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NEWS AND VIEWS

Opinion

These aren't the loci you'e looking for: Principles of effective SNP filtering for molecular ecologists

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Abstract

Sequencing reduced-representation libraries of restriction site-associated DNA (RADseq) to identify single nucleotide polymorphisms (SNPs) is quickly becoming a standard methodology for molecular ecologists. Because of the scale of RADseq data sets, putative loci cannot be assessed individually, making the process of filtering noise and correctly identifying biologically meaningful signal more difficult. Artefacts introduced during library preparation and/or bioinformatic processing of SNP data can create patterns that are incorrectly interpreted as indicative of population structure or natural selection. Therefore, it is crucial to carefully consider types of errors that may be introduced during laboratory work and data processing, and how to minimize, detect and remove these errors. Here, we discuss issues inherent to RADseq methodologies that can result in artefacts during library preparation and locus reconstruction resulting in erroneous SNP calls and, ultimately, genotyping error. Further, we describe steps that can be implemented to create a rigorously filtered data set consisting of markers accurately representing independent loci and compare the effect of different combinations of filters on four RAD data sets. At last, we stress the importance of publishing raw sequence data along with final filtered data sets in addition to detailed documentation of filtering steps and quality control measures.

KEYWORDS
conservation genetics, ecological genetics, landscape genetics, molecular evolution, population ecology, population genetics—empirical

1 | THE RISE OF RAD

Advances in sequencing technology coupled with increases in computational power have resulted in a shift towards genome-scale data analysis, for which data sets typically consist of thousands to tens of thousands of loci. At the same time, bioinformatic pipelines have become more user-friendly and accessible to scientists without extensive backgrounds in bioinformatics or programming. As a result, new analytical methods are rapidly being developed for studies assessing levels of population structure and genomic diversity, identifying and mapping quantitative trait loci (QTL), and screening for F_{ST} outliers putatively indicative of selection; increasingly, restriction site-associated DNA sequencing (RADseq)-derived single nucleotide polymorphisms (SNPs) are becoming the molecular marker of choice. RADseq methods are time- and cost-efficient techniques that utilize restriction enzymes to generate DNA fragments from which thousands of SNPs can be identified using next-generation sequencing. This set of methods does not require a fully sequenced reference genome as loci can be reconstructed de novo from sequencing reads, greatly widening the types of organisms that can be studied beyond traditional model species (Baird et al., 2008; Davey & Blaxter, 2010; Miller, Dunham, Amores, Cresko, & Johnson, 2007). In

addition to the original RADseq protocol (Miller et al., 2007), ddRAD (Peterson, Weber, Kay, Fisher, & Hoekstra, 2012), ezRAD (Toonen et al., 2013) and 2b-RAD (Wang, Meyer, McKay, & Matz, 2012) are commonly applied techniques. Despite differences between RADseq techniques and more traditional approaches, typically limited to data sets consisting of mitochondrial and/or nuclear loci (e.g., 10–100 microsatellite loci), all are unified by the assumption that the final data set consists of markers that each represents a single locus and that these loci are unlinked (freely recombining), a condition that must be met when allele and genotype frequencies are being used to infer biological processes.

Recent reviews have summarized differences between individual RADseq techniques, compared their respective advantages and disadvantages and pointed out some potential sources of genotyping error that can lead to biased data sets (Andrews et al., 2014; Puritz, Hollenbeck, & Gold, 2014; Puritz, Matz, et al., 2014). More effort, however, is required to establish widely accepted protocols to detect and remove putative markers that in reality do not represent single loci, identify and correct erroneous SNP calls, and assess genotyping error (but see Ilut, Nydam, & Hare, 2014; Li & Wren, 2014; Mastretta-Yanes et al., 2015). For other commonly used molecular markers such as AFLPs and microsatellites, sources of genotyping error (i.e. allelic dropout, null alleles, stuttering) and best practices to efficiently detect and correct for them are well established (Bonin et al., 2004), and standards of reporting regarding data quality control have been formalized. At present, published RADseq studies report (and practice) a wide array of data filtering and error detection procedures after variant calling, but many publications underreport quality control methods, making it difficult for the reader to assess data quality.

Generating SNP data sets using RADseq approaches involves three general steps: library preparation, bioinformatic processing, and filtering for data quality. It is important to realize that error potentially resulting in artefacts downstream can be introduced at any of these steps. The introduction of some error during technical stages is unavoidable; therefore, it is important to employ quality control steps that allow for the identification and reduction in error before the data set is analysed. Here, we briefly review and make recommendations on how to limit and detect common sources of technical artefacts during library preparation and bioinformatic processing and suggest a set of filtering strategies that can be employed to create a robust data set consisting of markers representing physically unlinked, correctly reconstructed loci (Table 1). Further, we apply different combinations of suggested filters to several RAD data sets and discuss the effectiveness of different filtering strategies.

2 | MINIMIZING ARTEFACTS ASSOCIATED WITH LIBRARY PREPARATION

The goal of library preparation for a typical RADseq experiment is to consistently sample the same set of fragments with sufficient coverage to correctly identify all alleles present at each locus across all individuals within and across sequencing runs. In this context, "library" refers to a set of RADseq fragments isolated from a given number of individuals that are barcoded and sequenced together on a single lane. Common technical artefacts introduced during library preparation include (a) coverage effects, (b) locus drop-in/dropout, (c) PCR artefacts and (d) library effects. Another common artefact, allele dropout, causes alleles to systematically remain unsampled due to physical properties of the genome, that is cut-site or length polymorphisms. Because allele dropout has a biological origin, it should be considered a biological artefact that cannot be technically mitigated but rather can only be managed during bioinformatic processing (discussed in detail in Section 4.3). In contrast, technical artefacts are associated with technical choices made by researchers and thus can be limited by careful planning during library preparation, as discussed below.

2.1 | Coverage effects: DNA quality, quantity and restriction digestion

RADseq methods, with the possible exception of recently developed hybrid enrichment methods (Schmid et al., 2017; Suchan et al., 2016), require high-molecular-weight DNA to ensure consistent digestion using restriction enzymes. Compared to other molecular markers, RADseq protocols also require greater amounts of DNA (up to 500 ng), and while there is some flexibility in how much DNA is used, lower starting amounts of DNA increase the risks of low-quality data. Inconsistent digestions can be due to partially degraded DNA, inhibitors present in the reaction (usually left over from extraction) and star activity of the enzymes (i.e., cleavage of noncanonical recognition sequences). This is problematic because inconsistent recovery of all fragments produces downstream variance in coverage and/or missing data among loci within and between libraries (Graham et al., 2015). To help ensure consistent digestions, researchers should use high-fidelity versions of restriction enzymes and perform trial digestions to determine adequate concentrations and sufficient digestion times. Quality control measures such as running digested samples on a fragment analyser or agarose gel can be implemented to compare digestion results. Unit definitions for enzymes and standard protocols are generally based on the digestion of purified λ-phage DNA; therefore, it is often advisable to use more enzymes than manufacturer's guidelines suggest. In addition, purifying genomic DNA before digestion can remove inhibitors (e.g., phenol or pigments) carried over from extraction.

When read depth per locus per individual (hereafter "coverage") is insufficient, alleles may not be detected. Coverage effects may occur when initial DNA quality differs among individuals or standardization of the amount of DNA prior to pooling is inconsistent, resulting in an unequal distribution of sequenced reads among individuals and loci. The use of high-sensitivity quantification kits and standardization of DNA quantity prior to enzyme digestion and again prior to adapter ligation can help to mitigate this issue. In the same way,

TABLE 1 Overview of described potential issues in raw RAD data sets, their causes and strategies for technical and bioinformatic mitigation

Issue	Potential causes	Technical mitigation	Bioinformatic mitigation
Inconsistent sequencing of loci	• Low-quality genomic DNA • Inconsistent digestions • Locus drop-in/dropout during size selection	• Consistent digestion across samples • Precise pooling of samples • Quality control size selection	• Genotype call rate/missing data filters • Mean minimum depth filter
Coverage effects (*false homozygotes*)	• Read depth too low to successfully recover both alleles	• Choose enzyme combination, fragment size and number of individuals based on desired read depth per locus & individual	• Depth filters (genotype, mean/variance per locus) • Excess homozygosity filter
Null alleles (*false homozygotes*)	• Length/size polymorphism results in nonamplification of allele	• Biological origin: effect cannot be minimized	• Excess homozygosity filter • Depth filters (genotype, mean/variance per locus • Difficult to distinguish from coverage effects (difficult to account for)

Clustering error (*artifactual contigs*)	• Oversplit or overclustering during de novo locus assembly	• Test range of parameters to identify best value for percentage similarity to split
		• Choose conservative threshold (risk overclustering to avoid oversplitting)
			• Oversplit loci cannot be efficiently detected and removed
			• Identify overclustered loci based on:
			  ○ Excess depth
			  ○ Excess heterozygosity
			  ○ Haplotyping

