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Genomic methods take the plunge: recent advances in high-throughput sequencing of marine mammals

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Abstract

The dramatic increase in the application of genomic techniques to non-model organisms over the past decade has yielded numerous valuable contributions to evolutionary biology and ecology, many of which would not have been possible with traditional genetic markers. We review this recent progression with a particular focus on genomic studies of marine mammals, a group of taxa that represent key macroevolutionary transitions from terrestrial to marine environments and for which available genomic resources have recently undergone notable rapid growth. Genomic studies of non-model organisms utilize an expanding range of approaches, including whole genome sequencing, restriction site-associated DNA sequencing, array-based sequencing of single nucleotide polymorphisms and target sequence probes (e.g., exomes), and transcriptome sequencing. These approaches generate different types and quantities of data, and many can be applied with limited or no prior genomic resources, thus overcoming one traditional limitation of research on non-model organisms. Within marine mammals, such studies have thus far yielded significant contributions to the fields of phylogenomics and comparative genomics, as well as enabled investigations of fitness, demography, and population structure. Here we review the primary options for generating genomic data, introduce several emerging techniques, and discuss the suitability of each approach for different applications in the study of non-model organisms.

Keywords: RADseq, SNP array, target sequence capture, whole genome sequencing, RNAseq, non-model organisms

Introduction

Recent advances in sequencing technologies, coincident with dramatic declines in cost, have increasingly enabled the application of genomic sequencing in non-model systems (Ekblom and Galindo 2011; Ellegren 2014). These advances in molecular technologies have in many ways begun to blur the distinction between model and non-model organisms (Armengaud et al. 2014). Non-model organisms (NMOs) have traditionally been defined as those for which whole-organism experimental manipulation is rarely, if ever, possible due to logistical and/or ethical constraints (Ankeny and Leonelli 2011). Further, NMOs have typically been characterized by limited genomic resources, but this is becoming increasingly less so as the number of NMO reference genomes grows rapidly, for example through efforts like the Genome 10K Project (Koepfli et al. 2015). In fact, in some taxonomic orders, we are approaching the point at which all species have at least one representative reference genome available for a closely related species (Fig 1).

Despite the limitations of working with NMOs, including potentially small sample sizes, low DNA quantity, and limited information on gene function, genetic and genomic investigations of NMOs have yielded numerous valuable contributions to understanding their evolutionary biology and ecology. For the past several decades, traditional genetic markers such as microsatellites and short fragments of mitochondrial DNA (e.g., the control region) have been extensively used in molecular ecology. These markers, which typically evolve under neutral expectations, have proven useful for identifying population structure and reconstructing population demographic history (Hedrick 2000). However, the power of such studies is limited by the number of markers that can feasibly be evaluated using traditional approaches. The advent of low-cost high-throughput sequencing has led to dramatic increases in the number of neutral markers that can be evaluated, in many cases improving our power to resolve fine-scale or cryptic population structure in species with high dispersal capability (e.g., Corander et al. 2013) and improving the accuracy of estimating some (though not all) demographic parameters (Li and Jakobsson 2012; Shafer et al. 2015). Importantly, high-throughput sequencing has also further enabled genomic studies of non-neutral processes in NMOs, for example, characterizing both deleterious and adaptive variation within and across species (Stinchcombe and Hoekstra 2008;

Künstner et al. 2010). It is increasingly evident that genomic analyses of NMOs can and have provided important insights that could not be identified with traditional genetic markers.

Many molecular ecologists now face the challenge of deciding which of the broad range of genomic approaches to apply to their study systems. Here we review the primary options for generating genomic data and their relative suitability for different applications in the study of NMOs. We focus on marine mammals, which represent several mammalian clades with notably rapid growth in available genomic resources in recent years. This growth is clearly evident in both publication rate (Fig 2) and the rise in number and size of genomic sequences deposited in public resources (Fig 3). We comprehensively review the literature on marine mammal genomics, highlighting recent trends in methodology and applications, and then describe in detail the molecular approaches that are most commonly applied to studies of NMO genomics. Our hope is that this review will highlight the promise of genomics for NMOs and offer guidance to researchers considering the application of genomic techniques in their non-model study system of choice.

Why study marine mammal genomics?

Marine mammals represent key macroevolutionary transitions from terrestrial to marine environments (McGowen et al. 2014) and accordingly are an exemplary system for investigating the evolution of several morphological and physiological adaptations (Foote et al. 2015) associated with locomotion (Shen et al. 2012), sight (Meredith et al. 2013), echolocation (Parker et al. 2013; Zou and Zhang 2015), deep diving (Mirceta et al. 2013), osmoregulation (Ruan et al. 2015), and cognition (McGowen et al. 2012). Furthermore, studies of marine mammal evolution to date have characterized several unique aspects of their genome evolution that merit further investigation, including low genomic diversity and a relatively slow molecular clock, especially in cetaceans (Jackson et al. 2009; McGowen et al. 2012; Zhou et al. 2013). As many cetacean species are highly mobile with no obvious physical geographic barriers to dispersal, they provide a unique opportunity to study the role of behavior and culture in shaping population structure and genetic diversity (Riesch et al. 2012; Carroll et al. 2015; Alexander et al. 2016). Though highly mobile, many marine mammals exhibit evidence of local adaptation; for example, several species show parallel divergent morphological and behavioral adaptations to coastal and pelagic environments (Moura et al. 2013; Louis et al. 2014; Viricel and Rosel 2014). These species may be studied across ocean basins as emerging examples of ecological adaptation and speciation (Morin et al. 2010a).

Beyond their value as systems of evolutionary study, many marine mammals are also of broader interest relating to their historical and present conservation status. Many marine mammal populations share histories of dramatic decline due to hunting and other human impacts. Genomics provides a promising tool with which to expand our insights into these historical population changes, which so far primarily have been derived from archival review and traditional genetic approaches (Ruegg et al. 2013; Sremba et al. 2015). More recently, since the implementation of national and international protections, many marine mammal populations have partially or fully recovered (Magera et al. 2013), yet the conservation status of certain marine mammal populations remains of concern. Such vulnerable populations could benefit greatly from an improved understanding of their genetic diversity and evolution, especially in ways that can inform predictions of adaptive capacity to anthropogenic pressures and expand the toolkit for conservation policy (Garner et al. 2016; Taylor and Gemmell 2016).

Recent trends in marine mammal genomics

We conducted a meta-analysis of the peer-reviewed marine mammal genomics literature to evaluate trends in publication rates across research methodologies and aims. A search of the Web of Science database using the term “genom*” and one of the following terms indicating study species - “marine mammal”, “pinniped”, “seal”, “sea lion”, “sea otter”, “whale”, “dolphin”, “polar bear”, “manatee” - identified 825 records on December 11, 2015. We excluded 77% of the search results that were not directly related to genomic studies in marine mammal systems. The remaining 101 articles that were relevant to marine mammal genomics were further categorized by primary research methodology and general research aim. A subset of these articles is described briefly in Supplemental Table 1.

From the early 1990s through 2015, published literature in the field shifted from an early focus on mitogenome sequencing to more sequence-intensive approaches, such as transcriptome and whole genome sequencing (Figs 2 and 4). This trajectory closely follows trends in sequencing technologies, from Sanger sequencing of short- and long-range PCR products for mitogenome sequencing (Arnason et al. 1991) and SNP discovery (Olsen et al. 2011), to high-throughput sequencing of reduced-representation genomic libraries (RRLs) that consist of selected subsets of the genome (e.g., Viricel et al. 2014), to high-throughput sequencing of whole genomes with varying levels of depth, coverage, and contiguity. Today, high-throughput sequencing can be used both to generate high-quality reference genome assemblies (Yim et al. 2014; Foote et al. 2015; Humble et al. 2016) and to re-sequence whole genomes at a population scale (Liu et al. 2014a; Foote et al. 2016). Similarly, the scale of gene expression studies has increased from quantitative real-time PCR of candidate genes (Tabuchi et al. 2006) to microarrays containing hundreds to thousands of genes (Mancia et al. 2007) and high-throughput RNAseq that evaluates hundreds of thousands of contigs across the genome (Khudyakov et al. 2015b). As the cost of high-throughput sequencing continues to decline, we anticipate an increase in studies that sequence RRLs, whole genomes, and transcriptomes in NMOs at a population scale.

Marine mammal genomic studies thus far have primarily contributed to the fields of phylogenomics and comparative genomics (Fig 2, Table S1). Several of these comparative genomics studies have aimed to improve our understanding of the mammalian transition to an aquatic lifestyle and describe the evolutionary relationships within and among marine mammals and their terrestrial relatives (McGowen et al. 2014; Foote et al. 2015). Whereas such studies require only a single representative genome per species, an emerging class of studies applying genomic techniques at a population scale enables further investigations of fitness, demography, and population structure within species (Table S1). However, expanding the scale of genomic studies requires careful selection of an appropriate method for data generation and analysis from a growing number of approaches that are becoming available to non-model systems.

Data generation

Our review of marine mammal genomics highlights an increasing number of options for the generation and analysis of genomic data. Choosing which of these sequencing strategies to apply is a key step in any genomics study. Here we describe approaches that have been used successfully in order to help guide future studies of ecological, physiological, and evolutionary genomics in NMOs. Across data generation methods, we highlight approaches that can be used with limited or no prior genomic resources, overcoming one traditional challenge of genomic studies of NMOs (the need for a reference genome to which sequencing reads can be mapped). These methods produce a range in quantity and type of data output, from hundreds of SNPs to whole genome sequences, and from single individuals to population samples, reflecting the trade-off between number of samples and amount of data generated per sample.

Sample collection, storage and extraction

Prior to starting a genomic study, researchers must recognize that many recent methods for high-throughput sequencing require genetic material of much higher quality and quantity than techniques used to characterize traditional genetic markers. These more stringent sample requirements necessitate new standards for tissue sampling, storage, and DNA/RNA extraction. Ideally, samples should be collected from live or newly deceased individuals and stored at -80°C, or when this is not possible at -20°C in RNAlater, Trizol, ethanol, salt-saturated DMSO, or dry, depending on the intended application. Given the sensitivity of new sequencing methods, great care should be taken to minimize cross-contamination during sampling, as even minute amounts of genetic material from another individual can bias downstream analyses, for example variant genotyping and gene expression profiles. Choice of extraction method varies with sample type and study aim, but typically genomic methods require cleanup and treatment with RNase to yield pure extracts, whereas RNAseq methods require rigorous DNase treatment to remove genomic contamination that can bias expression results. Depending on the genomic methodology, target quantities for a final sample may range from as low as 50 ng of DNA for some RRL sequencing methods (Andrews et al. 2016) up to ~1 mg for sequencing the full set of libraries (of different insert sizes) necessary for high-quality genome assemblies (Ekblom and Wolf 2014). Most commercial RNAseq library preparation services require at least 500-1,000 ng of pure total RNA that shows minimal degradation as measured by capillary gel electrophoresis (RNA Integrity Number (RIN) ≥ 8). Samples should ideally consist of high molecular weight genetic material (with little shearing), though continuing molecular advances enable genomic sequencing even of low quantity or poor quality starting material. Extreme examples of the latter include successfully sequenced whole genomes from ancient material (e.g., Rasmussen et al. 2010; Meyer et al. 2012; Allentoft et al. 2015), including a more than 500,000-year-old horse (Orlando et al. 2013).

<u>Reduced-representation genome sequencing</u>

i. RADseq

Reduced-representation sequencing methods evaluate only a small portion of the genome, allowing researchers to sequence samples from a larger number of individuals within a given budget in comparison to sequencing whole genomes. Restriction site-associated DNA sequencing (RADseq) is currently the most widely used RRL sequencing method for NMOs (Davey et al. 2011; Narum et al. 2013; Andrews et al. 2016). RADseq generates sequence data from short regions adjacent to restriction cut sites and therefore targets markers that are distributed relatively randomly across the genome and occur primarily in non-coding regions. This method allows simultaneous discovery and genotyping of thousands of genetic markers for virtually any species, regardless of availability of prior genomic resources. Of greatest interest are variable markers, characterized either as single SNPs or phased alleles that can be resolved from the identification of several variants within a single locus.

The large number of markers generated by RADseq dramatically increases genomic resolution and statistical power for addressing many ecological and evolutionary questions when compared to studies using traditional markers (Table S1). For example, heterozygosity fitness correlations in harbor seals (*Phoca vitulina*) were nearly fivefold higher when using 14,585 RADseq SNPs than when using 27 microsatellite loci (Hoffman et al. 2014). A recent study on the Atlantic walrus (*Odobenus rosmarus rosmarus*) using 4,854 RADseq SNPs to model demographic changes in connectivity and effective population size associated with the Last Glacial Maximum (Shafer et al. 2015) both supported and extended inferences from previous studies using traditional markers (Shafer et al. 2010; Shafer et al. 2014).

Furthermore, RADseq can provide sufficient numbers of markers across the genome to identify genomic regions influenced by natural selection. These analyses require large numbers (thousands to tens of thousands) of markers to ensure that some markers will be in linkage disequilibrium with genomic regions under selection and to minimize false positives, particularly under non-equilibrium demographic scenarios (Narum and Hess 2011; De Mita et al. 2013; Lotterhos and Whitlock 2014). Extreme demographic shifts, as experienced by many marine mammal populations (e.g., killer whales, Foote et al. 2016), can drive shifts in allele frequencies that confound the distinction of drift and selection and make it difficult to detect genomic signatures of selection (Poh et al. 2014). Proof of concept of the application of RADseq for identifying genomic signatures of selection in wild populations was demonstrated in three-spined sticklebacks (*Gasterosteus aculeatus*), for which analyses of over 45,000 SNPs (Hohenlohe et al. 2010) identified genomic regions of known evolutionary importance associated with differences between marine and freshwater forms (Colosimo et al. 2005; Barrett et al. 2008). RADseq studies with similar aims in marine mammals have resulted in comparatively sparser sampling of SNPs (<10,000), likely due to both methodological differences and generally low genetic diversity particularly among cetaceans. Nonetheless, genomic regions associated with resistance to harmful algal blooms in common bottlenose dolphins (*Tursiops truncatus*) were identified across multiple pairwise comparisons using 7,431 RADseq SNPs (Cammen et al. 2015), and genomic regions associated with habitat use and resource specialization in killer whales (*Orcinus orca*) were identified using 3,281 RADseq SNPs (Moura et al. 2014a). Some of these RADseq SNPs associated with diet in killer whales were later also confirmed as occurring in genomic regions of high differentiation and reduced diversity consistent with a signature of selection identified in a study utilizing whole genome re-sequencing (Foote et al. 2016). It will remain important for further studies of genomic signatures of selection in NMOs to carefully consider which approach will generate a sufficiently large number of SNPs to accurately identify the range of putatively neutral F_{ST} values (and thus outliers) given the demographic history of the population (Lotterhos and Whitlock 2014).

Numerous laboratory methods have been developed for generating RADseq data (reviewed in Andrews et al. 2016), with the most popular library preparation methods currently being the original RAD (Miller et al. 2007; Baird et al. 2008), Genotyping by Sequencing (GBS, Elshire et al. 2011; Poland et al. 2012), and double digest RAD (ddRAD, Peterson et al. 2012). All RADseq methods share the common goal of sequencing regions adjacent to restriction cut sites across the genome, but differ in technical details, such as the number and type of restriction enzymes used, the mechanisms for reducing genomic DNA fragment sizes, and the strategies for attaching sequencing adapters to the target DNA fragments. For example, both the original RAD method and GBS use a single enzyme digest, but the original RAD method uses a rare-cutting enzyme and mechanical shearing to reduce DNA fragment size (Baird et al. 2008), whereas GBS uses a more frequent-cutting enzyme and relies on preferential PCR amplification of shorter fragments for indirect size selection (Elshire et al. 2011). These modifications lead to differences across methods in the time and cost of library preparation, the number and lengths of loci produced, and the types of error and bias present in the resulting data. Different RADseq methods will be better suited to different research questions, study species, and research budgets, and therefore researchers embarking on a RADseq study should carefully consider the suitability of each method for their individual projects. Further details on the advantages and disadvantages of each method are described in Andrews et al. (2016).

ii. SNP arrays

An alternative high-throughput reduced-representation genotyping approach involves the use of custom arrays designed to capture and sequence targeted regions of the genome. Such array-based approaches may provide certain advantages over RADseq, including the ability to easily estimate genotyping error rates, scalability to thousands of samples, lower requirements for DNA quantity/quality and technical effort, greater comparability of markers across studies, and the ability to genotype SNPs within candidate genomic regions. However, unlike RADseq, array-based techniques require prior knowledge of the study system's genome or the genome of a closely related species, which remains unavailable for some NMOs. Furthermore, SNP arrays must take into account the potential for ascertainment bias (e.g., Malenfant et al. 2015), whereas RADseq avoids ascertainment bias by simultaneously discovering and genotyping markers.

