (as either 0 or 1) and then calculate the covariance and distance between every pair of individuals from these allele frequencies. This simple approach has been shown to work well for datasets with very low sequencing depth and uneven coverage across samples (see section 4.2 and ANGSD manual). PCAngsd (Meisner & Albrechtsen, 2018), in contrast, estimates the covariance matrix from posterior genotype probabilities while correcting for potential violation of the Hardy-Weinberg equilibrium.

Model-based clustering methods that estimate admixture proportions of each sample assuming a model of discrete ancestral populations are also implemented in several software programs using GLs as input. These include NGSAdmix (Skotte, Korneliussen, & Albrechtsen, 2013) and Ohana (Cheng, Racimo, & Nielsen, 2019). They both adopt a maximum likelihood implementation of the classic STRUCTURE model, (Pritchard, Stephens, & Donnelly, 2000; Tang, Peng, Wang, & Risch, 2005), but differ in their optimization approaches. PCAngsd implements a different approach, which uses an intermediate output from its PCA analysis as a starting point for model-based clustering. PCAngsd has been shown to outperform NGSAdmix in runtime without strongly compromising its inference accuracy, making it potentially more suitable for larger datasets (Meisner & Albrechtsen, 2018).

Selection scans: Several of these clustering programs also implement selection scan approaches that do not require a priori grouping of individuals, as their general strategy is to locate outlier loci that exhibit patterns of genetic variation among individuals that are highly different from the genome-wide average. For example, PCAngsd (Meisner & Albrechtsen, 2018; Meisner, Albrechtsen, & Hanghøj, 2021) implements the FastPCA method by (Galinsky et al., 2016) in a GL framework and in Ohana, SNPs that exhibit a significantly different covariance structure can be identified as potentially under selection.

Genome-wide association studies (GWAS): Multiple statistical frameworks have been developed to take genotype uncertainty into account in scans for genotype-phenotype associations. GWAS often require large sample sizes to gain sufficient power, and a lcWGS/GL-based approach provides an opportunity to maximise the number of individuals studied in a cost-efficient way. Several GL-based GWAS approaches implemented in ANGSD have shown power to discover meaningful associations, including in the presence of population structure (Jørsboe & Albrechtsen, 2019; Skotte et al., 2012). These methods range from simple case / control associations for identifying variants associated with binary phenotypes (Kim et al., 2011) to the analyses of quantitative traits with incorporation of covariates (Skotte et al. 2012; Jørsboe & Albrechtsen 2019). The maximum likelihood approach recently developed by (Jørsboe & Albrechtsen, 2019) also explicitly estimates the effect size of each locus.

Linkage disequilibrium (LD): LD estimation has many important applications, for example relating to inference of population size, demographic history, selection, and discovery of structural variants (Slatkin, 2008). In addition, since many downstream analyses make assumptions about the independence of genomic loci, LD estimation is essential for pruning lists of loci to avoid inclusion of strongly linked loci. Several approaches have been developed to estimate LD from GLs (i.e. taking genotype uncertainty into account), with examples being GUS-LD (Bilton et al., 2018) and ngsLD (Fox, Wright, Fumagalli, & Vieira, 2019). Unfortunately, the computational complexity of GUS-LD is too high for it to be practical for whole genome data, but ngsLD has a more efficient algorithm and has different built-in functionalities to limit its computational complexity (e.g. restricting LD estimation between SNPs within a set distance, setting a minor allele frequency filter, etc.), and comparative evaluation has indicated that ngsLD tends to show less bias at low read depths (1-2x) than GUS-LD (Bilton et al., 2018; Fox et al., 2019).

Other types of analysis: In addition to the examples discussed above, many other specialized software packages have been developed to account for genotype uncertainty in various types of inference, including estimation of relatedness among individuals (Korneliussen & Moltke, 2015; Link et al., 2017), parentage inference (Whalen, Gorjanc, & Hickey, 2019) and pedigree analysis (Snyder-Mackler et al., 2016), estimation of individual inbreeding coefficients (Link et al., 2017; Vieira, Fumagalli, Albrechtsen, & Nielsen, 2013) and identity-by-descent tracts (Vieira, Albrechtsen, & Nielsen, 2016), tests for introgression such as computation of ABBA-BABA/D-statistic (Korneliussen et al., 2014), and construction of linkage maps (Rastas, 2017). More examples are listed in Table 2. It is also important to note that samples sequenced to low-coverage of the nuclear genome typically have very high sequencing depth across the mitochondrial genome due to its much higher copy number in each cell, enabling recovery of high-confidence full mitochondrial genome sequences for each individual (see e.g. Therkildsen & Palumbi 2017). LcWGS thus provides a cost-effective way to generate full mitochondrial genome sequences for hundreds of individuals, enabling unprecedented resolution for phylogeographic analysis (Lou et al., 2018; Margaryan et al., 2020).

3.4. Population-level analyses

When individual samples can be grouped into discrete populations or categories based on either prior information (e.g. sampling location or experimental treatment) or results from individual-level population structure analyses (e.g. model-based clustering), analyses can be conducted at the population level.

Allele frequency estimation: The estimation of population-specific allele frequencies is essential for most population genomic studies as it is a required input for many downstream analyses. Many programs, such as ANGSD or ATLAS, can estimate minor allele frequencies for each site using a maximum-likelihood or Bayesian approach (Kim et al., 2011; Kousathanas et al., 2017). Since population-specific estimates are obtained by running the program, e.g. ANGSD, on each population separately, it is crucial for users to explicitly define the same alleles

as major and minor in each population to avoid inadvertently computing the frequency of opposite alleles in different populations.

Site frequency spectrum (SFS): The population-specific SFS is another population genomic parameter essential for many downstream analyses. A challenge in estimating the SFS with low-coverage data is that low-frequency SNPs are less likely to be identified as polymorphic and therefore an SFS directly estimated from allele frequencies at identified SNP positions can be biased towards intermediate frequencies. To get around this issue, ANGSD estimates the SFS by using the sample allele frequency (SAF) likelihoods to formulate the likelihood function of the SFS, which the program then optimizes (Nielsen et al., 2012). Depending on the availability of an outgroup or ancestral reference genome, the inferred SFS can either be folded or unfolded and ANGSD can estimate the SFS jointly for up to four populations (Nielsen et al., 2012). This approach can correct for the bias caused by low-coverage data, but its performance can be sensitive to the choice of underlying GL model (Korneliussen et al., 2014), also see Section 4.1). Another important limitation is that the runtime of the algorithm currently implemented in ANGSD grows quadratically with the number of samples and it can become impractical to run across the whole genome if the sample size is very large. One strategy is to estimate SFS by chromosomes or in smaller windows and sum them up in the end. Implementation of a faster algorithm (Han, Sinsheimer, & Novembre, 2015) may also be included in future ANGSD releases (Fumagalli, personal communication).

Genetic diversity and neutrality test statistics within a single population: Derived estimators for genome-wide genetic diversity θ, such as nucleotide diversity π and Watterson's estimator, can be directly calculated from the population-specific SFS. These estimators of θ can also be computed within genomic windows from window-specific SFS and subsequently, different neutrality test statistics (e.g. Tajima's D) that evaluate the skewness of SFS in each genomic window can be calculated. Individual heterozygosity estimates can be obtained by estimating the SFS for individuals (rather than populations). All these diversity statistics can be computed based on an infinite sites model implemented in ANGSD. In contrast, ATLAS (Kousathanas et al., 2017) bases its θ estimation on a model that allows for back mutations (Felsenstein, 1981), which can be more appropriate when working with ancient samples. Regardless of the method used, it is important to note that when generating diversity estimates, non-variable sites should be included in the calculation, and therefore minimum minor allele frequency filters or SNP p-value filters should not be used.

Genetic differentiation between populations: In addition to estimates of *within*-population diversity, the genetic differentiation *between* populations can be estimated with a variety of different statistics, from simply quantifying the allele frequency difference to more complex statistics such as relative genetic differentiation (F_{ST}) and absolute genetic divergence (d_{xy}). Various estimators of F_{ST} can be computed from GL data using ANGSD, ngsTools (Fumagalli et al., 2013), or vcflib (see Supplementary text for more detail). vcflib can also estimate pF_{ST}, which, contrary to what the name suggests, is not an F_{ST} estimator, but a statistic that quantifies the significance of allele frequency differences between populations in face of genotype uncertainty (Domyan et al., 2016). In contrast to F_{ST}, no established method to estimate d_{xy} from GLs has, to

our knowledge, been included in major software packages. Various custom scripts have been shared (see e.g. https://github.com/mfumagalli/ngsPopGen/tree/master/scripts, https://github.com/marqueda/PopGenCode/blob/master/dxy_wsfs.py). Note, however, that d_{xy} may be over-estimated with these scripts so they should be used only for inspecting the relative distribution of d_{xy} across the genome (Foote et al., 2016) and not to make inferences based on its absolute values.

Other analyses based on derived statistics: In addition to the methods that work directly with the GLs, many other types of population-level analysis can be conducted based on the derived statistics mentioned above. For example, several commonly used software tools can use allele frequency matrices as input to infer population relationships and potential gene flow (e.g. Treemix (Bradburd, Coop, & Ralph, 2018; Pickrell & Pritchard, 2012) and conStruct (Bradburd et al., 2018; Pickrell & Pritchard, 2012)), perform selection scans (e.g BayPass or WFABC (Foll & Gaggiotti, 2008; Foll, Shim, & Jensen, 2015; Gautier, 2015)), association analyses (e.g. BayPass) or variance partitioning analyses (e.g. RDA (Forester, Lasky, Wagner, & Urban, 2018)). To run these programs, population-level allele frequencies are estimated as explained above (e.g. using ANGSD), but have to be transformed into the appropriate input format using custom scripts. Similarly, the population-specific or multi-dimensional SFS estimated from ANGSD can be used to infer demographic history (e.g. δaδi (Excoffier & Foll, 2011; Gutenkunst, Hernandez, Williamson, & Bustamante, 2009), fastsimcoal2 (Excoffier & Foll, 2011; Gutenkunst et al., 2009)), or to explicitly control for the effect of demography in selection scans (e.g. SweepFinder2 (DeGiorgio, Huber, Hubisz, Hellmann, & Nielsen, 2016)). Both locus-specific neutrality test statistics and F_{ST} values can be used in selection scans (e.g. outFlank (Whitlock & Lotterhos, 2015)), and genome-wide F_{ST} estimates can be used, for example, to test for isolation by distance (Mantel test) or to estimate effective migration surfaces (e.g. EEMS (Petkova, Novembre, & Stephens, 2016)). Furthermore, Ancestry_HMM (Medina, Thornlow, Nielsen, & Corbett-Detig, 2018) and ancestryinfer (Schumer, Powell, & Corbett-Detig, 2020) can infer local ancestry across the genome without called genotypes, although they require detailed SNP information for reference populations. Using derived statistics as input data can be a powerful approach to expand the available toolbox for lcWGS. However, unlike the GL-based programs listed in the rest of this section and Table 2, this approach does not carry uncertainty about parameter estimation downstream. Accordingly, if summary statistics rather than GLs are used as input for analysis, p-values etc. should be interpreted with caution and in light of the expected precision given the sample size and sequencing depth (see section 4).