Artifactual SNPs (*false heterozygotes*)	• PCR artefacts • Sequencing error • Read mapping	• High-fidelity polymerase • Minimize PCR cycles • Incorporating adapters with random nucleotides for duplicate identification	• Mapping quality ratio • Allele balance • Strand bias • Properly paired reads • Locus quality/depth ratio
Library effects	Differences between libraries: • Size selection • Coverage • Sequencing lanes/machines	• Randomize samples to decouple signal • Technical replicates	• Identify and remove affected loci using PCA/DAPC • Ensure loci consistently amplified between libraries
Linkage	• Multiple SNPs on same contig • Fragments physically linked	• Biological origin: cannot be minimized	• SNP thinning • Haplotyping • Test for linkage disequilibrium

pooling too many individuals on a sequencing lane can result in systematic low read depth across all samples and loci. This can be avoided by reducing the number of individuals per sequencing lane or by adjusting the size selection window and enzymes used to decrease the number of targeted fragments. For loci affected by coverage effects, false homozygote calls will result in biased allele frequency estimates, which may cause genomic diversity to be underestimated, F_{ST}, and effective population size to be incorrectly estimated, and an increase in false positives/negatives in F_{ST}-outlier tests (Arnold, Corbett-Detig, Hartl, & Bomblies, 2013; Gautier et al., 2012).

2.2 | Locus drop-in/dropout due to size selection

Size selection is a crucial step to ensure consistent sampling of the same set of fragments across ddRAD libraries. The magnitude of the variance in the distribution of fragment lengths between libraries is dependent on the method used for size selection (Puritz, Hollenbeck, & Gold, 2015). Two commonly employed methods are manual gel cutting and automated size selection (e.g., Pippin Prep) . While the latter is expected to increase the accuracy and precision of size selection, there can still be inconsistencies caused by factors including the salt concentration of the loaded samples and variable ambient laboratory temperature that can result in changes in the size distribution of eluted fragments. Size selection anomalies can therefore result in fragments dropping in or out of the targeted size window for individually prepared libraries. To ensure consistent fragment recovery, it is important to make sure that both means and variances the mean and variance of fragment size distributions are similar across runs. Because small fragments may be amplified preferentially, libraries with wider variances may have suboptimal coverage for larger fragments as compared to libraries with less variance even if the mean fragment size is comparable. Thus, it is important to implement quality control steps to determine whether the selected fragments fall into the expected distribution given the targeted size window. For example, a fragment analyser or high-resolution electrophoresis gel can be used to determine the actual length of the fragments retained in each library prior to sequencing.

2.3 | PCR artefacts

With the exception of proposed PCR-free protocols (e.g., ezRAD; Toonen et al., 2013) and protocols performing PCR before size selection (Elshire et al., 2011), the final step of library preparation is PCR amplification, during which artefacts may also be introduced. These can be classified as (a) PCR error, including PCR chimeras, heteroduplexes and *Taq* polymerase error that could be exponentially propagated during PCR cycling, and (b) PCR bias, the preferential amplification of shorter fragments and those with higher GC content. PCR artefacts can be minimized by using high-fidelity polymerase and high annealing temperatures to limit copy error, reducing the number of cycles to minimize PCR bias and providing sufficient extension time based on fragment size. In addition, several authors have recommended the incorporation of barcodes with degenerate bases to aid in detection and removal of PCR duplicates (Schweyen, Rozenberg, & Leese, 2014; Tin, Rheindt, Cros, & Mikheyev, 2015), that is reads stemming from the same fragment template, which artificially increase read depth and therefore increase confidence in a SNP call despite not actually representing independent observations. At last, multiple reactions can be completed with fewer cycles and combined into a final product to further mitigate PCR error and bias.

2.4 | Library effects

One of the principal benefits of reduced-representation sequencing techniques is the reproducibility of the library preparation process. In theory, repeating the process with the same restriction enzymes and size selection window should consistently yield the same set of fragments. In practice, however, subtle differences between experiments, frequently beyond the control of the researcher, can result in a situation where different sets of fragments are sequenced and/or coverage differs greatly among libraries ("library effects"). Library effects can be caused by a number of factors including differences in reagents and protocols used, ambient laboratory temperature, poor accuracy and/or precision of size selection, and differences in DNA pool quality and/or concentration (Bonin et al., 2004). While not all library effects can be avoided, measures can be implemented to reduce the impact of library effects and identify the most severely affected markers.

The most effective ways to decouple the putative biological signal from patterns introduced by library effects are by (a) randomly allocating individuals from different treatments or geographic localities across libraries and (b) including technical replicates (repeated samples) across libraries (Meirmans, 2015). Randomizing samples across libraries broadly diminishes the chances that artifactual signal will be confused as a biologically meaningful pattern, while also allowing for downstream identification and removal of library effects. By performing a PCA, or similar analysis, with data grouped by library and identifying and examining those markers most associated with axes discriminating libraries, library effects can be mediated by removing biased loci (Figure 1). When studies incorporate multiple libraries prepared at different times and under different conditions and sequenced on multiple lanes, including a subset of individuals across libraries ("technical replicates") should be standard practice. Incorporating these technical replicates enables a direct comparison of genotypes across libraries, allowing for the identification of loci that are consistently sampled with sufficient coverage to identify both alleles and loci exhibiting systematic genotyping errors. Implementing the randomization of individuals and including the technical replicates during the library preparation stage are crucial for identifying library effects during bioinformatic processing and data filtering.

3 | MINIMIZING ARTEFACTS ASSOCIATED WITH BIOINFORMATICS

During bioinformatic processing of RADseq data in the absence of a fully sequenced and assembled genome, reads are first clustered into contigs (contiguous sequence alignments) with the goal that each contig should represent a single locus. Second, reads are clustered or aligned at each reconstructed locus to identify and call SNPs for each individual. Artefacts most commonly introduced at this stage are (a) clustering errors, that is the chosen values for the parameters of the clustering algorithm result in undersplitting or oversplitting of putative loci, and (b) artifactual SNPs resulting from mapping errors or failure to identify PCR error or sequencing error.

3.1 | Clustering error

One of the main advantages of RADseq methods is the fact that loci can be assembled de novo, that is without a draft genome. The critical step in generating markers that accurately represent these loci is the clustering of sequences into contigs that each represent a single locus (Ilut et al., 2014). Several pipelines for marker reconstruction exist, including *Stacks* (Catchen, Hohenlohe, Bassham, Amores, & Cresko, 2013), *PyRAD* (Eaton, 2014), *dDocent* (Puritz, et al., 2014; Puritz, Matz, et al., 2014) and *AftrRAD* (Sovic, Fries, & Gibbs, 2015), each of which differs slightly in the strategies and methods employed. While the algorithmic details of each pipeline are different, they all make the assignment of putative homology (orthology) of fragments based on the number of mismatches or percentage similarity. Efficacy of this technique requires that the maximum divergence among alleles at a given locus is smaller than the minimum divergence among loci (Ilut et al., 2014). Undersplitting occurs when sequence similarity thresholds are too low such that multiple loci are combined into a single cluster forming multilocus contigs. The formation of multilocus contigs will occur more frequently with paralogs, repetitive elements and otherwise superficially similar sequences in the genome. These multilocus contigs can inflate the mean estimated heterozygosity. Conversely oversplitting occurs when sequence similarity thresholds are too high, causing alleles of the same locus to be split into two or more contigs. Oversplitting results in deflation of mean estimated heterozygosity. Picking similarity thresholds that result in no over- or undersplitting is not possible because every genome contains elements that will suffer over-

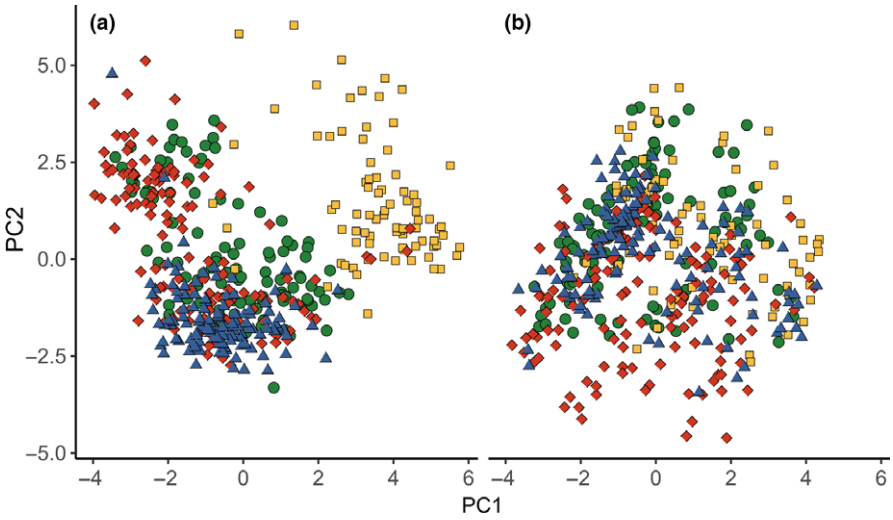

FIGURE 1 Library effects (adapted from Puritz et al., 2015). PCA of RAD data set combining four libraries (yellow squares, red diamonds, blue triangles and green circles) before (a) and after (b) correcting for library effects by removing affected markers

or undersplitting at every threshold selected (Ilut et al., 2014). However, it is generally better to err on the side of undersplitting, because methods to identify and remove multilocus contigs are more effective than those for identifying oversplit loci (Ilut et al., 2014; Mastretta-Yanes et al., 2015; Willis, Hollenbeck, Puritz, Gold, & Portnoy, 2017). In addition, understanding differences between bioinformatic pipelines is critical to properly clustering the data. For example, Puritz et al. (*in prep*) found that rates of oversplitting vary between *dDocent*, *PyRAD*, *Stacks* and *AftrRAD* across various combinations of parameters. Because effective thresholds for clustering will depend on the bioinformatic pipeline and vary by organism, enzyme and data set, researchers should test parameters to identify values where oversplitting is minimized.

