Fantastic beasts and how to sequence them:

genomic approaches for obscure model organisms

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Summary:
Application of genomic approaches to "obscure model organisms" (OMOs), meaning species with little or no genomic resources, enables increasingly sophisticated studies of genomic basis of evolution, acclimatization and adaptation in real ecological contexts. Here, I highlight sequencing solutions and data handling techniques most suited for genomic analysis of OMOs.

Glossary:

- **Allele Frequency Spectrum, AFS** (same as Site Frequency Spectrum, SFS): histogram of the number of segregating variants depending on their frequency in one or more populations.
- **Restriction site-Associated DNA (RAD) sequencing**: family of diverse genotyping methods that sequence short fragments of the genome adjacent to recognition site(s) for specific restriction endonuclease(s).
- **Linkage Disequilibrium (LD)**: in this review, correlation of genotypes at a pair of markers across individuals.
- **LD block**: typical distance between markers in the genome across which their genotypes remain correlated.
- **Genome scan:** profiling of genotypes along the genome looking for unusual patterns. Often used to look for signatures of natural selection or introgression.
- **"Denser-than-LD" genotyping**: genotyping of several polymorphic markers per LD block.
- **Highly contiguous reference**: genome or transcriptome reference sequence containing the least amount of fragmentation.
- **Phased data**: data showing which SNP alleles belong to the same homologous chromosome copy.
- **Cross-tissue gene expression analysis**: looking for individual-specific shifts in gene expression detectable across multiple tissues. Such shifts are predominantly genetic in nature.

The focus of this review is mainly on the type of sequencing data required and how to obtain it in the most cost-efficient way rather than on analytical approaches. That said, I could not help but mention some highly promising analytical methods that are not yet broadly adopted by OMO researchers, such as demographic inference based on allele frequency spectra and annotation-independent analyses of gene expression data.

I will start with the summary of general types of questions in OMO studies and corresponding data types required. We might be interested in the following four layers of genomic information, each requiring a specific type of experimental and reference data:

1. Genome-wide patterns of neutral variation. This data can elucidate population structure, population sizes, and migration rates, as well as changes of these parameters through time. This analysis benefits from high quality genotype calls but does not require dense genome coverage; it can even be performed in the absence of reference genome.

2. Regions in the genome particularly affected by non-drift processes (natural selection, introgression, etc). This type of analysis, typically referred to as "genome scanning", takes genome-wide neutral variation as baseline and looks for regions in the genome exhibiting highly dissimilar patterns. It requires "denser-than-LD" genotyping and a highly contiguous reference (see Glossary) to make sure no signal is overlooked.

3. Genome-wide gene expression, an extremely information-rich resource reflecting both environmental and genetic variation. Streamlined transcript counting methods represent a cost-efficient alternative to the industry-standard RNA-seq for generating quantitative data. Analysis of gene expression does not require a genome reference, although a transcriptome reference must be generated at some point. The reference does not have to be highly contiguous.

4. Epigenetics, here limited to DNA methylation. A variety of methods have been recently developed that can generate data for DNA methylation analysis. For vertebrates, genome reference is needed, but for other animals or plants, in which DNA methylation is much less prevalent and predominantly occurs in exons, transcriptome or exome presents a good cost-efficient alternative. The reference does not have to be highly contiguous.

Genome-wide neutral variation

Allele Frequency Spectrum analysis

Neutral genetic markers are traditionally analyzed using summaries of allele frequency differences between populations, such as F_{ST}. The large amount of markers accessible through next-generation sequencing opened up the possibility to dramatically enhance this approach by modeling the evolution of the whole allele frequency spectrum (AFS, see Glossary). AFS represents a rich source of information to fit alternative models with time-resolved population sizes and migration rates as parameters (Box 1) based on coalescent simulations (*fastsimcoal2*, [1]), diffusion approximation (*dadi*, [2]), or ordinary differential equations (*moments*, [3]). Model selection is then based on likelihood ratio tests or Akaike information criterion. The new *moments* method is particularly promising, as it is substantially faster than its predecessors and includes built-in bootstrap, demographic model plotting, and capacity to analyze up to five populations simultaneously. It is also very helpful that *moments* inherits the python code structure well familiar to *dadi* practitioners.