To identify SNPs for NMO array development, researchers must rely on existing genomic resources or generate new reference sequences, in the form of whole or reduced-representation genomes or transcriptomes (Hoffman et al. 2012; Malenfant et al. 2015). When a whole genome reference assembly is available for the target species or a related species, multiplex shotgun sequencing can facilitate the rapid discovery of hundreds of thousands of SNPs for array development. This SNP discovery approach involves high-throughput sequencing of sheared genomic DNA that can be sequenced at a low depth of coverage (i.e., low mean read depth across the genome) if suitable genotype likelihood-based methods (O'Rawe et al. 2015) are used to identify polymorphic sites. Thus, this approach is less restrictive in terms of DNA quality. For example, shotgun sequencing of 33 Northeast Atlantic common bottlenose dolphins, which included degraded DNA collected from stranded specimens, on one Illumina HiSeq2000 lane of 100 bp single-end sequencing identified 440,718 high-quality SNPs (M. Louis unpublished data). Such dense sampling of SNPs is essential for studies of population genomics that require a large number of markers, such as for inferences of demographic history (Gutenkunst et al. 2009; Excoffier et al. 2013; Liu and Fun 2015) and selective sweeps (Chen et al. 2010). Once a set of putative markers has been identified, hybridization probes can be designed from their flanking sequences and printed onto a SNP array. The two principal SNP genotyping platforms supporting thousands to millions of SNPs are the Illumina Infinium iSelect® and Affymetrix Axiom® arrays.

The use of SNP arrays in NMOs has thus far been somewhat limited, potentially due to low SNP validation rates (Chancerel et al. 2011; Helyar et al. 2011), issues of ascertainment bias (Albrechtsen et al. 2010; McTavish and Hillis 2015), and cost of SNP discovery. However, using both SNP data and whole genome sequence from the Antarctic fur seal (*Arctocephalus gazella*), Humble et al. (2016) recently demonstrated that careful filtering based on SNP genomic context prior to array development has the potential to substantially increase assay success rates. Further, ascertainment bias can be reduced by selecting samples for SNP discovery that span the geographic range of populations that will be target sequenced (Morin et al. 2004). By accounting for ascertainment bias, Malenfant et al. (2015) were able to demonstrate population structure in Canadian polar bears (*Ursus maritimus*) more clearly using a 9K SNP array than 24 microsatellite markers.

iii. Target sequence capture

Target sequence capture (TSC, also called target enrichment, direct selection, or Hyb-seq) has many of the same advantages and disadvantages as the array-based SNP approaches described above, but differs in library preparation, sequencing platform, and resulting sequence data. While SNP arrays genotype single variable positions, TSC can be used to sequence selected short fragments. With TSC, researchers can amplify and sequence up to a million target probes on solid-state arrays, and even more if in-solution arrays are used. This gives the user the ability to choose to sequence many samples in parallel (Cummings et al. 2010), as many as 100-150 per

Illumina HiSeq lane, or to sequence many regions per individual. Recent advances in target enrichment, such as genotyping in thousands (Campbell et al. 2015), anchored hybrid enrichment (Lemmon et al. 2012), and target capture of ultraconserved elements (UCEs, Faircloth et al. 2012; McCormack et al. 2012), have further increased the number of regions and individuals that can be sampled in a single lane. In addition, UCEs overcome the need for a reference genome, enabling their wide application across many NMOs (though designing custom probe sets from closely related species will remain preferable in many cases (Hancock-Hanser et al. 2013)). Although a number of methodological variants have been developed and optimized (Bashiardes et al. 2005; Noonan et al. 2006; Hodges et al. 2009; Cummings et al. 2010; Mamanova et al. 2010; Hancock-Hanser et al. 2013), TSC generally relies on hybridization and amplification of specially prepared libraries consisting of fragmented genomic DNA. Many companies offer kits for TSC, such as Agilent (SureSelect) and MYcroarray (MYbaits), with MYcroarray specifically marketing their kits for use with NMOs.

The most common use of TSC has been the capture of whole exomes in model organisms, including humans (Ng et al. 2009). However, as costs have plummeted, TSC is increasingly being used in investigations of NMOs. TSC is particularly useful in sequencing ancient DNA, where it can enrich the sample for endogenous DNA content relative to exogenous DNA (i.e., contamination) and thereby increase the relative DNA yield (Ávila-Arcos et al. 2011; Enk et al. 2014). For example, TSC has been used to generate mitogenome sequences from subfossil killer whale specimens originating from the mid-Holocene for comparison with modern lineages (Foote et al. 2013). TSC was also recently utilized to compare >30 kb of exonic sequence from museum specimens of the extinct Steller's sea cow (*Hydrodamalis gigas*) and a modern dugong (*Dugong dugon*) specimen to investigate evolution within Sirenia (Springer et al. 2015). Springer et al. (2016) further used TSC to examine gene evolution related to dentition across edentulous mammals, including mysticetes. Finally, TSC of both exonic and intronic regions has been used to assess genetic divergence across cetacean species (Hancock-Hanser et al. 2013; Morin et al. 2015). These studies show the potential use of TSC across evolutionary timescales for population genomics, phylogenomics, and studies of selection and gene loss across divergent lineages (Table S1).

Whole genome sequencing

Beyond advances enabled by the reduced-representation methods presented above, our power and resolution to elucidate evolutionary processes, including selection and demographic shifts, can be further increased by sequencing whole genomes.

i. Reference genome sequencing

At the time of publication, there are 12 publicly available[1] whole (or near-whole) marine mammal genomes of varying quality representing 10 families, including 7 cetaceans (Fig 1A), 3 pinnipeds (Fig 1B), the West Indian manatee (*Trichechus manatus*), and the polar bear. The first sequenced marine mammal genome was that of the common bottlenose dolphin, which was originally sequenced to ~2.5x depth of coverage using Sanger sequencing (Lindblad-Toh et al. 2011). This genome was later improved upon by adding both 454 and Illumina HiSeq data (Foote et al. 2015). Other subsequent marine mammal genomes were produced solely using Illumina sequencing and mate-paired or paired-end libraries with varied insert sizes (Miller et al. 2012; Zhou et al. 2013; Yim et al. 2014; Foote et al. 2015; Keane et al. 2015; Kishida et al. 2015; Humble et al. 2016).

Whole genome sequencing has been used to address many issues in marine mammal genome evolution, usually by comparison with other existing mammalian genomes. Biological insights discussed in the genome papers listed above include the evolution of transposons and repeat elements, gene evolution and positive selection, predicted population structure through time, SNP validation, molecular clock rates, and convergent molecular evolution (Table S1). For example, analyses of the Yangtze river dolphin (*Lipotes vexillifer*) genome confirmed that a bottleneck occurred in this species during the last period of deglaciation (Zhou et al. 2013). In addition, following upon earlier smaller-scale studies (e.g., Deméré et al. 2008; McGowen et al. 2008; Hayden et al. 2010), genomic analyses have confirmed the widespread decay of gene families involved in olfaction, gustation, enamelogenesis, and hair growth in some cetaceans (Yim et al. 2014; Kishida et al. 2015). Perhaps the most widespread use of whole genome studies

[1] These genomes are available on NCBI's online genome database or Dryad, but they have not all been published. As agreed upon in the Fort Lauderdale Convention, the community standard regarding such unpublished genomic resources is to respect the data generators' right to publish with these data first.

has been the use of models of selection to detect protein-coding genes that show evidence of natural selection in specific lineages. A recent study by Foote et al. (2015) extended this approach to investigate convergent positive selection among cetaceans, pinnipeds, and sirenians. This study exemplifies a trend in recent genomic studies that sequence multiple genomes to address a predetermined evolutionary question, in this case, the molecular signature of aquatic adaptation.

In addition to these evolutionary insights that typically stem from a comparative genomics approach, the development of high-quality reference genome assemblies provide an important resource that facilitates mapping of reduced-representation genomic data (see previous section) as well as short-read sequencing data with relatively low depth of coverage (see following section). These data types can be generated at relatively low cost on larger sample sizes enabling population-scale genomic studies. In many cases, genome assemblies from closely related species are sufficient for use as a reference. Particularly among marine mammals, given their generally slow rate of nucleotide divergence, it is therefore likely unnecessary to sequence a high-quality reference genome assembly for every species. Instead, resources could be allocated toward population-scale studies, including genome re-sequencing efforts.

ii. Population-level genome re-sequencing

In contrast to reference genome sequencing that today often exceeds 100x mean read depth and typically combines long- and short-insert libraries to generate high-quality assemblies for one to a few individuals, genome re-sequencing studies aim to achieve only ≥2x mean read depth on tens to hundreds of individuals from short-insert libraries whose reads are anchored to existing reference assemblies. Despite the inherent trade-offs between cost, read depth, coverage, and sample size, genome re-sequencing of large numbers of individuals for population-level inference can be conducted at a relatively low cost. In the past five years, several influential studies have used genome re-sequencing to advance our understanding of the genomic underpinnings of different biological questions in model systems. For example, population genomics of *Heliconius* butterflies highlighted the exchange of genes between species that exhibit convergent wing patterns (The *Heliconius* Genome Consortium 2012); whole genome re-sequencing of three-spined sticklebacks highlighted the re-use of alleles in replicated divergences associated with ecological speciation and local adaptation (Jones et al. 2012); and combined population genomics and phylogenomics have identified regions of the genome associated with variation in beak shape and size in Darwin's finches (Lamichhaney et al. 2015).

To date only two marine mammal population genomics studies using whole genome re-sequencing have been published. These studies involved re-sequencing the genomes of 79 individuals from three populations of polar bears (Liu et al. 2014a) and 48 individuals from five evolutionarily divergent ecotypes of killer whale (Foote et al. 2016). The findings of Foote et al. (2016) confirmed results of population differentiation that had previously been established using traditional genetic markers (Morin et al. 2010a). However, the study also provided new insights into the demographic history, patterns of selection associated with ecological niche, and evidence of episodic ancestral admixture that could not have been obtained using traditional markers.

Several new resources have made such population genomic studies economically possible for a greater number of NMOs, including the availability of reference genome assemblies (see section above), relatively low-cost high-throughput sequencing (further increases in throughput expected with the new Illumina HiSeq X Ten (van Dijk et al. 2014)), and crucially, the development of likelihood-based methods that allow estimation of population genetic metrics from re-sequencing data (Fumagalli et al. 2013; O'Rawe et al. 2015). One last consideration is the ease of laboratory methods necessary to generate whole genome re-sequencing data when compared to other methods such as RADseq or TSC. DNA simply needs to be extracted from the samples and, using proprietary kits, built into individually index-amplified libraries that are equimolarly pooled and submitted for sequencing.

Many population genomic analyses are based on the coalescent model that gains most information from the number of independent genetic markers, not the number of individuals sampled. Sample sizes of ~10 individuals are usually considered sufficient (Robinson et al. 2014) and have been standard in many genome-wide studies in the eco-evolutionary sciences (Ellegren et al. 2012; Jones et al. 2012). Thus, sampling fewer individuals by whole genome re-sequencing is a salient approach that allows us to consider many more gene trees, whilst continuing to provide robust estimates of per-site genetic metrics (e.g., F_{ST}). The robustness of inference from data with low mean read depth across the genome was recently confirmed using a comparison of per-site F_{ST} estimates for the same sites from high-depth (≥20x) RADseq data and low-depth (≈2x) whole genome re-sequencing data in pairwise comparisons between the same two killer whale ecotypes (Foote et al. 2016).

Beyond the increased power afforded by sequencing more polymorphic sites, whole genome re-sequencing also allows inference of demographic history from the genome of even just a single individual by identifying Identical By Descent (IBD) segments and runs of homozygosity (Li and Durbin 2011; Harris and Nielsen 2013). For example, Liu et al. (2014a) found evidence for ongoing gene flow from polar bears into brown bears after the two species initially diverged. Genome re-sequencing of sufficient numbers of individuals also facilitates haplotype phasing, which has many applications, including the detection of ongoing selective sweeps (Ferrer-Admetlla et al. 2014) and the inference of demographic history of multiple populations based on coalescence of pairs of haplotypes in different individuals (Schiffels and Durbin 2014). However, haplotype phasing typically requires genomic data with higher mean read depth (~20x) from tens of individuals (though recent advances in genotype imputation suggest success with data of lower mean read depth (VanRaden et al. 2015)). Thus far, phasing has been restricted to relatively few NMO studies, and no marine mammal studies to the best of our knowledge.

Transcriptome sequencing

In comparison with the DNA-based genomic approaches described above, RNA-based genomic approaches are a relatively new and emerging application in NMOs such as marine mammals. Transcriptomics by RNA sequencing (RNAseq) can rapidly generate vast amounts of information regarding genes and gene expression without any prior genomic resources. This approach can resolve differences in global gene expression patterns between populations, individuals, tissues, cells, and physiological or environmental conditions, and can yield insights into the molecular basis of environmental adaptation and speciation in wild animals (Wolf 2013; Alvarez et al. 2015). Furthermore, RNAseq is a valuable tool for resource development, for example as a precursor to designing SNP and TSC arrays (e.g., Hoffman et al. 2012). However, applying RNAseq to NMOs requires several unique considerations in comparison to the DNA-based methods described above. Most importantly, the labile nature of gene transcription and high detection sensitivity of RNAseq have the potential to amplify transcriptional "noise" and are thus extremely sensitive to experimental design.

If the experimental goal is to capture a comprehensive transcriptome profile for a study organism, multiple tissues from individuals of varied life history stages should be sampled. However, if the aim is to characterize transcriptional responses to physiological or environmental stimuli, efforts should focus on minimizing variability in individuals and sampling conditions (Wolf 2013). For differential expression analyses, pairwise comparisons should be made within the same individual if at all possible (e.g., before and after treatment, between two developmental stages). As RNAseq only captures a 'snapshot' of gene expression in time, repeated sampling or time-course studies are necessary to obtain a more complete picture of cellular responses to the condition(s) in question (Spies and Ciaudo 2015). Sampling and sequencing depth requirements will depend on the study design. Simulation studies have shown that a minimum of 5-6 biological replicates sequenced at a depth of 10-20 million reads per sample is necessary for differential expression analysis (Liu et al. 2014b; Schurch et al. 2015). RNAseq can also be used for biomarker development to expand molecular toolkits for NMOs without sequenced genomes (Hoffman et al. 2013). In this case, higher sequencing depths of 30-60 million reads per sample are recommended for SNP discovery and genotyping (De Wit et al. 2015).

Following sequence generation, transcript annotation remains a challenge for NMOs without reference transcriptomes or genomes. *De novo* transcriptomes can be annotated through detection of assembled orthologs of highly conserved proteins, but these analyses remain limited by the quality of reference databases. As a result, NMO transcriptomes are biased in favor of highly conserved terrestrial mammal genes and therefore provide an incomplete understanding of animal adaptations to natural environments (Evans 2015). For example, while 70.0% of northern elephant seal (*Mirounga angustirostris*) skeletal muscle transcripts had BLASTx hits to mouse genes, only 54.1% of blubber transcripts could be annotated due to poor representation of this tissue in terrestrial mammal reference proteomes (Khudyakov et al. 2015b).

To date, RNAseq has been used for gene discovery and phylogenomics analyses in Antarctic fur seal (Hoffman 2011; Hoffman et al. 2013), polar bear (Miller et al. 2012), Indo-Pacific humpback dolphin (*Sousa chinensis* (Gui et al. 2013)), spotted seal (*Phoca largha* (Gao et al. 2013)), bowhead whale (*Balaena mysticetus* (Seim et al. 2014)), narrow-ridged finless porpoise (*Neophocaena asiaeorientalis* (Ruan et al. 2015)), and humpback whale (*Megaptera novaeangliae* (Tsagkogeorga et al. 2015)) (Table S1). Due to the challenges of repeated sampling of wild marine mammals, few studies have examined cetacean or pinniped transcriptome responses to environmental or experimental stimuli. The majority of such functional gene expression studies have used microarrays (Mancia et al. 2008; Mancia et al. 2012; Mancia et al. 2015); however, RNAseq has been employed to profile sperm whale (*Physeter macrocephalus*) skin cell response to hexavalent chromium (Pabuwal et al. 2013) and free-ranging northern elephant seal skeletal muscle response to an acute stress challenge (Khudyakov et al. 2015a; Khudyakov et al. 2015b). With decreasing sequencing costs and improvements in bioinformatics tools, RNAseq has the potential to accelerate molecular discoveries in marine mammal study systems and supplement existing functional genomics approaches.

Emerging techniques

In addition to the relatively proven NMO genomic data generation techniques described above, a suite of emerging techniques is entering the field, with exciting promise for exploration of existing and new research areas. For example, high-throughput shotgun sequencing is increasingly being used to identify genetic material from multiple species in a single sample (metagenomics and metatranscriptomics), rather than focus on characterizing variation in a single target individual. These multi-species approaches can be used, for example, to characterize diet from fecal samples (Deagle et al. 2009) and to investigate microbiomes (Nelson et al. 2015), objectives with implications for improving our understanding of both basic ecology and health in natural populations of NMOs. Furthermore, high-throughput sequencing of environmental DNA dramatically increases the throughput of NMO detection in environmental (e.g., seawater) samples (Thomsen et al. 2012), using degenerate primers for multi-species detection rather than requiring the design and implementation of numerous single-species protocols (Foote et al. 2012).

