



## REVIEW

## A field ornithologist's guide to genomics: Practical considerations for ecology and conservation

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### ABSTRACT

Vast improvements in sequencing technology have made it practical to simultaneously sequence millions of nucleotides distributed across the genome, opening the door for genomic studies in virtually any species. Ornithological research stands to benefit in three substantial ways. First, genomic methods enhance our ability to parse and simultaneously analyze both neutral and non-neutral genomic regions, thus providing insight into adaptive evolution and divergence. Second, the sheer quantity of sequence data generated by current sequencing platforms allows increased precision and resolution in analyses. Third, high-throughput sequencing can benefit applications that focus on a small number of loci that are otherwise prohibitively expensive, time-consuming, and technically difficult using traditional sequencing methods. These advances have improved our ability to understand evolutionary processes like speciation and local adaptation, but they also offer many practical applications in the fields of population ecology, migration tracking, conservation planning, diet analyses, and disease ecology. This review provides a guide for field ornithologists interested in incorporating genomic approaches into their research program, with an emphasis on techniques related to ecology and conservation. We present a general overview of contemporary genomic approaches and methods, as well as important considerations when selecting a genomic technique. We also discuss research questions that are likely to benefit from utilizing high-throughput sequencing instruments, highlighting select examples from recent avian studies.

*Keywords:* adaptation, birds, conservation units, eDNA, landscape genomics, next-generation sequencing, population history, single-nucleotide polymorphisms

### Una guía de campo ornitológica de la genómica: consideraciones prácticas para ecología y conservación

#### RESUMEN

Grandes avances en la tecnología de secuenciación han hecho que sea práctico secuenciar simultáneamente millones de nucleótidos distribuidos a través del genoma, abriendo la puerta a estudios genómicos en prácticamente cualquier especie. Las investigaciones ornitológicas se benefician de tres modos sustanciales. Primero, los métodos genómicos mejoran nuestra habilidad para analizar sintácticamente y a la vez explorar las regiones genómicas tanto neutrales como no-neutrales, brindando de este modo evidencias de evolución adaptativa y divergencia. Segundo, la cantidad total de información de secuencias generada por las plataformas actuales de secuenciación permite aumentar la precisión y resolución de los análisis. Tercero, la secuenciación de alto rendimiento puede favorecer las aplicaciones que se enfocan en un pequeño número de loci que de otro modo son excesivamente caras, demandan mucho tiempo y son técnicamente difíciles usando métodos tradicionales de secuenciación. Estos avances han mejorado nuestra habilidad para entender procesos evolutivos como la especiación y la adaptación local, pero también brindan muchas aplicaciones prácticas en las disciplinas de ecología de poblaciones, seguimiento de la migración, planificación de la conservación, análisis de dieta y ecología de las enfermedades. Esta revisión brinda una guía para los ornitólogos de campo interesados en incorporar el enfoque genómico en sus programas de investigación, poniendo énfasis en las técnicas relacionadas a ecología y conservación. Presentamos una visión general de los enfoques y métodos genómicos contemporáneos, así como consideraciones importantes para el momento de seleccionar una técnica genómica. También se discuten preguntas de investigación que pueden beneficiarse de la utilización de instrumentos de secuenciación de alto rendimiento, destacando ejemplos seleccionados a partir de estudios recientes de aves.

*Palabras clave:* adaptación, ADN ambiental, aves, genómica del paisaje, historia poblacional, polimorfismos de un sólo nucleótido, secuenciación de próxima generación, unidades de conservación

The field of genomics has grown dramatically since the 1990s, driven largely by the development of new sequencing technologies born of the Human Genome Project (Collins et al. 1998, Collins and McKusick 2001) and the completion of other model genomes (Goffeau et al. 1996, Adams et al. 2000, Mouse Genome Sequencing Consortium 2002, Hillier et al. 2004). Sequencing projects can now be accomplished relatively quickly and inexpensively, making accessible the acquisition of genomic data for virtually any organism (Ellegren 2008, Schuster 2008, Glenn 2011).

Many excellent reviews have highlighted the expanded capabilities and potential advantages of genomic approaches for studies of non-model organisms. While some focus on the molecular and biochemical innovations of new sequencing technologies (Mardis 2008, Shendure and Ji 2008, Metzker 2010), others discuss how genomic approaches can expand insights in ecology and evolution (Eisen and Fraser 2003, Rokas and Abbot 2009, Rice et al. 2011, Pavey et al. 2012) as well as conservation genetics (Ryder 2005, Kohn et al. 2006, Primmer 2009, Allendorf et al. 2010, Avise 2010, Ouborg et al. 2010, Steiner et al. 2013). Additionally, several recent papers have considered the impact of new genomic techniques on ornithological research (Romanov et al. 2009, Lerner and Fleischer 2010, Kraus and Wink 2015, Toews et al. 2016), yet these have largely targeted readers with some background understanding of genetics or genomics. Here, we provide a focused overview of genomic methods and applications most relevant to avian ecology and conservation, with a specific orientation toward field ornithologists with little or no prior experience with molecular techniques.

Advancements in high-throughput sequencing technologies have the potential to move ornithological research forward in three important ways. First, whereas traditional genetic markers have typically been anonymous with respect to their position and function within the genome, sequencing at the whole-genome level provides an ability to parse and simultaneously analyze both neutral and non-neutral (i.e. affecting fitness) genomic regions, thereby providing insight into potentially adaptive genetic variation (e.g., local adaptation to different environments; Holder-egger et al. 2006, Kohn et al. 2006, Allendorf et al. 2010). Second, the sheer quantity of sequence data generated by a single run on a modern sequencing instrument enables substantial proportions of the genome to be sampled more quickly and at lower cost than has previously been feasible. In many cases, this increase in the number of loci examined (e.g.,  $10^3$ – $10^5$  single-nucleotide polymorphisms [SNPs]), in comparison to typical panels of “traditional” genetic markers (e.g., 10–20 microsatellite loci), can lead to greater precision and accuracy of population-genetic parameter estimates (for an illustrative example, see Appendix A). Third, high-

throughput sequencing can improve the efficiency of applications that focus on a small number of loci, rather than the whole genome, in many individuals or species; otherwise, such applications can be prohibitively expensive, time-consuming, and technically difficult. For example, new sequencing capabilities can be employed to simultaneously identify ecological communities from DNA in a single environmental sample (e.g., water, soil, or feces).

Avian species are likely to be particularly well suited for the new genomic approaches. Compared to other vertebrate taxa, birds have relatively small (mean  $\approx$  1.45 billion base pairs [bp]; Gregory 2005) and compact genomes (Organ et al. 2007), which reduces the effort required for whole-genome sequencing and analysis. Indeed, the complete genomes of 48 avian species, representing all extant neognath orders, were recently sequenced and published in a massive coordinated effort (Zhang et al. 2014a; for summaries, see Zhang et al. 2014b, Joseph and Buchanan 2015), with even more ambitious plans currently underway to generate draft genome sequences for taxa spanning 240 avian families (Zhang et al. 2015). Consequently, the implementation of new genomic applications for essentially all avian species will now benefit from the availability of whole-genome resources from a closely related species. Accordingly, genomic approaches have been rapidly adopted and developed within the fields of avian phylogenetic systematics, speciation, and hybridization (for reviews, see Lerner and Fleischer 2010, Kraus and Wink 2015, Toews et al. 2016). However, the application of genomic tools in conservation has been somewhat slower (Shafer et al. 2015), despite numerous potential applications in population ecology, migration tracking, conservation planning, diet analyses, and disease ecology. In our experience, this disparity is often due in part to numerous technical challenges common to avian field studies, including low or variable DNA sample quality, prohibitions on capture and invasive tissue-sampling techniques, the absence of established genomic resources for rare or threatened species, or simply the lack of experience and fluency with rapidly advancing molecular and bioinformatic techniques.

The aim of this review is to provide a practical guide for field ornithologists interested in incorporating genomics into their research program. We begin by defining “genomics” and describing the wide array of approaches and methods available, followed by a discussion of practical considerations when designing a genomic study. Next, we discuss research questions that can be addressed with existing technologies, with select examples from recent avian research. We focus on research questions derived from avian ecology and conservation, and not as much on questions purely related to evolution, yet our review will serve as a resource to any researcher interested in learning basic tools and applications in genomics. A glossary of relevant terms is provided in Appendix B.