Experimental data

The data required for AFS analysis is several thousand biallelic neutral single nucleotide polymorphisms (SNPs). Ideally, SNPs must not be closely physically linked in the genome to represent independent data points, although it is fully appropriate to analyze linked SNPs with AFS methods. The lack of requirement for contiguous SNP coverage makes various flavors of restriction site-associated DNA (RAD) sequencing (recently reviewed in [4,5], see Glossary) well suited for this analysis. In our experience, *dadi* [2] and *moments* [3] work robustly with 5-10 thousand SNPs (a typical RAD output) when analyzing populations individually or in pairs. Fitting models with three (*dadi*) or more (*moments, fastsimcoal2*) populations might be problematic with this relatively low number of SNPs but is usually not required for OMOs (Box 1). Recent population size changes are often of special interest in OMOs; since they predominantly affect rare alleles, their robust detection requires 20 or more high-quality genotypes per population [6]. This preference for more individuals rather than more SNPs per individual is an additional factor that makes cost-efficient RAD the approach of choice for AFS-based analysis. That said, relatively low number of independent (unlinked) SNPs generated by some RAD protocols might limit the power of the AFS analysis, and a good subject for a future study would be the effect of the number of unlinked SNPs on AFS model selection and uncertainties of parameter estimates. In this regard it is worth noting that RAD flavors differ considerably in the number of unlinked loci in the genome that they interrogate [4,5].

For demographic inference, the AFS data must be filtered to exclude potential sites under selection. Whichever test is used to identify such sites (for example, Bayescan, [7]), for their removal the false discovery rate should be set as high as 0.5 to ensure purging of the majority of non-neutral sites. Although under this setting half of the removed sites would be neutral, their removal will not affect the overall AFS as long as the removed fraction does not comprise more than 1-2% of the total number of sites.

Genotyping quality

In diploids, the most common genotyping error is missing one of the alleles in a heterozygote (i.e., a false homozygote call); and the next most common error is missing the whole SNP locus entirely. Both these "missing data" errors are due to insufficient sequencing coverage, the problem that is pervasive in today's OMO studies. Such errors strongly affect AFS in the region of rare alleles, which is unfortunate since rare alleles are the most informative about recent population history [6,8]. A telltale sign of poor heterozygote calling is under-representation of singletons, but frequencies of doubletons and higher-order frequency bins are also distorted, which has strong effect on AFS itself and inferred demographic parameters until mean sequencing coverage approaches ~10x [9]. When coverage is 10x or higher a good way to filter data is to select SNPs genotyped in >90-95% of samples [10]; importantly for RAD approach, this would select SNPs that are unlikely to be affected by null alleles due to mutation in the restriction endonuclease recognition site [4]. For obvious reasons, for AFS analysis genotype calls should never be quality-filtered based on allele frequencies (for example, retaining only

132 variants that are detected in a minimum of two individuals or requiring minor allele frequency to
133 exceed some cutoff). A robust empirical way to evaluate the consistency of genotype calls is to
134 compare results for independently processed biological samples of the same genotype [11]. Such
135 genotyping replicates are quite feasible in RAD and are also useful to identify true SNPs for
136 training variant quality score recalibration model of the GATK pipeline [12]. For low-coverage
137 data (<10x), a general solution is provided by the *ANGSD* package [13], which generates AFS as
138 well as other population genetic statistics based on genotype likelihoods without actually calling
139 genotypes [14]. This method generates unbiased single-population AFS even with 2x coverage
140 [9]. Still, there is a concern that high variation in coverage across samples and populations might
141 affect *ANGSD* statistics; to avoid this potential issue it is recommended to discard the lowest-
142 coverage outliers and down-sample reads from highest-coverage outliers (J. Ross-Ibarra, pers.
143 comm.).
144
145 *PCR duplicates*
146
147 Presence of PCR duplicates in many early RAD applications has been repeatedly highlighted as a
148 source of genotyping errors [4,15] due to induced over-dispersion of read counts among alleles
149 and loci. Interestingly, the proportion of PCR duplicates does not depend on the number of PCR
150 cycles performed during library preparation. Instead, it depends on the ratio between the number
151 of reads sequenced (N_r) and the number of unique fragments present in the sample prior to PCR
152 (N_o): the fraction of duplicates is the same as expected when sampling N_r from N_o with
153 replacement. Fortunately, PCR duplicates are easy to identify and remove using degenerate tags
154 ligated to RAD fragments prior to amplification [16]. Most present-day RAD protocols now
155 implement this simple deduplication procedure, including the current version of 2bRAD [11].
156
157 *Genome reference for AFS analysis*
158
159 A great advantage of RAD-based AFS analysis for OMOs is that SNPs can be called based on
160 RAD reads themselves, without the need for genome reference. Several *de novo* RAD genotyping
161 pipelines have been developed, such as STACKS, pyRAD, and UNEAK (see references in [4])
162 that work for most RAD flavors, plus a similarly structured pipeline for 2bRAD
163 (https://github.com/z0on/2bRAD_denovo) that takes into account the fact that in 2bRAD either
164 strand of the locus can be sequenced. Still, using a reference genome to call RAD genotypes
165 provides three important advantages. First, it identifies physically linked (and thus potentially
166 non-independent) groups of SNPs, to be resampled as units during AFS bootstrap. The second
167 advantage is particularly important for OMOs sampled in the field: mapping to reference genome
168 automatically discards reads from contaminant DNA sources (viruses, bacteria, ingested food,
169 symbionts etc). To be able to discard such contaminants in *de novo* RAD pipeline the experiment
170 must include at least one sample generated from a clean source and consider only the RAD loci
171 observed in that sample.
172
173 The third advantage of reference-based genotyping is the possibility to discriminate between
174 ancestral and derived SNP alleles, to attain the best power of AFS-based inference. Counter-
175 intuitively, the best reference for AFS analysis is not a genome of the species under investigation