4. Experimental design: The tradeoffs between sequencing depth per sample and total number of samples analyzed

With a finite sequencing budget, do we learn more about a population from adding more sequencing depth to each individual or stretching the sequencing effort over more individuals? Several previous studies have used simulated data to address this question (e.g. Buerkle & Gompert, 2013; Fumagalli, 2013; Nevado et al., 2014). In general, these studies have found that

sampling many individuals at 1x or 2x read depth provides more accurate estimates of many population parameters than higher read depth for fewer individuals. However, both the simulation (e.g. Haller & Messer, 2019; Huang, Li, Myers, & Marth, 2012) and the GL-based data analysis toolboxes (e.g. Fumagalli, Vieira, Linderoth, & Nielsen, 2014; Korneliussen et al., 2014; Meisner & Albrechtsen, 2018) have evolved rapidly since these studies were conducted, and a more up-to-date evaluation is now needed. Here, we used simulated data to compare common types of population genomic inference under a wide range of sample size and sequencing depth combinations, including depths<1x, which were not explicitly evaluated in earlier studies. Full details about all the simulations and analyses can be found in the supplementary methods and Table S2, and our entire simulation and analysis pipeline is available on GitHub (https://github.com/therkildsen-lab/lcwgs-simulation).

4.1. Population genomic inference for single populations

We simulated an isolated population that has reached mutation-drift equilibrium, and evaluated the accuracy of lcWGS in inferring key population genomic parameters, including allele frequencies, the SFS, θ, Tajima's D, and linkage disequilibrium (LD) under different experimental designs (Fox et al., 2019; Haller & Messer, 2019; Huang et al., 2012; Korneliussen et al., 2014). As expected, more sequencing data is always better and the accuracy in allele frequency estimation consistently increases with both higher sample size and coverage (as measured by the r^2 values in Figure 3). The number of false negative SNPs (i.e. true SNPs in the population that fail to be identified) similarly decreases with higher sample size and higher coverage (Figure S1). Importantly, however, distributing the same total sequencing effort (i.e. the product of sample size and coverage) across more samples, with each sample receiving lower coverage (i.e. going from bottom left to top right in Figure 3) also consistently improves allele frequency estimation, even when each sample is sequenced at a coverage as low as 0.25x. This is because each allele is less likely to be sequenced more than once with lower per-sample coverage, and thus the effective sample size is higher.

Consistent with what the authors of ANGSD have previously shown (Korneliussen et al., 2014), we found that the GL model used for SFS-based inference can strongly influence its result. With the Samtools GL model, Watterson's θ is systematically underestimated when the average coverage is low (≤4x), although Tajima's θ (π) estimates are more robust (Figure S2). Consequently, Tajima's D tends to be overestimated (Figure S3). In contrast, when the GATK GL model is used, Watterson's θ, Tajima's θ, and Tajima's D can all be accurately estimated even at coverage as low as 0.5x (Figure S2, S3). The two GL models differ in performance because both the GATK model and our simulation model assume that each base quality score reflects an independent and unbiased measurement of the probability of sequencing error (Huang et al., 2012; McKenna et al., 2010), whereas the Samtools model assumes that if one sequencing error occurs at a certain locus, subsequent errors are more likely (Li, 2011; Li et al., 2009). As a result, with the Samtools model, lower-frequency mutations are less likely to be identified as polymorphic sites and more likely to be interpreted as sequencing errors when the coverage is low. This leads to an underestimation of the number of singleton mutations, and therefore Watterson's θ tends to be underestimated, at least for our simulated data. We note

that these low-frequency SNPs have minimal impact on many other population genomic analyses and, in fact, are often filtered out, so we do not expect strong discrepancies between the two GL models in most types of analysis. We also stress that the sequencing errors modeled in our simulations may not accurately represent the sequencing error profile in real life, so our result should not be interpreted as a recommendation of one GL model over the other.

Lastly, we found that although relative estimates of LD (which may be adequate for many uses, e.g. for the identification of LD blocks or LD pruning) could reliably be obtained with per-sample coverage of 1-2x, higher per-sample coverage (e.g. ≥4x) would be required to get precise and accurate estimates of LD (e.g. for demographic inference) even with sample size as large as 160 (Figure S4, S5, Fox et al. 2019).

Box 2. Performance of lcWGS vs. Pool-seq in allele frequency estimation

A key advantage of lcWGS over Pool-seq is that each sequencing read can be assigned to an individual so we can detect uneven sequencing coverage and account for it in parameter estimation. But does it matter in practice when the contribution of each individual to the sequencing pool is roughly equal? With our simulated data, we found that a lcWGS analysis approach that accounts for individual-level GLs consistently provides slightly more accurate allele frequency estimates than Pool-seq analysis (which ignores individual-level information), even when the total amount of sequence is exactly equal for all individuals (Figure 4). This is because the sampling variance inherent to next-generation sequencing creates stochastic variation in the sequencing depth for each individual at each locus. In practice, inaccuracies due to measurement and pipetting errors, variation in DNA quality, and sequencing biases make it almost impossible to ensure the optimal scenario of even amounts of sequence among samples (Figure S6, Schlötterer, Tobler, Kofler, & Nolte, 2014), further enhancing the value of being able to account for sample overrepresentation with individually barcoded reads (Figure S7-S8).

4.2. Inference of spatial structure

To evaluate the power of different lcWGS sampling designs in detecting population structure, we simulated a metapopulation consisting of nine subpopulations located on a three-by-three grid that have reached mutation-drift-migration equilibrium. We first examined a scenario in which gene flow among subpopulations is low (0.25 effective migrants between neighboring subpopulations per generation). In this scenario, the spatial structure among subpopulations can be correctly inferred from PCA even with extremely low sample size (5 samples per subpopulation) and coverage (0.125x coverage per sample; Figure 5A). In addition, migrant individuals and hybrids, when included in the sample, can be identified in the PCA (Figure 5A), which would not be possible with a Pool-seq design.

We then increased the level of gene flow (1 effective migrant between subpopulations every generation). As expected, the power of PCA to resolve the weaker spatial structure slightly declines, but interestingly, small sample size causes a greater loss of power than low coverage

does (Figure 5B). Subpopulations fail to form discrete clusters in the PCA space when the sample size per population is 5, unless the coverage is 2x or higher per sample. On the other hand, with a sample size of 10, the correct spatial structure can be inferred with a coverage as low as 0.125x (i.e. a per-population coverage of only 1.25x; Figure 5B). The reason we can push the per-sample coverage so low is that PCA depends on reliable covariance estimation between some, but not all pairs, of samples in the dataset. To get reliable covariance estimates in a sample pair, both samples need to have at least 1x coverage at some informative SNPs. As sample size increases, the number of all available sample pairs increases quadratically, and the number of sample pairs for which enough informative SNPs are shared also increases quadratically. Therefore, the overall population structure is more likely to be correctly extrapolated from these sample pairs. We also note that, due to computational limitations, our simulations are based on only a single 30Mb chromosome. Since the power of PCA depends on the number of informative SNPs shared between pairs of samples, with a larger genome size, even lower sequencing depth and/or sample size would be required to resolve the spatial structure among subpopulations, given the same SNP density as simulated here (see Figure S9 for an example of this). Lastly, we found that ANGSD (Korneliussen et al., 2014), the results of which are presented here, outperforms PCAngsd (Meisner & Albrechtsen, 2018) in scenarios with low sample size (e.g. ≤10 samples per population) or very low coverage (e.g. ≤0.25x per sample) (Figure S10-11).

4.3. Scans for divergent selection in the face of gene flow

A primary advantage of lcWGS compared to reduced-representation sequencing approaches is the increased resolution for genome scans for signatures of selection, for example in the form of outlier SNPs that show elevated levels of differentiation between populations. To evaluate how experimental design affects our ability to detect outliers, we simulated two populations connected by gene flow that are strongly affected by divergent selection. We estimated F_{ST} between the two populations from lcWGS data to identify the loci under selection (details in the supplementary material).

We first examined a scenario where the size of each population is large ($N_e = 5x10^4$) and gene flow is high (5 effective migrants per generation). In this scenario, seven SNPs under divergent selection, along with their neighboring neutral SNPs, show highly elevated F_{ST} values compared to the genome-wide background, creating a distinct pattern of narrow genomic islands of divergence (Figure 6) (Turner, Hahn, & Nuzhdin, 2005). This F_{ST} landscape can be recovered from lcWGS data with a total sequencing coverage ≥10x in each population (e.g. 40 samples per population and 0.25x coverage per sample, Figure 6). For a given total sequencing effort, however, we observe an increase in background F_{ST} when fewer samples are sequenced (e.g. 40 samples each at 0.25x vs. 5 samples per population and 2x coverage per sample), which can lead to more false positive signals in the outlier detection (Figure 6). The same conclusions hold in a scenario with smaller N_e ($N_e = 10^4$) and lower gene flow (2.5 effective migrants per generation) (Figure S12, S13).