3.2 | Artifactual SNPs

Artifactual SNPs, those that do not exist in the actual genome but are called from mapped reads, may be the result of erroneous read clustering/mapping, PCR error and/or sequencing error. Because the rate of sequencing error varies by platform employed, chemistry and read length, the typical user cannot control all error introduced at this stage; therefore, it is important to account for sequencing error during bioinformatic analysis. FASTQ format sequence reads include Phred scale quality scores that indicate the probability of a base call being correct. The quality score, Q, equals $-10 \log_{10} P$, with P being the probability of a base-calling error; for example, $Q = 30$ corresponds to the expectation that 1 in 1000 base calls will be incorrect, that is the probability of a correct base call is 99.9%. Quality scores can be used during bioinformatic processing to trim low-quality sections from the beginnings and/or ends of reads or to eliminate reads entirely; failure to do so can affect mapping quality downstream and/or introduce artifactual SNPs. In the same way, library effects may be introduced at this stage if sequence data are not carefully assessed for quality (especially at the 3′ and 5′ ends) and properly trimmed. A Phred-like quality score is also used by several variant callers, including FreeBayes and GATK (Depristo et al., 2011; Garrison & Marth, 2012), to determine the probability of a SNP call being real or artifactual.

4 | FILTERING SNP DATA

Despite attempts to limit the introduction of technical artefacts during library preparation and bioinformatic processing, SNP data sets require rigorous filtering because the inclusion of only a few incorrectly genotyped loci in a data set can create a significant, misleading signal (Davey et al., 2013; Li & Wren, 2014; Meirmans, 2015; Puritz, et al., 2014; Puritz, Matz, et al., 2014). This is especially important for Fst-outlier detection to determine loci potentially under selection because signal caused by genotyping error is likely to stand out in pattern and magnitude from the signal produced by the background SNP data (Hendricks et al., 2018; Xue et al., 2009). Full postprocessing exploration of each data set should include an evaluation of the quality of each locus and individual, the confidence in both SNP calls and genotypes, and whether specific loci are likely to be multilocus contigs. This should involve generating frequency distributions of parameters including missing data per locus and individuals, read depth and heterozygosity to determine appropriate threshold values for these parameters. In addition, the comparison of multiple filtered data sets generated using different parameter values provides guidance for which combinations of thresholds retain the most loci while minimizing artefacts.

Beyond identifying parameters and threshold values that best identify and remove specific types of artefacts, other important considerations include the order in which filters are applied, whether individual genotypes should be selectively coded as missing (e.g., due to insufficient coverage) or entire loci removed, whether specific SNPs or entire SNP-containing contigs should be removed, and whether threshold values should be applied across the entire data set or separately across biologically meaningful groups, for example geographic sampling locations or, to mitigate library effects, separately across individuals grouped, for example, by library/sequencing

lane. In addition, every data set will be unique in terms of the number and quality of samples/sequencing runs, and differences in the protocols employed (e.g., enzyme combinations, targeted coverage) and this means that individual data sets will differ in terms of missing data, coverage, etc. Therefore, while certain parameters should always be considered during filtering, the exact steps employed and the applied thresholds will be specific to each data set.

To illustrate the effects of various filtering strategies and parameter thresholds, we employed six different filtering schemes (FS) across four different data sets (Hollenbeck, 2016; O'Leary, Hollenbeck, Vega, Gold, & Portnoy, 2018; Portnoy et al., 2015; Puritz, Gold, & Portnoy, 2016). All data sets were created using the *dDocent* pipeline and differ in terms of the focal organism, type of reference used to map reads, the type of reads and the number of libraries sequenced (Supporting information Table S1). The red snapper data set (Puritz et al., 2016) consists of previously published data that have been recalled against a fully sequenced draft genome consisting of large contigs (154,064 contigs; N50 = 233,156 bp; total length 1.23 Gb), while the other three were assembled de novo as previously published. For all FS, we first filtered genotypes, loci and individuals. Then, because most researchers analyse data sets of biallelic SNPs, as a final step we decomposed multinucleotide variants and retained only SNPs. Details of full FS are available in Table 2, and fully annotated scripts for filtering are available at https://github.com/sjoleary/SNPFILT. The results of these FS are discussed in the following sections to illustrate suggested filters.

4.1 | Low-quality loci versus low-quality individuals

Filtering parameters used to identify loci and individuals that did not sequence well include genotype call rate per locus (i.e., proportion of individuals a locus is called in) and missing data per individual, as well as genotype depth and the mean depth per locus, that is mean number of reads at a given locus across individuals. For data sets characterized by high levels of missing data (e.g., red snapper, Figure 2), applying hard thresholds can result in retaining little to no loci in the filtered data set. For example, for the red snapper data set, setting hard cut-offs retaining only loci with genotype call rates >95% and individuals with <25% missing data leads to a final data set of only 10 SNPs on three contigs in 262 individuals (raw data set contains 1,106,387 SNPs on 25,168 contigs for 282 individuals, Table 3).

As an alternative strategy, starting with low cut-off values for missing data (applied separately per locus and individual) and iteratively and alternately increasing them may result in more high-quality loci and individuals being retained. For example, in the red snapper data set, first removing low-confidence genotypes by filtering for minimum genotype read depth >5, SNP quality score >20, minor allele count >3 and minimum mean read depth per locus >15 changes the distribution of missing data per locus and individual and decreases the mean missing data from approximately 75% to 35% (Compare Figure 2a, b with c, d). Then, iteratively increasing the stringency of allowed missing data (final threshold values of a 95%

TABLE 2 Detailed description of six different filtering schemes applied to example data sets, and the order of the rows indicates the order in which filters are applied. Applied filters are designed to remove loci with low-confidence SNP calls (minimum genotype read depth (minDP), SNP quality score (Qual), mean read depth per locus across all individuals (meanDP), minor allele count (mac), missing data (allowed missing data per individual (imiss), genotype call rate (number of individuals that have been called for a given locus (geno)) and INFO filters as described in the manuscript

Filter	FS 1	FS 2	FS 3	FS 4	FS 5	FS 6
Low-confidence SNP calls			minDP > 5	minDP > 5	minDP > 5	minDP > 5
			Qual > 20	Qual > 20	Qual > 20	Qual > 20
			meanDP > 15	meanDP > 15	meanDP > 15	meanDP > 15
			mac < 3	mac < 3	mac < 3	
Missing data				geno > 50%	geno > 50%	geno > 50%
				imiss < 90%	imiss < 90%	imiss < 90%
				geno > 60%	geno > 60%	geno > 60%
				imiss < 70%	imiss < 70%	imiss < 70%
				geno > 70%	geno > 70%	geno > 70%
				imiss < 50%	imiss < 50%	imiss < 50%
INFO filters					Allele balance	Allele balance
					Quality/depth ratio	Quality/depth ratio
					Mapping quality ratio	Mapping quality ratio
					Strandedness	Strandedness
					Properly paired status	Properly paired status
					High depth/quality ratio	High depth/quality ratio
Missing data	geno > 95%	imiss < 25%	geno > 95%	imiss > 25%	imiss > 25%	imiss > 25%
	imiss < 25%	geno > 95%	imiss < 25%	geno < 95%	geno < 95%	geno < 95%

genotype call rate and 25% allowed missing data per individual) results in 9,478–12,056 SNPs on 1,626–1,680 contigs and 187–189 individuals being retained (Table 3), depending on the FS outlined in Table 2. This occurs because poor-quality individuals tend to deflate genotype call rates in otherwise acceptable loci, and poor-quality loci increase missing data in otherwise acceptable individuals. Applying an iterative filtering strategy consistently results in more loci and individuals being retained overall, even in data sets consisting of individuals sequenced on a single sequencing lane for which the initial distributions of missing data per locus and individuals are more favourable (Figure 3). For example, after removing low-confidence loci from the flounder data set as described above and then setting a hard cut-off for a genotype call rate of >95% and allowed missing data per individual of <25% result in a data set consisting of 15,682 SNPs on 3,802 contigs over 170 individuals, while iterative filtering results in data sets consisting of 18,663–24,103 SNPs on 4,789–5,341 contigs over 164–167 individuals (Table 3).