176 but a genome of a related outgroup species, separated from the focal one by a few million years
177 of evolution, because the SNP state as in the outgroup can be assumed to represent the ancestral
178 state (e.g., [17]). If the reads are mapped to the same-species genome, to identify ancestral states
179 of the variants a single well-sequenced RAD sample of an outgroup taxon could be included. The
180 analysis will then be limited to sites that can be successfully genotyped both in ingroup and
181 outgroup; in effect, the result is going to be the same as when mapping the reads from whole
182 project to an outgroup's genome. Although some proportion of ancestral states will be
183 misidentified due to incomplete lineage sorting, convergence or technical artifacts, this error is
184 easy to account for by including a single additional parameter into the model, specifying the
185 proportion of the AFS that needs to be flipped when predicting the data (e.g., [18]). The reference
186 for AFS does not have to be highly contiguous; the contigs should be just long enough to cover a
187 typical LD block for meaningful bootstrapping.
188
189 **Genome scanning**
190
191 Since outlier regions by definition occupy only a small portion of the genome and typically do not
192 form a single cluster, their confident detection requires "denser-than-LD" genotyping (see
193 Glossary). It has been argued that in most situations, RAD-like approaches would sample the
194 genome too sparsely to satisfy this requirement [19,20]. Although many successful genome scans
195 based on RAD have been published [21], RAD cannot be recommended for genome scanning
196 since it inevitably leaves considerable fraction of the genome unexplored. Even when LD is
197 known to be extensive enough for RAD to produce "denser-than-LD" genotyping, a better
198 solution might be to take full advantage of the extended LD and go instead for low coverage
199 whole-genome sequencing (WGS) followed by imputation, to obtain full-genome phased data
200 (Table 1).
201
202 The types of sequencing approaches for genome scanning with their pros and cons are
203 summarized in Table 1. Importantly, all of them require highly accurate reads mapped to a
204 reference for confident SNP detection, making short Illumina reads the genotyping data type of
205 choice. Some of the very promising approaches that have not yet been fully adopted for OMOs
206 are exome-seq and ultra-low whole genome sequencing (WGS) with imputation. Exome-seq used
207 to be a prerogative of model organisms because of the need for exome-capture platform
208 development, but it has recently been shown that OMO exome can be captured just as efficiently
209 using bead-bound normalized cDNA obtained from the OMO itself (EecSeq Puritz 2017). Such
210 "home-made exome" sequencing could become an excellent alternative to RAD since it would
211 interrogate essentially all the interpretable genetic variation for a comparably low cost. Ultra-low
212 WGS with imputation used to require extensive reference haplotype panels available only for
213 well-established model organisms. However, several methods have been recently developed
214 (most notably STITCH, [22]) that can impute phased genotypes and correct genotyping errors in
215 ultra-low coverage data without relying on reference panels. Still, their applicability for each new
216 OMO must be experimentally confirmed because the success of imputation critically depends on
217 multiple polymorphisms occurring within a typical LD block, and whether this is so is not known
218 for OMOs *a priori*. Demographic events such as strong recent bottleneck, domestication, or
219 recent colonization would make imputation more efficient because of more extensive LD and