## A Brief Genomics Primer

**Defining “genomic” approaches.** Generally speaking, the distinction between “genetic” and “genomic” approaches is, to a large extent, quantitative—genetic methods examine one or a handful of loci, whereas genomic methods typically query orders of magnitude more loci distributed across the genome. With sufficiently dense numbers of loci examined, genomic analyses are thus expected to better capture patterns of variation at the whole-genome level. The ability to carry out genomic studies in non-model systems has been greatly facilitated by the development of “second-generation” (e.g., Illumina SBS, Applied Biosystems SOLiD, Roche 454) and, more recently, “third-generation” (e.g., PacBio RS II, Oxford Nanopore) sequencing platforms that routinely generate anywhere from hundreds of thousands to several million nucleotides of sequence data per instrument run. We note, however, that the designation of “genomics” is not exclusive to projects utilizing such instruments; there are numerous examples of studies that have assayed relatively large numbers of markers using older “genetic” approaches (e.g., Hansson et al. 2012), albeit often at considerable effort and expense. Further, as discussed below, “genomic” techniques can also be particularly useful for “genetic” applications that are inherently focused on only a small fraction of the genome. Regardless, the increasing reliability, accessibility, and affordability of sequencing instruments has encouraged a proliferation of new genomic techniques. In the following, we attempt to briefly characterize the range of approaches relevant for studies of avian ecology and conservation. We note that this is not an exhaustive survey of genomic methods, but rather an introduction to currently prominent genomic approaches that are likely useful for assaying relatively large numbers of genetic markers across the genome. Recent reviews have addressed genomic applications for avian systematics and phylogenetics (McCormack et al. 2013, Kraus and Wink 2015) as well as speciation research (Toews et al. 2016); thus, methods tailored for these purposes will not be discussed in depth here.

**Spectrum of genomic approaches.** Advances in sequencing technology have greatly reduced barriers to whole-genome sequencing, yet for many applications in ecology and conservation, sequencing every nucleotide position within the genome is often neither necessary nor warranted. Consequently, the majority of genomic approaches employed today involve some form of subsampling, with the goal of capturing overall patterns of variation at the whole-genome level, but at the same time reducing the overall size, complexity, and costs of the data generated. The diversity of available genomic methods (Table 1) can thus be categorized by the proportion of, and distribution within, the genome that is represented in the final dataset.

On one end of the spectrum, reduced-representation approaches use various techniques for subsampling a fraction of positions within the genome (Good 2011), commonly exploiting the action of restriction enzymes to cut genomic DNA molecules into fragments. A proportion of those fragments are subsequently sequenced on a second-generation sequencing platform, followed by alignment of sequences to detect SNPs (for a review, see Davey et al. 2011). First detailed by Baird et al. (2008), restriction-associated DNA sequencing (RAD-Seq) and related protocols (e.g., GBS, Elshire et al. 2011; ddRAD, Peterson et al. 2012; 2b-RAD, Wang et al. 2012; RESTseq, Stolle and Moritz 2013; ezRAD, Toonen et al. 2013) have attracted the most attention for ornithological applications because of their relatively simple and inexpensive laboratory preparations and their ability to “tune” the number of markers sequenced per individual by selection of enzymes with different cut-site frequencies within the genome (thereby affecting the size and number of DNA fragments subsequently sequenced). The resulting SNP markers are presumed to be randomly distributed throughout the genome and to be mostly neutral and anonymous (unless a reference genome is available; see below).

An alternative approach to reducing genome complexity relies on the process of transcription to subsample the genome. Most notably, RNA sequencing (RNA-Seq; Wang et al. 2009) refers to techniques in which the entire population of messenger RNA (mRNA) transcripts is isolated from tissues and subjected to reverse transcription to generate complementary DNA (cDNA), which is subsequently sequenced on a high-throughput platform. The resulting transcriptome can then be assembled *de novo* (i.e. without a reference genome; e.g., Grabherr et al. 2011, Finseth and Harrison 2014), though, in most cases, alignment of the assembly to a closely related reference genome (e.g., Van Bers et al. 2012) has been shown to greatly improve the quality of resulting SNPs within it (for a review, see De Wit et al. 2015). Unlike the anonymous loci produced from RAD-Seq approaches, SNPs identified from RNA-Seq are associated with expressed genes; thus, these methods can facilitate downstream identification of genes if functional characterization of observed variation is of particular interest to the study question. Furthermore, because the number of sequence reads obtained will be proportional to the abundance of different transcripts in the tissue sampled (except in protocols involving library normalization prior to sequencing; e.g., Christodoulou et al. 2011), RNA-Seq can simultaneously provide quantitative information for gene expression analysis (Wang et al. 2009).

A third approach to genome reduction involves selective enrichment of the genomic library for particular loci of interest (for reviews, see Cosart et al. 2011, Good 2011). Briefly, so-called targeted capture methods involve the use

**TABLE 1.** Spectrum of genomic approaches relevant for avian ecology and conservation, including resource/data requirements, potential applications, relative time requirements (for sample preparation and analyses), and relative costs.

Approach	Genome Representation	Species-specific Reference Genome Required?			Bioinformatics /HPC Resource Requirements			Relative Time Requirements		Relative Costs		Applications
		no	recommended	recommended	low	moderate	high	low	high	per sample	per locus	
RAD-Seq	anonymous neutral loci	no		varies	low	low	low	low	low	low	high	population genetics, marker discovery
RNA-Seq	transcriptome	recommended		high	moderate	moderate	moderate	moderate	moderate	moderate	moderate	population genetics, expression analysis
targeted/exon capture	targeted regions, e.g., exons	recommended		varies	high	high	high	high	high	high	high	population genetics, genic variation
whole-genome (re)sequencing	whole genome(s)	yes (re-sequencing)		high	high	low	high	low	low	high	low	coalescent modeling, genome-wide association study

of custom oligonucleotide probe sets (either attached to magnetic beads in solution or printed on custom microarrays) that are complementary to the regions of interest, most often exons. After binding to the target regions in the sample DNA, captured fragments are isolated from the solution, amplified using polymerase chain reaction (PCR), then sequenced on a high-throughput instrument. Thus, targeted capture methods are closely related to amplicon sequencing, in which the PCR products of specific loci are sequenced directly on high-throughput instruments. However, whereas amplicon sequencing is typically focused on relatively small numbers of loci (e.g., 10s–100s), targeted capture experiments generally query several orders of magnitude greater numbers of loci across the genome. Like RNA-Seq, targeted capture protocols often focus sequencing effort on gene exons, thereby providing a more direct route to downstream functional characterization of genetic variation compared to anonymous markers. However, whereas RNA-Seq methods sequence the entire transcriptome, sequence capture protocols can be tailored to target a particular subset of genes of interest. As such, the custom design of capture probe sets generally benefits from a high-quality reference genome for the study species or a closely related taxon, though some advances (e.g., Bi et al. 2012) have been made in techniques for designing targeted capture experiments in non-model species without a reference genome (for a review, see Jones and Good 2016).

At the opposite end of the spectrum from reduced-representation approaches, whole-genome sequencing aims to sequence nearly every position within the nuclear and mitochondrial genomes. This is accomplished chiefly through shotgun sequencing, in which relatively short reads (e.g., 50–300 bp for Illumina SBS platforms) from across the genome are sequenced with some degree of replication, referred to as “read depth” (e.g., 5× read depth indicates that each nucleotide position in the genome is, on average, sequenced 5 times within the dataset), then bioinformatically aligned *in silico* (i.e. using computer algorithms) to reconstruct the contiguous target DNA sequence. Recent advances in high-throughput sequencing and bioinformatics have dramatically reduced the time and cost required for whole-genome sequencing and *de novo* genome assembly in non-model organisms (Ekblom and Wolf 2014, Ellegren 2014). Perhaps most significantly, the increases in sequence read length afforded by third-generation sequencing instruments (e.g., 10–15 kbp for PacBio RS II) promise to greatly improve genome quality by enabling the assembly to span highly repetitive regions, which have traditionally presented the single greatest impediment to efficient assembly using shorter-read-length (i.e. <100 bp) datasets (English et al. 2012, Huddleston et al. 2014). Additionally, recently developed novel genomic library techniques (e.g., Kuleshov et al.