A second broad area of emerging interest moves beyond the study of variation at the DNA and RNA levels to examine epigenetic effects of histone modification on gene regulation and evolution. Epigenomic studies often examine changes in DNA methylation in association with processes such as cancer and ageing. Such approaches, from targeted gene to genome-wide, have only very recently and not yet frequently been applied in NMOs. Polanowski et al. (2014) used a targeted gene approach to examine changes in DNA methylation in age-associated genes, previously identified in humans and mice, in humpback whales of known age. The most informative markers were able to estimate humpback whale ages with standard deviations of approximately 3-5 years, demonstrating the potential transferability of these approaches from model to non-model organism. Villar et al. (2015) utilized a genome-wide approach – chromatin immunoprecipitation followed by high-throughput sequencing (ChIPseq) – to examine gene regulatory element evolution across mammals, including four species of cetaceans. This study identified highly conserved gene regulatory elements based on their histone modifications (H3K27ac and H3K4me3), showed that recently evolved enhancers were associated with genes under positive selection in marine mammals, and identified unique *Delphinus*-specific enhancers. Finally, reduced-representation epigenomic approaches have also been developed (Gu et al. 2011), and although they have not yet been used in marine mammals to our knowledge, these techniques could facilitate future studies of how changes in DNA methylation patterns affect other biological processes, such as stress levels or pregnancy.

Data analysis

Following the generation of genomic data, researchers must select the most appropriate genomic analysis (i.e., bioinformatics) pipelines, which often differ significantly from those used in traditional genetic studies of NMOs. The choice of analysis pipeline will depend on multiple factors including the availability of a reference genome, the level of diversity within the dataset (e.g., single- or multi-species), the type of data generated (e.g., single- or paired-end), and the computing resources available. The computational needs, both in terms of hardware and competency in computer science, for analysis of genomic data typically far exceed those necessary for traditional genetic markers. On the smaller end of the spectrum, one lane of 50 bp single-end sequencing on an Illumina HiSeq 2500 can produce tens of gigabytes of data, while data files associated with a single high-quality vertebrate genome may reach hundreds of gigabytes in size (Ekblom and Wolf 2014). Computing resources necessary for the analysis of these genomic datasets can range from ~10 gigabytes for a pilot study using a reduced-representation sequencing approach to over a terabyte for whole genome sequence assembly (Ekblom and Wolf 2014). Fortunately, university computing clusters, cloud-based (Stein 2010) and high-performance computing clusters (e.g., XSEDE; Towns et al. 2014), and open web-based platforms for genomic research (e.g., Galaxy; Goecks et al. 2010) are becoming increasingly accessible. Furthermore, new pipelines are continuously being developed and improved, and there are a growing number of resources aimed at training molecular ecologists and evolutionary biologists in computational large-scale data analysis (Andrews and Luikart 2014; Belcaid and Toonen 2015; Benestan et al. 2016). We provide an indicative list of the current, most commonly used analysis pipelines that are specific to each data generation method in Table 1. Here we briefly summarize current genomic data analysis pipelines and discuss considerations that are likely to be similar across multiple data generation methods.

Genomic data analysis often involves multiple steps, and the choice of analysis tool for each step can greatly affect the outcome, with different tools producing different (though usually overlapping) sets of results (e.g., Schurch et al. 2015). All analyses begin by evaluating data quality, trimming sequences if necessary to remove erroneous nucleotides (MacManes 2014), and implementing appropriate data quality filters (e.g., phred scores, read length, and/or read depth). Raw reads also need to be demultiplexed based on unique barcodes if pools of individuals were sequenced in a single lane. Analyses then usually proceed in a *de novo* or genome-enabled manner, depending on available resources. Briefly, sequences can be compared (e.g., to identify variants) by mapping all reads to a reference genome or *de novo* assembling stacks of sequences putatively derived from the same locus based on sequence similarity. *De novo* methods are sensitive to sequencing error, as well as true genetic variation, and therefore can erroneously assemble polymorphic sequences as separate loci or transcripts, requiring further filtering to remove redundancy. The opposite problem can also occur in both *de novo* and reference mapping approaches, where two distinct loci (e.g., paralogous loci) may assemble as a single locus or map to the same reference location. Researchers should therefore recognize the inherent trade-offs when carefully selecting their thresholds for acceptable levels of variation within and among loci.

Considerations relevant to the selection of subsequent downstream analyses are specific to the type of data generated and the research objective. For example, RADseq analysis pipelines differ in the algorithms used to genotype variants (Table 1). Similarly, there are several gene expression analysis pipelines for RNAseq data that compare transcript abundance between samples (Table 1). Analysis of TSC data usually uses standard *de novo* assemblers (e.g., Trinity, Velvet); these assemblers can be run using packages such as PHYLUCE (Faircloth 2015), which is designed specifically for use with ultraconserved elements. Unfortunately, for most analyses, there are no unifying recommendations currently available and researchers must evaluate several approaches, each with their own advantages and disadvantages, in order to select the most appropriate tool for their particular experiment and system. Furthermore, we can expect that the recommendations for analysis tools will continue to evolve as new programs become available in the future.

Guidelines for data quality control and sharing

With rapid growth in sequencing platforms and bioinformatics analysis pipelines comes the need to extend existing principles (e.g., Bonin et al. 2004) on quality control, analysis, and transparency. General recommendations for sample and data handling, library preparation, and sequencing have been discussed elsewhere (Paszkiewicz et al. 2014). We therefore focus on the need to produce guidelines on data quality evaluation and reporting for genomic data (e.g., Morin et al. 2010b). A primary challenge in this area is that quality metrics vary widely across sequencing technologies. Yet, regardless of sequencing platform, the quality of sequencing reads must be evaluated (e.g., using FastQC; Andrews 2010) and reported.

Best practices guidelines for reference genome sequencing and RNAseq data generation, analysis, and reporting are available from the human-centric ENCODE consortium (www.encodeproject.org). These include minimum depth of sequencing and number and reproducibility of biological replicates. For RNAseq experiments, evaluation of *de novo* assembly quality remains a challenge. Suggested quality metrics include percentage of raw reads mapping back to the assembly and number of assembled transcripts with homology to known proteins (MacManes 2016). Emerging tools such as Transrate (Smith-Unna et al. 2015) attempt to integrate these and other metrics into a comprehensive assembly quality score.

In contrast, there is not yet any standard way to estimate or report error rates with RADseq or genome re-sequencing methods (but see Mastretta-Yanes et al. 2015; Fountain et al. 2016). Recommendations to improve confidence in genotyping include using methods that account for population-level allele frequencies when calling individual genotypes, mapping reads to reference genomes rather than *de novo* assembly (Nadeau et al. 2014; Fountain et al. 2016), filtering out PCR duplicates (Andrews et al. 2014), identifying and removing markers in possible repeat regions, and filtering data to include only those with high read depth (>10-20x per locus per individual) (Nielsen et al. 2011). Other analysis methods, such as robust Bayesian methods and likelihood-based approaches that account for read quality in calculations of posterior probabilities of genotypes and per-site allele frequencies utilizing the sample mean site frequency spectrum as a prior (Fumagalli et al. 2013), can account for uncertainty and/or error in the data, and are therefore suitable for use with low to moderate read depths (2-20x per locus; e.g., Han et al. 2015; O'Rawe et al. 2015).

Due to the large number of analysis tools that are available, data quality and reproducibility ultimately depend on methods and data transparency. All raw sequencing reads should be publicly archived, for example deposited in the NCBI Sequence Read Archive. Many journals, including the *Journal of Heredity* (Baker 2013), now also require that primary data supporting the published results and conclusions (e.g., SNP genotypes, assemblies) be publicly archived in online data repositories (e.g., Dryad). We further recommend making public the analysis pipelines, scripts (e.g., using GitHub), and additional outputs, as appropriate, in order for analyses to be fully reproducible and transparent, which is the cornerstone of the scientific method (Nosek et al. 2015).

Future directions

As demonstrated here for one group of mammalian taxa, the rapid growth of the field of non-model genomics has been both impressive and empowering. As we approach a point of relative saturation in reference genomes, we anticipate an increase in population-scale genomic studies that produce lower depth or coverage datasets per individual but across larger sample sizes. In addition (or alternatively), we hope to see increasing efforts to sequence reference transcriptomes and improve NMO genome annotation in ways beyond the inherently limited approach of comparison to gene lists from a few model organisms. Population-scale genomic studies will facilitate greater ecological understanding of natural populations, while efforts to improve annotation will address persistent limitations in our understanding of gene function for NMOs. Ultimately, improving our understanding of local adaptation, adaptive potential, and demographic history through the use of genomic toolkits such as those described here is likely to have important implications for the future conservation of these populations.

Advances in sequencing technologies and analytical tools will no doubt continue, in some cases drawing on established techniques in model organisms, posing both new opportunities and new challenges for researchers in NMO genomics. Likely the most persistent challenge will remain selecting the data generation and experimental design that is most appropriate for the respective research objective. Our review identified few cases that exhibit relative dominance of a single methodology and analytical pipeline (e.g., RADseq and STACKS, RNAseq and Trinity); rather, more often we found a diversity of approaches even within each category of data generation. In fact, such diversity of approaches has its benefits, with each approach promoting its own advantages (and limitations). Overall, our reflections on lessons learned from the past decade of NMO genomics in one well-studied group of mammalian taxa clearly demonstrate the value, increased ease, and future promise of applying genomic techniques across a wide range of non-model species to gain previously unavailable insights into evolution, population biology, and physiology on a genome-wide scale.

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Table 1. Current and commonly used tools for analysis of genomic data generated in non-model organisms. Please note that this list is not exhaustive and new computational tools are continuously being developed.

Computational Tool	Purpose	Strengths/Weaknesses	Reference
*RADseq**			
STACKS	quality filtering, *de novo* assembly or reference-aligned read mapping, variant genotyping	scalable (new data can be compared against existing locus catalog); flexible filtering and export options; recently implemented a gapped alignment algorithm to process insertion-deletion (indel) mutations; secondary algorithm adjusts SNP calls using population-level allele frequencies; compatible with input data from multiple RADseq methods	Catchen et al. (2011; 2013), http://catchenlab.life.illinois.edu/stacks/
PyRAD	quality filtering, *de novo* assembly, read mapping, variant genotyping	efficiently processes indel mutations, thus optimal for analysis of highly divergent species; high speed and quality of paired-end library assemblies; compatible with input data from multiple RADseq methods	Eaton (2014)
TASSEL-GBS	quality filtering, reference-aligned read mapping, variant genotyping	optimized for single-end data from large sample sizes (tens of thousands of individuals) with a reference genome; performs genome-wide association studies	Glaubitz et al. (2014)
dDocent	quality trimming, *de novo* assembly, read mapping, variant genotyping	beneficial in analysis of paired-end data; identifies both SNP and indel variants; most appropriate for ezRAD and ddRAD data	Puritz et al. (2014)
AftrRAD	quality filtering, *de novo* assembly, read mapping, variant genotyping	identifies both SNP and indel variants; computationally faster than STACKS and PyRAD	Sovic et al. (2015)
Array-based high-throughput sequencing			
Affymetrix Axiom™ Analysis Suite, Illumina® GenomeStudio	genotype scoring	visualization of genotype clusters; quality scores assigned to genotype calls allow user-specific filtering; manual editing possible	
Whole genome sequencing			
AdapterRemoval v2, Trimmomatic	trim raw sequences	remove adapter sequences and low-quality bases prior to assembly	Bolger et al. (2014), Schubert et al. (2016)
ALLPATHS-LG, PLATANUS, SOAPdenovo	*de novo* genome assembly	designed for short-read sequences of large heterozygous genomes	Li et al. (2010), Gnerre et al. (2011), Kajitani et al. (2014)
AUGUSTUS, GenomeScan, MAKER2	gene annotation	highly accurate evidence-driven or BLASTX-guided gene prediction (Yandell and Ence 2012)	Yeh et al. (2001), Stanke et al. (2006), Holt and Yandell (2011)

Bowtie, bwa	read mapping	rapid short-read alignment with compressed reference genome index, but limited number of acceptable mismatches per alignment (Flicek and Birney 2009)	Langmead et al. (2009), Li and Durbin (2009)
SAMtools	data processing, variant calling	multi-purpose tool that conducts file conversion, alignment sorting, PCR duplicate removal, and variant (SNP and indel) calling for SAM/BAM/CRAM files	Li et al. (2009)
GATK	data processing and quality control, variant calling	suitable for data with low to high mean read depth across the genome; initially optimized for large human datasets, then modified for use with non-model organisms	McKenna et al. (2010), DePristo et al. (2011)
ANGSD/NGStools	data processing, variant calling, estimation of diversity metrics, population genomic analyses	suitable for data with low mean read depth, including palaeogenomic data; allow downstream analyses such as D-statistics and SFS estimation	Fumagalli et al. (2014), Korneliussen et al. (2014)
RNAseq			
Fastx Toolkit, Trimmomatic	trim raw sequences	remove erroneous nucleotides from reads prior to assembly	MacManes (2014)
khmer diginorm, Trinity normalization	*in silico* read normalization	reduce memory requirements for assembly, but can result in fragmented assemblies and collapse heterozygosity	Brown et al. (2012); Haas et al. (2013)
Trinity	*de novo* and genome-guided transcriptome assembly	accurate assembly across conditions, but requires long runtime if normalization is not used (Zhao et al. 2011)	Haas et al. (2013)
bowtie, bowtie2, STAR	read alignment to genome or transcriptome assembly	required for many downstream analyses, but bowtie is computationally intensive and all produce very large output BAM files	Langmead et al. (2009), Dobin et al. (2013)
eXpress, kallisto, RSEM, Sailfish, Salmon	estimation of transcript abundance	RSEM requires computationally intensive read mapping back to the assembly; the others are faster streaming alignment, quasi-alignment, or alignment-free algorithms	Li and Dewey (2011), Patro et al. (2015)
DESeq, DESeq2, edgeR	differential expression analysis	exhibit highest true positive and lowest false positive rates in experiments with smaller sample sizes (Schurch et al. 2015)	Anders and Huber (2010), Robinson et al. (2010), Love et al. (2014)
blast2GO, Trinotate	functional annotation of assembled transcripts	complete annotation pipelines including gene ontology and pathway enrichment analyses	Conesa et al. (2005), Haas et al. (2013)

* This is a non-exhaustive list of software that focuses on *de novo* loci assembly and genotype calling for RADseq data, as many practitioners working on NMOs will not have access to a reference genome. Other programs (e.g., GATK and ANGSD) that undertake genotype calling using reference-aligned loci are described in the whole genome sequencing section.

Figure 1

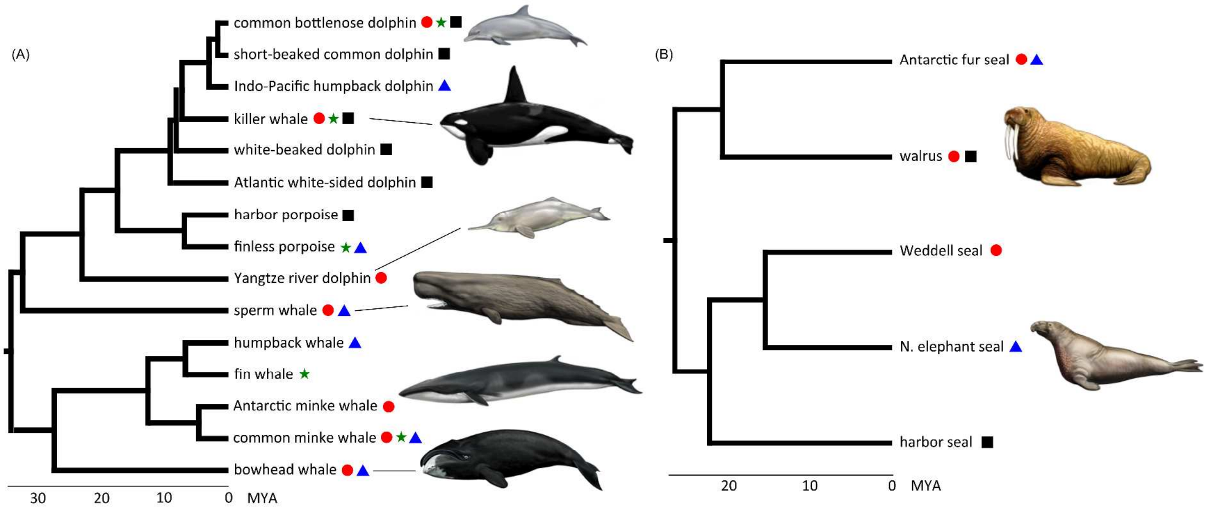


Figure 1. Phylogenetic tree showing current genomic resources available for (A) cetaceans and (B) pinnipeds; relationships and branch lengths are based on molecular dating estimates from McGowen et al. (2009), McGowen (2011), and Higdon et al. (2007). Scale is in millions of years ago (MYA). Red circles indicate species with high-quality reference genomes; green stars indicate whole genome re-sequencing data; blue triangles indicate transcriptomes (generated by microarray or RNAseq); and black squares indicate RADseq data.