220 small number of founding haplotypes [22], and conversely, in large outbred populations
221 imputation will be less accurate and might require sequencing of a very large number (thousands)
222 of individuals. The accuracy of imputation can be evaluated by sequencing a few individuals at
223 high coverage (>10x) to generate high-confidence genotype calls and then attempting to impute
224 them based on sub-sampled read sets to emulate low coverage. It must be noted that it is
225 inappropriate to measure imputation accuracy by imputing genotype calls masked in high-quality
226 datasets (as in, for example, [23]): masked data do not contain false homozygote calls and
227 therefore do not correctly represent the real-life situation.
228
229 **Gene expression**
230
231 There are many aspects to gene expression, of which I here focus on just one: abundance
232 or protein-coding (polyadenylated) transcripts. The reason is that transcript abundance is by far
233 the most interpretable and it can be very easily analyzed in OMOs.
234
235 *Counting transcripts instead of resequencing them*
236
237 Typical RNA-seq [24] resequences the whole transcriptome in each sample, but there is a
238 much more economic way to count abundances of protein-coding transcripts: sequence just a
239 single fragment per each transcript molecule and count reads corresponding to each gene. TagSeq
240 [25], for example, sequences a single randomly generated fragment near the 3'-end of the
241 transcript, which is the most economic use of sequencing effort and removes bias towards longer
242 transcripts. In a recent benchmarking study TagSeq was actually more accurate than the standard
243 RNA-seq in measuring transcript abundances, despite nearly tenfold lower cost [26]. More
244 recently introduced QuantSeq [27] is conceptually similar to TagsSeq: it also sequences a single
245 randomly generated fragment near the 3'-end of each transcript but has a different library
246 preparation procedure, implemented as a kit from Lexogen (https://www.lexogen.com/quantseq-
247 3mrna-sequencing/). Bioinformatics analysis for both TagSeq ad QuantSeq is highly simplified
248 compared to typical RNA-seq. TagSeq was originally designed for OMOs and so its pipeline uses
249 transcriptome rather than genome as a reference to attribute reads to genes
250 (https://github.com/z0on/tag-based_RNAseq). One notable feature of the current version of
251 TagSeq pipeline is that it includes removal of PCR duplicates based on adaptor-derived
252 degenerate tags [11], similarly to 2bRAD and for the same reason – to avoid PCR-associated
253 over-dispersion or read counts.
254
255 *Analysis of gene expression "beyond gene lists"*
256
257 The unfortunate tradition that OMO research inherits from the biomedical field is putting
258 too much emphasis on possible functional implications of expression changes of specific genes.
259 For OMOs, this is bound to remain inconclusive because gene annotations are often absent,
260 tentative or based predominantly on similarity to human genes, which may or may not serve the
261 same function in the OMO. Even greater problem is interpretation bias: too often researchers
262 focus primarily on genes that "make sense" and ignore the rest. This leads to conclusions

263 reflecting predominantly the researchers' idea of what *should* be going on rather than what is
264 actually happening.
265
266 Table 2 lists alternative ways of objective analysis of gene expression data that are
267 enabled by the large sample sizes feasible with TagSeq or QuantSeq. They either do not require
268 gene annotations or rely sufficiently general functional summaries to be robust to occasional
269 missing or mis-annotations. Particularly useful for OMOs are analyses that use gene expression
270 patterns as anonymous multivariate readouts to compare and classify samples, such as principal
271 coordinate analysis (PCoA) or differential analysis of principal components (DAPC). Related
272 multivariate analyses to visualize and classify genome-wide gene expression data, recently
273 reviewed in [28], have become the mainstream tool of single-cell RNA-seq, where they are used
274 to discover cell types and quantify differences between them. With appropriate experimental
275 design, in OMOs these analyses can lead to much more definitive biological conclusions than
276 studies scrutinizing long lists of differentially expressed genes passing a certain significance
277 cutoff.
278
279 *Gene expression as functional summary of genotype*
280
281 Gene expression is best known for its context-dependence reflecting phenotypic plasticity, which
282 is the view inherited from biomedical research dealing with genetically uniform models. In
283 natural populations, one of the most important sources of gene expression variation is genetic
284 difference among individuals, manifested as context-*in*dependent, individual-specific deviations
285 in gene expression. This is easy to demonstrate in clonally replicated organisms such as corals. In
286 two reciprocal transplantation experiments performed on different coral species in different
287 oceans, stable between-genotype differences accounted for more than 50% of total gene
288 expression variation despite transplantation of clonal fragments for up to a year to highly
289 dissimilar sites [29,30]. In non-clonal model organisms such as mice or humans, the best
290 demonstration of the effect of genetic variation on gene expression are abundant differences in
291 expression between alleles of the same gene [31,32]. In humans, fixed between-population
292 differences are exemplified by hundreds of genes that are differentially expressed between
293 African and European Americans [33]. All this suggests that gene expression can be a proxy of
294 not only phenotypic plasticity and acclimatization, but of genetic variation and adaptation. A
295 major advantage of the use of gene expression for these types of studies is that gene expression
296 integrates over many functionally relevant variants in the genome and thus represents a
297 condensed functional summary of the genotype [34].
298
299 In humans, nearly half of all genetic variants affecting gene expression have detectable effects in
300 all tissues [32], and so one feasible way to separate genotype-specific gene expression from
301 context-dependent variation might be to perform "cross-tissue" comparison (see Glossary) to
302 isolate body-wide expression shifts [35]. In the coming years, cross-tissue or similar analysis is
303 likely to become a major approach to study functional genetic variation in natural populations.
304
305