2014, Putnam et al. 2016, Zheng et al. 2016) hold new promise for increased quality and sequencing performance for improved *de novo* assemblies, though the broad-scale feasibility and accessibility of such approaches remain to be established.

Birds are particularly well suited for whole-genome sequencing because of their relatively small and compact genomes (i.e. low frequency of repetitive elements, shortened introns, and intergenic distances; Organ et al. 2007), as evidenced by the rapid pace at which new avian reference genomes are currently published (Zhang et al. 2014a). Indeed, a growing number of examples have demonstrated the feasibility of whole-genome resequencing—that is, sequencing populations of genomes to evaluate intraspecific and interspecific variation at the whole-genome level—in both domesticated (e.g., Rubin et al. 2010, Shapiro et al. 2013) and wild avian systems (e.g., Poelstra et al. 2014, Burri et al. 2015, Lamichhaney et al. 2015). However, for many purposes (e.g., traditional population genetics or paternity analysis) that require neutral markers in only modest numbers (i.e. <100s to 1,000s of loci), the effort and expense for whole-genome (re)sequencing may not be warranted. In these cases, whole-genome sequencing data might be utilized for designing high-density genotyping assays such as SNP arrays (e.g., Kranis et al. 2013), which are custom DNA microarrays capable of genotyping hundreds of thousands of SNPs from multiple individuals within a single experiment, though the setup costs for such an approach will often be prohibitively expensive and require a priori knowledge of SNP allelic variation in the study species. Alternatively, whole-genome sequencing at low read depth (1–5×) may still be useful for discovery of more “traditional” genetic markers, such as microsatellites that are subsequently utilized for more economical, PCR-based population genotyping (Castoe et al. 2012, Grohme et al. 2013).

### Which Genomic Tools Should I Choose?

Despite the increased accessibility of genomic tools, adopting new genomic methods for one’s study system still represents a considerable undertaking and, for most researchers, involves nontrivial commitments of time and resources. Given the bewildering array of genomic approaches available, how should one go about selecting the most appropriate method? Below, we discuss several key practical considerations.

**Research application.** A critical consideration when selecting a genomic approach is the types of genetic inferences required for the study. For applications that require neutral anonymous loci, such as population structure analysis, inbreeding assessment, or inference of kinship, the hundreds to thousands of SNPs typically generated from a RAD-Seq experiment are likely to be

more than sufficient. However, if functional characterization of genetic variation is an important research objective, methods that focus sequencing effort on coding regions (e.g., RNA-Seq or targeted sequence capture) will be more appropriate. Targeted sequence capture offers an added aspect of flexibility in that, while typically designed to bind exonic regions, the capture probes can also be designed to include any number of noncoding sequences, limited only by the availability of reference genome resources (Bi et al. 2012). For research questions concerned with variation in genome structure (e.g., chromosomal inversions; Tuttle et al. 2016) or with detecting evidence of recent bouts of natural selection (i.e. selective sweeps), whole-genome resequencing will generally provide the greatest degree of resolution.

**Costs and time.** For most laboratories, budget and time limitations will likely constrain which genomic techniques will be feasible. For most projects, sequencing will represent the single greatest expense; thus, an important decision is how to most efficiently allocate sequencing effort (Table 1). Consequently, reduced-representation approaches such as RAD-Seq will likely be most cost-effective for studies involving large numbers of individuals (100s–1,000s), since these methods subsample the genome, resulting in relatively lower costs per individual. At the opposite end of the spectrum, whole-genome resequencing will cover a far greater proportion of positions within the genome, yielding lower costs per base sequenced but at significantly greater costs per individual included in the dataset. RNA-Seq can also provide economical genomic sequencing, though this approach generally requires more time for both library preparation and bioinformatics analysis than other reduced-representation methods. Likewise, targeted-sequence-capture experiments can be finely tuned to economize sequencing effort only on loci of interest, but they require substantial up-front resource investment for design and synthesis of capture probe sets and involve more complicated bioinformatics analyses. However, for long-term studies or those involving large numbers of samples, these setup costs could potentially be amortized over the course of the project.

**Availability of a reference genome.** For nearly all of the genomic approaches discussed above, access to a species-specific reference genome can significantly improve genotyping accuracy, bioinformatic efficiency, and functional genetic inference (Davey et al. 2011). While reduced-representation methods like RAD-Seq have gained popularity for study species that lack genome resources, the ability to align short sequence fragments to a reference genome can increase confidence in the SNPs identified (e.g., by helping to distinguish between duplicate gene sequences and polymorphisms; Ilut et al. 2014). Moreover, in cases where a reference genome with

annotated genes is available, mapping short sequence reads from reduced-representation approaches can yield information regarding genes located in the same genomic region as observed SNPs. Aligning reads to a species-specific reference genome will produce similar benefits for RNA-Seq and targeted (exon) capture protocols, though these methods may also accommodate use of a reference genome from a related taxon, given the relative high sequence conservation expected for coding regions (Jones and Good 2016). Similarly, whole-genome assembly has been demonstrated to benefit from utilizing a high-quality reference genome from a closely related species, particularly when there is insufficient sequencing read depth for efficient *de novo* assembly (Card et al. 2014, Wang et al. 2014). However, while access to a reference genome is likely to benefit a broad range of genomic techniques, the high degree of gene synteny and conserved chromosomal structure observed among bird genomes (Zhang et al. 2014b), together with the growing number of reference genomes available across all avian orders (Jarvis et al. 2014), suggests that, in many cases, researchers adopting new methods may be able to circumvent the need to generate their own species-specific reference.

**Sample quality.** Generally speaking, genomic methods tend to query significantly greater proportions of the genome than traditional molecular marker techniques (e.g., microsatellites, mitochondrial control regions) and are, consequently, more sensitive to samples with contamination (e.g., environmental DNA sources), low concentrations, and/or degraded DNA/RNA. Fortunately for field ornithologists, standard whole-blood sampling methods (e.g., brachial venipuncture and collection with capillary tubes) typically provide sufficient quantities of DNA due to the presence of nucleated red blood cells in birds. However, in situations restricted to noninvasive sampling (e.g., from shed feathers, discarded eggshells, or museum skins), sample quality and quantity may constrain the range of potential genomic methods possible and are, therefore, important considerations.

Whole-genome sequencing for *de novo* genome assembly typically involves large quantities of high-purity DNA, ideally obtained from a single individual (Ekblom and Wolf 2014). As an example, a total of ~60 µg of DNA was required for *de novo* sequencing of an estimated 1.1-gigabase-pair avian genome, consisting of sequences from 3 Illumina HiSeq and 50 PacBio single-molecule real-time (SMRT) libraries (~125× and ~25× sequencing coverage, respectively; K. P. Oh personal observation), though other sequencing strategies may require as much as 1 mg of starting DNA. It is generally possible to obtain such quantities of DNA from standard whole-blood samples of at least 100 µL, a collection amount that is typically approved by animal care and use committees for birds as small as 10 g. Furthermore, long-read technologies such as

PacBio SMRT sequencing, which are critical for creating high-quality genome assemblies with minimal gaps, require high-molecular-weight DNA (Kim et al. 2014); thus, fragmented or highly degraded samples will not be suitable for most *de novo* sequencing applications.

By definition, reduced-representation approaches such as RAD-Seq and targeted sequence-capture sample the genome in a more fragmented manner and, therefore, can typically accommodate a greater range of DNA quality than is required for whole-genome sequencing. Nevertheless, evidence suggests that high levels of sample degradation can lead to dramatic reductions in efficiency and sequence quality. Utilizing a RAD-Seq approach, Graham et al. (2015) recently demonstrated that incubating tissue samples at room temperature for 96 hr prior to DNA extraction (a scenario intended to simulate potential sample-handling conditions in the field) resulted in an average of 96.5% reduction in variable sites (SNPs) identified per individual, compared to samples that were processed immediately. However, comparatively low to moderate levels of sample neglect (24–48 hr at room temperature) showed no significant reductions in numbers of loci genotyped or accuracy of SNP calling (Graham et al. 2015). Likewise, targeted sequence-capture methods have been successfully utilized to generate high-density, genome-wide SNP markers for population genetic analyses using historical (~100 yr old) museum skins (Bi et al. 2013), which traditionally have posed a challenge for PCR-based methods because of extensive levels of DNA degradation. Interestingly, one recent approach has proposed combining aspects of both RAD-Seq and targeted capture. Briefly, *hyRAD* (Suchan et al. 2016) leverages relatively simple and inexpensive RAD-Seq techniques using high-quality DNA samples to generate libraries that, in turn, serve as probes for targeted enrichment of lower-quality DNA, thereby increasing the efficiency of RAD-Seq in degraded samples while avoiding the time and expenses associated with custom probe development. Overall, a variety of reduced-representation approaches are likely to present attractive options for laboratories analyzing samples with low concentrations or variable-quality DNA.