Figure 2

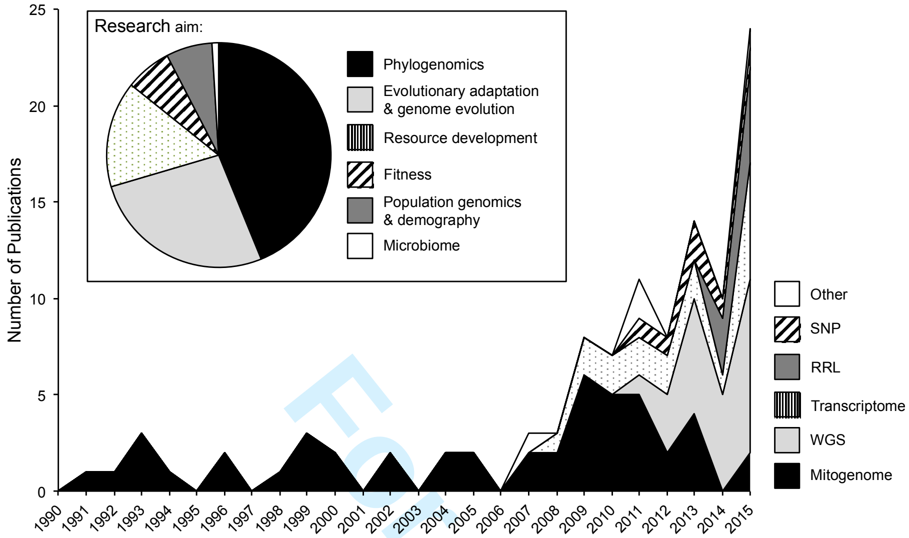


Figure 2. Number of marine mammal genomics publications from 1990 to 2015, categorized by primary methodology and research aim. Genomic methodologies include high-throughput single nucleotide polymorphism (SNP) genotyping and sequencing of mitogenomes, whole genomes (WGS), transcriptomes (generated by microarray or RNAseq), and reduced-representation genomic libraries (RRL). The "Other" category includes studies of microbiomes, BAC libraries, and large (~100) gene sets.

Figure 3

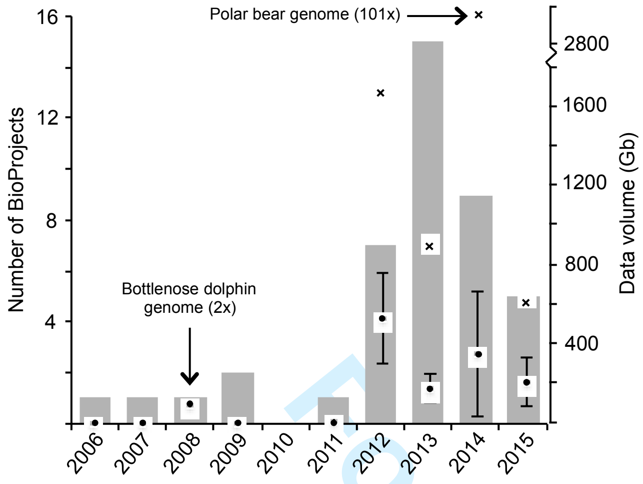
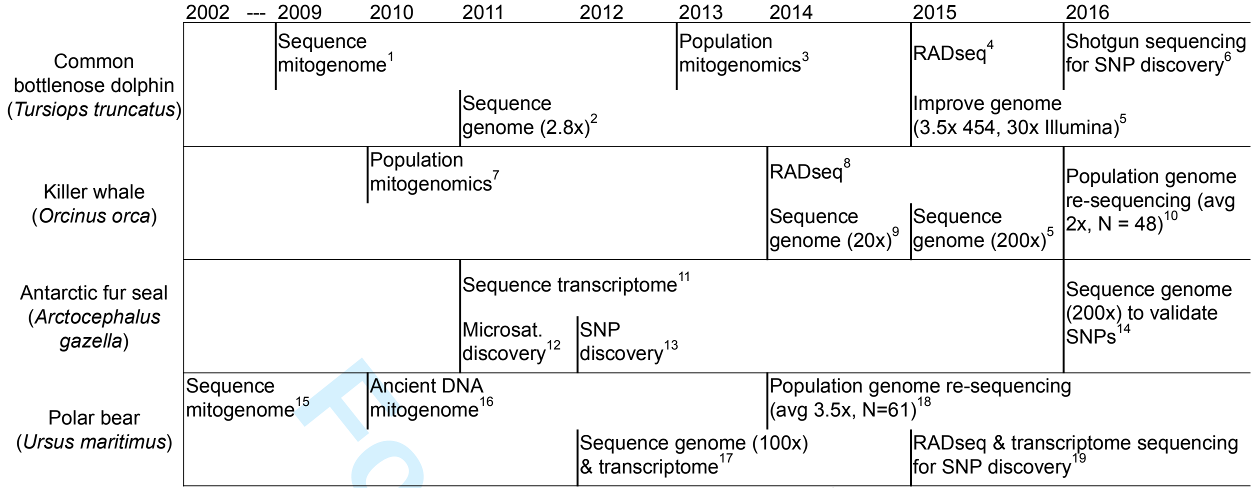


Figure 3. Number of BioProjects (gray bars) related to marine mammal genomics submitted from 2006 to 2015 to an online public database maintained by NCBI. Early BioProjects were largely microarray datasets. The number of projects created each year, as well as the yearly average (black dots ± SE) and maximum (×) size of data submitted in each BioProject, increased dramatically after 2011, reflecting advances in high-throughput sequencing technologies that facilitated their use in non-model systems.

Figure 4



2002 --- 2009 2010 2011 2012 2013 2014 2015 2016

Common bottlenose dolphin (*Tursiops truncatus*)

Sequence mitogenome[1]

Sequence genome (2.8x)[2]

Population mitogenomics[3]

RADseq[4]

Improve genome (3.5x 454, 30x Illumina)[5]

Shotgun sequencing for SNP discovery[6]

Killer whale (*Orcinus orca*)

Population mitogenomics[7]

RADseq[8]

Sequence genome (20x)[9]

Sequence genome (200x)[5]

Population genome re-sequencing (avg 2x, N = 48)[10]

Antarctic fur seal (*Arctocephalus gazella*)

Sequence transcriptome[11]

Microsat. discovery[12]

SNP discovery[13]

Sequence genome (200x) to validate SNPs[14]

Polar bear (*Ursus maritimus*)

Sequence mitogenome[15]

Ancient DNA mitogenome[16]

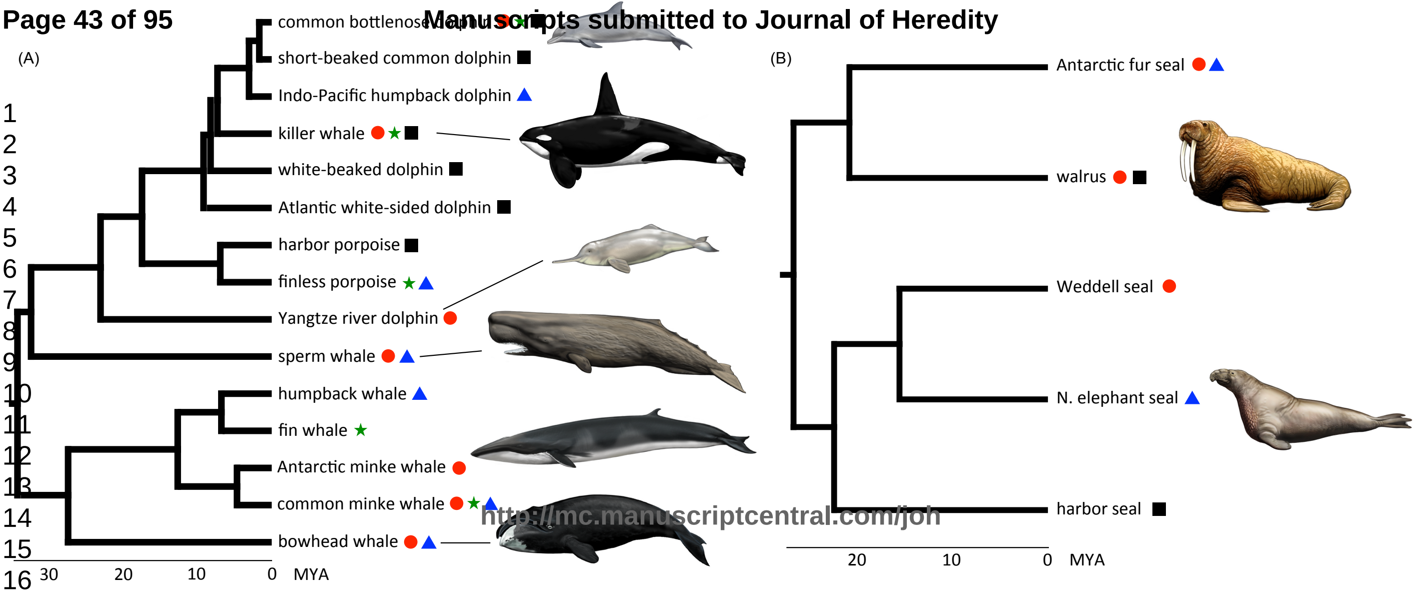
Sequence genome (100x) & transcriptome[17]

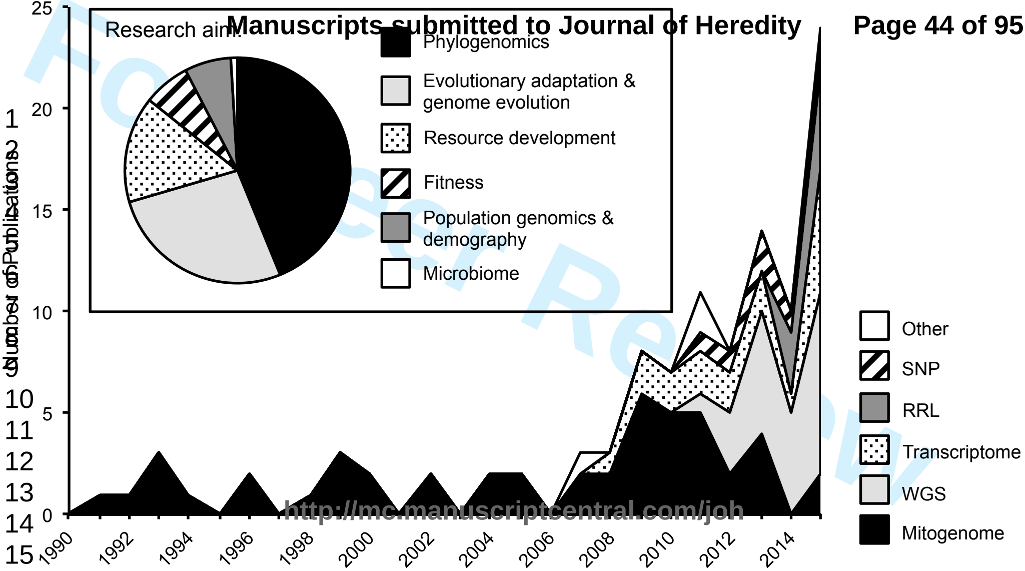
Population genome re-sequencing (avg 3.5x, N=61)[18]

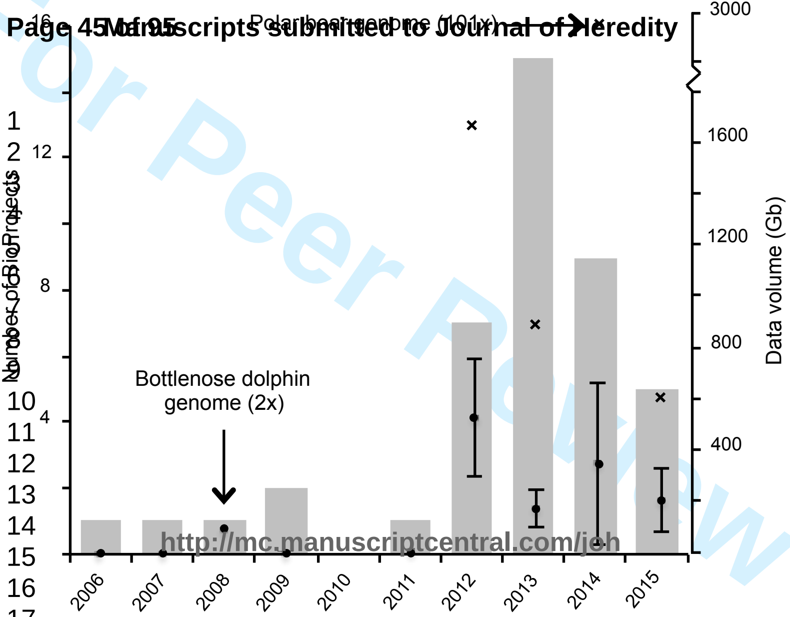
RADseq & transcriptome sequencing for SNP discovery[19]

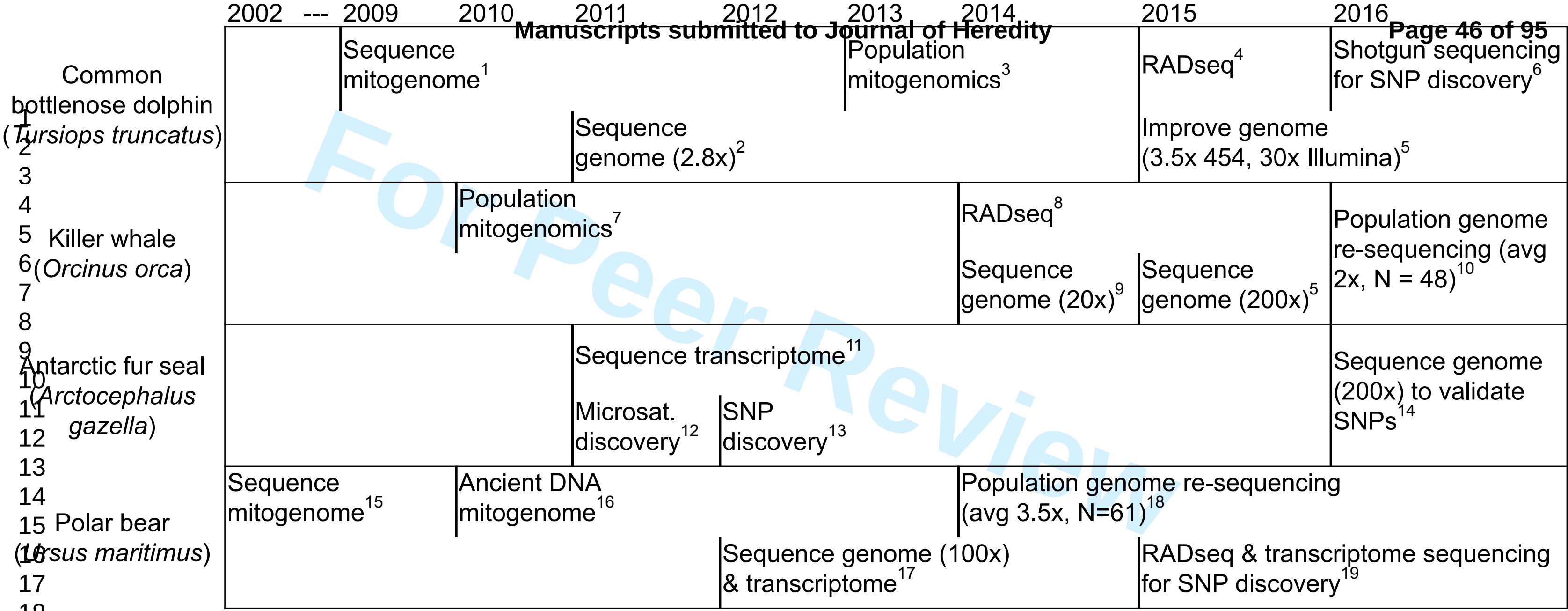
1) Xiong et al. 2009; 2) Lindblad-Toh et al. 2011; 3) Moura et al. 2013; 4) Cammen et al. 2015; 5) Foote et al. 2015; 6) Louis et al. unpubl. data; 7) Morin et al. 2010; 8) Moura et al. 2014a; 9) Moura et al. 2014b; 10) Foote et al. 2016; 11) Hoffman 2011; 12) Hoffman and Nicholas 2011; 13) Hoffman et al. 2012; 14) Humble et al. 2016; 15) Arnason et al. 2002; 16) Lindqvist et al. 2010; 17) Miller et al. 2012; 18) Liu et al. 2014; 19) Malenfant et al. 2015

Figure 4. Timelines depicting the independent progression of genomic studies for four representative marine mammal species. Trajectories show the common progression for non-model species from mitogenome sequencing to whole genome sequencing, as well as from sequencing reference specimens to population-scale genomic sequencing. In addition, the timelines reveal the utility of genomic and transcriptomic sequencing for subsequent genetic marker development.


(A)
common bottlenose dolphin ● ★ ■
short-beaked common dolphin ■
Indo-Pacific humpback dolphin ▲
killer whale ● ★ ■
white-beaked dolphin ■
Atlantic white-sided dolphin ■
harbor porpoise ■
finless porpoise ★ ▲
Yangtze river dolphin ●
sperm whale ● ▲
humpback whale ▲
fin whale ★
Antarctic minke whale ●
common minke whale ● ★ ▲
bowhead whale ● ▲
30
20
10
0
MYA
(B)
Antarctic fur seal ● ▲
walrus ● ■
Weddell seal ●
N. elephant seal ▲
harbor seal ■
20
10
0
MYA


Research aim:
Phylogenomics
Evolutionary adaptation & genome evolution
Resource development
Fitness
Population genomics & demography
Microbiome
Number of Publications
0
5
10
15
20
25
1990
1992
1994
1996
1998
2000
2002
2004
2006
2008
2010
2012
2014
Other
SNP
RRL
Transcriptome
WGS
Mitogenome


Polar bear genome (101x)
Bottlenose dolphin genome (2x)
Number of BioProjects
16
12
8
4
Data volume (Gb)
3000
1600
1200
800
400
2006
2007
2008
2009
2010
2011
2012
2013
2014
2015


2002

2009
2010
2011
2012
2013
2014
2015
2016
Common bottlenose dolphin (*Tursiops truncatus*)
Sequence mitogenome1
Population mitogenomics3
RADseq4
Shotgun sequencing for SNP discovery6
Sequence genome (2.8x)2
Improve genome (3.5x 454, 30x Illumina)5
Killer whale (*Orcinus orca*)
Population mitogenomics7
RADseq8
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Sequence genome (200x)5
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RADseq & transcriptome sequencing for SNP discovery19

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Table 1. Current and commonly used tools for analysis of genomic data generated in non-model organisms. Please note that this list is not exhaustive and new computational tools are continuously being developed.