4.2 | Confidence in SNP identification

The ability to filter loci depends on the pipeline used to reconstruct and genotype loci and the set of parameters reported. As previously mentioned, variant callers such as FreeBayes report Phred-like quality scores for variants (SNPs) indicating the confidence in the SNP call being correct. In the same way, users can set a minimum genotype depth below which genotypes are coded as missing to determine the minimum number of reads that need to be present at each locus to be confident that false homozygotes are excluded from the data (for further discussion, see Section 4.3).

Further, users often choose to set a minor allele count to remove potentially artifactual SNP calls. For example, a minor allele count of three requires an allele to be observed in at least two individuals (homozygote and heterozygote). It is common practice to assume that loci with a minor allele frequency < 5% are not informative at a population level and to remove them from data sets. It is unfortunate that this strategy will remove true rare alleles from data sets that could be informative in understanding fine-scale patterns of connectivity and local adaptation. Because minor and private alleles can be vital to accurately drawing inferences about past demographic events (e.g., genetic bottlenecks), elucidating fine-scale population structure, understanding patterns of local adaptation and analysing shifts in frequency spectra (Cubry, Vigouroux, & François, 2017; O'Connor et al., 2015; Slatkin, 1985), being able to distinguish between true minor alleles and genotyping error would allow for better analysis of data sets. Carefully applying the filters as discussed in this section can allow users to make this distinction, as illustrated by comparing the difference between data sets created using specific filters before and after applying a minor allele count threshold.

4.3 | Confidence in genotypes: allele dropout/coverage effects

While artifactual SNPs as described above will result in genotyping error (individuals called heterozygous for alleles that do not exist), genotyping error at real SNPs may also occur. Allele dropout and coverage effects can lead to unsampled alleles and individuals incorrectly genotyped as homozygotes. Whereas coverage effects can be technically mitigated by setting a target number of reads per individual, per locus based on the total number of reads expected on each sequencing lane and the number of fragments excepted, allele dropout is an unavoidable artefact of using restriction enzymes and size selection during library preparation. For targeted fragments to be amplified and sequenced, adapters must be correctly ligated to the "sticky" ends left by the enzymes, but polymorphisms may occur in the enzyme recognition site (cut-site polymorphisms) resulting in alleles that are not cut by the restriction enzymes. In the same way,

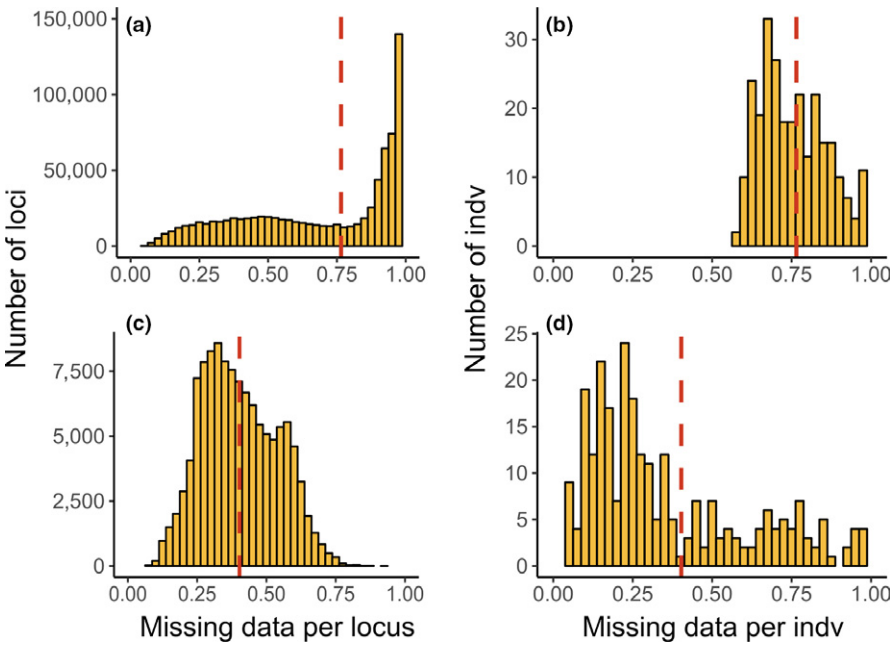

FIGURE 2 Missing data per locus and individual (indv), respectively, for unfiltered red snapper data set (a, b) and after coding genotypes with <5 reads as missing and removing low-quality loci with SNP quality score <20 and minimum mean depth <15 reads (c, d). Red dashed line indicates mean proportion of missing data

TABLE 3 Comparison of the number of SNPs, contigs (cont) and individuals (indv) in the raw data sets and number (proportion) retained in each data set for six different filtering schemes (FS) as described in Table 2

	Red drum			Red snapper			Southern flounder			Bonnethead sharks		
	SNPs	Cont	Indv	SNPs	Cont	Indv	SNPs	Cont	Indv	SNPs	Cont	Indv
Raw	430,466 (100%)	33,170 (100%)	623 (100%)	110,6387 (100%)	25,168 (100%)	282 (100%)	447,144 (100%)	39,950 (100%)	175 (100%)	590,322 (100%)	154,382 (100%)	134 (100%)
FS-1	120,803 (28%)	8,797 (26%)	598 (95%)	10 (0%)	3 (0%)	262 (93%)	81,193 (18%)	7,629 (18%)	172 (98%)	83,471 (14%)	29,295 (19%)	128 (96%)
FS-2	272,396 (63%)	19,863 (59%)	249 (40%)	0 (0%)	0 (0%)	0 (0%)	142,168 (31%)	10,837 (27%)	107 (61%)	309,276 (52%)	66,680 (43%)	29 (21%)
FS-3	15,893 (3%)	3,171 (9%)	602 (97%)	0 (0%)	0 (0%)	282 (0%)	15,682 (4%)	3,869 (10%)	170 (97%)	4,252 (1%)	2,140 (1%)	128 (96%)
FS-4	22,040 (5%)	4,244 (12%)	591 (95%)	11,800 (1%)	1,680 (7%)	189 (67%)	24,103 (5%)	5,341 (13%)	164 (94%)	15,668 (3%)	7,757 (5%)	119 (89%)
FS-5	12,541 (2%)	3,754 (11%)	588 (94%)	9,478 (1%)	1,626 (6%)	188 (67%)	18,663 (4%)	4,789 (12%)	167 (95%)	13,491 (2%)	7,526 (5%)	119 (89%)
FS-6	15,407 (4%)	3,882 (12%)	587 (94%)	12,056 (1%)	1,662 (7%)	187 (66%)	23,451 (5%)	5,251 (13%)	167 (95%)	21,433 (3%)	11,033 (7%)	120 (90%)

length polymorphisms (insertion–deletions, "indels") may result in allele dropout when alleles fall outside the selected size window. In either case, the result is allele-specific sequencing failure.

Allele dropout cannot be avoided by optimizing standard laboratory procedures, but can be accounted for during filtering by removing genotypes below a certain threshold of minimum reads and by identifying loci with high variance in read depth among individuals (Cooke et al., 2016; Davey et al., 2013). Low coverage can result in false homozygotes because the number of reads may not be high enough to successfully call both alleles. Loci can be filtered based on a threshold of minimum mean depth per locus and users can code individuals' genotypes at specific loci as missing if they fall below a minimum depth threshold that reflects the number of reads required to confidently call homozygotes. This increases the confidence in individual genotypes and results in the removal of loci that consistently have genotypes not called with high confidence across individuals. It is unfortunate that, during filtering, it is difficult to distinguish between allele dropout and coverage effects because they create similar patterns of missing data, variance in depth and excess homozygosity. In both cases, failure to remove potentially affected loci causes the introduction of false homozygotes and may result in biased estimates of population genetic parameters based on allele frequencies and heterozygosity (DaCosta & Sorenson, 2014; Gautier et al., 2012), although the magnitude of this bias will vary depending on the magnitude of the true biological signal in the data.