By contrast, RNA-Seq methods have been shown to be particularly sensitive to mRNA degradation (Gallego Romero et al. 2014), which occurs rapidly upon collection unless samples are immediately stored in a stabilizing reagent such as *RNAlater* (Qiagen, Valencia, California, USA). Even after RNA is successfully extracted, stringent laboratory protocols must be observed to avoid sample degradation from contamination by ubiquitous environmental RNases (Nagalakshmi et al. 2010). Thus, RNA-Seq and related transcriptome-based methods will likely be best suited for studies with close access to controlled laboratory facilities.

**Bioinformatics and computing resources.** In the face of continuing improvements in sequencing yield afforded by second- and third-generation sequencing instruments, access to sufficient computational resources and bioinformatics expertise will increasingly represent significant bottlenecks for many researchers. Fortunately, most commercial and university-based sequencing centers now offer some degree of bioinformatic analysis service, ranging from basic sequence processing all the way through SNP genotyping and full genome assembly. For laboratories seeking to build their own analysis capabilities, the development and sophistication of open-source bioinformatic software have largely kept pace with sequencing advancements, though the majority of packages require some basic fluency in Linux command-line operations (for an example of typical bioinformatics workflow, see Appendix C Figure 2). For RAD-Seq experiments, several analysis pipelines—such as *RADtools* (Baxter et al. 2011), *Stacks* (Catchen et al. 2013), *dDocent* (Puritz et al. 2014), *PyRAD* (Eaton 2014), and *TASSEL-GBS* (Glaubitz et al. 2014)—have attracted particular attention because of their user-friendly interfaces and adaptability to various protocols. There are also a number of both commercial (e.g., *CLC Genomics Workbench*, Qiagen) and open-source bioinformatic packages (e.g., *Galaxy*, <https://galaxyproject.org>; Giardine et al. 2005) that offer intuitive graphical interfaces and consolidated analysis pipelines.

Although the computing resources required for efficient analysis of genomic datasets may often exceed the capacity of typical consumer-oriented desktop computers, a growing number of institutions and sequencing facilities offer their users remote access to high-performance computing environments, and recent efforts have explored the utility of cloud computing for intensive bioinformatic analyses (Schatz et al. 2010). These are likely to be attractive options for many ecologists and conservation biologists who only occasionally require analysis capabilities. For laboratories that anticipate longer-term needs, purchase of a dedicated bioinformatics workstation may be warranted. The recommended specifications for such a system will vary according to the particular application, but there are some general guidelines that should be considered. First, it is increasingly common for bioinformatic programs to incorporate some degree of parallel processing, in which large computational jobs are split into smaller tasks that can be simultaneously analyzed, substantially reducing analysis times. Thus, computers with greater numbers of central processing units (CPUs) and/or multi-core CPUs will often be advantageous. Additionally, manipulation of sequence data is often memory intensive, so relatively large amounts of RAM are generally favored. Finally, raw sequencing data-files from high-throughput sequencing tend to be relatively large (e.g., approximately 200–500 gigabytes, uncompressed, for a single lane of paired-end

sequencing from an Illumina HiSeq 2500) and, thus, require considerable hard disk space for storage, preferably with some degree of redundancy (i.e. RAID storage) for proper archiving. With these parameters in mind, specifications for a suitable workstation may range from relatively modest (e.g., 8 processors, 64 gigabytes of memory, 2 terabytes of hard disk storage) for small projects (e.g., a single RAD-Seq experiment) to considerably more powerful systems (e.g., 32 processors, 512 gigabytes of RAM, and 10 terabytes of disk storage) for more resource-intensive analyses (e.g., *de novo* assembly, whole-genome resequencing). Ultimately, selecting an appropriate configuration will depend on multiple factors, including the relative importance of analysis speed vs. hardware costs, the long-term research direction, and the level of bioinformatic expertise available.

### Ornithological Genomic Applications

The advent of genetic techniques provided biologists an ability to ask previously intractable questions about the evolution and demography of natural populations. Genomic approaches allow researchers to query the entire genome and, for that reason, have the potential to enhance and expand on research topics in a number of respects. Below, we highlight several promising ways in which genomics can be applied to questions in avian ecology and conservation.

**Identifying adaptive genetic variation.** Understanding the genetic basis of adaptation is a common goal for many studies in population biology. Moreover, identifying populations that are genetically distinct as a consequence of adaptive evolution, for instance due to divergent natural selection across distinct climates or habitats (e.g., Manthey and Moyle 2015), has become a key consideration for conservation practices. One of the primary advances offered by genomic approaches is the ability to examine variation across nearly the entire genome and potentially detect regions that are subject to natural or sexual selection (Allendorf et al. 2010, Manel et al. 2010, Oleksyk et al. 2010). A number of methods have been developed to identify genomic targets of selection using high-density SNP datasets (e.g., generated from a RAD-Seq experiment). Here, we briefly detail 4 of the most common strategies that are typically applied to intraspecific field studies on birds, which involve performing (1) within-population scans of genetic variation, (2) interpopulation outlier tests, (3) phenotype–genotype correlations, and (4) environmental association analyses. On their own, none of these methods can conclusively demonstrate that a locus is involved with adaptive variation. Nevertheless, they can be a useful first step toward identifying candidate genes—especially in wild populations, which pose challenges for many traditional methods of identifying the molecular basis of adaptation (e.g., quantitative-trait-locus experi-

ments; but see Slate et al. 2010). If promising candidate genes are identified using one method, other approaches can be used to further test for a signature of selection and corroborate the findings of the initial analysis (De Mita et al. 2013).

One general strategy for detecting targets of selection involves the evaluation of classical population-genetic parameters, originally developed for within-population analysis of one or a handful of loci, with genomic datasets (for a review, see Oleksyk et al. 2010). These methods often employ a “sliding window” approach, in which the parameter of interest is calculated within a predefined interval (“window”), which is iteratively recalculated across the entire genomic dataset (e.g., Rubin et al. 2010). Windows containing loci that have been subject to recent positive selection (i.e. a “selective sweep”) are expected to exhibit characteristic “signatures” of selection compared to nonselected regions, including reduced heterozygosity (Oleksyk et al. 2008), an abundance of rare allelic variants (i.e. Tajima’s *D*-test; Tajima 1989, Nielsen et al. 2005), and extended genetic linkage disequilibrium surrounding the target locus (e.g., Sabeti et al. 2002). However, in practice, the reliability of these tests is often limited by the confounding effects of demographic processes such as changing population sizes; thus, the practicality for studies of wild populations remains questionable.

More commonly, between-population comparisons involving outlier tests can provide an initial step for field-based studies seeking to identify regions of the genome under selection. This approach focuses on among-group differences and applies methods aimed at differentiating between (1) neutral loci, defined as loci for which all genotypes have the same fitness; and (2) outlier loci, which exhibit a significant departure from background, genome-wide levels of divergence (i.e. are statistically deviated from a model of natural evolution; Beaumont and Nichols 1996, Beaumont and Balding 2004, Gompert and Buerkle 2011, Whitlock and Lotterhos 2015). Under ideal conditions, these tests have the potential to identify loci under selection with a minimal false-positive rate (Gompert and Buerkle 2011). However, they have limited power when selection is weak, when only a single population is subject to divergent selection, or when background divergence is low or high (which makes it difficult to detect loci under balancing and divergent selection, respectively; Gompert and Buerkle 2011, Narum and Hess 2011). Cautious interpretation is also required because genome-wide variation in patterns of divergence could be partially attributable to variation in recombination rates, rather than variation in the mode or strength of selection (Cruickshank and Hahn 2014). This method can be applied to SNP datasets generated through any number of methods, including (but not limited to) RAD-Seq and whole-genome resequencing. It has been used widely in



both avian and non-avian taxa to identify outlier loci at the intraspecific level (i.e. among populations or subspecies; Moen et al. 2008, Nielsen et al. 2009, Prunier et al. 2011, Haynes and Latch 2012, Limborg et al. 2012, Delmore et al. 2015, Wenzel et al. 2015) or the interspecific level (i.e. among closely related taxa; Backström et al. 2010, Lavretsky et al. 2015).