4.4. The optimal experimental design depends on study goals

Perhaps unsurprisingly, our simulation results suggest that there is not a single lcWGS experimental design that is ideal for all purposes. Instead, the optimal design depends on the goals, system, and budget of a study. For many common types of population genomic inference (e.g. allele frequency estimation, population structure analysis, genetic differentiation between populations), higher accuracy can be achieved by spreading a given sequencing effort thinly across more samples (Figure 3, 5, 6). There are, however, some notable exceptions. For example, inference that depends heavily on low-frequency alleles (e.g. Watterson's θ, Tajima's D) can be very sensitive to the chosen GL model when per-sample sequencing coverage is low, so until we have a better understanding of which GL models best fit the empirical data, sequencing each sample with relatively higher coverage (e.g. >4x) might generate more robust results for these types of analyses (Figure S2, S3). Similarly, the methods that are currently available for LD estimation with lcWGS data can generate biased estimates when the coverage is lower than 4x (Figure S4, S5), but note that reliable relative estimates of LD can be obtained at lower coverage.

It is important to keep in mind that tradeoff exists between sample size and per-sample depth: with a given budget, the higher per-sample sequencing depth needed for robust estimation of the SFS (e.g. for demographic inference using δaδi) or absolute values of e.g. Tajima's D or LD will likely compromise the accuracy for other estimates, e.g. of allele frequencies or F_{ST} outliers. Accordingly, researchers must carefully consider what types of inference are most essential to their study goals and strike an appropriate balance. Based on our results here and those from previous studies, we provide some general guidelines to lcWGS experimental design in Table 4. For more targeted guidance, we also encourage researchers to build on our simulation pipeline (https://github.com/therkildsen-lab/lcwgs-simulation) to optimize the experimental design for their specific studies.

Box 3. Performance of lcWGS vs. RAD-seq in selection scans

Compared to lcWGS, RAD-seq has the advantage of being able to generate high-confidence genotype calls, but suffers from a sparser coverage of the genome, which can result in missed signals in selection scans (Lowry et al., 2017). Here, we simulated RAD-seq data for our two divergent selection scenarios with a range of realistic sample sizes and RAD tag densities. In the scenario with larger population size and higher gene flow, we found that even with a large sample size and a much higher marker density than typically used (128 RAD tags per Mb), RAD-seq picked up some, but tended to miss several of the narrow F_{ST} peaks. With a lower, much more commonly used marker density (e.g. 8 tags per Mb), the majority of the selection-induced peaks would be missed, regardless of sample size (Figure 7). In the scenario where the population size is smaller and gene flow is lower, RAD-seq is more likely to sample SNPs within the true F_{ST} peaks due to the stronger linked selection, but because of the higher background noise in these scenarios, it still struggles to detect distinct F_{ST} peaks (Figure S14). These findings are consistent with a growing number of empirical examples where RAD-seq missed signatures of selection clearly detected with WGS data (see introduction).

5. Application to empirical data

To supplement our simulation-based evaluation of lcWGS inference with an exploration of how sequencing depth affects the identification of polymorphic sites, population structure analysis and detection of outlier loci in empirical data, we subsampled and re-analysed previously published whole genome sequencing data from the Neotropical butterfly *Heliconius erato* (Van Belleghem et al., 2017). The *H. erato* radiation comprises several subspecies that show a vast visual diversity in Müllerian mimicry related to wing patterns, and many of the underlying candidate genes have been identified (Reed et al., 2011; Van Belleghem et al., 2017). For example, the *optix* gene has been shown to control the red band phenotype in multiple *Heliconius* species and accordingly shows strong differentiation among subspecies with different band patterns (Reed et al., 2011; Van Belleghem et al., 2017). We subsampled resequencing data (originally average coverage of 11x ± 2.3x per individual) mapped to the *H. erato demophoon* (v1) to coverage depths of 8x, 4x, 2x, 1x, 0.5x and 0.25x (see supplementary text) and analysed them in a GL framework. For simplicity, we focus on results for 8x, 2x and 0.5x coverage, as results from 4x and 1x are very similar to 8x and 2x, respectively (see supplementary Figure S15).

First, we found a positive correlation between the number of variable sites identified during SNP identification in ANGSD and the mean genome-wide sequencing coverage (Figure 8a; quadratic function: $r^2 = 0.98$, p=0.00099). Across all 51 individuals used in the final analyses, the number of SNPs identified with a p-value threshold of 1e-6 ranged from 12,266 at 0.5x coverage to 14,851,731 at a mean coverage depth of 8x. It has to be noted though, that the number of detected SNPs depends on the p-value threshold, and for a dataset with a mean per-individual coverage of 0.25x a lower p-value threshold would have to be used to identify any SNPs at all (Figure 8).

Second, we reconstructed the population structure using PCA, performed on covariance matrices estimated using random read sampling in ANGSD (see supplementary methods). The PCA showed a very similar clustering pattern for all datasets regardless of coverage level, with populations grouping into three distinct clusters corresponding to the geographic origin of samples (Central America, East of Andes, West of Andes; Figure 8b). One subspecies (*H. erato hydara*) sampled from two geographic regions was split over two clusters. On a finer population structure scale, we observed a slightly wider spread of data points at the lowest coverage (0.5x), although the general clustering was comparable to higher coverages.

Lastly, comparing the genetic differentiation between *H. erato* subspecies with (n=28) and without (n=23) the red bar phenotype (Van Belleghem et al., 2017), we recovered the well-characterized F_{ST} peak around the *optix* gene at per-individual coverages as low as 1x (Figure 8c) (Van Belleghem et al., 2017). At 0.5x coverage, we were restricted to estimating F_{ST} within fewer genomic windows compared to higher coverages (112 50kb windows at 0.5x vs 255 50kb windows at >1x along scaffold 1801), leading to much sparser window coverage across the scaffold and therefore a noisier signal (Figure 8c). However, even at this low resolution, we

781 detected one differentiated genomic window in the optix region, albeit the estimated F_{ST} was
782 elevated at 0.5x (F_{ST} ~0.6) compared to higher coverages (F_{ST} ~0.4).
783
784 Overall, these results suggest that even at a comparatively low individual sequencing coverage
785 of 0.5-1x and moderate sample sizes of 20-30 per population, we can detect population
786 structure and recover distinct peaks of differentiation across the genome in empirical data.
787
788
789 ## Box 4. Using imputation to bolster genotype estimation from lcWGS
790
791 The majority of current population genomic inference methods, including all the lcWGS methods
792 discussed in this paper so far, consider data on a SNP-by-SNP basis and accordingly ignore all
793 the information contained in the surrounding haplotype structure. Imputation can be used to
794 boost genotyping accuracy by leveraging LD patterns between variants to identify shared
795 stretches of chromosome and incorporate information from flanking alleles to infer missing or
796 low-confidence genotypes (Li et al., 2011; Pasaniuc et al., 2012). Imputation has been used
797 extensively to obtain genotype calls from low-coverage data in humans and agricultural species,
798 but has seen limited application in non-model species because most imputation methods, such
799 as Beagle and findhap (Browning & Yu, 2009; VanRaden, Sun, & O'Connell, 2015), rely on
800 externally generated haplotype reference panels, which are unavailable for most species. In
801 contrast, the more recently developed program STITCH imputes directly from sequence read
802 data without reference panels, and has been shown to perform well when sample sizes are
803 large (n>2000; (Davies, Flint, Myers, & Mott, 2016). However, sample sizes of this magnitude
804 are not achievable in many studies, especially for rare or elusive species. To evaluate the utility
805 of imputation without reference panels with sample sizes more typical of studies of non-model
806 species, we simulated three populations with varying levels of genetic diversity and LD, tested
807 combinations of sequencing depths and sample sizes, and identified the conditions under which
808 reference panel-free imputation is likely to bolster genomic analyses of lcWGS data.
809
810 **Imputed genotype accuracy**
811 We simulated three populations characterized by 1) low diversity, high LD (Ne = 1,000, r = 0.5
812 cM/Mb); 2) medium diversity, medium LD (Ne = 10,000, r = 0.5 cM/Mb); and 3) medium
813 diversity, low LD (Ne = 10,000, r = 2.5). For each population, we subsampled 25, 100, 250, 500
814 or 1000 individuals and simulated sequence reads to average depths of 1x, 2x and 4x per
815 sampled individual. We compared genotype dosages for all SNPs with minor allele
816 frequency>0.05 imputed without reference panels in Beagle v.3.3.2 and STITCH v.3.6.2, to
817 those estimated without imputation in ANGSD v.0.931 (see the supplementary text and Table
818 S2 for details on simulations, genotype dosage estimation and imputation).
819
820 Our analysis suggests that using imputation without reference panels does improve population
821 genomic inference under certain circumstances. Imputation was most effective under the low
822 diversity, high LD scenario (Figure 9A). Under this scenario, genotype dosages imputed in
823 STITCH from large sample sizes (n≥500) sequenced at 1x coverage were highly correlated with
824 true genotypes (r^2>0.94), and all experimental designs with sample sizes ≥100 showed a

substantial improvement in genotype estimation (Figure 9A). In the medium diversity and medium LD population, larger sample sizes were necessary to achieve similar imputation accuracy (e.g., n=1000 was needed for r^2=0.95; Figure 9B). Performance was markedly worse in the populations with medium diversity, low LD, but there was nonetheless an improvement when imputing from large sample sizes (n≥250) or greater sequencing depths (≥2x) compared to genotypes called without imputation (Figure 9C).

Considerations for using imputation in non-model systems

Choosing whether to apply imputation to real-world data will depend on the details of the study system and the experimental design. In general, imputation accuracy increases with SNP density and LD between SNPs (de Bakker, Neale, & Daly, 2010; Shi et al., 2018), and our results suggest that populations with lower LD (even those with greater SNP density) require greater sample sizes and/or coverage to achieve the same imputation accuracy. For populations with higher LD, STITCH can substantially boost genotype accuracy for samples sequenced at 1x coverage, provided sample sizes are adequate (n≥100). When coverage is higher (≥2x), Beagle tends to perform similarly to or even outperform STITCH. However, for populations with lower LD, the improvement in genotype accuracy by imputation may be small unless sample sizes are ≥1000 and/or coverage is ≥2x for the conditions tested here; at smaller sample sizes or lower coverage, the potential benefit of imputation for low LD populations may not warrant the computational time.