Hence, it is important to consider the statistical model being used for variant calling and how the model relates to read depth. For example, FreeBayes and GATK (Depristo et al., 2011; Garrison & Marth, 2012) are Bayesian callers that integrate data across all samples when determining genotypes, meaning lower read depth genotypes can be called with greater accuracy. This is in contrast to genotyping models implemented in STACKS or PyRAD (Catchen, Amores, Hohenlohe, Cresko, & Postlethwait, 2011; Eaton, 2014), which genotype individuals one at a time without the ability to integrate data across samples until genotyping is completed. Finally, when deviations from Hardy–Weinberg proportions are not expected, chi-square tests of Hardy–Weinberg expectations for individual loci within demes can also indicate heterozygote deficits that may indicate allele dropout.

4.4 | Identification of multilocus contigs

Multilocus contigs can be identified by assessing distributions of read depth, excess heterozygosity and the number of haplotypes observed per each individual at each marker (Ilut et al., 2014; Li & Wren, 2014; Willis et al., 2017). In general, total or mean read depth per locus should be approximately normally distributed. Loci with coverage falling well above this distribution may be reads clustered or mapped from multiple loci. Loci with excess coverage are best identified by generating a frequency distribution of coverage and choosing thresholds, for example two times the mode (Willis et al., 2017) or the 90th quantile (https://github.com/jpuritz/dDocent/blob/master/scripts/dDocent_filters; Figure 4). Appropriate

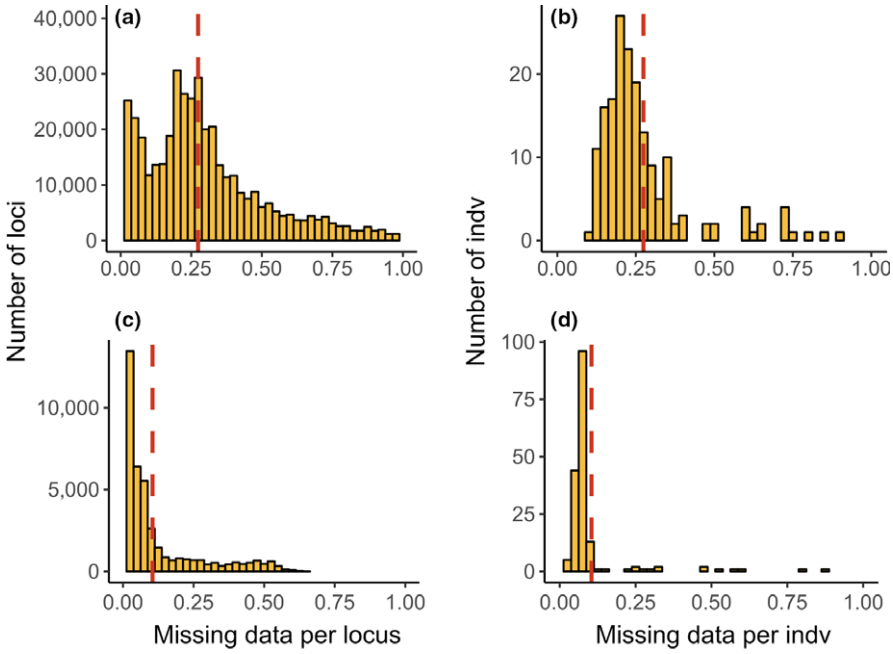

FIGURE 3 Missing data per locus and individual, respectively, for unfiltered southern flounder data set (a, b) and after coding genotypes with <5 reads as missing and removing low-quality loci with SNP quality score <20 and minimum mean depth <15 reads (c, d). Red dashed line indicates mean proportion of missing data

thresholds will vary between data sets and species. Because fixed or near-fixed differences may exist between nonorthologous loci, multilocus contigs often have an excess number of heterozygotes (Hohenlohe, Amish, Catchen, Allendorf, & Luikart, 2011; Willis et al., 2017). *VCFtools* (Danecek et al., 2011) provide a statistical framework for assessing heterozygote excess via a chi-square test of Hardy–Weinberg expectations for VCF files. Finally, reads in multilocus contigs often exhibit more than two haplotypes per individual, and therefore, loci can be removed based on a threshold for the number of individuals with excess haplotypes (Ilut et al., 2014; Willis et al., 2017). While each of these filters applied alone may catch many or even the majority of multilocus contigs, the most effective strategy to remove multilocus contigs appears to be applying each filter in parallel and removing markers flagged by any of the three filters (Willis et al., 2017).

4.5 | INFO-flag filtering of vcf files

FreeBayes and other multisample variant callers create annotated output files (VCF files) containing additional data pertaining to individual SNPs, coded as "INFO" flags. Using utilities such as *VCFtools* (Danecek et al., 2011), the suite of tools from *vcflib* (https://github.com/vcflib/vcflib), and simple PERL and BASH scripting, it is possible to create custom filters based on these flags. Li and Wren (2014) investigated false heterozygote calls on a SNP data set generated from a haploid genome and estimated that the raw data set contained one erroneous call in 10–15 kb. After implementing a set of filters based on the INFO flags, the genotyping error rate was reduced to one in 10–200 kb. The INFO flag filters include allele balance, mapping quality ratio, reads mapped as proper pairs, strand bias and the relationship of read depth to quality score.

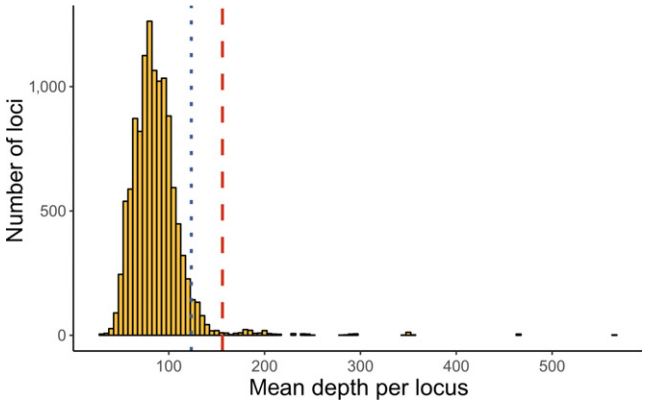

FIGURE 4 Distribution of mean depth per locus across all loci for red snapper data set after removing low-confidence/low-quality loci (minimum genotype depth >3, SNP quality score >20, minor allele count >3, mean minimum depth across all individuals >15) and iterative filtering of missing data to final threshold of genotype call rate >95% and allowed missing data per individual <25%. Blue dotted line indicates 95% percentile (123.5) and red dashed line 2× the mode (156) as potential cut-offs to remove loci with excessively high depth indicative of multilocus contigs following Willis et al. (2017)

Allele balance (AB) compares the number of reads for the reference allele to the number of reads for the alternate allele across heterozygotes. The expected allele balance is 0.5; large deviations may indicate false heterozygotes due to coverage effects, multilocus contigs or other artefacts. Figure 5 shows AB for a raw data set and for data sets that have been filtered for low-quality genotypes, loci and individuals. In both unfiltered and filtered data sets, loci with high/low AB are present, indicating that problematic loci will remain unless AB is explicitly filtered for.