Another option, genome-wide association studies (GWAS), involves scanning a dense panel of SNPs to detect regions of the genome that are correlated with fitness-related phenotypic traits (Hirschhorn and Daly 2005, Marchini et al. 2007, Svishcheva et al. 2012, Schielzeth and Husby 2014). This approach was originally developed to pinpoint the genetic basis of disease in humans, but it can also be applied to non-model organisms to study phenotypic traits of interest (e.g., Johnston et al. 2011, Hecht et al. 2013). For instance, a series of studies recently identified multiple candidate genomic regions that may underlie variation in clutch size in Collared Flycatchers (*Ficedula albicollis*). Ellegren et al. (2012) sequenced the genome of one *F. albicollis* at 85× coverage and conducted population whole-genome resequencing of 9 *F. albicollis* and 10 *F. hypoleuca* (a close relative, the Pied Flycatcher) at 6× coverage; the resulting genomic data were used to identify 13 million variable sites in the genome of *Ficedula*. Kawakami et al. (2014) then developed a custom chip to efficiently genotype 45,138 SNPs, focusing on loci that were variable in Collared Flycatchers. This enabled Husby et al. (2015) to conduct a GWAS by genotyping SNPs in 313 females for which data were available on clutch size (from a long-term study population) and testing for an association between variation at SNP loci and variation in clutch size. They identified 3 SNP sites that were significant predictors of variation in clutch size, work that will form the foundation of future efforts to identify candidate genes and understand the functional consequences of variation in those regions of the genome.

Another suite of methods seeks to identify adaptive loci by testing for associations between genomic variation and environmental variables (e.g., temperature, elevation, habitat type; Joost et al. 2007, Manel et al. 2010, Frichot et al. 2013, Guillot et al. 2014). These methods hinge on the availability of relevant environmental data (Manel et al. 2010), and they can be especially powerful when environmental variation is decoupled, at least somewhat, from patterns of neutral genomic divergence (De Mita et al. 2013). One drawback, however, is that environmental variables are often correlated with one another, so it can be challenging to pinpoint the most important environmental driver (Joost et al. 2007). Nevertheless, these methods can be a useful first step toward understanding the molecular basis of local adaptation to variation in environmental conditions (e.g., Eckert et al. 2009, Narum et al. 2010). For

example, Manthey and Moyle (2015) applied 2 different methods—latent factor mixed modeling (Frichot et al. 2013) and a Bayesian model implemented by the software BAYENV2 (Günther and Coop 2013)—to identify SNP sites that covaried with climatic variables in White-breasted Nuthatches (*Sitta carolinensis*).

**Landscape genomics.** In addition to identifying and understanding patterns of local adaptation, genomic data can expand our understanding of how environmental conditions and landscape features influence dispersal (i.e. gene flow)—and, hence, the degree to which populations are genetically isolated from one another (Fraser and Bernatchez 2001, Manel et al. 2003, Segelbacher et al. 2010). Fewer genomic resources are typically required for these types of studies (e.g., compared to GWAS). A SNP dataset consisting of hundreds to a few thousand unlinked loci is usually sufficient, and a reference genome is helpful but not required. Numerous methods are available to visualize and test for spatial variation in genomic data. For example, individuals can be assigned to population groupings using the Bayesian clustering algorithm fast-STRUCTURE (Raj et al. 2014), and spatial genomic variation can be summarized using spatial principal component analysis (Jombart et al. 2008). These methods have the potential for identifying finer-scale genomic structure than was previously detectable using genetic methods, given the increased number of loci available in genomic studies (e.g., Vincent et al. 2013, De Kort et al. 2014). One example of this increased power in an avian system comes from a recent genomic study of Corsican Blue Tits (*Cyanistes caeruleus*): Szulkin et al. (2016) detected restricted gene flow between 2 habitat types located <6 km from one another. Birds in those 2 habitat types were previously found to exhibit divergent life-history and morphological characteristics (Blondel et al. 1999, 2006), but neutral genetic analyses (based on microsatellite data) lacked the power to detect genetic differences at the same fine spatial scale (Porlier et al. 2012).

**Migration ecology.** Practical methods for reliably tracking the movements of migratory birds and linking breeding and nonbreeding populations (i.e. migratory connectivity) have long presented a major challenge for ornithologists. While there have been considerable advances in the development of tracking technologies (e.g., light-level geolocators; Stutchbury et al. 2009), many of those approaches still require that birds fitted with tracking devices can be recaptured at a later date to retrieve the data. However, when different populations have distinguishing genetic compositions, DNA collected from migrating birds can be used to assign individuals to a source population. For this application, studies have largely relied on a small number of genetic markers (e.g., mitochondrial DNA [mtDNA] haplotypes, microsatellites),

often in combination with other geographically variable markers like stable isotopes (Kelly et al. 2005, Boulet et al. 2006); but for most species, these markers offered limited geographic resolution. As previously discussed, newer sequencing methods have the potential to provide finer resolution, not only because of the greater power afforded by large numbers of loci, but also because of the added benefit of incorporating loci subject to divergent selection (Allendorf et al. 2010).

One drawback of this approach is that it requires a priori knowledge of population genomic structure across a species' breeding range. Unlike tracking with stable isotopes in feathers, where the same isotopic map can be applied to different species (Hobson and Wassenaar 1997, Bowen et al. 2005), a species-specific map of spatial genomic variation is required for application to migration tracking. This may not be possible for some species because of logistical and/or resource constraints associated with sampling and genotyping. It is also worth noting that, even with a broad spatial sampling, this approach would have limited utility for species that exhibit little or no population genomic structure. Kraus et al. (2013) analyzed genomic variation in Mallards (*Anas platyrhynchos*) at 363 SNPs across the species' circumpolar range and found a complete lack of population structure, suggesting that this species would not be a suitable candidate for genomic-based migration tracking.

The utility of this approach has been demonstrated in at least one study to date: Ruegg et al. (2014) applied genomic data to map the migratory movements of Wilson's Warblers (*Cardellina pusilla*). Previous studies had used mtDNA (Kimura et al. 2002), microsatellites (Clegg et al. 2003), and amplified fragment length polymorphisms (Irwin et al. 2011) in attempts to unravel migratory connectivity in this Nearctic–Neotropical migrant, but they were only able to resolve 2 clades on the western and eastern sides of the species' breeding range. Ruegg et al. (2014) developed a finer map of population structure using genomic data. First, they performed RAD-Seq on samples from 22 individuals from a range of breeding locations and identified 96 highly divergent SNP loci. Next, they developed a SNP assay for those 96 diagnostic markers and genotyped 1,626 individuals sampled during different stages throughout the annual migratory cycle. This resulted in a detailed map of genomic variation across the species' breeding range, which was utilized to assign individuals sampled throughout the year to a broadly defined breeding population (e.g., northwestern North America). Future work is needed to improve the resolution of spatial variation using samples from more regions, but this study provides a useful example of the potential application of genomic data for tracking the year-round movements of migratory birds.

**Population demography and history.** Genomic analysis can also be applied to understanding population demography and history. Numerous methods have been developed for microsatellite data that allow researchers to (1) quantify genetic diversity (e.g., Aparicio et al. 2006), (2) estimate effective population size (e.g., Tallmon et al. 2008, Waples and Do 2010), and (3) test for evidence of historical population bottlenecks (e.g., Cornuet and Luikart 1996, Luikart et al. 1999, Garza and Williamson 2001). Genomic methods can improve the accuracy and precision of those types of analyses by generating data at a greater number of loci (Luikart et al. 2003, Allendorf et al. 2010). Genome-wide SNP data are now relatively easy to ascertain for this purpose through reduced-representation approaches, as demonstrated in studies on Greater Sage-Grouse (*Centrocercus urophasianus*), Gunnison Sage-Grouse (*C. minimus*), and Plain Xenops (*Xenops minutus*) (Harvey and Brumfield 2015, Oyler-McCance et al. 2015a). Genomic data for these analyses also can be obtained through targeted capture (Bi et al. 2013), which may be particularly useful for analyzing museum specimens and other samples that suffer from lower DNA quality. To date, only a few avian studies have examined historical genomic information using museum specimens (Besnard et al. 2015, Parks et al. 2015, McCormack et al. 2016).