Imputation provides another potential benefit for spreading sequencing effort thinly among many individuals in some circumstances. As our results have shown, by leveraging LD information from all samples, imputation can to some extent make up for the genotype uncertainty inherent in lcWGS data. For example, in the high LD population, genotypes imputed in STITCH from 1000 samples sequenced at 1x coverage were only slightly lower in accuracy (r^2=0.975) than for 500 samples at 2x coverage (r^2=0.981) and 250 samples at 4x coverage (r^2=0.982). For many questions where a large sample size is necessary to achieve adequate power, such as GWAS, what can be gained from increased sample size could readily outweigh the minimal loss in genotype accuracy. In addition, for some GWAS methods, the remaining genotype uncertainty can be incorporated directly into the analysis (Skotte et al., 2012; Jørsboe & Albrechtsen, 2019).

Because the performance of imputation varies with the LD and diversity of populations, a priori information on population history may help researchers anticipate how well imputation will perform. A set of “true genotypes” (e.g. from high-depth samples) and quality metrics output by the imputation programs (Browning & Yu, 2009; Davies et al., 2016) can also be used. Populations with small Ne or that have experienced recent bottlenecks, such as threatened or endangered species, will have higher genome-wide LD (Hayes, Visscher, McPartlan, & Goddard, 2003; Waples & Do, 2010), making them potentially good systems for applying imputation if relatively large sample sizes (e.g. ≥100 for the scenarios simulated here) can be obtained. Where pedigree information is available, methods that incorporate the pedigree into imputation can be used (e.g. Ros-Freixedes, Whalen, Gorjanc, Mileham, & Hickey, 2020; Whalen, Ros-Freixedes, Wilson, Gorjanc, & Hickey, 2018). Finally, although imputation has been mainly applied to regular short-read data, the haplotype reconstruction step could be

greatly simplified by long-read or linked-read data that is becoming increasingly available (see section 7).

6. Current limitations and future developments

Despite the many strengths of lcWGS, there are also clear limitations to this data type. Here, we outline key constraints that researchers should consider before adopting the approach and discuss prospects for overcoming these constraints in the future.

Not suitable for analysis requiring genotype calls: It is important to stress that the potential for improved inference accuracy by spreading sequencing effort thinly over many individuals is only realized if the resulting uncertainty about individual genotypes is accounted for statistically in downstream analyses, with approaches such as those reviewed in section 3. As discussed, hard-calling genotypes from lcWGS data remains likely to bias inference regardless of how large the sample size is, so lcWGS data is not well-suited for analysis types or downstream software that require genotypes as input, unless imputation can provide more accurate genotype calls (see Box 4 for details). However, as outlined in section 3, GL-based inference frameworks are available for most major types of population genomic analysis and many additional approaches are under development.

Lack of user-friendly interface and documentation: Unfortunately, a key barrier to the wider adoption of lcWGS has been a lack of user-friendly interfaces and sparse documentation for programs that handle GL data. Accordingly, these tools are only accessible to users with prior expertise in bioinformatics, and the development of workflows often requires a substantial time investment. We hope that this beginner's guide can be part of the effort to increase the accessibility of lcWGS We are also aware that efforts are underway to develop a graphical front-end to ANGSD, which should make this powerful and versatile software package accessible to a broader set of researchers (Fumagalli, pers. comm).

Computational demands: Another practical limitation is the often much greater computational cost of GL-based methods compared to genotype-based methods. For example, SFS estimation from GLs in ANGSD is computationally intensive with very large sample sizes, which may be prohibitive for researchers without access to high-performance computational resources. New, more efficient algorithms (e.g. Han et al., 2015) and strategies for analyzing smaller sections of the genome in turn (see section 3) may alleviate some of these constraints, but the computational demands for analysis should definitely be considered, especially for researchers transitioning to lcWGS after working with much smaller datasets such as RADseq.

Flaws and gaps in the current toolbox: Although tremendous progress has been made in the development of methods and tools for the analysis of lcWGS data over the past decade, some key analytical challenges remain. One important issue is the potential sensitivity to the choice of GL model in some types of analyses (see sections 4.1 and Box 4 in Fuentes-Pardo & Ruzzante, 2017). A better understanding of which GL models best match the real error structures

generated by different sequencing platforms is essential for more robust inference from low-coverage data. In addition, alignment error is not taken into account in any of the current GL models, which could be problematic for genomes with high repeat content or for poor-quality reference genomes. The current analysis framework implemented in most software packages is also centered on the analysis of diploid organisms; extension to an arbitrary ploidy level would expand its usefulness for working with haploid and polyploid organisms, and key parts of this framework have already been developed (Blischak et al., 2018). There also remain types of analysis for which GL-based methods are not yet available. However, new analytical approaches for lcWGS data also continue to emerge. GL-based equivalents to some established approaches, such as implementation of the Pairwise Sequentially Markovian Coalescent (PSMC) model, are currently under development (ngsPSMC [https://github.com/ANGSD/ngsPSMC]).

Analysis susceptible to batch artifacts: LcWGS data have great potential for reusability because the possibility to combine different datasets does not depend on the selection of the same restriction enzyme or markers. However, lcWGS could be particularly susceptible to batch effects when different datasets are combined. As mentioned earlier, some GL-based approaches are heavily dependent on the accurate modeling of the error structure in the data, which can vary between sequencing batches. For example, the sequencing error could be overestimated in one batch and underestimated in another (Lou et al. in prep), leading to artificial differences between batches that could confound real biological signals. Many of these batch effects can be mitigated with simple bioinformatic approaches, although extra care needs to be taken (Lou et al. in prep).

Limited ability to phase lcWGS data: A major limitation is that no bioinformatic solution is yet available to allow accurate phasing of lcWGS data without a reference panel, therefore prohibiting haplotype-based analyses. Haplotype data are a rich source of information, e.g. for inference of local ancestry tracks across the genome, demographic histories, or ongoing selective sweeps (see Leitwein, Duranton, Rougemont, Gagnaire, & Bernatchez, 2020) for a detailed overview). Despite major technological advances, long-read sequencing that can recover haplotype information remains too costly for typical population genomic studies. However, the recent development of an affordable linked-read low-coverage sequencing approach (Meier et al., 2020) promises to open many new opportunities for haplotype-based inference on a population scale by enabling efficient phasing and imputation of low-coverage linked-read data without a reference panel. Phased haplotype data will provide substantial improvement in imputation performance compared to the short-insert lcWGS data explored in Box 4, and make completely new types of analysis possible with lcWGS data.

Limitations for small sample sizes and very large genomes: LcWGS will not be an optimal solution for all study systems. In particular, for species that are rare or difficult to collect (e.g. endangered or elusive species), it may be impossible to obtain adequate sample sizes for accurately estimating population genomic parameters with lcWGS (see section 4). In these cases, many types of analysis, such as demographic history, diversity, selective sweeps and inbreeding levels, can be performed based on deep sequencing of the genome of a few or even

just a single individual (e.g. Li & Durbin, 2011). For species with extremely large genomes (e.g. many amphibians and pine species), whole genome sequencing may also remain impractical at any sequencing depth from a cost or data storage/handling perspective, and reduced representation approaches such as RAD-seq or targeted sequence capture may be preferable (Burgon et al., 2020; McCartney-Melstad, Mount, & Shaffer, 2016). For targeted methods like sequence capture, low-coverage sequencing of larger sample sizes and associated GL-based analysis can, similar to WGS, confer distinct advantages over sequencing fewer individuals at higher depth (e.g. Snyder-Mackler et al., 2016; Nina O. Therkildsen et al., 2019; Warmuth & Ellegren, 2019; Wilder et al., 2020).

7. Conclusion

In conclusion, although some limitations still exist for the use of lcWGS, this approach offers many advantages over reduced-representation sequencing or pooled WGS approaches and is ripe for broader implementation. We are excited about how its cost-effectiveness democratizes population-scale whole genome analysis, which until recently was only available to well-funded research groups working on model species. The ability to obtain full genome data for hundreds of individuals even on modest research budgets, and the rapidly expanding toolbox for versatile analysis of lcWGS data now makes it an increasingly promising approach for molecular ecology, conservation and evolutionary biology research. We hope this guide will inspire broader adoption to expedite the exploration of genomic variation across the tree of life.

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Data availability

All scripts used to generate the analysis presented in this manuscript will be available in a GitHub repository release deposited in Zenodo. The NCBI SRA accession numbers for the Heliconius data re-analyzed in this project is available in Table S3.

Author contributions

NOT conceived of the project. All the authors designed the research jointly and collaborated to compile the overview of available methods. RNL simulated the test data and performed the comparative analysis for different experimental designs, AJ performed the analysis of the

empirical data and designed the graphics, and APW performed the imputation analysis. All the authors provided input on all analyses and wrote the manuscript together.

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Table 1. Total cost per sample for both library preparation and sequencing based on November 2020 price levels (rounded up to nearest dollar)

Genome size (Gb)	Cost per sample (USD)* 1x coverage	2x coverage	Example organisms
0.2	11(3)	13(5)	Fruit fly, Honeybee, Arabidopsis
0.65	16(8)	24(16)	Atlantic silverside, Stickleback, Eastern oyster
1	20(12)	32(24)	Zebra finch, Chicken, Purple sea urchin
3	44(36)	79(71)	Human, Atlantic salmon, African clawed frog

*Cost estimates do not include labor and assume that samples are sequenced efficiently on a HiSeq X Ten system. The assumed costs break down to 8 USD per library (Therkildsen & Palumbi, 2017) and 1,300 USD per lane generating 110 Gb sequence data (see supplementary methods for estimates of initial investment costs). The numbers in brackets show the cost of sequencing only (i.e. the approximate total cost with a cheap homebrew library preparation method (see section 2.2)).

Table 2. List of published software for the analysis of lcWGS data. References for each software can be found in the main text (Section 3) or in the supplementary material.