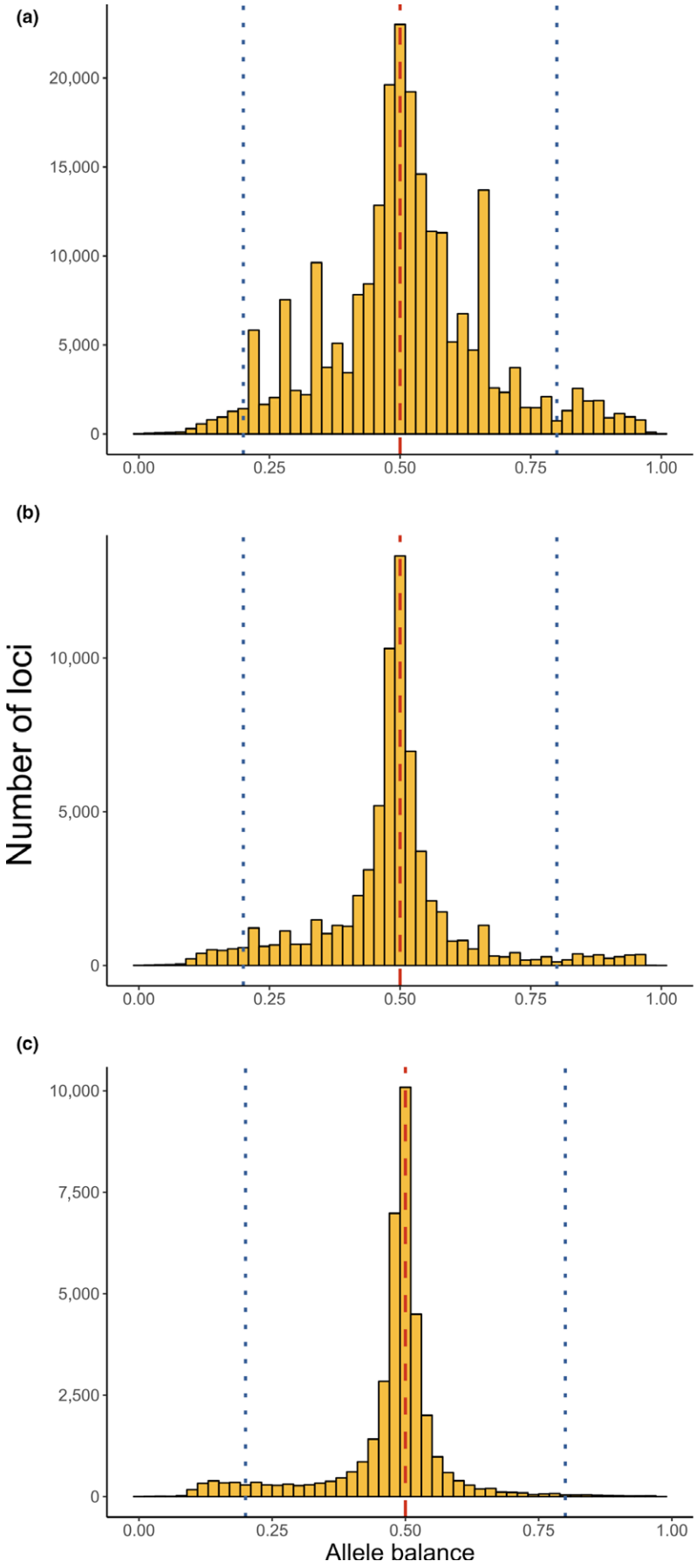

FIGURE 5 Allele balance in heterozygous genotypes (proportion of reads corresponding to the reference allele) for (a) unfiltered red drum data set, (b) data set with genotype read depths <3 reads coded as missing and loci with SNP quality score <20, mean depth <15 reads and/or >30% missing data removed, and (c) data set filtered as (b) and loci with a minor allele count <3 removed in addition. Except for minor sampling error, reference and alternate allele should be supported by the same number of reads, that is allele balance should be 0.5 (red dashed line); values away from this indicate potential anomalies. The blue dotted lines indicate default cut-off values of 0.2 and 0.8 implemented in dDocent_filters (https://github.com/jpuritz/dDocent/blob/master/scripts/dDocent_filters)

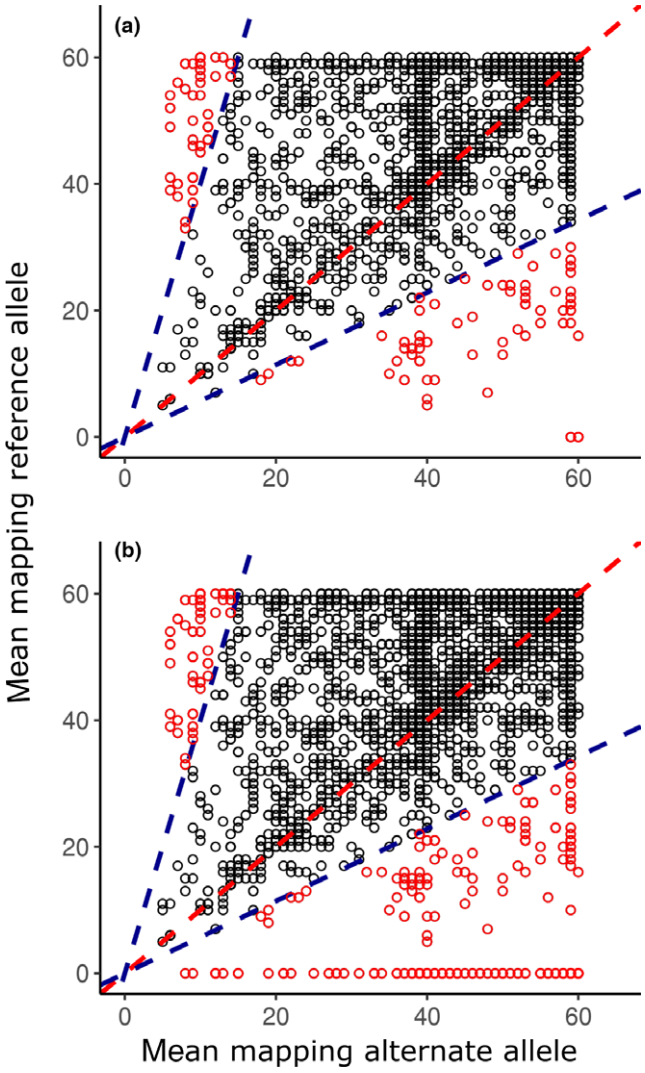

FIGURE 6 Ratio of mean mapping quality scores for the reference and alternate allele for southern flounder data set. (a) Genotypes with <5 reads have been coded as missing and loci with SNP quality score <20, mean read depth <15 reads, >30% missing data and/or and minor allele count of <3 removed; (b) same data set without applying minor allele count filter. Red dashed line indicates loci with mapping quality ratio of 1, that is the further away the larger the discrepancy between the mapping quality of the reference and alternate allele. Blue dashed lines indicate cut-off values for the ratio of mean mapping quality score of 0.25 and 1.75 (alternate to reference allele) as implemented in dDocent_filters (https://github.com/jpuritz/dDocent/blob/master/scripts/dDocent_filters) to remove loci with high discrepancy of mapping quality for the alleles of a given locus (indicated in red below the dashed line)

Reads supporting either allele in a heterozygote should have similar mapping quality values; the ratio of mapping quality between alleles therefore should be approximately one. The mapping quality of a read is the probability of a given read mapping similarly well to another location in the reference; reads stemming from paralogous or multicopy loci should therefore have reduced mapping quality, as they will map similarly well to multiple locations in the reference. Hence, systematically large discrepancies between the mapping quality for reads supporting the reference and alternate alleles at a SNP may be indicative of read mapping errors, due to repetitive elements, paralogs or multilocus contigs. Users should remove loci where reads supporting the alternative allele have a substantially lower mapping quality compared to reads supporting the reference allele. For example, *dDocent_filters* (https://github.com/jpuritz/dDocent/blob/master/scripts/dDocent_filters), a companion script to the dDocent pipeline, suggests a lower threshold of 0.25 (Figure 6). In the same way, reads supporting the reference allele are expected to have high mapping quality scores, thus limiting how much higher the mapping quality of reads supporting the alternative allele can become. Therefore, high ratios only occur when mapping quality of reads supporting the reference allele are low, resulting in a need for an upper threshold value (default 1.75 for *dDocent_filters;* Figure 6). Users are encouraged to assess their data sets to identify appropriate cut-offs. Standard filtering steps do not remove all loci with biased mapping quality ratios (Figure 6). As mentioned in Section 4.2, assessing mapping quality ratios has the added benefit that it can help to identify minor alleles that are not true alleles (Figure 6b), allowing researchers to retain true minor alleles that may contain an important biological signal.

For paired-end libraries, artefacts can also be identified by examining the properly paired status of reads and potential strand bias. The forward and reverse reads of a known pair should always map to the same contig; improper read pairing, in which forward and reverse reads of a known pair map to different contigs, indicates mapping anomalies such as multicopy or improperly assembled loci. Strand bias describes the relationship between forward and reverse reads and SNP calls at a given locus. For most paired-end RADseq libraries, the forward and reverse reads do not overlap because the actual RAD fragments will be too long. For example, a 350-bp RAD fragment characterized with 125 bp pair-end reads will have 100 bp of uncharacterized, intervening sequence. Therefore, a given SNP should only be apparent on either the forward or reverse read. Calls of the same SNP in both forward and reverse reads often indicate mapping anomalies. However, the implications of this criterion depend on read length and fragment length and therefore the expected overlap of paired reads in a given data set.

At last, the relationship between SNP quality score and read depth should be assessed; these measures should be positively correlated, because, theoretically, increasing read depth should decrease the likelihood of false homozygous calls (Li & Wren, 2014). Users may choose to apply a general threshold value for the ratio of locus quality to read depth and/or apply a separate SNP quality score threshold value for loci with high read depth. For example, *dDocent_filters* (https://github.com/jpuritz/dDocent/blob/master/scripts/dDocent_filters), a companion script to the dDocent pipeline, implements this by considering SNPs with a depth > mean + 1 standard deviation as high coverage and then removing high coverage SNPs for which the quality score is less than two times the read depth (Figure 7, Li & Wren, 2014).