306 **Epigenetics**
307
308 Among many covalent chromatin modifications I will discuss DNA methylation since it currently
309 receives the most attention in OMOs. Still it must be mentioned that in plants histone
310 methylation appears be no less and perhaps even more involved in acclimatization and
311 transgenerational plasticity [36]. While vertebrates show high methylation throughout the genome,
312 invertebrates and plants methylate their genomes sparsely and mostly in protein-coding regions
313 (so-called gene body methylation, GBM, [37]). The function of his ubiquitous and evolutionarily
314 ancient DNA modification remains unclear [38,39] and the greatest challenge in the next few
315 years will be to decipher it. The most important questions are: (i) Does GBM affect gene
316 expression? (ii) Can it be modified on ecological timescale, to achieve acclimatization to a novel
317 environment? (iii) Can acquired changes in GBM be transmitted across generations? If the
318 answers to all three questions are "yes", then we have a mechanism for transgenerational
319 inheritance of acquired traits, which is an exciting (albeit tentative, [40]) possibility. Table 3
320 summarizes the methods for generating DNA methylation data. If every gene in the genome has
321 to be interrogated, MBD-seq and meDIP provide the best resolution for sequencing effort [38]. If
322 the goal of the study is to characterize general methylation patterns rather than identify specific
323 genes, highly cost-efficient solutions are provided by RRBS-seq and methylRAD. For studies
324 requiring single base resolution, the best approach appears to be direct detection by PacBio or
325 ONT – however, these exciting developments still require validation in complex genomes.
326
327 **Generating a reference sequence**
328
329 For all approaches described here, the accuracy of the reference sequence in terms of per-base
330 error rate must only be high enough to allow unambiguous mapping of high-accuracy (Illumina)
331 reads. The gold standard of genome sequence quality, Q30 or 99.9% accuracy, would not provide
332 any benefit compared to a rough draft accuracy of 99%. Occasional errors in the reference would
333 manifest themselves as SNPs that are not polymorphic in the analyzed samples and therefore
334 irrelevant for analysis. This is the same reason why it is possible to use a genome of a related
335 species as a reference.
336
337 For AFS analysis, which does not require highly contiguous reference, even a rough genome draft
338 that can be assembled from a single lane worth of 150b paired-end reads from Illumina HiSeq
339 would be suitable. However, substantially better options are now becoming available for a
340 comparably low price tag. The technology offered by 10x Genomics [41] attaches specific
341 barcodes to short reads originating from the same long DNA fragment, which allows assembling
342 Illumina HiSeq data into very long haplotypes. The two single-molecule long-read "third-
343 generation sequencing" methods, Single Molecule Real Time (SMRT) sequencing by PacBio and
344 nanopore sequencing by ONT, produce reads with broad length distribution, including
345 exceedingly long ones (tens to hundreds of kilobases) resulting in a qualitatively more contiguous
346 genome assemblies [42–45] (Table 4, see [43] for recent benchmarking study of assembly
347 pipelines). At the moment of this writing, read accuracy and cost of data for PacBio (Sequel
348 system) and ONT (R9 flow cell) were equivalent; PacBio generated higher proportion of long
349 reads than ONT; however, PacBio's library prep required ten fold more high-quality DNA than

ONT. Both for PacBio and ONT it is critically important to obtain high molecular weight DNA in fragments exceeding 20kb in length. For new OMOs, it is also essential to confirm that the DNA is accessible to enzymatic modifications by trying to digest it with a frequent-cutting restriction endonuclease.