Analysis type		Software						
Analysis	**Method**	**ANGSD**	**Atlas**	**MAPGD**	**vcflib**	**ngsTools†**	**PCAngsd**	**Specialised software**
SNP identification		✓	✓					BaseVar, EBG, Freebayes, GATK, Reveel, etc.
Population structure	PCA	✓				✓	✓	
	Individual genetic distance	✓	✓			✓		skmer
	Local PCA							lostruct*
	Admixture						✓	Entropy, evalAdmix, ngsAdmix, Ohana
Selection scan	PCA-based; ancestry-corrected						✓	Ohana
Association analysis		✓						SNPTEST
Linkage disequilibrium				✓		✓		GUS-LD, PopLD
Individual relatedness	Relatedness			✓			✓	ngsRelate
	Parentage							AlphaAssign
	Pedigree analysis							WHODAD
Inbreeding	Inbreeding coefficient		✓		✓	✓	✓	ngsRelate
	IBD tracts					✓		
	Runs of homozygosity							bcftools roh
Ancestry relationships	D-statistics/ABBA-BABA	✓	✓		✓			
Linkage map construction								Lep-MAP3
Allele frequency estimation		✓	✓	✓				
Site frequency spectrum		✓				✓		
Within population genetic diversity	θ estimators (e.g. Watterson's, π)	✓	✓			✓		
Within population neutrality stats	e.g. Tajima's D, Fay & Wu's H	✓						
Individual level genetic diversity	Individual heterozygosity	✓	✓	✓				heterozygosity-em
Population differentiation	F_{ST}	✓			✓	✓		
	dxy					✓		
Allele frequency differentiation	pFst				✓			
Hardy-Weinberg equilibrium		✓		✓			✓	
Structural variants								svgem
Quality score recalibration		✓	✓					

Ploidy inference								HMMploidy
Genotype imputation								Beagle, LB-Impute, LinkImput, loimpute, NOISYmputer, STITCH, etc.

† ngsTools is a collection of loosely-connected programs including ngsSim, ngsF, ngsPopGen, ngsUtils, ngsDist, ngs-HMM, and ngsLD

* lostruct can be used together with custom scripts that perform the PCA e.g. in PCAngsd.

Table 3. Key data filters to consider in the analysis of lcWGS data

Category	Filter	Recommendation
General filters	Base quality	Base quality scores are factored into the calculation of genotype likelihoods, so if they accurately reflect the probability of sequencing error, bases with low scores also carry useful information. However, base quality scores are sometimes miscalibrated, so noise may be reduced if bases with scores below a threshold, e.g. 20, are either trimmed off prior to analysis or ignored.
	Mapping quality	Mapping quality is not considered in genotype likelihood estimation in currently available tools, so it is often advisable to remove low-confidence and/or non-uniquely mapped reads prior to analysis. Filtering out reads that do not map in proper pairs should also further increase confidence in reads being mapped to the correct location, but could cause biases in regions with structural variation
	Minimum depth and/or number of individuals	To avoid sites with low or confounding data support in downstream analysis, minimum depth and/or minimum individual filters can be used to exclude sites with much reduced sequencing coverage compared to the rest of the dataset (e.g. regions with low mapping rates, such as repetitive sequences). Appropriate thresholds will vary between data sets, but could e.g. be to exclude sites with read data for <50% of individuals (globally or within each population), or with <0.8x average depth across individuals.
	Maximum depth	Maximum depth filters are used to exclude sites with exceptionally high coverage (e.g. regions that are susceptible to dubious mapping, such as copy number variants or paralogs). Common maximum depth thresholds are one or two standard deviations above the median genome-wide depth.
	Duplicate reads	PCR duplicates can give inflated impressions of how many unique molecules have been sequenced, which - particularly in the presence of preferential amplification of one allele - could bias genotype likelihood estimation. We therefore recommend removing duplicate reads prior to any analysis.
	Indels	Reads mapped to indels are frequently misaligned, especially if the ends of reads span an indel. To avoid false SNPs, we recommend either realigning reads covering an indel or excluding bases flanking indels

Filters on polymorphic sites*	p-value	The significance threshold (often in the form of maximum p-value) can be adjusted to fine-tune the sensitivity of polymorphism detection, with lower p-values leading to fewer, but higher-confidence, SNP calls. A commonly used cut-off is 10-6
	SNPs with more than two alleles	Most software programs for downstream analyses assume that all SNPs are biallelic, so SNPs with more than two alleles can be filtered out in the SNP identification step to avoid violation of such assumptions.
	Minimum minor allele frequency (MAF)	For many types of analysis, e.g. PCA, admixture analysis, detection of F_{ST} outliers and estimation of LD, low-frequency SNPs are uninformative and can even bias results. For those types of analysis, imposing a minimum MAF filter of 1-10% can substantially speed up computation time. Appropriate thresholds depend on coverage, sample size (how many copies does a MAF threshold correspond to) and the type of downstream analysis.
Restrict analysis to a predefined site list	List of global SNPs	For comparison of parameter estimates for multiple populations, it is important to ensure that data are obtained for a shared set of sites and that SNP polarization (which allele we track the frequency of) is consistent. For programs like ANGSD where population-specific estimates are obtained by analyzing the data from each population separately, a good strategy is to first conduct a global SNP calling with all samples and restrict population-specific analysis to those SNPs with consistent major and minor allele designations and no MAF or SNP p-value filter (because that would give “missing data” if a site is fixed in a particular population).

* Note that no SNP significance or minimum MAF threshold should be used to estimate genetic diversity (e.g. theta and the SFS) as all sites contain relevant information. This also applies to the estimation of the absolute values of dxy.

Table 4. Experimental design recommendations for different types of population genomic analyses using lcWGS data

Type of analyses	Examples	Recommendations on experimental design
Allele frequency and differentiation	Allele frequency trajectory, BayPass, F_{ST} (as implemented in vcflib), pFst	Prioritize larger sample sizes, ≥10 samples per population, ≥10x coverage per population (Figure 3, 4)
SFS-based analyses (absolute estimation of rare-allele-dependent metrics)	Absolute estimation of Watterson's θ, Tajima's D, individual heterozygosity δaδi	Prioritize higher coverage per sample, >4x coverage per sample, ≥5 samples per population (Figure S2, S3)
SFS-based analyses (relative estimation of rare-allele-dependent metrics, or non-rare-allele-dependent metrics)	Relative estimation of Watterson's θ and Tajima's D (e.g. for outlier scan) π, d_{xy}, F_{ST} (as implemented in ANGSD)	Prioritize larger sample sizes, ≥10 samples per population, ≥10x coverage per population (Figure 6, S2-3, S10-11)
Population structure	PCA, admixture	Prioritize larger sample sizes, ≥10 samples per population, extremely low per-sample coverage (e.g. 0.125x, Figure 5, S9) or highly uneven per-sample coverage (e.g. 0.5-6x, Skotte et al. 2013) could be viable
Absolute estimation of linkage disequilibrium	LD decay rate, demographic inference	Prioritize higher coverage per sample, ≥4x coverage per sample, ≥20 samples per population (Figure S4, S5; Bilton et al., 2018; Fox et al., 2019; Maruki & Lynch, 2014)
Relative estimation of linkage disequilibrium	LD pruning, LD block identification	Per-sample coverage as low as 1x could be viable, ≥20x coverage per population (Figure S4, S5)
Genotype imputation without reference panels	STITCH, Beagle	STITCH: prioritize larger sample size (≥500) over per-sample coverage (1x could be sufficient) Beagle: prioritize higher per-sample coverage (≥2x) over sample sizes (≤250 could be sufficient) (Figure 9)

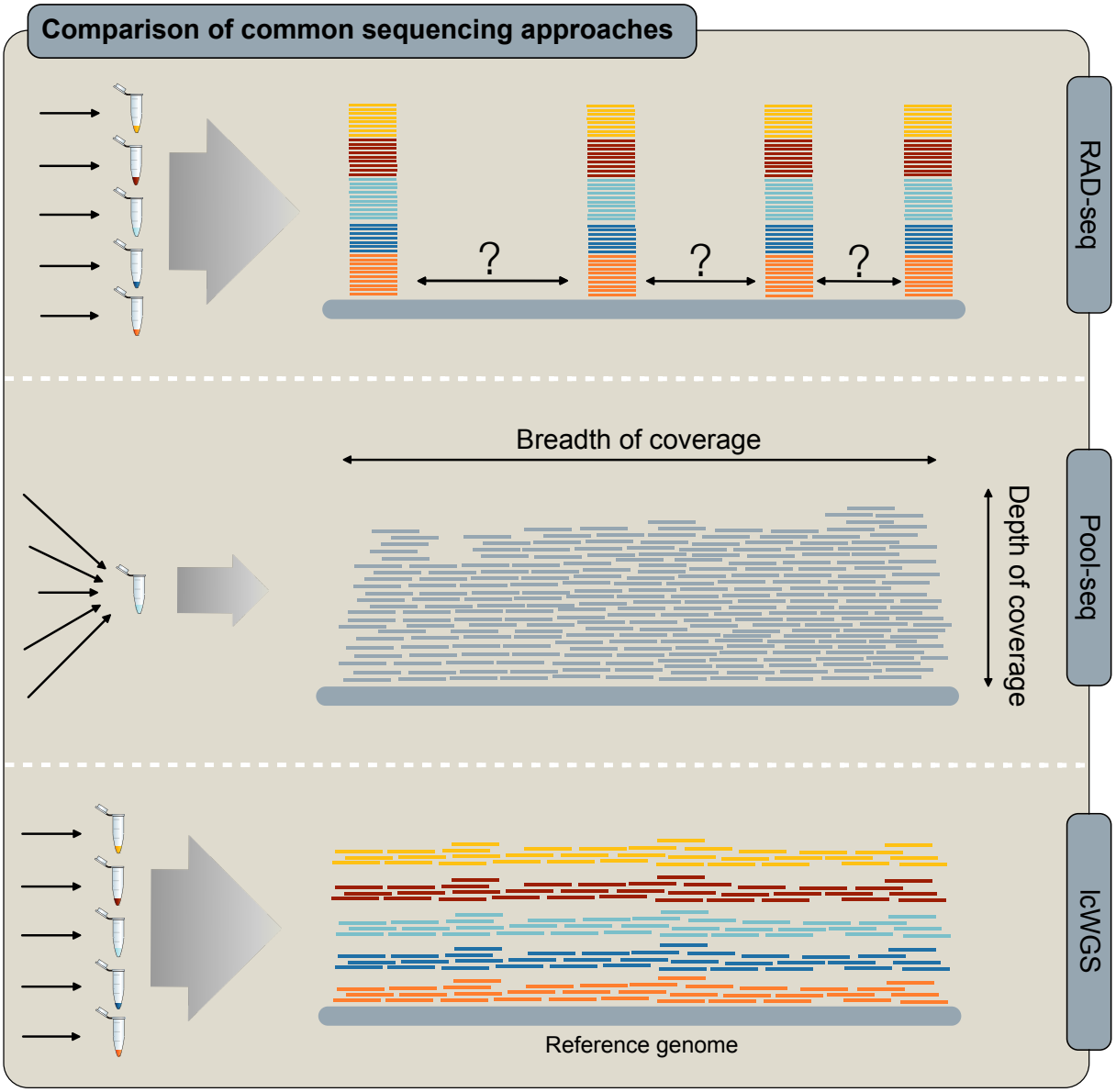

Figure 1. Diagram showing the distribution of sequencing reads mapped to a reference genome under **(A)** a RAD-seq, **(B)** a Pool-seq, and **(C)** a lcWGS design.