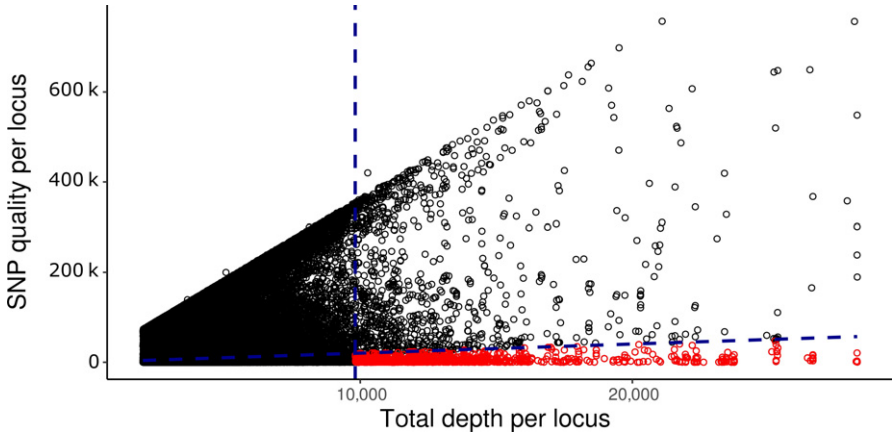

FIGURE 7 Comparison of SNP quality score and total depth per locus for the bonnethead shark data set. Vertical blue dashed line identifies loci with high depth (mean + 1 standard deviation). Loci with a quality score <2× the depth at that locus are below the diagonal blue dashed line (indicated in red)

5 | PHYSICAL LINKAGE

After filtering, most RADseq data sets will generally contain sets of SNPs located on the same contig. SNPs located within a few hundred base pairs of each other are generally physically linked (Hohenlohe, Bassham, Currey, & Cresko, 2012; Miyashita & Langley, 1988), whereas most commonly used analyses assume that all genetic markers are independent. Due to the fact that RAD methods randomly sample the genome; it is possible that selected fragments are linked as well and users should, where appropriate, test for linkage disequilibrium between loci to avoid biasing results. Treating physically linked SNPs as independent markers provides biased results, including false signals of population structure. A common method to remove this bias is to retain only one SNP from each contig ("thinning"). This is an appropriate strategy but one that reduces the information content of a given marker if multiple SNPs are contained on a single contig. Another way to deal with physical linkage is to infer haplotypes for each contig based on the combination of filtered SNPs within paired reads (Willis et al., 2017). This strategy will produce the same number of markers as thinning, but many markers will be multiallelic; therefore, haplotyping manages physical linkage while preserving the total information content of the data set.

6 | CONCLUSIONS & OUTLOOK (ON THE IMPORTANCE OF REPRODUCIBLE RESEARCH)

With the shift from data sets consisting of markers for tens to hundreds of microsatellite loci to several thousand SNP-containing loci, bioinformatic processing has become the only viable means of ensuring data quality. If careful quality control is implemented, RAD methods are a powerful instrument in the molecular ecologist's toolbox to assess levels of population structure, connectivity and local adaptation in nonmodel species for which genomic resources might not yet be available. Many studies currently report very few details pertaining to quality control methods applied to the output from SNP calling pipelines beyond very basic filtering, frequently limited to the removal of markers and/or individuals with low coverage or high levels of missing data. Enabling this under-reporting is a lack of clear quality control standards. Nevertheless, it is incumbent upon the authors to document data preparation and quality control steps and make these available to the scientific community along with raw data sets to ensure that data analyses are transparent and fully reproducible (Leek & Peng, 2015; Peng, 2014).

Here, we have provided a discussion of several of the places that errors and artefacts may be introduced into RADseq datasets and provided recommendations for how to minimize, detect and account for these artefacts from the laboratory through bioinformatic and filtering stages. We hope that these recommendations facilitate discussion on standardization of quality control in RAD-based population genomic data sets. While a detailed description of each filtering step would exhaust available space for Methods section of a manuscript, researchers should include detailed procedures in the supplementary material and deposit custom scripts in public data or code repositories (e.g., O'Leary et al., 2018; Portnoy et al., 2015; Puritz et al., 2016). Further, platforms such as GitHub (http://github.com) allow for convenient archiving as well as assigning DOIs (digital object identifiers) to make code citable. A description of processing should accompany data sets archived in readily interpretable formats, along with the associated metadata, and consist of the tools (name and version) and exact parameters used for processing. In addition to making data analysis fully transparent and reproducible, this will allow developed approaches to be applied to other data sets and facilitate the development of new and better approaches in the application of genomics to molecular ecology.

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DATA ACCESSIBILITY

Annotated scripts for filtering are available at https://github.com/sjoleary/SNPFILT along with information to obtain versions of published data sets used to illustrate filtering principle set forth in this manuscript.

REFERENCES

Andrews, K. R., Hohenlohe, P. A., Miller, M. R., Hand, B. K., Seeb, J. E., & Luikart, G. (2014). Trade-offs and utility of alternative RADseq methods: Reply to Puritz et al. *Molecular Ecology*, *23*, 5943–5946. https://doi.org/10.1111/mec.12964

Arnold, B., Corbett-Detig, R. B., Hartl, D., & Bomblies, K. (2013). RADseq underestimates diversity and introduces genealogical biases due to nonrandom haplotype sampling. *Molecular Ecology*, *22*(11), 3179–3190. https://doi.org/10.1111/mec.12276

Baird, N. A., Etter, P. D., Atwood, T. S., Currey, M. C., Shiver, A. L., Lewis, Z. A., … Johnson, E. A. (2008). Rapid SNP discovery and genetic mapping using sequenced RAD markers. *PLoS ONE*, *3*(10), 1–7. https://doi.org/10.1371/journal.pone.0003376

Bonin, A., Bellemain, E., Eidesen, P. B., Pompanon, F., Brochmann, C., & Taberlet, P. (2004). How to track and assess genotyping errors in population genetics studies. *Molecular Ecology*, *13*(11), 3261–3273. https://doi.org/10.1111/j.1365-294X.2004.02346.x

Catchen, J. M., Amores, A., Hohenlohe, P., Cresko, W., & Postlethwait, J. H. (2011). Stacks: Building and genotyping loci de novo from short-read sequences. *G3: Genes|Genomes|Genetics*, *1*(3), 171–182. https://doi.org/10.1534/g3.111.000240

Catchen, J., Hohenlohe, P. A., Bassham, S., Amores, A., & Cresko, W. A. (2013). Stacks: An analysis tool set for population genomics. *Molecular Ecology*, *22*(11), 3124–3140. https://doi.org/10.1111/mec.12354

Cooke, T. F., Yee, M. C., Muzzio, M., Sockell, A., Bell, R., Cornejo, O. E., … Kenny, E. E. (2016). GBStools: A statistical method for estimating allelic dropout in reduced representation sequencing data. *PLoS Genetics*, *12*(2), e1005631. https://doi.org/10.1371/journal.pgen.1005631

Cubry, P., Vigouroux, Y., & François, O. (2017). The empirical distribution of singletons for geographic samples of DNA sequences. *Frontiers in Genetics*, *8*, 139. https://doi.org/10.3389/fgene.2017.00139

DaCosta, J. M., & Sorenson, M. D. (2014). Amplification biases and consistent recovery of loci in a double-digest RAD-seq protocol. *PLoS ONE*, *9*(9), e106713. https://doi.org/10.1371/journal.pone.0106713

Danecek, P., Auton, A., Abecasis, G., Albers, C. A., Banks, E., DePristo, M. A., … Durbin, R. (2011). The variant call format and VCFtools. *Bioinformatics*, *27*(15), 2156–2158. https://doi.org/10.1093/bioinformatics/btr330

Davey, J. L., & Blaxter, M. W. (2010). RADseq: Next-generation population genetics. *Briefings in Functional Genomics*, *9*(5–6), 416–423. https://doi.org/10.1093/bfgp/elq031

Davey, J. W., Cezard, T., Fuentes-Utrilla, P., Eland, C., Gharbi, K., & Blaxter, M. L. (2013). Special features of RAD sequencing data: Implications for genotyping. *Molecular Ecology*, *22*(11), 3151–3164. https://doi.org/10.1111/mec.12084

Depristo, M. A., Banks, E., Poplin, R., Garimella, K. V., Maguire, J. R., Hartl, C., … Daly, M. J. (2011). A framework for variation discovery and genotyping using next-generation DNA sequencing data. *Nature Genetics*, *43*(5), 491–501. https://doi.org/10.1038/ng.806

Eaton, D. A. R. (2014). PyRAD: Assembly of de novo RADseq loci for phylogenetic analyses. *Bioinformatics*, *30*(13), 1844–1849. https://doi.org/10.1093/bioinformatics/btu121

Elshire, R. J., Glaubitz, J. C., Sun, Q., Poland, J. A., Kawamoto, K., Buckler, E. S., & Mitchell, S. E. (2011). A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. *PLoS ONE*, *6*(5), e19379. https://doi.org/10.1371/journal.pone.0019379

Garrison, E., & Marth, G. (2012). Haplotype-based variant detection from short-read sequencing. *PLoS ONE*, *11*(3), e0151651. https://doi.org/arXiv:1207.3907 [q-bio.GN]