An alternative approach for inferring historical population trends involves the examination of sequence data across the genome of one or more individuals (Li and Durbin 2011, Parks et al. 2015). Such data can be generated with reduced-representation, targeted-capture, or whole-genome sequencing or resequencing methods. This approach is based on coalescent theory, which seeks to model the evolution of observed genetic variation retrospectively (backward through time), based on a set of basic population-genetic parameters, including effective population size. Although this can be accomplished with a small number of loci, statistical resolution is greatly improved by access to high-quality genomic sequence data (Li and Durbin 2011, Schiffels and Durbin 2014). Several recent examples utilizing whole-genome data highlight this potentially powerful method for examining changes in population size over long timescales for species of conservation concern (e.g., Cho et al. 2013, Zhao et al. 2013, McManus et al. 2015). Examples from avian systems include a study by Zhan et al. (2013), which compared historical population trends in the Peregrine Falcon (*Falco peregrinus*) and Saker Falcon (*F. cherrug*) and showed that both species underwent severe population bottlenecks followed by expansion. Unlike the Saker Falcon, the Peregrine Falcon has undergone a second, more recent bottleneck potentially related to habitat loss driven by climate change (Zhan et al. 2013). Similar analyses have been completed for the Adélie Penguin (*Pygoscelis adeliae*), Emperor Penguin (*Aptenodytes forsteri*), Scarlet

Macaw (*Ara macao*), and Northern Bobwhite (*Colinus virginianus*) (Halley et al. 2014, Li et al. 2014).

**Delineating conservation units.** Population genomic data are useful for delineating intraspecific conservation units. One of the most commonly employed designations of intraspecific diversity is the evolutionarily significant unit (ESU), which is an important tool in conservation because it helps guide management efforts and, in many jurisdictions, legal protection at the intraspecific level (Waples 1995). The concept was originally framed around the goal of identifying intraspecific units that were evolutionarily independent and adaptively divergent (Ryder 1986, Waples 1995). However, with the ready availability of genetic data in the 1990s, there was a shift toward delineating ESUs solely on the basis of neutral genetic divergence (Moritz 1994). More recently, conservation biologists have argued for a reversion to the original ESU definition and, hence, for a greater emphasis on adaptive differences between populations (Crandall et al. 2000, Fraser and Bernatchez 2001, Rader et al. 2005). However, one of the main obstacles to applying that ESU concept has been a lack of data on adaptive divergence in non-model organisms. Advances in genomic technology have the potential to change that, because we now have an unprecedented ability to simultaneously examine evolutionary independence and adaptive divergence in non-model organisms using data from both neutral and adaptive regions of the genome (Funk et al. 2012). Data for this purpose could be acquired using any number of methods, including RAD-Seq, whole-genome sequencing, and targeted capture. Ultimately, the use of genomic data to delineate ESUs will not only be helpful for conserving distinct populations, but could also contribute to the design of management strategies aimed at limiting or facilitating movement between populations. For instance, knowledge of adaptive genetic distinctiveness would give managers an ability to design translocations that conserve adaptations to local environmental conditions (Storfer 1999). This application has not been applied in birds, but there are several good examples in the fisheries literature (Coleman et al. 2013, Lemay et al. 2013, Hemmer-Hansen et al. 2014, Larson et al. 2014).

**Physiological responses to stress.** Transcriptome sequencing, notably RNA-Seq, promises to greatly improve the ability of researchers to understand physiological responses to biotic and abiotic stressors, both naturally occurring (e.g., seasonal thermal changes; Stager et al. 2015) and those of anthropogenic origin (e.g., environmental toxins; Schwartz and Bronikowski 2013). By providing measures of relative changes in gene expression in response to exposure to stressors, these analyses not only yield insights into the molecular basis of these responses, but may also serve as biological indicators for monitoring ecosystem health (Isaksson 2015). Thermal

stress has been examined in this way for a number of aquatic species (Kenkel et al. 2013, Smith et al. 2013, Gleason and Burton 2015) that are amenable to experimental manipulation. To date, however, most avian studies that have utilized transcriptome profiling in response to stress have focused on domesticated poultry (e.g., Li et al. 2011); its utility for studies of wild avian study systems has yet to be fully realized.

**Captive breeding.** Genomic techniques may also hold promise for supporting captive-breeding programs for imperiled species, which are often established with the goal of not only protecting the remaining individuals but also bolstering genetic diversity and fitness through selective breeding. In the past, conservation biologists have relied on pedigree analysis to inform captive breeding strategies (Ralls and Ballou 2004, Ivy et al. 2009), and genetic data (collected using microsatellite markers) have increasingly been used to augment these efforts (e.g., Wisely et al. 2003, Araki et al. 2007). Genomic methods have the potential to provide improved resolution for estimates of kinship and genomic diversity, and they offer the added benefit of directly addressing inbreeding (Allendorf et al. 2010). For instance, chondrodystrophy, a lethal disorder affecting the highly endangered California Condor (*Gymnogyps californianus*), was identified in the captive-breeding program, with autosomal recessive transmission (Ralls et al. 2000). Therefore, carriers of the disorder could be identified only through the production of affected chicks. Romanov et al. (2006, 2009) compared California Condor genomic sequences with those from the chicken genome to help identify and characterize candidate loci associated with the chondrodystrophy mutation that can be used to identify carrier status in the breeding population.

### Ornithological Genomic Applications: Non-avian Sources

Although most genomic applications in ornithology will understandably focus on avian DNA, there are several growing research areas that extend the investigation of DNA to non-avian sources. Such research often utilizes DNA collected on or near birds to identify other key players in that species' ecology, such as prey, pathogens, and symbionts. This is accomplished primarily by extracting DNA from a sample (e.g., fecal samples, gut contents, water), then amplifying and sequencing (i.e. amplicon sequencing) a diagnostic region of DNA that is known to be present in diverse organisms, and subsequently comparing these results to a multispecies reference database. Broadly referred to as "genetic barcoding," this process—while not "genomic," in that it is inherently focused on only a small fraction of the genome—has been dramatically transformed by improved sequencing technologies. We can now identify multiple genomes (from multiple species) simultaneously from a single sample, using more efficient

methods that negate the need for cloning of PCR products followed by traditional Sanger sequencing. In the examples below, we highlight areas in avian research that are likely to benefit from these advances.

**Diet.** Genetic analysis of diet can be accomplished with DNA isolated from stomach contents or noninvasively from feces or regurgitated materials (Jarman et al. 2004, Deagle et al. 2009, Pompanon et al. 2012). This process involves amplifying a specific portion of that DNA (through PCR) using universal primers that are highly conserved across most organisms (e.g., the cytochrome *b* and CO1 regions of the mtDNA for animal diet items and P6 loop of the chloroplast *trnL* intron for plants; reviewed in Pompanon et al. 2012). After PCR amplification, each amplicon (PCR product) is then sequenced using second-generation sequencing techniques and compared to a database of known sequences for putative diet items (an approach known as “metabarcoding”). These methods have been applied in mammals and reptiles (Brown et al. 2012, Shehzad et al. 2012, Bergmann et al. 2015), but there are few examples in the ornithological literature to date. A recent food-chain study of the Atlantic Puffin (*Fratercula arctica*) by Bowser et al. (2013) used metabarcoding from feces-derived DNA to compare the diet of adults and chicks. Interestingly, the same sequence data also provided insight into the stomach content of the primary prey species, the Atlantic herring (*Clupea harengus*). The results of the study thus provided unique insight into food-chain dynamics, revealing the immediate prey of the puffin (the herring) as well as the plankton consumed by the herring (Bowser et al. 2013).