For genome scanning, gene expression, or invertebrate DNA methylation analyses targeting protein-coding sequences (exome) genome sequence might not be the best reference; instead, a highly contiguous transcriptome assembly would be preferable. Until now the standard way to generate a *de novo* transcriptome was to perform high-coverage RNA-seq and assemble the results with Trinity [46]. In the coming years, it is expected that even higher-quality and lower-cost OMO transcriptomes would be generated by PacBio or ONT sequencing of full-length cDNA (or, for ONT, direct mRNA sequencing). The long-read capacity of these technologies would essentially obviate the need for assembly, leaving only the sequence correction procedure to be performed.

Finally, which tissue or body part to sample for sequencing? For genome sequencing it does not matter much as long as contamination by other DNA sources can be kept to a minimum, but for *de novo* transcriptomics it is not a trivial question, as gene expression varies dramatically across tissues and life cycle stages. In mammals, there is definitely an organ of choice that expresses nearly all genes in the genome: testis. Rather unexpected transcriptome complexity in the testis is putatively due to chromatin re-packaging during spermatogenesis, which results in genome-wide transcription leakage [47]. If so, testis might be a good choice for *de novo* transcriptomics not only for mammals but for any organism that produces compact sperm.

Note on data sharing

As we have seen, the best power of ecological genomics in OMOs is achieved using a genome or transcriptome reference. Every new reference dataset enables new biological questions, and the whole OMO field will get a great boost if these resources are promptly shared. Please consider rapidly sharing your reference data, at least as soon as the initial preprint of your paper is posted to bioRxiv and ideally sooner, by distributing the link to data through research-related email list or professional twitter feed.

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Box 1: AFS models.

In the world of OMOs we are usually dealing with samples from many populations, which would be hard or impossible to model simultaneously; moreover, there are usually many populations left unsampled. To infer meaningful demographic parameters in a sparsely sampled system of many populations, a practical solution is to perform two-dimensional AFS analysis of all population pairs [10]. Typical hypotheses and corresponding tests are:

- Are the two populations demographically separate?
    - compare model with split to model without split, under which the two compared populations are regarded as independent samples from the same population.
- If yes, is there still gene flow between them?
    - compare split models with and without migration.
- If yes, is the gene flow symmetric or asymmetric?
    - compare split model with two potentially different migration rates to a split model with a single symmetrical migration rate.
- Was population size stable or went through changes in the past?
    - compare single-population model involving population size change in the past to a standard neutral model.

Simple command-line scripts for AFS plotting and running basic pairwise models in *moments* can be found here: https://github.com/z0on/AFS-analysis-with-moments. To access the full potential of *moments*, however, the user is expected to compose python scripts of their own.

547 **Table 1. Genotyping approaches for genome scanning.**

Approach	Features	Pros	Cons
Exome-seq [48,49]	Isolates and sequences only the protein-coding portion of genome.	Dense coverage of genes guarantees that coding variants and variants linked to cis-regulatory mutations are discovered.	Other (arguably less important) types of variation are not profiled (e.g., distant enhancers).
RNA-seq [50,51]	Sequences RNA.	Same as exome sequencing.	Genotyping quality of a gene depends on expression level. Allele-specific expression affects accuracy of heterozygote calls.
Pool-seq [52,53]	Sequences pooled DNA from multiple individuals from each population.	Dense whole-genome coverage with confident determination of allele frequencies in populations.	No possibility for individual–based analysis (such as STRUCTURE) or validation based on genotype-phenotype association across individuals. Must be confident in *a priori* population designations.
Low-coverage whole-genome sequencing (WGS) [54]	Sequences individual genomes at ~1-4x coverage.	Dense whole genome coverage at individual level.	Per-site genotypes are unreliable because of missing data; must use uncertainty-aware analysis such as *ANGSD*.
Ultra-low coverage WGS with imputation [22]	Sequences individual genomes at <2x coverage, imputes missing genotypes and corrects false homozygote calls	Dense whole genome coverage at individual level, phased data enables haplotype-based analysis	Rare alleles (minor allele frequency<0.05) are missed. Requires large sample sizes (depending on LD, hundreds or thousands of individuals). Accuracy of imputation must be experimentally validated for every new OMO.