Computational Tool	Purpose	Strengths/Weaknesses	Reference
*RADseq**			
STACKS	quality filtering, *de novo* assembly or reference-aligned read mapping, variant genotyping	scalable (new data can be compared against existing locus catalog); flexible filtering and export options; recently implemented a gapped alignment algorithm to process insertion-deletion (indel) mutations; secondary algorithm adjusts SNP calls using population-level allele frequencies; compatible with input data from multiple RADseq methods	Catchen et al. (2011; 2013), http://catchenlab.life.illinois.edu/stacks/
PyRAD	quality filtering, *de novo* assembly, read mapping, variant genotyping	efficiently processes indel mutations, thus optimal for analysis of highly divergent species; high speed and quality of paired-end library assemblies; compatible with input data from multiple RADseq methods	Eaton (2014)
TASSEL-GBS	quality filtering, reference-aligned read mapping, variant genotyping	optimized for single-end data from large sample sizes (tens of thousands of individuals) with a reference genome; performs genome-wide association studies	Glaubitz et al. (2014)
dDocent	quality trimming, *de novo* assembly, read mapping, variant genotyping	beneficial in analysis of paired-end data; identifies both SNP and indel variants; most appropriate for ezRAD and ddRAD data	Puritz et al. (2014)
AftrRAD	quality filtering, *de novo* assembly, read mapping, variant genotyping	identifies both SNP and indel variants; computationally faster than STACKS and PyRAD	Sovic et al. (2015)
Array-based high-throughput sequencing			
Affymetrix Axiom™ Analysis Suite, Illumina® GenomeStudio	genotype scoring	visualization of genotype clusters; quality scores assigned to genotype calls allow user-specific filtering; manual editing possible	
Whole genome sequencing			
AdapterRemoval v2, Trimmomatic	trim raw sequences	remove adapter sequences and low-quality bases prior to assembly	Bolger et al. (2014), Schubert et al. (2016)
ALLPATHS-LG, PLATANUS, SOAPdenovo	*de novo* genome assembly	designed for short-read sequences of large heterozygous genomes	Li et al. (2010), Gnerre et al. (2011), Kajitani et al. (2014)
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SAMtools	data processing, variant calling	multi-purpose tool that conducts file conversion, alignment sorting, PCR duplicate removal, and variant (SNP and indel) calling for SAM/BAM/CRAM files	Li et al. (2009)
GATK	data processing and quality control, variant calling	suitable for data with low to high mean read depth across the genome; initially optimized for large human datasets, then modified for use with non-model organisms	McKenna et al. (2010), DePristo et al. (2011)
ANGSD/NGStools	data processing, variant calling, estimation of diversity metrics, population genomic analyses	suitable for data with low mean read depth, including palaeogenomic data; allow downstream analyses such as D-statistics and SFS estimation	Fumagalli et al. (2014), Korneliussen et al. (2014)
RNAseq			
Fastx Toolkit, Trimmomatic	trim raw sequences	remove erroneous nucleotides from reads prior to assembly	MacManes (2014)
khmer diginorm, Trinity normalization	*in silico* read normalization	reduce memory requirements for assembly, but can result in fragmented assemblies and collapse heterozygosity	Brown et al. (2012); Haas et al. (2013)
Trinity	*de novo* and genome-guided transcriptome assembly	accurate assembly across conditions, but requires long runtime if normalization is not used (Zhao et al. 2011)	Haas et al. (2013)
bowtie, bowtie2, STAR	read alignment to genome or transcriptome assembly	required for many downstream analyses, but bowtie is computationally intensive and all produce very large output BAM files	Langmead et al. (2009), Dobin et al. (2013)
eXpress, kallisto, RSEM, Sailfish, Salmon	estimation of transcript abundance	RSEM requires computationally intensive read mapping back to the assembly; the others are faster streaming alignment, quasi-alignment, or alignment-free algorithms	Li and Dewey (2011), Patro et al. (2015)
DESeq, DESeq2, edgeR	differential expression analysis	exhibit highest true positive and lowest false positive rates in experiments with smaller sample sizes (Schurch et al. 2015)	Anders and Huber (2010), Robinson et al. (2010), Love et al. (2014)
blast2GO, Trinotate	functional annotation of assembled transcripts	complete annotation pipelines including gene ontology and pathway enrichment analyses	Conesa et al. (2005), Haas et al. (2013)

* This is a non-exhaustive list of software that focuses on *de novo* loci assembly and genotype calling for RADseq data, as many practitioners working on NMOs will not have access to a reference genome. Other programs (e.g., GATK and ANGSD) that undertake genotype calling using reference-aligned loci are described in the whole genome sequencing section.

Cammen_SupMat_TableS1 - Marine mammal genomics - *JHered*

Table S1. Broad applications of genomic tools in studies of non-model organisms are provided with concrete examples of research areas drawn from the field of marine mammal genomics. The number of loci used in each study provides an estimate of the scope of the respective genomic tools and study, but represents the outcome of several filtering steps from raw sequence data that vary across studies. Further details of each method can be found in the listed references. Please note that this is not an exhaustive list. GBS: Genotyping by Sequencing; RADseq: restriction site-associated DNA sequencing; SNP: single nucleotide polymorphism; TSC: target sequence capture; WGS: whole genome sequencing.

Method	# loci	Research area	Reference
Evolutionary genomics: describe evolutionary history and adaptation			
Mitogenome sequencing	Mitogenome	Cetacean phylogenomics	McGowen et al. (2009)
TSC	Mitogenome	Comparison of sub-fossil and modern killer whales	Foote et al. (2013)
TSC	>30kb coding sequence	Evolution of Sirenia	Springer et al. (2015)
WGS	Whole genome	Yangtze river dolphin genome analysis	Zhou et al. (2013)
WGS	Whole genome	Minke whale genome analysis	Yim et al. (2014)
WGS	Whole genome	Bowhead whale genome analysis	Keane et al. (2015)
WGS	Whole genome	Analysis of convergent evolution in marine mammal lineages	Foote et al. (2015)
WGS	10,025 coding sequences	Positive selection in common bottlenose dolphin genome	McGowen et al. (2012)
WGS	Sensory genes	Analysis of gene loss in olfaction and taste in Antarctic minke whale	Kishida et al. (2015)
Genome re-seq	Whole genome	Speciation and adaptation in brown and polar bears	Liu et al. (2014)
Transcriptomics	9,395 genes	Evolution of longevity in bowhead whales	Seim et al. (2014)
Transcriptomics	103,077 unigenes	Osmoregulatory divergence in narrow-ridged finless porpoise	Ruan et al. (2015)
Population genomics: characterize population structure and investigate demography			
RADseq	3,281 SNPs	Killer whale ecotype divergence	Moura et al. (2014)
RADseq (GBS)	24,996 loci; 4,854 SNPs	Historical demography in Atlantic walrus	Shafer et al. (2015)
TSC	Mitogenome and 43-118 nuclear loci	Phylogeography and population genomics of cetaceans	Hancock-Hanser et al. (2013); Morin et al. (2015)
Genome re-seq	Whole genome	Demographic history, population differentiation, and ecotype divergence in killer whales	Foote et al. (2016)

Cammen_SupMat_TableS1 - Marine mammal genomics - *JHered*

Adaptation genomics: describe relationships between genomic variation and fitness			
RADseq	83,148 loci; 14,585 SNPs	Effect of inbreeding depression on parasite infection in harbor seals	Hoffman et al. (2014)
RADseq	129,494 loci; 7,431 SNPs	Common bottlenose dolphin adaptation to harmful algal blooms	Cammen et al. (2015)
Transcriptomics	11,286 contigs	Sperm whale skin cell response to hexavalent chromium	Pabuwal et al. (2013)
Transcriptomics	164,966 contigs	Physiological stress response in northern elephant seals	Khudyakov et al. (2015a; 2015b)
Develop molecular resources			
RADseq	3,595 loci	Comparison of short-beaked common dolphin and harbor porpoise	Viricel et al. (2014)
Shotgun sequencing	440,718 SNPs	SNP discovery in Northeast Atlantic common bottlenose dolphins	M. Louis (unpubl. data)
WGS	144 SNPs	SNP validation in Antarctic fur seal	Humble et al. (2016)
Transcriptomics	23,096 contigs; 144 SNPs	Gene and SNP discovery in Antarctic fur seal	Hoffman et al. (2011; 2012; 2013)
Transcriptomics & RADseq	9,000 SNPs	Development of SNP array for polar bear and demonstration of utility in population genomics	Malenfant et al. (2015)

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Genomic methods take the plunge: recent advances in high-throughput sequencing of marine mammals

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Abstract

The dramatic increase in the application of genomic techniques to non-model organisms over the past decade has yielded numerous valuable contributions to evolutionary biology and ecology, many of which would not have been possible with traditional genetic markers. We review this recent progression with a particular focus on genomic studies of marine mammals, a group of taxa that represent key macroevolutionary transitions from terrestrial to marine environments and for which available genomic resources have recently undergone notable rapid growth. Genomic studies of non-model organisms utilize an expanding range of approaches, including ~~low- and high-coverage~~ whole genome sequencing, restriction site-associated DNA sequencing, array-based ~~high-throughput~~ sequencing of single nucleotide polymorphisms and target sequence probes (e.g., exomes), and transcriptome sequencing. These approaches generate different types and quantities of data, and many can be applied with limited or no prior genomic resources, thus overcoming one traditional limitation~~s~~ of research on non-model organisms. Within marine mammals, such studies have thus far yielded significant contributions to the fields of phylogenomics and comparative genomics, as well as enabled investigations of fitness, demography, and population structure~~ in natural populations~~. Here~~,~~ we review the primary options for generating genomic data, introduce several emerging techniques, and discuss the suitability of each approach for different applications in the study of non-model organisms.

Keywords: RADseq, SNP array, target sequence capture, whole genome sequencing, RNAseq, non-model organisms

Introduction

Recent advances in sequencing technologies, coincident with dramatic declines in cost, have increasingly enabled the application of genomic sequencing in non-model systems (Ekblom and Galindo 2011; Ellegren 2014). These advances in molecular technologies have in many ways begun to blur the distinction between model and non-model organisms (Armengaud et al. 2014). Non-model organisms (NMOs) have traditionally been defined as those for which whole-organism experimental manipulation is rarely, if ever, possible due to logistical and/or ethical constraints (Ankeny and Leonelli 2011). Further, NMOs have typically been characterized by limited genomic resources, but this is becoming increasingly less so as the number of NMO reference genomes grows rapidly, for example through efforts like the Genome 10K Project (Koepfli et al. 2015). In fact, in some taxonomic orders, we are approaching the point at which all species have at least one representative reference genome available for a closely related species (Fig 1).

Despite the limitations of working with NMOs, including potentially small sample sizes, low DNA quantity, and limited information on gene function, genetic and genomic investigations of NMOs have yielded numerous valuable contributions to understanding their evolutionary biology and ecology. For the past several decades, traditional genetic markers such as microsatellites and short fragments of mitochondrial DNA (e.g., the control region) have been extensively used in molecular ecology. These markers, which typically evolve under neutral expectations, have proven useful for identifying population structure and reconstructing population demographic history (Hedrick 2000). However, the power of such studies is limited by the number of markers that can feasibly be evaluated using traditional approaches. The advent of low-cost high-throughput sequencing has led to dramatic increases in the number of neutral markers that can be evaluated, in many cases improving our power to resolve fine-scale or cryptic population structure in species with high dispersal capability (e.g., Corander et al. 2013) and improving the accuracy of estimating some (though not all) demographic parameters (Li and Jakobsson 2012; Shafer et al. 2015). Importantly, high-throughput sequencing has also further enabled genomic studies of non-neutral processes in NMOs, for example, characterizing both deleterious and adaptive variation within and across species (Stinchcombe and Hoekstra 2008;

Künstner et al. 2010). It is increasingly evident that genomic analyses of NMOs can and have provided important insights that could not be identified with traditional genetic markers.

Many molecular ecologists now face the challenge of deciding which of the broad range of genomic approaches to apply to their study systems. Here we review the primary options for generating genomic data and their relative suitability for different applications in the study of NMOs. We focus on marine mammals, which represent several mammalian clades with notably rapid growth in available genomic resources in recent years. This growth is clearly evident in both publication rate (Fig 2) and the rise in number and size of genomic sequences deposited in public resources (Fig 3). We comprehensively review the literature on marine mammal genomics, highlighting recent trends in methodology and applications, and then describe in detail the molecular approaches that are most commonly applied to studies of ~~non-model~~NMO genomics. Our hope is that this review will highlight the promise of genomics for NMOs and offer guidance to researchers considering the application of genomic techniques in their non-model study system of choice.

Why study marine mammal genomics?

Marine mammals represent key macroevolutionary transitions from terrestrial to marine environments (McGowen et al. 2014) and accordingly are an exemplary system for investigating the evolution of several morphological and physiological adaptations (Foote et al. 2015) associated with locomotion (Shen et al. 2012), sight (Meredith et al. 2013), echolocation (Parker et al. 2013; Zou and Zhang 2015), deep diving (Mirceta et al. 2013), osmoregulation (Ruan et al. 2015), and cognition (McGowen et al. 2012). Furthermore, studies of marine mammal evolution to date have characterized several unique aspects of their genome evolution that merit further investigation, including low genomic diversity and a relatively slow molecular clock, especially in cetaceans (Jackson et al. 2009; McGowen et al. 2012; Zhou et al. 2013). As many cetacean species are highly mobile with no obvious physical geographic barriers to dispersal, they provide a unique opportunity to study the role of behavior and culture in shaping population structure and genetic diversity (Riesch et al. 2012; Carroll et al. 2015; Alexander et al. 2016).~~ Finally,~~ T~~t~~hough highly mobile, many marine mammals exhibit evidence of local adaptation; for example, several species show parallel divergent morphological and behavioral adaptations to coastal and pelagic environments (Moura et al. 2013; Louis et al. 2014; Viricel and Rosel 2014). These species may be studied across ocean basins as emerging examples of ecological adaptation and speciation (Morin et al. 2010a).

Beyond their value as systems of evolutionary study, many marine mammals are also of broader interest relating to their historical and present conservation status. Many marine mammal populations share histories of dramatic decline due to hunting and other human impacts. Genomics provides a promising tool with which to expand our insights into these historical population changes, which so far primarily have been derived from archival review and traditional genetic approaches (Ruegg et al. 2013; Sremba et al. 2015). More recently, since the implementation of national and international protections, many marine mammal populations have partially or fully recovered (Magera et al. 2013), yet the conservation status of certain marine mammal populations remains of concern. Such vulnerable populations could benefit greatly from an improved understanding of their genetic diversity and evolution, especially in ways that can inform predictions of adaptive capacity to anthropogenic pressures and expand the toolkit for conservation policy (Garner et al. 2016; Taylor and Gemmell 2016).

Recent trends in marine mammal genomics

We conducted a meta-analysis of the peer-reviewed marine mammal genomics literature to evaluate trends in publication rates across research methodologies and aims. A search of the Web of Science database using the term “genom*” and one of the following terms indicating study species - “marine mammal”, “pinniped”, “seal”, “sea lion”, “sea otter”, “whale”, “dolphin”, “polar bear”, “manatee” - identified 825 records on December 11, 2015. We excluded 77% of the search results that were not directly related to genomic studies in marine mammal systems. The remaining 101 articles that were relevant to marine mammal genomics were further categorized by primary research methodology and general research aim. A subset of these articles is described briefly in Supplemental Table 1.

From the early 1990s through 2015, published literature in the field ~~has~~ shifted from an early focus on mitogenome sequencing to more sequence-intensive approaches, such as transcriptome and whole genome sequencing (Figs 2 and 4). This trajectory closely follows trends in sequencing technologies, from Sanger sequencing of short- and long-range PCR products for mitogenome sequencing (Arnason et al. 1991) and SNP discovery (Olsen et al. 2011), to high-throughput sequencing of reduced-representation genomic libraries (RRLs) that consist of selected subsets of the genome (e.g., Viricel et al. 2014), to high-throughput sequencing of whole genomes with varying levels of depth, ~~of~~ coverage, and contiguity. Today, high-throughput sequencing can be used both to generate high-~~-~~quality reference genome assemblies (Yim et al. 2014; Foote et al. 2015; Humble et al. 2016) and to re-sequence whole genomes at a population scale (Liu et al. 2014a; Foote et al. 2016). Similarly, the scale of gene expression studies has increased from quantitative real-time PCR of candidate genes (Tabuchi et al. 2006) to microarrays containing hundreds to thousands of genes (Mancia et al. 2007) and high-throughput RNAseq that evaluates hundreds of thousands of contigs across the genome (Khudyakov et al. 2015b). As the cost of high-throughput sequencing continues to decline, we anticipate an increase in studies that sequence RRLs, whole genomes, and transcriptomes in NMOs at a population scale.