**Environmental DNA.** Genomic approaches can be used as an indirect method of species detection. Cellular material (e.g., skin, feces, urine) shed by organisms into the environment (referred to as “environmental DNA,” or eDNA) can be amplified and sequenced using second-generation sequencing to determine the presence of species that may be rare or otherwise difficult to detect. Thus far, water and soil samples have been the primary sources of genetic material for eDNA studies. For example, Thomsen et al. (2012) sequenced eDNA in seawater samples to investigate the composition of marine fish communities. In addition to 15 different fish species, 4 bird species were also detected. Given that many bird species are relatively easy to monitor through sight or sound and already have comprehensive monitoring systems in place (e.g., Breeding Bird Survey and Christmas Bird Counts), eDNA-based approaches may be most useful to ornithologists as a way to detect non-avian species (e.g., to quantify prey availability for a piscivorous bird). That said, eDNA could also be applied to the detection of ephemeral, rare, or cryptic bird species, such as those visiting stopover sites along migration routes or using known resources (e.g., ponds, roost sites).

**Avian gut microbiomes.** Birds house a diverse array of gut microorganisms that influence their health and physiology (Waite and Taylor 2015). Investigations into the diversity and functions of avian microbiomes are now much more feasible because of advances in sequencing. Similar to the diet analysis discussed above, avian microbiomes can be characterized by amplifying the 16S rRNA gene from bacteria and Archaea present in the host’s gastrointestinal tract using metabarcoding and second-generation sequencing techniques. Avian gut-microbiome research to date has been focused on describing variation in microbial communities along the gastrointestinal tract, investigating the effects of different diets and the age of hosts on microbiome diversity and composition, and examining the effects of factors like captivity, treatment by antibiotics, and colonization by pathogens (for a detailed review, see Waite and Taylor 2015). While the majority of these studies have focused on domestic birds such as chicken and turkey (e.g., Bjerrum et al. 2006, Stanley et al. 2012, Danzeisen et al. 2013), several studies have characterized the microbiomes of wild birds. Wienemann et al. (2011) found differences in bacterial microbiotas between wild and captive Western Capercaillies (*Tetrao urogallus*) and also found seasonal differences in wild Western Capercaillies that are likely associated with highly specialized seasonal diets. In a study of Black-Legged Kittiwakes (*Rissa tridactyla*), van Dongen et al. (2013) compared the cloacal microbiomes of chick and adult Black-Legged Kittiwakes and found that the gastrointestinal tracts differed with age, suggesting that bacterial assemblages of chicks are more variable yet eventually transition into a more stable state in adults.

**Avian epidemiology and zoonoses.** Given the migratory nature of many bird species, bird-borne pathogens have the potential to spread readily among continents, although many avian diseases show substantial spatial variation (Bensch and Åkesson 2003, Fuller et al. 2012). Land conversion and the introduction of nonnative host species may have exacerbated emergent avian disease in the past, yet climate change is now thought to be one of the most significant factors underlying recent outbreaks of avian disease (Fuller et al. 2012, Van Hemert et al. 2014). As such, avian ecologists may be interested in which species (or individuals) carry pathogens and what the route of the spread of disease may be across continents. While the detection of pathogens is often achieved using PCR-based methods (e.g., Duckworth et al. 2003), contemporary sequencing platforms can be used to sequence the pathogen itself. Because viruses consist of a segmented RNA genome that evolves relatively quickly through genetic reassortment events, phylogenetic investigation of virus genomes can delineate genetic variation and document reassortment events, which can thereby be used to trace global

transmission routes (Lei and Shi 2011). Dusek et al. (2014), for example, tested waterfowl and gulls in Iceland for avian influenza and sequenced the virus using second-generation sequencing. They detected viruses entirely of American origin, viruses entirely of Eurasian origin, and viruses with mixed lineages, thereby highlighting the importance of the North Atlantic as a movement corridor for avian influenza between Europe and North America (Dusek et al. 2014). Additionally, advances in transcriptome sequencing (RNA-Seq) have recently yielded new insights for understanding host immune responses in birds to infection by pathogens (Videvall et al. 2015).

### Conclusion

Ornithologists interested in ecology and conservation have much to gain by taking advantage of genomic techniques. There is no doubt that learning and keeping pace with new advances in sequencing technology and bioinformatic analyses is challenging. However, genomic methods can offer a substantial step forward, greatly expanding the types of questions that can now be answered. Contemporary sequencing approaches not only allow for the expansion of the amount of the genome examined (thereby providing better estimates of important parameters of interest) and the potential to identify and differentiate multiple genomes in a given sample, but also are particularly useful for beginning to identify the genetic basis of adaptation. Furthermore, genomic techniques provide an unprecedented avenue for exploring an individual's response to outside stressors such as changing environmental conditions, environmental contaminants that lead to physiological stress, or a novel infectious disease. Ornithologists are in a unique position to leverage the plethora of recently developed avian genomic resources, along with existing ecological and behavioral data on birds, to begin to understand mechanistic relationships that have previously been elusive.

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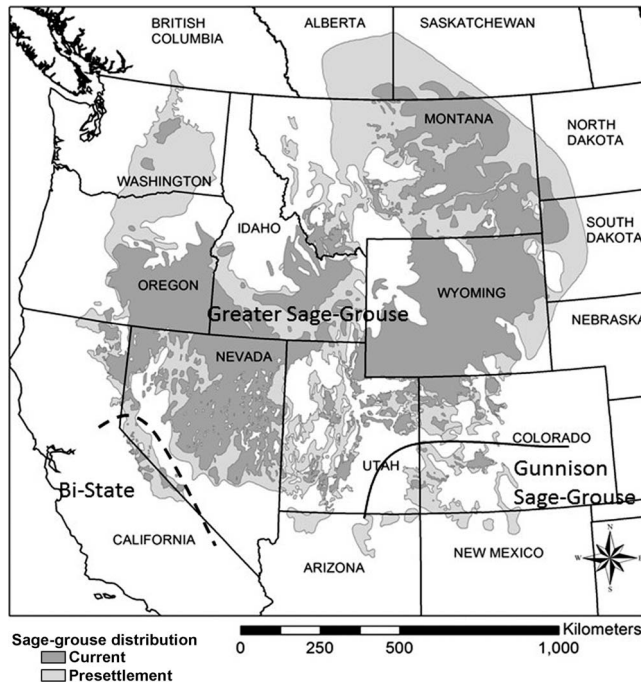
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## APPENDIX A

### Reexamining Patterns of Genetic Variation in Sage-grouse Using Genomic Methods

Sage-grouse (*Centrocercus* spp.) are iconic, declining inhabitants of sagebrush habitats in western North America and are of considerable conservation concern (Figure 1). Greater Sage-Grouse (*Centrocercus urophasianus*) differ from Gunnison Sage-Grouse (*C. minimus*) both behaviorally and morphologically (Young et al. 2000). Over the past decade, population genetic analyses of sage-grouse based on a relatively small number of microsatellite loci have been used to guide management and help delineate the 2 distinct species (Oyler-McCance et al. 1999, 2005). A parapatric group of Greater Sage-Grouse along the border of California and Nevada (“Bi-State”) was also found to be genetically distinct. Compared to other Greater Sage-Grouse populations, the Bi-State population exhibits a similar level of neutral genetic divergence as the Gunnison Sage-Grouse, yet it lacks the morphological and behavioral differences present between the 2 species (Taylor and Young 2006, Oyler-McCance et al. 2014). This has led to lingering confusion over the taxonomic status of the Bi-State population.



**FIGURE 1.** Current (light gray) and presettlement (dark gray) distributions of sage-grouse (from Schroeder et al. 2004). The boundary for the Bi-State population of Greater Sage-Grouse is delineated by the dotted line, and the boundary for the Gunnison Sage-Grouse distribution is delineated by the solid line.

Genomic information helped to resolve this taxonomic uncertainty and to better understand the nature of genetic divergence among the 3 groups. Oyler-McCance et al. (2015a) used a reduced-representation approach (RAD-Seq) to identify >11,000 single-nucleotide polymorphisms (SNPs) among the 3 groups of sage-grouse. Contrary to previous findings with traditional genetic markers, they found much higher differentiation between Gunnison and Greater sage-grouse than within Greater Sage-Grouse (e.g., Bi-State population vs. populations in the remainder of the species' range). They also mapped each SNP site onto the chicken (*Gallus gallus*) genome and found that the most highly divergent SNPs (between Greater and Gunnison sage-grouse) were located on the Z (sex) chromosome and that genetic diversity on the Z in both species was reduced compared to autosomes (i.e. non-sex chromosomes; Oyler-McCance et al. 2015b). Greater divergence on the Z chromosome could be the result of selection (including sexual selection) or of genetic drift associated with a genetic bottleneck related to the speciation event. These recent studies highlight the added value of genomic approaches by providing a better characterization of patterns of genetic variation in sage-grouse and insights into the mechanisms underlying speciation in these birds.