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550 **Table 2. Gene expression analyses not relying on accurate gene annotations**

Analysis	What does it do	Software	Applications
Principal coordinate analysis based on Manhattan distances (sum of all log-fold changes across genes)	Characterizes overall transcriptome differences across experimental groups. Measures fraction of variation attributable to each experimental factor.	R: package *ape*, function *pcoa* [55] package *vegan,* function *adonis* [56]	[57,58]
Differential analysis of principal components (DAPC)	Quantifies transcriptome differences between samples with respect to specified multivariate axis. Good for quantifying overall gene expression plasticity.	R: package *adegenet* [59]	[29]
Weighted gene co-expression network analysis (WGCNA)	Identifies co-regulated groups of genes, which are linked to experimental factors and traits *post hoc*. Method of choice for complex experimental designs (>20 samples) with many quantitative traits measured.	R: package WGCNA [60]	[29,61,62]
Rank-based functional summaries of KOG (euKaryotic Orthologous Groups) classes	Reveals broad functional trends in gene expression. Particularly useful for OMOs since it tolerates sparse and inaccurate annotations. Its main use is for statistical comparison of highly diverse datasets, even from different species.	R: package *KOGMWU* [11].	[11,58]

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553 **Table 3. Methods for interrogating DNA methylation**

Method	Features	Pros	Cons
Whole-Genome Bisulfite Sequencing (WGBS) [63]	Sequences complete genome after bisulfite conversion	Complete characterization of 5me-cythosine methylation at single-base resolution	High coverage is required to obtain quantitative data. In non-vertebrate OMOs, much sequencing effort is wasted since most of genome is not methylated.
RRBS-seq [64]	Bisulfite sequencing of genome fragments adjacent to all (methylated and un-methylated) CCGG sites	Saves costs dramatically compared to WGBS.	Only a fraction of all CpG sites is interrogated. Complicated library preparation protocol. Sequencing effort is wasted on non-methylated sites.
MBD-seq [65], meDIP [66]	Pull-down and sequencing of methylated DNA.	Optimizes sequencing effort by focusing on methylated DNA.	Complicated library preparation protocol. Resolution equals the length of pulled-down fragments (~300-500b). Pull-down procedure is not absolutely efficient, many reads still correspond to un-methylated genome regions.
methylRAD [67]	Direct sequencing of genomic fragments adjacent only to the methylated CCGG and CCWGG sites.	Very simple library prep protocol. Highly cost-efficient due to focus on methylated sites only.	Only a fraction of all CpG sites is interrogated. New method, requires further benchmarking.
PacBio [68,69]	Direct detection of modified DNA bases during normal SMRT sequencing, based on polymerase lags.	Robust detection of 4-methylcytosine, 8-oxoguanine, and N6-methyladenine. Single-base resolution.	Same as WGBS. 5-methylcytosine, the most common methylation mark in animals, is not reliably detected.
ONT [70]	Direct detection of modified DNA bases during normal nanopore sequencing, based on conductivity changes.	Detects all marks, including 5-methylcytosine. Single-base resolution.	Same as WGBS.

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557 **Table 4. Assembly pipelines for PacBio and ONT reads**

Pipeline	Required coverage	Features	Pros	Cons
Canu +Quiver* [71]	>30x	Correct and trims reads before assembly.	Best accuracy at base, indel and assembly level.	Very computationally demanding for large genomes. Generates incomplete assemblies at low coverage.
Falcon + Quiver* [72]	>50x	Similar to Canu.	Standard for PacBio.	Very computationally demanding for large genomes. High reliance on reads >20kb. Highly incomplete assemblies at low coverage.
minimap + miniasm + racon [73,74]	<30x	Raw reads are assembled, correction is done post-assembly	Very fast even for large genomes. Works with lower coverage, shorter reads than Canu and Falcon.	The resulting accuracy is lower than with Canu + Quiver.
pilon [75]	NA (error correction method)	Performs additional correction post-assembly.	Boosts accuracy for any assembly.	Requires high-quality Illumina reads.

558 *Quiver is a consensus polishing software that is now replaced by Arrow to handle PacBio
559 Sequel data (https://github.com/PacificBiosciences/GenomicConsensus). Racon [74] can be used
560 instead of Quiver/Arrow [43].
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