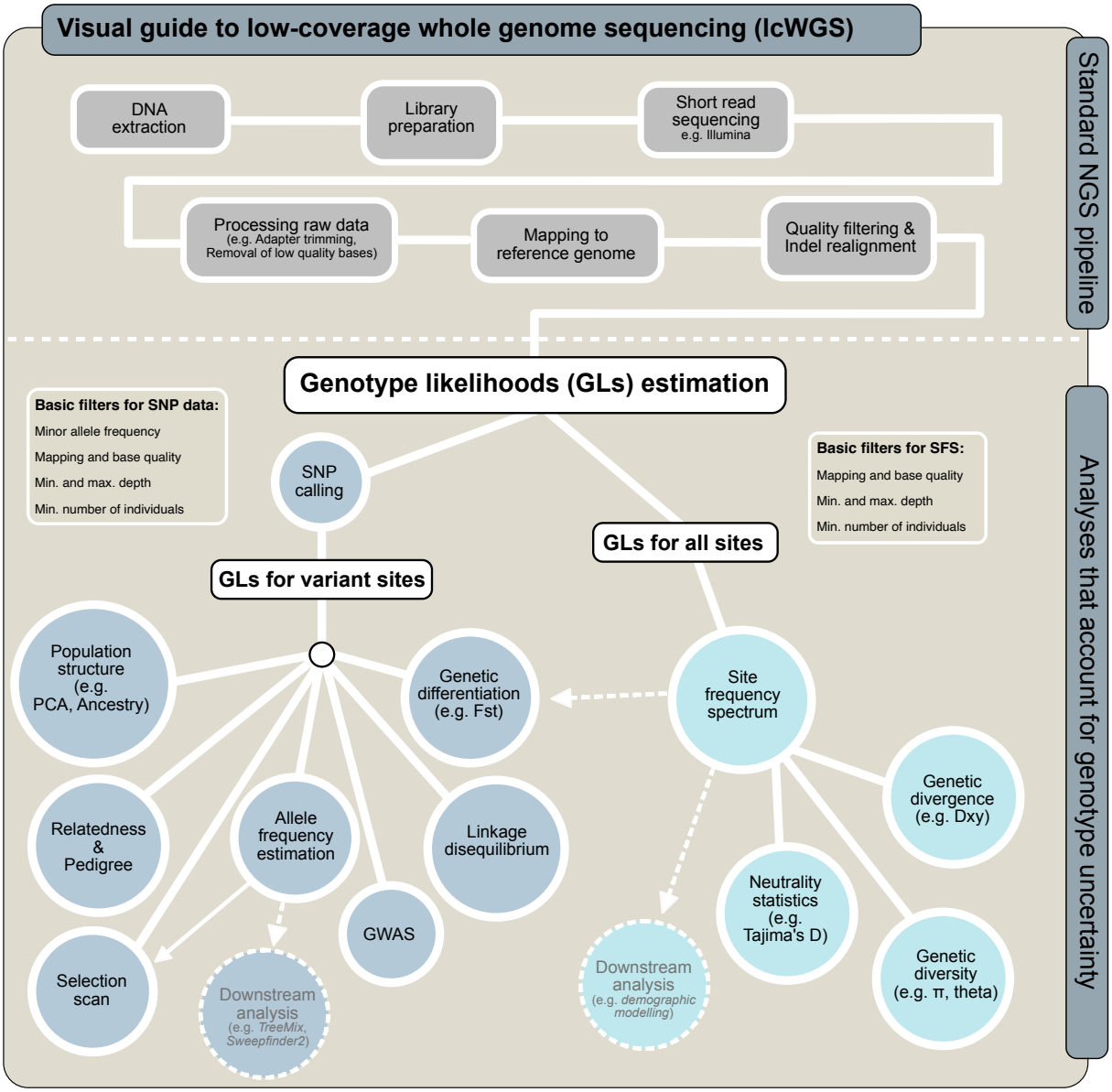

Figure 2. Diagram showing a typical computational pipeline for lcWGS data. **Top:** the data processing part of the pipeline, which is similar to the pipeline for other types of NGS data. **Bottom:** the data analysis part of the pipeline, which is based on a probabilistic framework using genotype likelihood to account for genotype uncertainty.

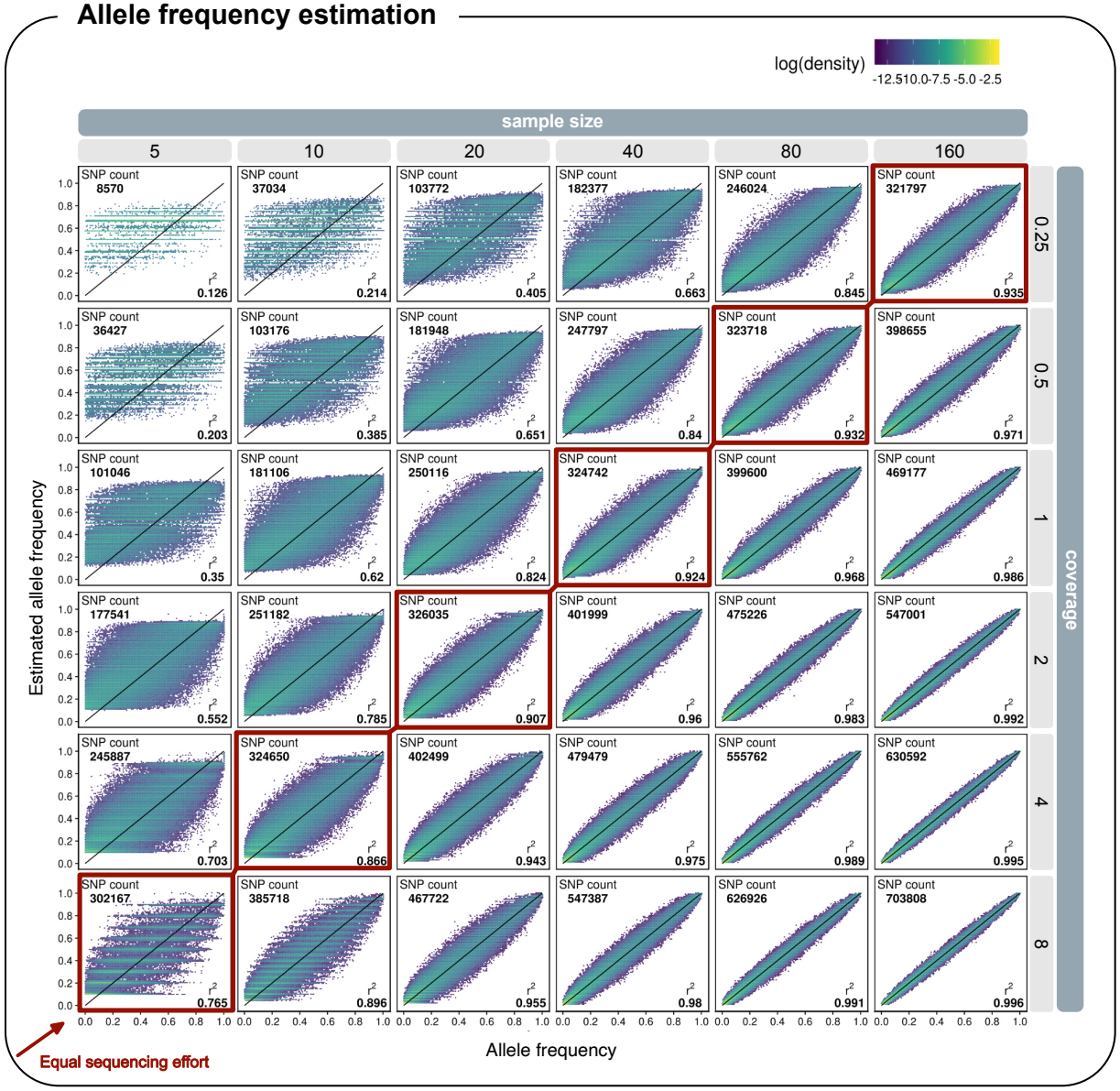

Figure 3. The estimated vs. true allele frequencies at all called SNPs (i.e. true positives + false positives) with lcWGS. Across the different facets, sample size increases from left to right, and coverage increases from top to bottom. The total sequencing effort remains the same along the diagonal from bottom left to top right. The color indicates the density of points in the area, with yellow corresponding to the highest density and dark blue corresponding to the lowest density. r^2 and the number of SNPs called (SNP count) are shown in each facet. The black line in each facet indicates the positions where the estimated allele frequency is equal to the true allele frequency. False negative SNPs are not included in this figure; their distribution is shown in Figure S1.