Marine mammal genomic studies thus far have primarily contributed to the fields of phylogenomics and comparative genomics (Fig 2, Table S1). Several of these comparative genomics studies have aimed to improve our understanding of the mammalian transition to an aquatic lifestyle and describe the evolutionary relationships within and among marine mammals and their terrestrial relatives (McGowen et al. 2014; Foote et al. 2015). Whereas such studies require only a single representative genome per species, an emerging class of studies applying genomic techniques at a population scale enables further investigations of fitness, demography, and population structure within ~~a~~ species (Table S1). However, expanding the scale of genomic studies requires careful selection of an appropriate method for data generation and analysis~~,~~ from a growing number of approaches that are becoming available to non-model systems.

Data generation

Our review of marine mammal genomics highlights an increasing number of options for the generation and analysis of genomic data. Choosing which of these sequencing strategies to apply is a key step in any genomics study. Here~~,~~ we describe approaches that have been used successfully in order to help guide future studies of ecological, physiological, and evolutionary genomics in NMOs. Across data generation methods, we highlight approaches that can be used with limited or no prior genomic resources, overcoming one traditional challenge of genomic studies of NMOs (the need for a reference genome to which sequencing reads can be mapped). These methods produce a range in quantity and type of data output, from hundreds of SNPs to whole genome sequences, and from single individuals to population samples, reflecting the trade-off between number of samples and amount of data generated per sample.

Sample collection, storage and extraction

Prior to starting a genomic study, researchers must recognize that many recent methods for high-throughput sequencing require genetic material of much higher quality and quantity than techniques used to characterize traditional genetic markers. These more stringent sample requirements necessitate new standards for tissue sampling, storage, and DNA/RNA extraction. Ideally, samples should be collected from live or newly deceased individuals and stored at -80°C, or when this is not possible at -20°C in RNAlater, Trizol, ethanol, salt-saturated DMSO, or dry, depending on the intended application. Given the sensitivity of new sequencing methods, great care should be taken to minimize cross-contamination during sampling, as even minute amounts of genetic material from another individual can bias downstream analyses, for example variant genotyping and gene expression profiles. Choice of extraction method varies with sample type and study aim, but typically genomic methods require cleanup and treatment with RNase to yield pure extracts, whereas RNAseq methods require rigorous DNase treatment to remove genomic contamination that can bias expression results. Depending on the genomic methodology, target quantities for a final sample may range from as low as 50 ng of DNA for some RRL sequencing methods (Andrews et al. 2016) up to ~1 mg for sequencing the full set of libraries (of different insert sizes) necessary for high-quality genome assemblies (Ekblom and Wolf 2014). Most commercial RNAseq library preparation services require at least 500-1,000 ng of pure total RNA that shows minimal degradation as measured by capillary gel electrophoresis (RNA Integrity Number (RIN) ≥ 8). Samples should ideally consist of high molecular weight genetic material (with little shearing), though continuing molecular advances enable genomic sequencing even of low quantity or poor quality starting material. Extreme examples of the latter include successfully sequenced whole genomes from ancient material (e.g., Rasmussen et al. 2010;

Meyer et al. 2012; Allentoft et al. 2015), including a more than 500,000-year-old horse (Orlando et al. 2013).

Reduced-representation genome sequencing

i. RADseq

Reduced-representation sequencing methods evaluate only a small portion of the genome, allowing researchers to sequence samples from a larger number of individuals within a given budget in comparison to sequencing whole genomes. Restriction site-associated DNA sequencing (RADseq) is currently the most widely ~~-~~used RRL sequencing method for NMOs (Davey et al. 2011; Narum et al. 2013; Andrews et al. 2016). RADseq generates sequence data from short regions adjacent to restriction cut sites and therefore targets markers that are distributed relatively randomly across the genome and occur primarily in non-coding regions. This method allows simultaneous discovery and genotyping of thousands of genetic markers for virtually any species, regardless of availability of prior genomic resources. Of greatest interest are variable markers, characterized either as single SNPs or phased alleles that can be resolved from the identification of several ~~SNPs~~ variants within a single locus.

The large number of markers generated by RADseq dramatically increases genomic resolution and statistical power for addressing many ecological and evolutionary questions when compared to studies using traditional markers (Table S1). For example, heterozygosity ~~-~~fitness ~~associations~~ correlations in harbor seals (*Phoca vitulina*) were nearly fivefold higher when using 14,585 RADseq SNPs than when using 27 microsatellite loci (Hoffman et al. 2014). A recent study on the Atlantic walrus (*Odobenus rosmarus rosmarus*) using 4,854 RADseq SNPs to model demographic changes in connectivity and effective population size associated with the Last Glacial Maximum (Shafer et al. 2015) both supported and extended inferences from previous studies using traditional markers (Shafer et al. 2010; Shafer et al. 2014).

Furthermore, RADseq can provide sufficient numbers of markers across the genome to identify genomic regions influenced by natural selection~~ in some cases~~. These analyses require large numbers (thousands to tens of thousands) of markers to ensure that some markers will be in linkage disequilibrium with genomic regions under selection and to minimize false positives, particularly under non-equilibrium demographic scenarios (Narum and Hess 2011; De Mita et al. 2013; Lotterhos and Whitlock 2014). Extreme demographic shifts, as experienced by many marine mammal populations (e.g., killer whales, Foote et al. 2016), can drive shifts in allele frequencies that confound the distinction of drift and selection and make it difficult to detect genomic signatures of selection (Poh et al. 2014). Proof of concept of the application of RADseq for identifying genomic signatures of selection in wild populations was demonstrated in three-spined sticklebacks (*Gasterosteus aculeatus*), for which analyses of over 45,000 SNPs (Hohenlohe et al. 2010) identified genomic regions of known evolutionary importance associated with differences between marine and freshwater forms (Colosimo et al. 2005; Barrett et al. 2008). RADseq studies with similar aims in marine mammals have resulted in comparatively sparser sampling of SNPs (<10,000), likely due to both methodological differences and generally low genetic diversity particularly among cetaceans. Nonetheless, genomic regions associated with resistance to harmful algal blooms in common bottlenose dolphins (*Tursiops truncatus*) were identified across multiple pairwise comparisons using 7,431 RADseq SNPs (Cammen et al. 2015), and genomic regions associated with habitat use and resource specialization in killer whales (*Orcinus orca*) were identified using 3,281 RADseq SNPs (Moura et al. 2014a). Some of these RADseq SNPs associated with diet in killer whales were later also confirmed as occurring in genomic regions of high differentiation and reduced diversity consistent with a signature of selection identified in a study utilizing ~~low-coverage~~ whole genome re-sequencing (Foote et al. 2016). It will remain important for further studies of genomic signatures of selection in NMOs to carefully consider which approach~~es~~ will generate a sufficiently large number of SNPs to accurately identify the range of putatively neutral F_{ST} values (and thus outliers) given the demographic history of the population (Lotterhos and Whitlock 2014).

Numerous laboratory methods have been developed for generating RADseq data (reviewed in Andrews et al. 2016), with the most popular library preparation methods currently being the original RAD (Miller et al. 2007; Baird et al. 2008), Genotyping by Sequencing (GBS, Elshire et al. 2011; Poland et al. 2012), and double digest RAD (ddRAD, Peterson et al. 2012). All RADseq methods share the common goal of sequencing regions adjacent to restriction cut sites across the genome, but differ in technical details, such as the number and type of restriction enzymes used, the mechanisms for reducing genomic DNA fragment sizes, and the strategies for attaching sequencing adapters to the target DNA fragments. For example, both the original RAD method and GBS use a single enzyme digest, but the original RAD ~~protocol~~ method uses a rare-cutting enzyme and mechanical shearing to reduce DNA fragment size (Baird et al. 2008), whereas GBS uses a more frequent-cutting enzyme and relies on preferential PCR amplification of shorter fragments for indirect size selection (Elshire et al. 2011). These ~~types of variation~~modifications lead to differences across methods in the time and cost of library preparation, the number and lengths of loci produced, and the types of error and bias present in the resulting data. Different RADseq methods will be better suited to different research questions, study species, and research budgets, and therefore researchers embarking on a RADseq study should carefully consider the suitability of each method for their individual projects. Further details on the advantages and disadvantages of each method are described in Andrews et al. (2016).

ii. SNP arrays

An alternative high-throughput reduced-representation genotyping approach involves the use of custom arrays designed to capture and sequence targeted regions of the genome. Such array-based approaches may provide certain advantages over RADseq, including the ability to easily estimate genotyping error rates, scalability to thousands of samples, lower requirements for DNA quantity/quality and technical effort, greater comparability of markers across studies, and the ability to genotype SNPs within candidate genomic regions. However, unlike RADseq, array-based techniques require prior knowledge of the study system's genome or the genome of a closely related species, which remains unavailable for some NMOs. Furthermore, SNP arrays must take into account the potential for ascertainment bias (e.g., Malenfant et al. 2015), whereas RADseq avoids ascertainment bias by simultaneously discovering and genotyping markers.

To identify SNPs for NMO array development, researchers must rely on existing genomic resources or generate new reference sequences, in the form of whole or reduced-representation genomes or transcriptomes (Hoffman et al. 2012; Malenfant et al. 2015). When a whole genome reference assembly is available for the target species or a related species, multiplex shotgun sequencing can facilitate the rapid discovery of hundreds of thousands of SNPs for array development. This SNP discovery approach involves high-throughput sequencing of sheared genomic DNA~~, which~~ that can be sequenced at a low depth of coverage (i.e., low mean read depth across the genome) if suitable genotype likelihood-based methods (O'Rawe et al. 2015) are used to identify polymorphic sites. Thus, this approach is less restrictive in terms of DNA quality. For example, shotgun sequencing of 33 Northeast Atlantic common bottlenose dolphins, which included degraded DNA collected from stranded specimens, on one Illumina HiSeq2000 lane of 100-bp single-end sequencing identified 440,718 high-quality SNPs (M. Louis unpublished data). Such dense sampling of SNPs is essential for studies of population genomics that require a large number of markers, such as for inferences of demographic history (Gutenkunst et al. 2009; Excoffier et al. 2013; Liu and Fun 2015) and selective sweeps (Chen et al. 2010). Once a set of putative markers has been identified, hybridization probes can be designed from their flanking sequences and printed onto a SNP array. The two principal SNP genotyping platforms supporting thousands to millions of SNPs are the Illumina Infinium iSelect® and Affymetrix Axiom® arrays.

The use of SNP arrays in NMOs has thus far been somewhat limited, potentially due to low SNP validation rates (Chancerel et al. 2011; Helyar et al. 2011), issues of ascertainment bias (Albrechtsen et al. 2010; McTavish and Hillis 2015), and cost of SNP discovery. However, using both SNP data and whole genome sequence from the Antarctic fur seal (*Arctocephalus gazella*), Humble et al. (2016) recently demonstrated that careful filtering based on SNP genomic context prior to array development has the potential to substantially increase assay success rates. Further, ascertainment bias can be reduced by selecting samples for SNP discovery that span the geographic range of populations that will be target-sequenced (Morin et al. 2004). By accounting for ascertainment bias, Malenfant et al. (2015) were able to demonstrate population structure in Canadian polar bears (*Ursus maritimus*) more clearly using a 9K SNP array than 24 microsatellite markers.

iii. Target sequence capture

Target sequence capture (TSC, also called target enrichment, direct selection, or Hyb-seq) has many of the same advantages and disadvantages as the array-based SNP approaches described above, but differs in library preparation, sequencing platform, and resulting sequence data. While SNP arrays genotype single variable positions, TSC can be used to sequence selected short fragments. With TSC, researchers can amplify and sequence up to a million target probes on solid-state arrays, and even more if in-solution arrays are used. This gives the user the ability to choose to sequence many samples in parallel (Cummings et al. 2010), as many as 100-150 per Illumina HiSeq lane, or to sequence many regions per individual. Recent advances in target enrichment, such as genotyping in thousands (Campbell et al. 2015), anchored hybrid enrichment (Lemmon et al. 2012), and target capture of ultra~~-~~conserved elements (UCEs, Faircloth et al. 2012; McCormack et al. 2012), have further increased the number of regions and individuals that can be sampled in a single lane. In addition, UCEs overcome the need for a reference genome, enabling their wide application across many NMOs (though designing custom probe sets from closely related species will remain preferable in many cases (Hancock-Hanser et al. 2013)). Although a number of methodological variants have been developed and optimized (Bashiardes et al. 2005; Noonan et al. 2006; Hodges et al. 2009; Cummings et al. 2010; Mamanova et al. 2010; Hancock-Hanser et al. 2013), TSC generally relies on hybridization and amplification of specially prepared libraries consisting of fragmented genomic DNA. Many companies offer kits for TSC, such as Agilent (SureSelect) and MYcroarray (MYbaits), with MYcroarray specifically marketing their kits for use with NMOs.

The most common use of TSC has been the capture of whole exomes in model organisms, including humans (Ng et al. 2009). However, as costs have plummeted, TSC is increasingly being used in investigations of NMOs. TSC is particularly useful in sequencing ancient DNA, where it can enrich the sample for endogenous DNA content relative to exogenous DNA (i.e., contamination) and thereby increase the relative DNA yield (Ávila-Arcos et al. 2011; Enk et al. 2014). For example, TSC has been used to generate mitogenome sequences from subfossil killer whale specimens originating from the mid-Holocene~~,~~ for comparison with modern lineages (Foote et al. 2013). TSC was also recently utilized to compare >30 kb of exonic sequence from museum specimens of the extinct Steller’s sea cow (*Hydrodamalis gigas*) and a modern dugong (*Dugong dugon*) specimen to investigate evolution within Sirenia (Springer et al. 2015). Springer et al. (2016) further used TSC to examine gene evolution related to dentition across edentulous mammals, including mysticetes. Finally, TSC of both exonic and intronic regions has been used to assess genetic divergence across cetacean species (Hancock-Hanser et al. 2013; Morin et al. 2015). These studies show the potential use of TSC across evolutionary time~~-~~scales for population genomics, phylogenomics, and studies of selection and gene loss across divergent lineages (Table S1).

Whole genome sequencing

Beyond advances enabled by the reduced-representation methods presented above, our power and resolution to elucidate evolutionary processes, including selection and demographic shifts, can be further increased by sequencing whole genomes.

i. ~~High-coverage R~~reference genome sequencing

At the time of publication, there ~~exist~~ are 12 publicly available[1] whole (or near-whole) marine mammal genomes of varying quality representing 10 families, including 7 cetaceans (Fig 1A), 3 pinnipeds (Fig 1B), the West Indian manatee (*Trichechus manatus*), and the polar bear. The first sequenced marine mammal genome was that of the common bottlenose dolphin, which was originally sequenced to ~2.5x depth of coverage using Sanger sequencing (Lindblad-Toh et al. 2011). This genome was later improved upon by adding both 454 and Illumina HiSeq data (Foote et al. 2015). Other subsequent marine mammal genomes were produced solely using Illumina sequencing and mate-paired or paired-end libraries with varied insert sizes (Miller et al. 2012; Zhou et al. 2013; Yim et al. 2014; Foote et al. 2015; Keane et al. 2015; Kishida et al. 2015; Humble et al. 2016).

Whole genome sequencing has been used to address many issues in marine mammal genome evolution, usually by comparison with other existing mammalian genomes. Biological insights discussed in the genome papers listed above include the evolution of transposons and repeat elements, gene evolution and positive selection, predicted population structure through time, SNP validation, molecular clock rates, and convergent molecular evolution (Table S1). For example, analyses of the Yangtze river dolphin (*Lipotes vexillifer*) genome confirmed that a bottleneck occurred in this species during the last period of deglaciation (Zhou et al. 2013). In addition, following upon earlier smaller-scale studies (e.g., Deméré et al. 2008; McGowen et al.

[1] These genomes are available on NCBI's online genome database or Dryad, but they have not all been published. As agreed upon in the Fort Lauderdale Convention, the community standard regarding such unpublished genomic resources is to respect the data generators' right to publish with these data first.

2008; Hayden et al. 2010), genomic analyses have confirmed the widespread decay of gene families involved in olfaction, gustation, enamelogenesis, and hair growth in some cetaceans (Yim et al. 2014; Kishida et al. 2015). Perhaps the most widespread use of whole genome studies has been the use of models of selection to detect protein-coding genes that show evidence of natural selection in specific lineages. A recent study by Foote et al. (2015) ~~has~~ extended this approach to investigate convergent positive selection among cetaceans, pinnipeds, and sirenians. This study exemplifies a trend in recent genomic studies~~, which~~ that sequence multiple genomes to address a predetermined evolutionary question, in this case, the molecular signature of aquatic adaptation.