Gautier, M., Gharbi, K., Cezard, T., Foucaud, J., Kerdelhué, C., Pudlo, P., … Estoup, A. (2012). The effect of RAD allele dropout on the estimation of genetic variation within and between populations. *Molecular Ecology*, *22*(11), 3165–3178. https://doi.org/10.1111/mec.12089

Graham, C. F., Glenn, T. C., Mcarthur, A. G., Boreham, D. R., Kieran, T., Lance, S., … Somers, C. M. (2015). Impacts of degraded DNA on restriction enzyme associated DNA sequencing (RADSeq). *Molecular Ecology Resources*, *15*(6), 1304–1315. https://doi.org/10.1111/1755-0998.12404

Hendricks, S., Anderson, E. C., Antao, T., Bernatchez, L., Forester, B. R., Garner, B., … Luikart, G. (2018). Recent advances in conservation and population genomics data analysis. *Evolutionary Applications*. https://doi.org/10.1111/eva.12659

Hohenlohe, P. A., Amish, S. J., Catchen, J. M., Allendorf, F. W., & Luikart, G. (2011). Next-generation RAD sequencing identifies thousands of SNPs for assessing hybridization between rainbow and westslope cutthroat trout. *Molecular Ecology Resources*, *11*(SUPPL. 1), 117–122. https://doi.org/10.1111/j.1755-0998.2010.02967.x

Hohenlohe, P. A., Bassham, S., Currey, M., & Cresko, W. A. (2012). Extensive linkage disequilibrium and parallel adaptive divergence across threespine stickleback genomes. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *367*(1587), 395–408. https://doi.org/10.1098/rstb.2011.0245

Hollenbeck, C. (2016). Genomic studies of red drum. Doctoral dissertation, Texas A&M.

Ilut, D. C., Nydam, M. L., & Hare, M. P. (2014). Defining loci in restriction-based reduced representation genomic data from nonmodel species: Sources of bias and diagnostics for optimal clustering. *BioMed Research International*, *2014*, 675158. https://doi.org/10.1155/2014/675158

Leek, J. T., & Peng, R. D. (2015). Opinion: Reproducible research can still be wrong: Adopting a prevention approach. *Proceedings of the National Academy of Sciences*, *112*(6), 1645–1646. https://doi.org/10.1073/pnas.1421412111

Li, H., & Wren, J. (2014). Toward better understanding of artifacts in variant calling from high-coverage samples. *Bioinformatics*, *30*, 2843–2851https://doi.org/10.1093/bioinformatics/btu356

Mastretta-Yanes, A., Arrigo, N., Alvarez, N., Jorgensen, T. H., Piñero, D., & Emerson, B. C. (2015). Restriction site-associated DNA sequencing, genotyping error estimation and de novo assembly optimization for population genetic inference. *Molecular Ecology Resources*, *15*(1), 28–41. https://doi.org/10.1111/1755-0998.12291

Meirmans, P. G. (2015). Seven common mistakes in population genetics and how to avoid them. *Molecular Ecology*, *24*(July), 3223–3231.

Miller, M. R., Dunham, J. P., Amores, A., Cresko, W. A., & Johnson, E. A. (2007). Rapid and cost-effective polymorphism identification and genotyping using restriction site associated DNA (RAD) markers. *Genome Research*, *17*(2), 240–248. https://doi.org/10.1101/gr.5681207

Miyashita, N., & Langley, C. H. (1988). Molecular and phenotypic variation of the white locus region in Drosophila melanogaster. *Genetics*, *120*(1), 199–212.

O'Connor, T. D., Fu, W., Mychaleckyj, J. C., Logsdon, B., Auer, P., Carlson, C. S., … Akey, J. M. (2015). Rare variation facilitates inferences of fine-scale population structure in humans. *Molecular Biology and Evolution*, *32*(3), 653–660. https://doi.org/10.1093/molbev/msu326

O'Leary, S. J., Hollenbeck, C. M., Vega, R. R., Gold, J. R., & Portnoy, D. S. (2018). Comparative genomics as a tool for restoration enhancement and culture of southern flounder, *Paralichthys lethostigma*. *BMC Genomics*, *19*(1), 163.

Peng, R. D. (2014). Reproducible research in computational science. *Science*, *334*, 1226–1227. https://doi.org/10.1126/science.1213847

Peterson, B. K., Weber, J. N., Kay, E. H., Fisher, H. S., & Hoekstra, H. E. (2012). Double digest RADseq: An inexpensive method for de novo SNP discovery and genotyping in model and non-model species. *PLoS ONE*, *7*(5), e37135. https://doi.org/10.1371/journal.pone.0037135

Portnoy, D. S., Puritz, J. B., Hollenbeck, C. M., Gelsleichter, J., Chapman, D., & Gold, J. R. (2015). Selection and sex-biased dispersal: The influence of philopatry on adaptive variation. *PeerJ*, *1–20*, https://doi.org/10.7287/peerj.preprints.1300v1

Puritz, J. B., Gold, J. R., & Portnoy, D. S. (2016). Fine-scale partitioning of genomic variation among recruits in an exploited fishery: Causes and consequences. *Scientific Reports*, *6*(1), 36095. https://doi.org/10.1038/srep36095

Puritz, J. B., Hollenbeck, C. M., & Gold, J. R. (2014). dDocent : A RADseq, variant-calling pipeline designed for population genomics of non-model organisms. *PeerJ*, *2*, e431. https://doi.org/10.7717/peerj.431

Puritz, J. B., Matz, M. V., Toonen, R. J., Weber, J. N., Bolnick, D. I., & Bird, C. E. (2014). Demystifying the RAD fad. *Molecular Ecology*, *23*, 5937–5942. https://doi.org/10.1111/mec.12965

Puritz, J. B., Hollenbeck, C. M., & Gold, J. R. (2015). Fishing for selection, but only catching bias: examining library effects in double-digest RAD data in a non-model marine species. In Plant and Animal Genome XXIII Conference, https://doi.org/10.6084/m9.figshare.1287474.v3

Schmid, S., Genevest, R., Gobet, E., Suchan, T., Sperisen, C., Tinner, W., & Alvarez, N. (2017). HyRAD-X, a versatile method combining exome capture and RAD sequencing to extract genomic information from ancient DNA. *Methods in Ecology and Evolution*, *8*, 1374–1388https://doi.org/10.1111/2041-210X.12785

Schweyen, H., Rozenberg, A., & Leese, F. (2014). Detection and removal of PCR duplicates in population genomic ddRAD studies by addition of a degenerate base region (DBR) in sequencing adapters. *Biological Bulletin*, *227*(2), 146–160. https://doi.org/10.1086/BBLv227n2p146

Slatkin, M. (1985). Rare alleles as indicators of gene flow. *Evolution*, *39*(1), 53–65. https://doi.org/10.2307/2408516

Sovic, M. G., Fries, A. C., & Gibbs, H. L. (2015). AftrRAD: A pipeline for accurate and efficient de novo assembly of RADseq data. *Molecular Ecology Resources*, *15*(5), 1163–1171. https://doi.org/10.1111/1755-0998.12378

Suchan, T., Pitteloud, C., Gerasimova, N. S., Kostikova, A., Schmid, S., Arrigo, N., … Alvarez, N. (2016). Hybridization capture using RAD probes (hyRAD), a new tool for performing genomic analyses on collection specimens. *PLoS ONE*, *11*(3), e0151651. https://doi.org/10.1371/journal.pone.0151651

Tin, M. M. Y., Rheindt, F. E., Cros, E., & Mikheyev, A. S. (2015). Degenerate adaptor sequences for detecting PCR duplicates in reduced representation sequencing data improve genotype calling accuracy. *Molecular Ecology Resources*, *15*(2), 329–336. https://doi.org/10.1111/1755-0998.12314

Toonen, R. J., Puritz, J. B., Forsman, Z. H., Whitney, J. L., Fernandez-Silva, I., Andrews, K. R., & Bird, C. E. (2013). ezRAD: A simplified method for genomic genotyping in non-model organisms. *PeerJ*, *1*, e203. https://doi.org/10.7717/peerj.203

Wang, S., Meyer, E., McKay, J. K., & Matz, M. V. (2012). 2b-RAD: A simple and flexible method for genome-wide genotyping. *Nature Methods*, *9*(8), 808–810. https://doi.org/10.1038/nmeth.2023

Willis, S. C., Hollenbeck, C. M., Puritz, J. B., Gold, J. R., & Portnoy, D. S. (2017). Haplotyping RAD loci: An efficient method to filter paralogs and account for physical linkage. *Molecular Ecology Resources*, *17*, 955–965https://doi.org/10.1111/1755-0998.12647

Xue, Y., Zhang, X., Huang, N., Daly, A., Gillson, C. J., Macarthur, D. G., … Tyler-Smith, C. (2009). Population differentiation as an indicator of recent positive selection in humans: An empirical evaluation. *Genetics*, *183*(3), 1065–1077. https://doi.org/10.1534/genetics.109.107722

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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