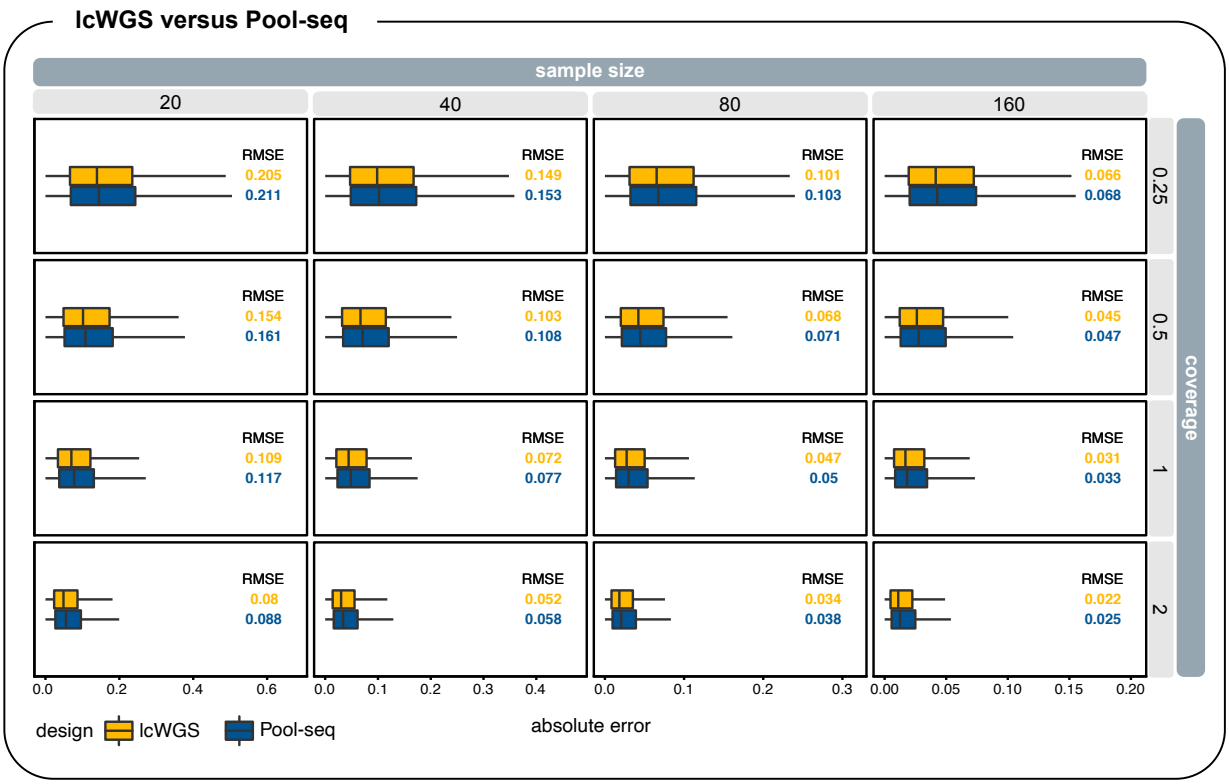

Figure 4. The error in allele frequency estimation with lcWGS (yellow) and Pool-seq (blue) data. The distribution of absolute errors (|estimated frequency - true frequency|) is shown with the box plots along the x-axis. The lower and upper hinges of the box plots show the interquartile ranges of absolute errors, and the whiskers extend to the largest or smallest values no further than 1.5 times the interquartile range. Outlier points are hidden. Across the different facets, sample size increases from left to right, and coverage increases from top to bottom. The total sequencing effort remains the same along the diagonal from bottom left to top right. The root mean squared error (RMSE) for the two sequencing designs are shown in each facet; note the differences in scale of the x-axes. False negative SNPs are not included in this figure; their distribution is shown in Figure S1.

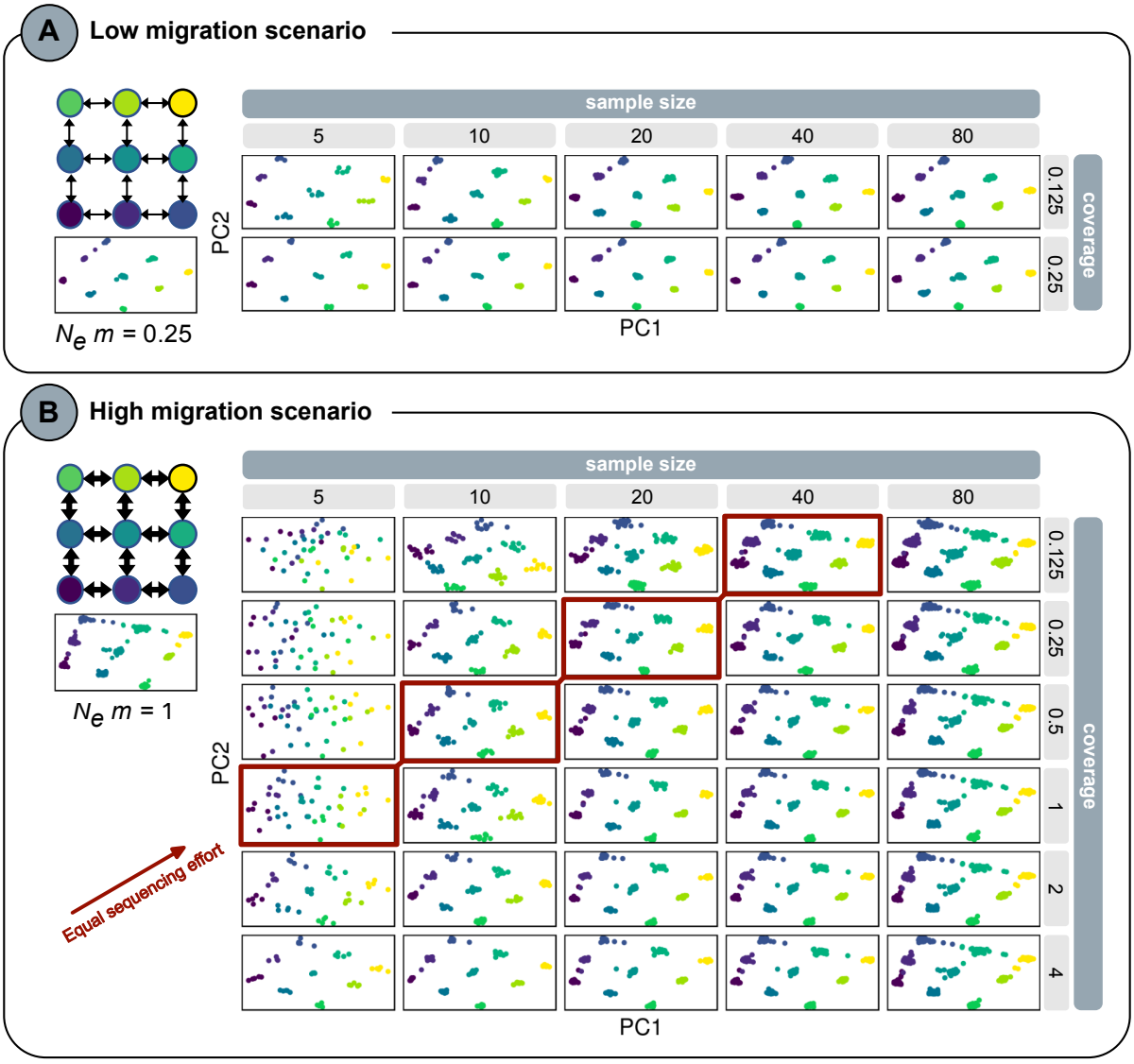

Figure 5. Patterns of spatial population structure inferred through principal component analysis (PCA) with lcWGS data. **(A)** A scenario with lower gene flow (an average of 0.25 effective migrants per generation). **(B)** A scenario with higher gene flow (an average of 1 effective migrant from one population to another every generation). Left: the true population structures being simulated; each node corresponds to a simulated population. Right: the first two principal components from the PCA with simulated lcWGS data; each point corresponds to an individual sample and its color corresponds to the population it is sampled from. Sample size per population increases across panels from left to right, and coverage per sample increases from top to bottom.