In addition to these evolutionary insights that typically stem from a comparative genomics approach, the development of high-~~-~~quality reference genome assemblies provide an important resource that facilitates mapping of reduced-representation genomic data (see previous section) as well as ~~relatively low-coverage,~~ short-read sequencing data with relatively low depth of coverage (see following section). These data types can be generated at relatively low cost on larger sample sizes enabling population-~~-~~scale genomic studies. In many cases, genome assemblies from closely related species are sufficient for use as a reference. Particularly among marine mammals, given their generally slow rate of nucleotide divergence, it is therefore likely unnecessary to sequence a high-~~-~~quality reference genome assembly for every species. Instead, resources could be allocated toward population-~~-~~scale studies, including ~~low-coverage~~ genome re-sequencing efforts.

ii. Population-level ~~low-coverage~~ genome re-sequencing

In contrast to ~~high-coverage~~reference genome sequencing that today often exceeds 100x mean read ~~coverage~~ depth and typically combines long- and short-insert libraries to generate high-quality assemblies for one to a few individuals, ~~low-coverage~~ genome re-sequencing studies ~~capitalize on existing reference assemblies and~~ aim to achieve only ≥2x ~~coverage~~ mean read depth on tens to hundreds of individuals from short-insert libraries ~~which~~ that ~~are then~~whose reads are anchored to ~~the~~ existing reference assemblies~~y~~. ~~Given~~ Despite the inherent trade-offs between cost, read depth, coverage, and sample size, ~~low-coverage~~ genome re-sequencing of large numbers of individuals for population-level inference can be conducted at a relatively low cost. In the past five years, several influential studies have used genome re-sequencing to advance our understanding of the genomic underpinnings of different biological questions in model systems. For example, population genomics of *Heliconius* butterflies highlighted the exchange of genes between species that exhibit convergent wing patterns (The *Heliconius* Genome Consortium 2012); whole genome re-sequencing of three-spined sticklebacks highlighted the re-use of alleles in replicated divergences associated with ecological speciation and local adaptation (Jones et al. 2012); and combined population genomics and phylogenomics have identified regions of the genome associated with variation in beak shape and size in Darwin's finches (Lamichhaney et al. 2015).

To date only two marine mammal population genomics studies using whole genome re-sequencing have been published. These studies involved re-sequencing the genomes of 79 individuals from three populations of polar bears (Liu et al. 2014a) and 48 individuals from five evolutionarily divergent ecotypes of killer whale (Foote et al. 2016). The findings of Foote et al. (2016) confirmed results of population differentiation that had previously been established using traditional genetic markers (Morin et al. 2010a). However, the study also provided new insights into the demographic history, patterns of selection associated with ecological niche, and evidence of episodic ancestral admixture that could not have been obtained using traditional markers.

Several new resources have made such population genomic studies economically possible for a greater number of NMOs, including the availability of~~ a~~ reference genome assemblies~~y~~ (see section above), relatively low-cost high-throughput sequencing (further increases in throughput expected with the new Illumina HiSeq X Ten (van Dijk et al. 2014)), and crucially, the development of likelihood-based methods that allow estimation of population genetic metrics from ~~low-coverage ~~re-sequencing data (Fumagalli et al. 2013; O'Rawe et al. 2015). One last consideration is the ease of laboratory methods necessary to generate whole genome re-sequencing data when compared to other methods such as RADseq or TSC. DNA simply needs to be extracted from the samples and, using proprietary kits, built into individually index-amplified libraries~~ using proprietary kits, which~~ that are ~~then ~~equimolarly pooled and submitted for sequencing.

Many population genomic analyses are based on the coalescent model that gains most information from the number of independent genetic markers, not the number of individuals sampled. Sample sizes of ~10 individuals are usually considered sufficient (Robinson et al. 2014) and have been standard in many genome-wide studies in the eco-evolutionary sciences (Ellegren et al. 2012; Jones et al. 2012). Thus, sampling fewer individuals ~~at lower coverage but for orders of magnitude more data~~by whole genome re-sequencing is a salient approach~~, which~~ that allows us to consider many more gene trees, whilst continuing to provide robust estimates of per-site genetic metrics (e.g., F_{ST}). The robustness of inference from ~~low-coverage~~ data with low mean read depth across the genome was recently confirmed using a comparison of per-site F_{ST} estimates for the same sites from high~~- coverage~~depth (≥20x) RADseq data and low-~~coverage~~depth (≈2x) whole genome re-sequencing data in pairwise comparisons between the same two killer whale ecotypes (Foote et al. 2016).

Beyond the increased power afforded by sequencing more polymorphic sites, whole genome re-sequencing also allows inference of demographic history from the genome of even just a single individual by identifying Identical By Descent (IBD) segments and runs of homozygosity (Li and Durbin 2011; Harris and Nielsen 2013). For example, Liu et al. (2014a) found evidence for ongoing gene flow from polar bears into brown bears after the two species initially diverged. Genome re-sequencing of sufficient numbers of individuals also facilitates haplotype phasing, which has many applications, including the detection of ongoing selective sweeps (Ferrer-Admetlla et al. 2014) and the inference of demographic history of multiple populations based on coalescence of pairs of haplotypes in different individuals (Schiffels and Durbin 2014). However, haplotype phasing ~~has~~ typically require~~s~~d genomic ~~higher coverage~~ data with higher mean read depth (~20x) from tens of individuals (though recent advances in genotype imputation suggest success with ~~lower coverage~~ data of lower mean read depth (VanRaden et al. 2015)). Thus far, phasing has been restricted to relatively few NMO studies, and no marine mammal studies to the best of our knowledge.

Transcriptome sequencing

In comparison with the DNA-based genomic approaches described above, RNA-based genomic approaches are a relatively new and emerging application in NMOs such as marine mammals.

Transcriptomics by RNA sequencing (RNAseq) can rapidly generate vast amounts of information regarding genes and gene expression without any prior genomic resources. This approach can resolve differences in global gene expression patterns between populations, individuals, tissues, cells, and physiological or environmental conditions, and can yield insights into the molecular basis of environmental adaptation and speciation in wild animals (Wolf 2013; Alvarez et al. 2015). Furthermore, RNAseq is a valuable tool for resource development, for example as a precursor to designing SNP and TSC arrays (e.g., Hoffman et al. 2012). However, applying RNAseq to NMOs requires several unique considerations in comparison to the DNA-based methods described above. Most importantly, the labile nature of gene transcription and high detection sensitivity of RNAseq have the potential to amplify transcriptional "noise" and are thus extremely sensitive to experimental design.

If the experimental goal is to capture a comprehensive transcriptome profile for a study organism, multiple tissues from individuals of varied life history stages should be sampled. However, if the aim is to characterize transcriptional responses to physiological or environmental stimuli, efforts should focus on minimizing variability in individuals and sampling conditions (Wolf 2013). For differential expression analyses, pairwise comparisons should be made within the same individual if at all possible (e.g., before and after treatment, between two developmental stages). As RNAseq only captures a 'snapshot' of gene expression in time, repeated sampling or time-course studies are necessary to obtain a more complete picture of cellular responses to the condition(s) in question (Spies and Ciaudo 2015). Sampling and sequencing depth requirements will depend on the study design. Simulation studies have shown that a minimum of 5-6 biological replicates sequenced at a depth of 10-20 million reads per sample is necessary for differential expression analysis (Liu et al. 2014b; Schurch et al. 2015). RNAseq can also be used for biomarker development to expand molecular toolkits for NMOs without sequenced genomes (Hoffman et al. 2013). In this case, higher sequencing depths of 30-60 million reads per sample are recommended for SNP discovery and genotyping (De Wit et al. 2015).

Following sequence generation, transcript annotation remains a challenge for NMOs without reference transcriptomes or genomes. *De novo* transcriptomes can be annotated through detection of assembled orthologs of highly conserved proteins, but these analyses remain limited by the quality of reference databases. As a result, NMO transcriptomes are biased in favor of highly conserved terrestrial mammal genes and therefore provide an incomplete understanding of animal adaptations to natural environments (Evans 2015). For example, while 70.0% of northern elephant seal (*Mirounga angustirostris*) skeletal muscle transcripts had BLASTx hits to mouse genes, only 54.1% of blubber transcripts could be annotated due to poor representation of this tissue in terrestrial mammal reference proteomes (Khudyakov et al. 2015b).

To date, RNAseq has been used for gene discovery and phylogenomics analyses in Antarctic fur seal (Hoffman 2011; Hoffman et al. 2013), polar bear (Miller et al. 2012), Indo-Pacific humpback dolphin (*Sousa chinensis* (Gui et al. 2013)), spotted seal (*Phoca largha* (Gao et al. 2013)), bowhead whale (*Balaena mysticetus* (Seim et al. 2014)), narrow-ridged finless porpoise (*Neophocaena asiaeorientalis* (Ruan et al. 2015)), and humpback whale (*Megaptera novaeangliae* (Tsagkogeorga et al. 2015)) (Table S1). Due to the challenges of repeated sampling of wild marine mammals, few studies have examined cetacean or pinniped transcriptome responses to environmental or experimental stimuli. The majority of such functional gene expression studies have used microarrays (Mancia et al. 2008; Mancia et al. 2012; Mancia et al. 2015); however, RNAseq has been employed to profile sperm whale (*Physeter macrocephalus*) skin cell response to hexavalent chromium (Pabuwal et al. 2013) and free-ranging northern elephant seal skeletal muscle response to an acute stress challenge (Khudyakov et al. 2015a; Khudyakov et al. 2015b). With decreasing sequencing costs and improvements in bioinformatics tools, RNAseq has the potential to accelerate molecular discoveries in marine mammal study systems and supplement existing functional genomics approaches.

Emerging techniques

In addition to the relatively proven NMO genomic data generation techniques described above, a suite of emerging techniques is entering the field, with exciting promise for exploration of existing and new research areas. For example, high-throughput shotgun sequencing is increasingly being used to identify genetic material from multiple species in a single sample (metagenomics and metatranscriptomics), rather than focus on characterizing variation in a single target individual. These multi-species approaches can be used, for example, to characterize diet from fecal samples (Deagle et al. 2009) and to investigate microbiomes (Nelson et al. 2015), objectives with implications for improving our understanding of both basic ecology and health in natural populations of NMOs. Furthermore, high-throughput sequencing of environmental DNA dramatically increases the throughput of NMO detection in environmental (e.g., seawater) samples (Thomsen et al. 2012), using degenerate primers for multi-species detection rather than requiring the design and implementation of numerous single-species protocols (Foote et al. 2012).

A second broad area of emerging interest moves beyond the study of variation at the DNA and RNA levels to examine epigenetic effects of histone modification on gene regulation and evolution. Epigenomic studies often examine changes in DNA methylation in association with processes such as cancer and ageing. Such approaches, from targeted gene to genome-wide, have only very recently and not yet frequently been applied in NMOs. Polanowski et al. (2014) used a targeted gene approach to examine changes in DNA methylation in age-associated genes, previously identified in humans and mice, in humpback whales of known age. The most informative markers were able to estimate humpback whale ages with standard deviations of approximately 3-5 years, demonstrating the potential transferability of these approaches from model to non-model organism. Villar et al. (2015) utilized a genome-wide approach – chromatin immunoprecipitation followed by high-throughput sequencing (ChIPseq) – to examine gene-regulatory element evolution across mammals, including four species of cetaceans. This study identified highly conserved gene-regulatory elements based on their histone modifications (H3K27ac and H3K4me3), showed that recently evolved enhancers were associated with genes under positive selection in marine mammals, and identified unique *Delphinus*-specific enhancers. Finally, reduced-representation epigenomic approaches have also been developed (Gu et al. 2011), and although they have not yet been used in marine mammals to our knowledge, these techniques could facilitate future studies of how changes in DNA methylation patterns affect other biological processes, such as stress levels or pregnancy.

Data analysis

Following the generation of genomic data, researchers must select the most appropriate genomic analysis (i.e., bioinformatics) pipelines, which often differ significantly from those used in traditional genetic studies of NMOs. The choice of analysis pipeline will depend on multiple factors including the availability of a reference genome, the level of diversity within the dataset (e.g., single- or multi~~-ple ~~species), the type of data generated (e.g., single~~-end vs.~~- or paired-end), and the computing resources available. The computational needs, both in terms of hardware and competency in computer science, for analysis of genomic data typically far exceed those necessary for traditional genetic markers. On the smaller end of the spectrum, one lane of 50 bp single-end sequencing on an Illumina HiSeq 2500 can produce tens of gigabytes of data, while data files associated with a single high-~~coverage ~~quality vertebrate genome may reach hundreds of gigabytes in size (Ekblom and Wolf 2014). Computing resources necessary for the analysis of these genomic datasets can range from ~10 gigabytes for a pilot study using a reduced-representation sequencing approach to over a terabyte for whole ~~-~~genome sequence assembly (Ekblom and Wolf 2014). Fortunately, university computing clusters, cloud-based (Stein 2010) and high-performance computing clusters (e.g., XSEDE; Towns et al. 2014), and open web-based platforms for genomic research (e.g., Galaxy; Goecks et al. 2010) are becoming increasingly accessible. Furthermore, new pipelines are continuously being developed and improved, and there are a growing number of resources aimed at training molecular ecologists and evolutionary biologists in computational large-scale data analysis (Andrews and Luikart 2014; Belcaid and Toonen 2015; Benestan et al. 2016). We provide ~~a limited~~an indicative list of the current, most commonly used analysis pipelines that are specific to each data generation method in ~~Supplemental ~~Table 1~~2~~. Here~~,~~ we briefly summarize current genomic data analysis pipelines and discuss considerations that are likely to be similar across multiple data generation methods.

Genomic data analysis often involves multiple steps, and the choice of analysis tool for each step can greatly affect the outcome, with different tools producing different (though usually overlapping) sets of results (e.g., Schurch et al. 2015). All analyses begin by evaluating data quality, trimming sequences if necessary to remove erroneous nucleotides (MacManes 2014), and implementing appropriate data quality filters (e.g., phred scores, read length, and/or read depth). Raw reads also need to be demultiplexed based on unique barcodes if pools of individuals were sequenced in a single lane. Analyses then usually proceed in a *de novo* or genome-enabled manner, depending on available resources. Briefly, sequences can be compared (e.g., to identify variants) by mapping all reads to a reference genome or *de novo* assembling stacks of sequences putatively derived from the same locus~~,~~ based on sequence similarity. *De novo* methods are sensitive to sequencing error, as well as true genetic variation, and therefore can erroneously assemble polymorphic sequences as separate loci or transcripts, requiring further filtering to remove redundancy. The opposite problem can also occur in both *de novo* and reference mapping approaches, where two distinct loci (e.g., paralogous loci) may assemble as a single locus or map to the same reference location. Researchers should therefore recognize the inherent trade-offs when carefully selecting their thresholds for acceptable levels of variation within and among loci.

Considerations relevant to the selection of subsequent downstream analyses are specific to the type of data generated and the research objective. For example, RADseq analysis pipelines differ in the algorithms used to genotype variants (Table 1~~S2~~). Similarly, there are several gene expression analysis pipelines for RNAseq data that compare transcript abundance between samples (Table 1~~S2~~). Analysis of TSC data usually uses standard *de novo* assemblers (e.g., Trinity, Velvet); these assemblers can be run using packages such as PHYLUCE (Faircloth 2015), which is designed specifically for use with ultraconserved elements. Unfortunately, for most analyses, there are no unifying recommendations currently available and researchers must evaluate several approaches, each with their own advantages and disadvantages, in order to select the most appropriate tool for their particular experiment and system. Furthermore, we can expect that the recommendations for analysis tools will continue to evolve as new programs become available in the future.

Guidelines for data quality control and sharing

With rapid growth in sequencing platforms and bioinformatics analysis pipelines comes the need to extend existing principles (e.g., Bonin et al. 2004) on quality control, analysis, and transparency. General recommendations for sample and data handling, library preparation, and sequencing have been discussed elsewhere (Paszkiewicz et al. 2014). We therefore focus on the need to produce guidelines on data quality evaluation and reporting for genomic data (e.g., Morin et al. 2010b). A primary challenge in this area is that quality metrics vary widely across sequencing technologies. Yet, regardless of sequencing platform, the quality of sequencing reads must be evaluated (e.g., using FastQC; Andrews 2010) and reported.

Best practices guidelines for ~~high-coverage whole~~reference genome sequencing and RNAseq data generation, analysis, and reporting are available from the human-centric ENCODE consortium (www.encodeproject.org). These include minimum depth of sequencing and number and reproducibility of biological replicates. For RNAseq experiments, evaluation of *de novo* assembly quality remains a challenge. Suggested quality metrics include percentage of raw reads mapping back to the assembly and number of assembled transcripts with homology to known proteins (MacManes 2016). Emerging tools such as Transrate (Smith-Unna et al. 2015) attempt to integrate these and other metrics into a comprehensive assembly quality score.

In contrast, there is not yet any standard way to estimate or report error rates with RADseq or ~~low-coverage~~ genome re-sequencing methods (but see Mastretta-Yanes et al. 2015; Fountain et al. 2016). Recommendations to improve confidence in genotyping include using methods that account for population--level allele frequencies when calling individual genotypes, mapping reads to reference genomes rather than *de novo* assembly (Nadeau et al. 2014; Fountain et al. 2016), filtering out PCR duplicates (Andrews et al. 2014), identifying and removing markers in possible repeat regions, and filtering data to include only those with high read depth (>10-20x per locus per individual) (Nielsen et al. 2011). Other analysis methods, such as robust Bayesian methods and likelihood--based approaches that account for read quality in calculations of posterior probabilities of genotypes and per-site allele frequencies utilizing the sample mean site frequency spectrum as a prior (Fumagalli et al. 2013), can account for uncertainty and/or error in the data, and are therefore suitable for use with low to moderate read depths (2-20x per locus; e.g., Han et al. 2015; O'Rawe et al. 2015).

Due to the large number of analysis tools that are available, data quality and reproducibility ultimately depend on methods and data transparency. All raw sequencing reads should be publicly archived, for example deposited in the NCBI Sequence Read Archive. Many journals, including the *Journal of Heredity* (Baker 2013), now also require that primary data supporting the published results and conclusions (e.g., SNP genotypes, assemblies) be publicly archived in online data repositories (e.g., Dryad). We further recommend making public the analysis pipelines, scripts (e.g., using GitHub), and additional outputs, as appropriate, in order for analyses to be fully reproducible and transparent, which is the cornerstone of the scientific method (Nosek et al. 2015).