## APPENDIX B

### Glossary

**adaptive genetic variation.** Variation that is related to the fitness of individuals; some genetic variants confer increased fitness in the local environment.

**amplicon.** A fragment of DNA or RNA that is replicated through polymerase chain reaction.

**amplified fragment length polymorphism.** A method of genotyping that uses restriction enzymes to cut the genome and polymerase chain reaction to selectively amplify DNA fragments associated with enzyme recognition sites.

**annotation.** The process by which genes and other features are identified within a genome or transcriptome, typically accomplished using bioinformatics software.

**bioinformatics.** The research discipline concerned with the application of computer science and statistics to analyze large and complex biological datasets, including genomic datasets.

**complementary DNA (cDNA).** Double-stranded DNA that is synthesized from a messenger RNA template.

**de novo assembly.** A computational process by which a whole-genome/transcriptome sequence is compiled by piecing together shorter nucleotide sequences (e.g., generated from a second-generation sequencing instrument—see below), without comparison to a reference genome.

**effective population size.** The number of individuals in a population that pass on their genes to the next generation.

**environmental DNA (eDNA).** DNA from cellular material (e.g., skin, feces, urine) shed by organisms into the environment.

**exon.** Part of the gene sequence that is present in the final messenger RNA prior to protein synthesis.

**gene.** A segment of DNA (representing a heritable unit of genetic information) that codes for a product such as a protein.

**genetic marker.** A specific fragment of DNA in the genome that is amplified and used to distinguish individuals, populations, and species.

**genetic methods.** Methods that examine one or only a handful of loci.

**genomic library.** A collection of DNA fragments or clones of fragments that represent a portion of or the entire genome(s) for an organism or group of organisms, typically constructed in preparation for sequencing.

**genomic methods.** Methods that examine loci across the entire genome.

**genotyping.** The process of identifying the genetic makeup of an individual by examining its DNA.

**high-throughput sequencing.** The process of sequencing DNA in a massively parallel way, producing hundreds of thousands to millions of nucleotides of sequence data in a short amount of time on a single instrument run.

**intron.** A section of noncoding DNA within a gene that is removed (spliced out) before RNA is translated into a protein.

**locus (plural: loci).** A distinct position within the genome; the exact physical location may be known (non-anonymous) or unknown (anonymous).

**messenger RNA (mRNA).** A template transcribed from DNA that is used to encode proteins.

**metabarcoding.** A method for rapidly identifying species in a sample by identifying species-specific sequences in highly conserved genetic regions.

**microsatellite.** Regions in the nuclear genome that are characterized by short, tandem repeats (e.g., AT repeated 20 times), useful as genetic markers due to high variability in repeat number among individuals.

**mitochondrial DNA (mtDNA).** DNA located in the mitochondria instead of in the cell nucleus, commonly sequenced for use in population genetic studies and phylogenetics.

**neutral genetic variation.** Variation that is not related to the fitness of individuals; can be used to infer the magnitude of neutral processes like gene flow and genetic drift.

**oligonucleotide probes.** A short sequence of DNA or RNA that is synthesized to be complementary to a specific region of DNA/RNA of interest, commonly utilized during genomic library preparation for target loci of interest.

**polymerase chain reaction (PCR).** A technique to generate many copies of a segment of DNA.

**read.** A contiguous stretch of DNA sequence data; read length is generally a property of the sequencing instrument utilized and typically ranges from 50 to 300 bp (for second-generation sequencing) and >1,000 bp (for third-generation sequencing)

**reduced representation.** A group of genomic-library-preparation techniques that employ various molecular methods (e.g., restriction enzymes) to subsample a small fraction of positions within the genome.

**reference genome.** The complete genome sequence of the species of interest (or one closely related) that can be used to improve genotyping accuracy, sequence assembly, and gene finding.

**repetitive region.** A sequence of DNA that is repeated multiple times within the genome.

**restriction-associated DNA sequencing (RAD-Seq).** A technique that involves subsampling the genome using

restriction enzymes, followed by high-throughput sequencing and alignment of sequences to identify SNPs.

**restriction enzyme.** A type of enzyme that recognizes and cuts DNA/RNA at specific short sequences of nucleotides referred to as “restriction sites” or “cut sites” (e.g., the enzyme EcoRI will cut DNA anywhere it finds the recognition sequence “AATT”).

**ribonuclease (RNase).** An enzyme that breaks down RNA into smaller pieces.

**RNA sequencing (RNA-Seq).** A technique in which the entire population of messenger RNA is isolated from tissues, reverse-transcribed into complementary DNA, and sequenced on a high-throughput instrument.

**Sanger sequencing.** A method developed in the 1970s to sequence a single fragment of DNA using a chain-termination process.

**second-generation sequencing.** A high-throughput sequencing approach that is capable of generating thousands to billions of DNA sequences in a single instrument run, typically with sequence read lengths of 50–300 bp; examples include Illumina HiSeq, Applied Biosystems SOLiD, and Roche 454.

**selection.** Differential survival and/or reproduction among individuals due to variation in phenotypes.

**sequencing depth/coverage.** A parameter in sequencing projects, typically expressed as “ $N\times$  coverage,” where  $N$  is the number of replicate times a single position within the genome is sequenced.

**shotgun sequencing.** Sequencing DNA that has been randomly sheared into many small fragments.

**single-nucleotide polymorphism (SNP).** Genetic variation at a single nucleotide position; commonly utilized as genetic markers for population genetics/genomics analyses.

**synteny.** Colocalization of genes on the same chromosome; commonly used to describe the relative order of groups of genes along a chromosome.

**targeted capture.** A genomic-library-preparation technique in which particular loci of interest are “captured” using complementary oligonucleotide “baits” and then amplified using PCR before sequencing.

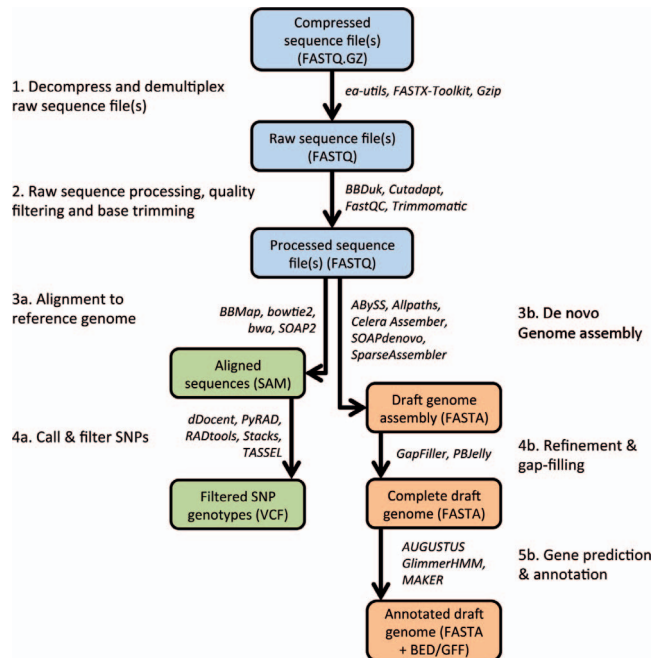
**third-generation sequencing.** High-throughput sequencing technology characterized by long sequence read lengths (>1,000 bp), often utilizing a single-molecule template DNA. Examples include the Pacific Biosciences RS II and the Oxford Nanopore Minion.

**transcription.** The first step in gene expression when DNA is copied into messenger RNA.

**transcriptome.** The complete set of messenger RNA molecules that are expressed by an organism.

**whole-genome sequencing.** Sequencing nearly every position within the nuclear and mitochondrial genomes.

## APPENDIX C



**Figure 2.** Examples of typical workflow for bioinformatic analysis of genomic sequence data. Colors correspond to preprocessing stages (blue), a typical reduced-representation sequencing (e.g., RAD-Seq) analysis pipeline (green), and a basic de novo genome-assembly pipeline (orange). Common data-file formats corresponding to each stage of analysis are shown in parentheses within each element; popular software packages for processing avian genomic data through each stage are provided (in italics) next to each transition arrow.