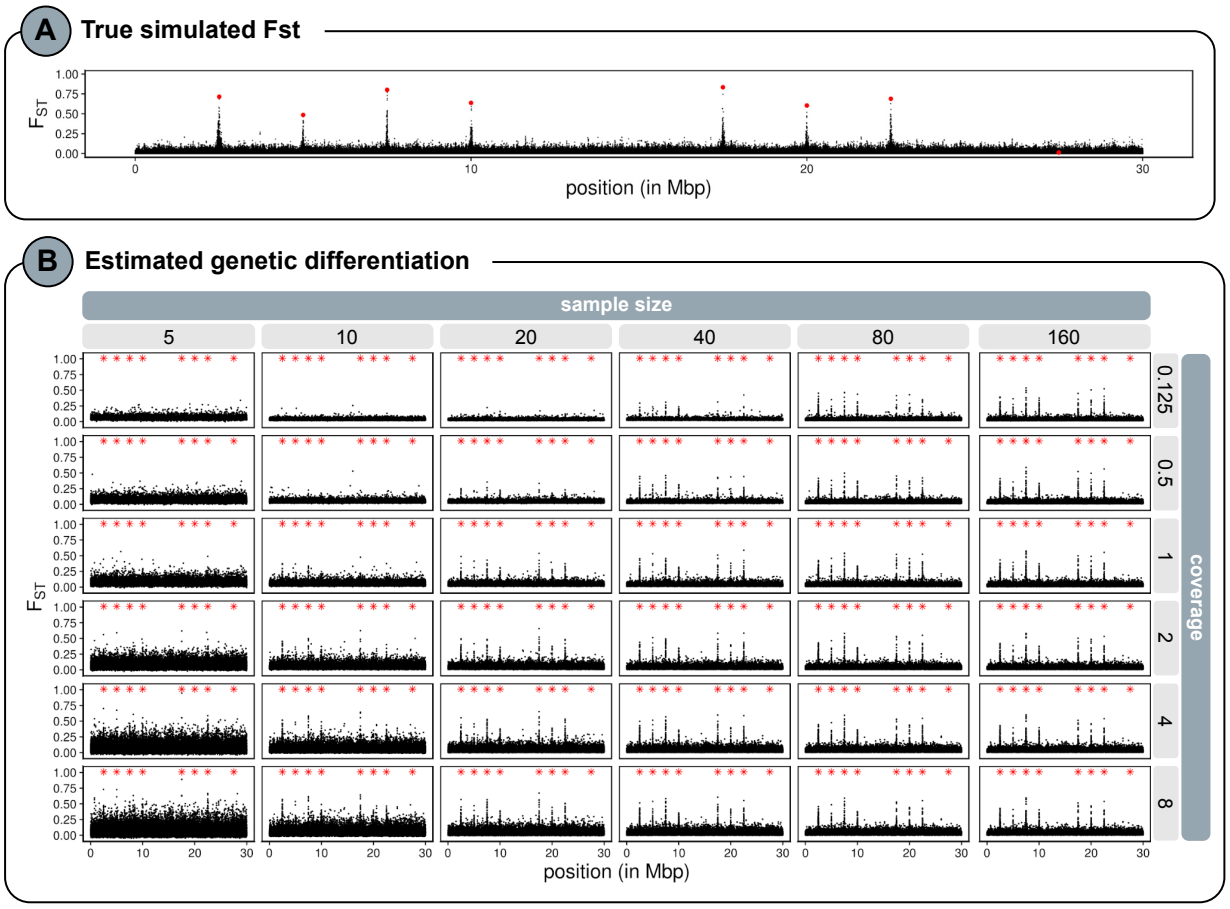

Figure 6. Genome-wide scan for divergent selection with lcWGS data. **(A)** The true per-SNP F_{ST} values along the chromosome between the two simulated populations. **(B)** The F_{ST} values inferred from lcWGS data in 1kb windows along the chromosome. Sample size per population increases from left to right, and coverage per sample increases from top to bottom. In **(A)**, the red points mark the position of SNPs under selection and the black points mark the neutral SNPs. In **(B)**, the black points mark both the selected and neutral SNPs, and the red asterisks only mark the positions of the selected SNPs (not their inferred F_{ST} values).

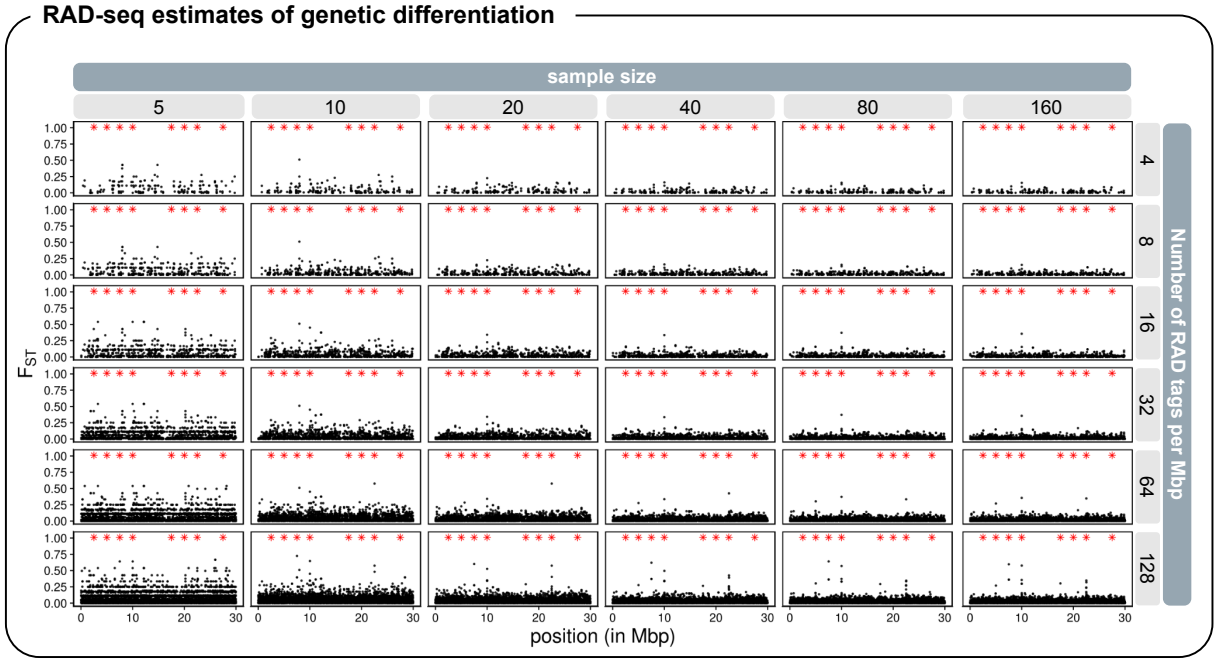

Figure 7. Genome-wide scan for divergent selection with RADseq data. The per-SNP F_{ST} values inferred from RADseq data are shown on the y axis and the SNP positions are shown on the x axis. Sample size per population increases from left to right, and RAD tag density increases from top to bottom. The black points mark both the selected and neutral SNPs, and the red asterisks only mark the positions of the selected SNPs (not their inferred F_{ST} values).

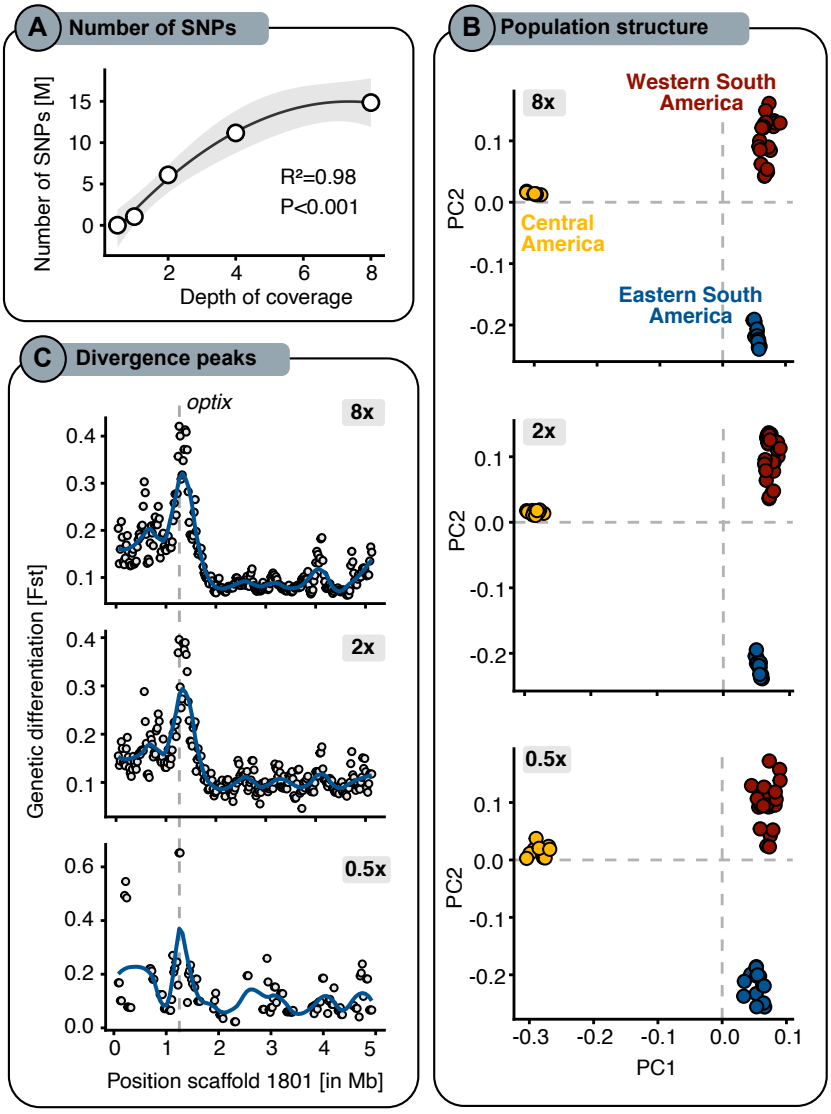

Figure 8. Application of genotype likelihoods to empirical data. **(A)** Correlation between the number of identified SNPs (in millions) with variation in depth of sequencing coverage in the downsampled *Heliconius* dataset. **(B)** Principal components analysis for three different coverages (8x, 2x and 0.5x) of 51 samples. Estimates of population structure are highly concordant across coverages. Subspecies are pooled and colored by their broader region of origin. **(C)** Estimates of genetic differentiation (F_{ST}) between pooled *Heliconius* subspecies with the red-bar phenotype (n=23) and without the red-bar phenotype (n=28) along the scaffold containing the causal *optix* candidate genes in 50kb sliding windows with 20kb steps. F_{ST} estimates are highly concordant between 8x and 2x coverage, but more sparse at 0.5x due to the lower number of identified variant sites.

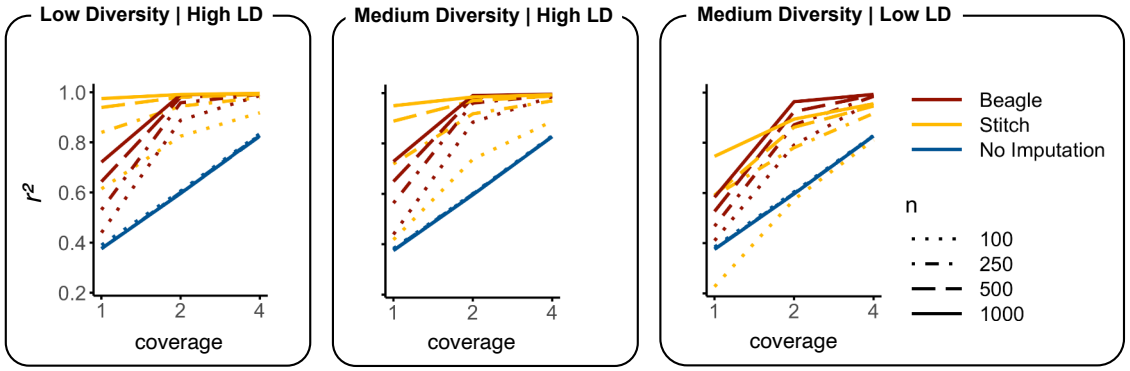

Figure 9. Genotype imputation in STITCH and Beagle compared to posterior genotypes estimated without imputation in three in populations with varying diversity and linkage disequilibrium. r^2 between true genotypes and estimated genotype dosages are shown for combinations of sample size (n; with increasing n indicated by more contiguous lines), sequencing coverage (x-axis) and method (line colors).