Future directions

As demonstrated here for one group of mammalian taxa, the rapid growth of the field of non-model genomics has been both impressive and empowering. As we approach a point of relative saturation in reference genomes, we anticipate an increase in population-scale genomic studies that produce lower depth or coverage datasets per individual but across larger sample sizes ~~relative to high-coverage sequencing of a few individuals of each species~~. In addition (or alternatively), we hope to see increasing efforts to sequence reference transcriptomes and improve NMO genome annotation in ways beyond the inherently limited approach of comparison to gene lists from a few model organisms. Population-scale genomic studies will facilitate greater ecological understanding of natural populations, while efforts to improve annotation will address persistent limitations in our understanding of gene function for NMOs. Ultimately, improving our understanding of local adaptation, adaptive potential, and demographic history through the use of genomic toolkits such as those described here is likely to have important implications for the future conservation of these populations.

Advances in sequencing technologies and analytical tools will no doubt continue, in some cases drawing on established techniques in model organisms, posing both new opportunities and new challenges for researchers in NMO genomics. Likely the most persistent challenge will remain selecting the data generation and experimental design that is most appropriate for the respective research objective. Our review identified few cases that exhibit relative dominance of a single methodology and analytical pipeline (e.g., RADseq and STACKS, RNAseq and Trinity); rather, more often we found a diversity of approaches even within each category of data generation. In fact, such diversity of approaches has its benefits, with each approach promoting its own advantages (and limitations). Overall, our reflections on lessons learned from the past decade of NMO genomics in one well-studied group of mammalian taxa clearly demonstrate the value, increased ease, and future promise of applying genomic techniques across a wide range of non-model species to gain previously unavailable insights into evolution, population biology, and physiology on a genome-wide scale.

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Table 1. Current and commonly used tools for analysis of genomic data generated in non-model organisms. Please note that this list is not exhaustive and new computational tools are continuously being developed.

Computational Tool	Purpose	Strengths/Weaknesses	Reference
*RADseq**			
STACKS	quality filtering, *de novo* assembly or reference-aligned read mapping, variant genotyping	scalable (new data can be compared against existing locus catalog); flexible filtering and export options; recently implemented a gapped alignment algorithm to process insertion-deletion (indel) mutations; secondary algorithm adjusts SNP calls using population-level allele frequencies; compatible with input data from multiple RADseq methods	Catchen et al. (2011; 2013), http://catchenlab.life.illinois.edu/stacks/
PyRAD	quality filtering, *de novo* assembly, read mapping, variant genotyping	efficiently processes indel mutations, thus optimal for analysis of highly divergent species; high speed and quality of paired-end library assemblies; compatible with input data from multiple RADseq methods	Eaton (2014)
TASSEL-GBS	quality filtering, reference-aligned read mapping, variant genotyping	optimized for single-end data from large sample sizes (tens of thousands of individuals) with a reference genome; performs genome-wide association studies	Glaubitz et al. (2014)
dDocent	quality trimming, *de novo* assembly, read mapping, variant genotyping	beneficial in analysis of paired-end data; identifies both SNP and indel variants; most appropriate for ezRAD and ddRAD data	Puritz et al. (2014)
AftrRAD	quality filtering, *de novo* assembly, read mapping, variant genotyping	identifies both SNP and indel variants; computationally faster than STACKS and PyRAD	Sovic et al. (2015)
Array-based high-throughput sequencing			
Affymetrix Axiom™ Analysis Suite, Illumina® GenomeStudio	genotype scoring	visualization of genotype clusters; quality scores assigned to genotype calls allow user-specific filtering; manual editing possible	
Whole genome sequencing			
AdapterRemoval v2, Trimmomatic	trim raw sequences	remove adapter sequences and low-quality bases prior to assembly	Bolger et al. (2014), Schubert et al. (2016)
ALLPATHS-LG, PLATANUS, SOAPdenovo	*de novo* genome assembly	designed for short-read sequences of large heterozygous genomes	Li et al. (2010), Gnerre et al. (2011), Kajitani et al. (2014)
AUGUSTUS, GenomeScan, MAKER2	gene annotation	highly accurate evidence-driven or BLASTX-guided gene prediction (Yandell and Ence 2012)	Yeh et al. (2001), Stanke et al. (2006), Holt and Yandell (2011)

Bowtie, bwa	read mapping	rapid short-read alignment with compressed reference genome index, but limited number of acceptable mismatches per alignment (Flicek and Birney 2009)	Langmead et al. (2009), Li and Durbin (2009)
SAMtools	data processing, variant calling ~~(SNP and indel discovery)~~	multi-purpose tool that conducts file conversion, alignment sorting, PCR duplicate removal, and variant (SNP and indel) calling for SAM/BAM/CRAM files	Li et al. (2009)
GATK	data processing and quality control, variant calling	suitable for ~~processing and analyses of~~ data with low to high mean read depth across the genome~~coverage data~~; initially optimized for large human datasets, then modified for use with non-model organisms	McKenna et al. (2010), DePristo et al. (2011)
ANGSD/NGStools	data processing, variant calling, estimation of diversity metrics, population genomic analyses	suitable for ~~processing and analyses of~~ data with low mean read depth, including ~~coverage and~~ palaeogenomic data; allow downstream analyses such as D-statistics and SFS estimation	Fumagalli et al. (2014), Korneliussen et al. (2014)
RNAseq			
Fastx Toolkit, Trimmomatic	trim raw sequences	remove erroneous nucleotides from reads prior to assembly	MacManes (2014)
khmer diginorm, Trinity normalization	*in silico* read normalization	reduce~~s~~ memory requirements for assembly, but can result in fragmented assemblies and collapse heterozygosity	Brown et al. (2012); Haas et al. (2013)
Trinity	*de novo* and genome-guided transcriptome assembly	accurate assembly across conditions, but requires long runtime if normalization is not used (Zhao et al. 2011)	Haas et al. (2013)
bowtie, bowtie2, STAR	read alignment to genome or transcriptome assembly	required for many downstream analyses, but bowtie is computationally intensive and all produce very large output BAM files	Langmead et al. (2009), Dobin et al. (2013)
eXpress, kallisto, RSEM, Sailfish, Salmon	estimation of transcript abundance	RSEM requires computationally intensive read mapping back to the assembly; the others are faster streaming alignment, quasi-alignment, or alignment-free algorithms	Li and Dewey (2011), Patro et al. (2015)
DESeq, DESeq2, edgeR	differential expression analysis	exhibit highest true positive and lowest false positive rates in experiments with smaller sample sizes (Schurch et al. 2015)	Anders and Huber (2010), Robinson et al. (2010), Love et al. (2014)
blast2GO, Trinotate	functional annotation of assembled transcripts	complete annotation pipelines including gene ontology and pathway enrichment analyses	Conesa et al. (2005), Haas et al. (2013)

* This is a non-exhaustive list of software that ~~include~~ focuses on *de novo* loci assembly and genotype calling for RADseq data, as many practitioners working on NMOs will not have access to a reference genome. Other programs (e.g., GATK and ANGSD) that undertake genotype calling using reference-aligned loci ~~only~~ are described in the whole genome sequencing section.

Figure 1

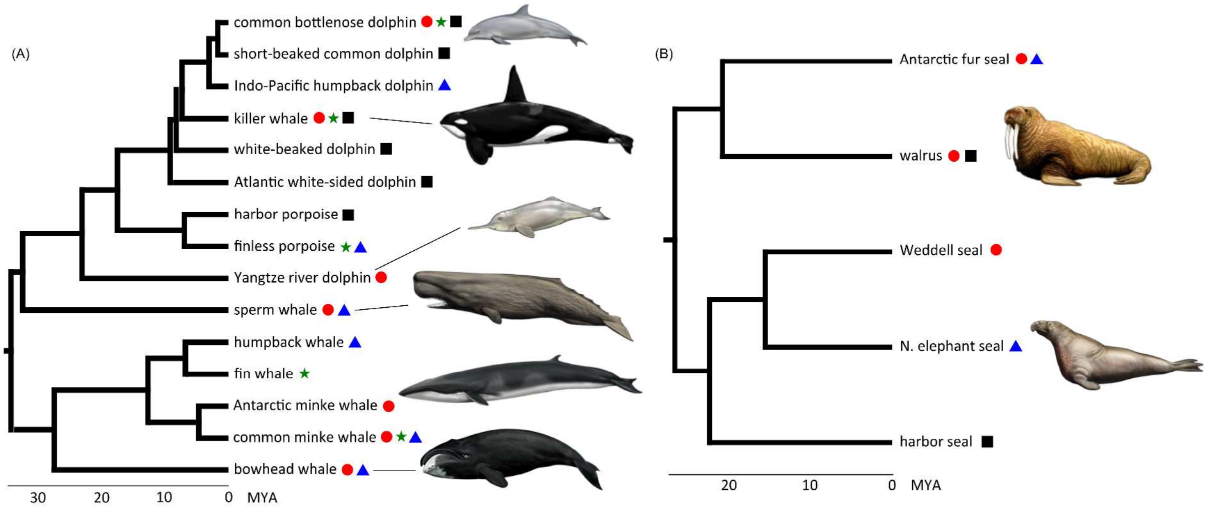


Figure 1. Phylogenetic tree showing current genomic resources available for (A) cetaceans and (B) pinnipeds; relationships and branch lengths are based on molecular dating estimates from McGowen et al. (2009), McGowen (2011), and Higdon et al. (2007). Scale is in millions of years ago (MYA). Red circles indicate species with high-~~coverage~~ quality ~~whole~~ reference genomes; green stars indicate ~~low-coverage~~ whole genome re-sequencing data; blue triangles indicate transcriptomes (generated by microarray or RNAseq); and black squares indicate RADseq data.

Figure 2

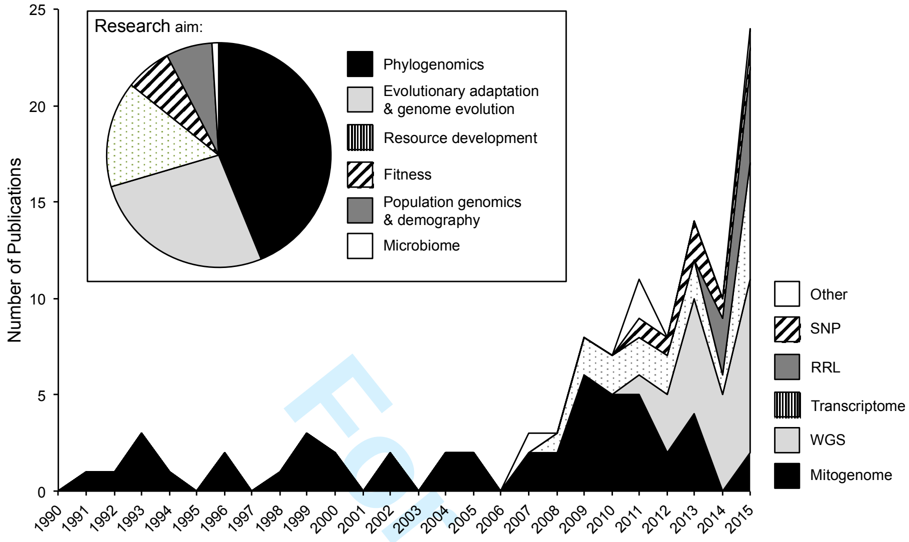


Figure 2. Number of marine mammal genomics publications from 1990 to 2015, categorized by primary methodology and research aim. Genomic methodologies include high-throughput single nucleotide polymorphism (SNP) genotyping and sequencing of mitogenomes, whole genomes (WGS), transcriptomes (generated by microarray or RNAseq), and reduced-representation genomic libraries (RRL). The "Other" category includes studies of microbiomes, BAC libraries, and large (~100) gene sets.

Figure 3

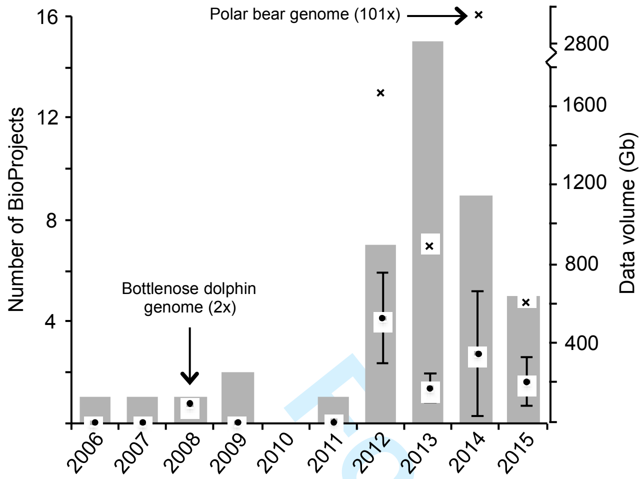
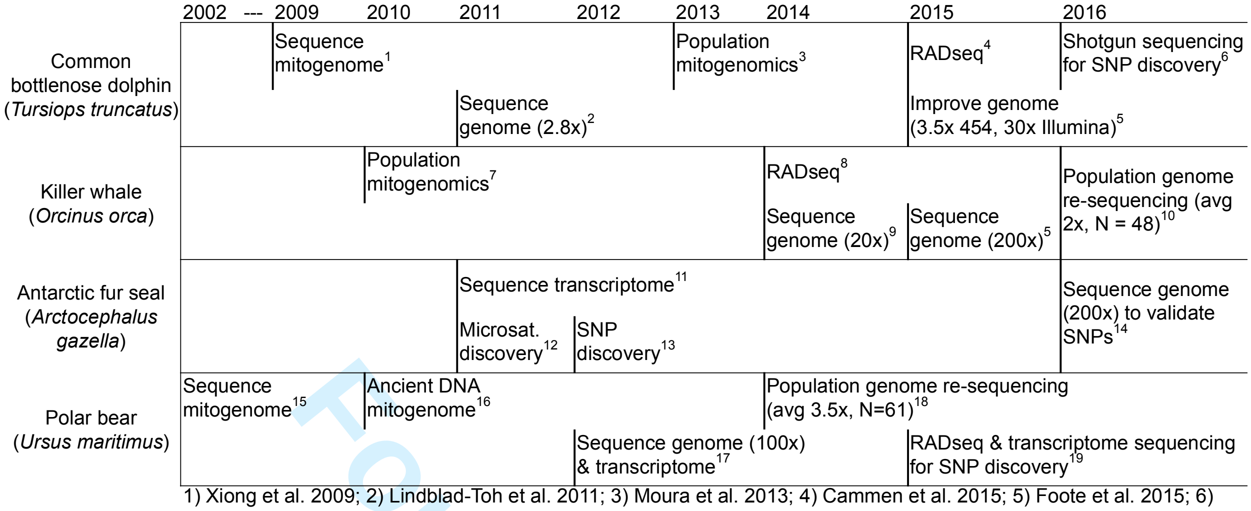


Figure 3. Number of BioProjects (~~shaded~~ gray bars) related to marine mammal genomics submitted from 2006 to 2015 to an online public database maintained by NCBI. Early BioProjects were largely microarray datasets. The number of projects created each year, as well as the yearly average (black dots ± SE) and maximum (×) size of data submitted in each BioProject, increased dramatically after 2011, reflecting advances in high-throughput sequencing technologies that facilitated their use in non-model systems.

Figure 4


2002

2009
2010
2011
2012
2013
2014
2015
2016
Common bottlenose dolphin (*Tursiops truncatus*)
Sequence mitogenome1
Population mitogenomics3
RADseq4
Shotgun sequencing for SNP discovery6
Sequence genome (2.8x)2
Improve genome (3.5x 454, 30x Illumina)5
Killer whale (*Orcinus orca*)
Population mitogenomics7
RADseq8
Sequence genome (20x)9
Sequence genome (200x)5
Population genome re-sequencing (avg 2x, N = 48)10
Antarctic fur seal (*Arctocephalus gazella*)
Sequence transcriptome11
Microsat. discovery12
SNP discovery13
Sequence genome (200x) to validate SNPs14
Polar bear (*Ursus maritimus*)
Sequence mitogenome15
Ancient DNA mitogenome16
Population genome re-sequencing (avg 3.5x, N=61)18
Sequence genome (100x) & transcriptome17
RADseq & transcriptome sequencing for SNP discovery19

1) Xiong et al. 2009; 2) Lindblad-Toh et al. 2011; 3) Moura et al. 2013; 4) Cammen et al. 2015; 5) Foote et al. 2015; 6) Louis et al. unpubl. data; 7) Morin et al. 2010; 8) Moura et al. 2014a; 9) Moura et al. 2014b; 10) Foote et al. 2016; 11) Hoffman 2011; 12) Hoffman and Nicholas 2011; 13) Hoffman et al. 2012; 14) Humble et al. 2016; 15) Arnason et al. 2002; 16) Lindqvist et al. 2010; 17) Miller et al. 2012; 18) Liu et al. 2014; 19) Malenfant et al. 2015

Figure 4. Timelines depicting the independent progression of genomic studies for four representative marine mammal species. Trajectories show the common progression for non-model species from mitogenome sequencing to whole genome sequencing, as well as from sequencing reference specimens to population-scale genomic sequencing. In addition, the timelines reveal the utility of genomic and transcriptomic sequencing for subsequent genetic marker development.