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INTRODUCTION

Single-nucleotide polymorphism (SNP) discovery and applications of SNP genotyping in nonmodel organisms

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Background

A supplemental issue on the topic of single-nucleotide-polymorphism-enabled (SNP) research in nonmodel organisms is especially timely. In this issue, organisms with reference genomes are considered to be 'model'; 'nonmodel' organisms are those whose genomes are yet to be sequenced. Advances in DNA sequencing and SNP genotyping have provided profound insights into the genetics of model organisms, but until recently, studies of nonmodel species lagged behind because of the scarcity of sequence and markers (see Fig. 1). In the past year, Tautz *et al.* (2010) and associated papers in supplemental issue of Molecular Ecology described a revolutionary transition from studies of 'molecular ecology' to studies of 'ecological genomics'. Concurrently, Allendorf *et al.* (2010) grappled with placing the new-found wealth of sequence and SNP information into a 'conservation genomics' context. This revolution in molecular genetics studies would have been difficult to forecast a few years ago.

Molecular genetic studies provide exceptional insight into relationships, migration and evolution of natural populations (Morin *et al.* 2004). During the origins of molecular ecology, in the 1960s and 1970s, it became clear that techniques such as allozyme electrophoresis would provide a basic framework for understanding species interactions and adaptation and for conserving natural genetic variability (Utter *et al.* 1966, 1974; Avise *et al.* 1975). Technical limitations at the time restricted both the ability to explore the dynamics of genetic diversity in species exhibiting low levels of variation as well as the direct analysis of adaptive variation in the wild.

During the following years, innovators began to dream of potential applications for conservation and management of economically exploited species that included using molecular markers to determine the population-of-origin of migrating animals (see papers in Ryman & Utter 1987; Waples & Aebersold 1990), an important focus of many papers in this issue. Recent decades were punctuated by improvements in molecular and statistical techniques that produced an array of tools relevant to ecological and evolutionary studies such as assignment tests, estimates of effective population size, fine-scale population structure, kinship analyses (e.g. Helyar *et al.* this issue; Waples & Waples this issue) and genome-wide surveys based upon an ever increasing resolution of individuals and populations.

The advantages of genotyping polymorphic SNPs with high-throughput assays have created much interest (Vignal *et al.* 2002; Brumfield *et al.* 2003; Morin *et al.* 2004; Schlötterer 2004). Until recently, however, the scarcity of available DNA sequence data for nonmodel species limited marker development. Further, because of comparatively low mutation rates, cross-species amplification of primers for SNP analyses did not yield the same results as for microsatellites. For example, Miller *et al.* (2010) tested the OvineSNP50 BeadChip, developed for domestic sheep, in two related ungulates and found only about 1% of the nearly 50 000 SNP loci to be polymorphic. Therefore, the SNP assays or probes developed for one species were not likely to be useful in others, even though primers may cross-amplify.

The current supplemental issue contains 22 papers that underline the advantages of SNPs, advocate the need for SNP research in nonmodel organisms, and chart advances in discovery and applications. Although progress is apparent across a broad array of taxa, most papers presented here focus upon species of fish. This outcome, beyond the bias of the workshop organizers, may be in part because of the well-developed multinational collaborations that coordinate the sharing of DNA

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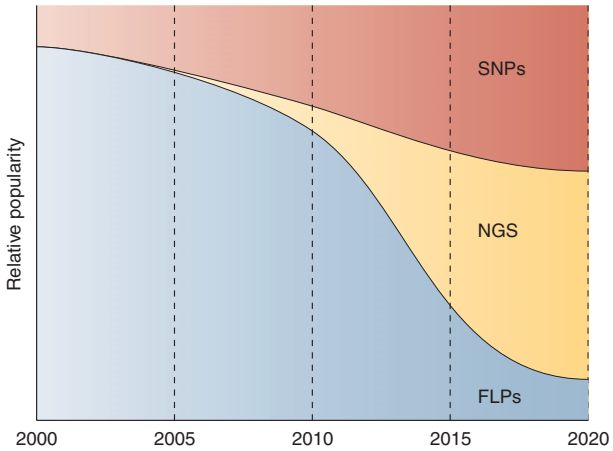

Fig. 1 Subjective view of the changing importance of genotyping strategies for nonmodel organisms built upon and expanding from Fig. 1 in Schlötterer (2004). The horizontal axis represents time, and the vertical axis corresponds to the relative importance of genotyping strategies. SNP, single-nucleotide polymorphism; NGS, next-generation sequencing of SNP polymorphisms; FLP, fragment length polymorphism. Popularity of SNP genotyping will continue to increase as more markers become available in nonmodel organisms. SNP genotyping of panels of 96–384 will be the mainstay of many studies of molecular ecology and population genetics. Some larger arrays will gain importance in some species. NGS strategies such as the sequencing of restriction site–associated DNA tags (Hohenlohe *et al.* this issue) will dominate SNP genotyping for population genomics studies of nonmodels. FLPs such as microsatellites and others will continue to have importance, especially in kinship or forensic studies where polyallelic variation is useful (e.g. Hauser *et al.* this issue; Smith *et al.* this issue).

data on migratory species (Seeb *et al.* 2007; Martinsohn & Ogden 2009) as well as the economic and cultural importance of some of these species that fuels research efforts.

SNP discovery

Chain-termination sequencing

SNP discovery in many nonmodel organisms, initiated in the 1990s, is still primarily performed with chain-termination (Sanger) sequencing. Many downstream applications focus upon genotyping a handful of loci, up to 96, on many thousands of individuals (Seeb *et al.* this issue; Templin *et al.* this issue). SNPs are often observed at a rate of about one per 300 bp in nonmodel organisms, so chain-termination sequencing usually yields one or more valid SNPs per sequencing read. While sequencing and validation require substantial work for each species, success rates are sufficient to meet these modest needs (see Table 1).

One approach to SNP discovery, in the absence of a reference genome, is through cloning and shotgun sequencing. Olsen *et al.* (this issue) describe a shotgun approach to discover 768 SNPs for population genetics study of the ringed seal *Pusa hispida hispida*. They point out that the chain-termination primers that they developed will be useful in a broader range of pinnipeds using the targeted gene approach (Aitken *et al.* 2004).

The targeted gene approach uses cross-amplification of primers to sequence and discover SNPs in closely related species. Cross-amplification can be used to screen several species at once (e.g. Smith *et al.* 2005); SNPs are often found in each, but they usually occur at different locations and require species-specific probes for high-throughput assays (Campbell & Narum this issue).

Expressed sequence tag (EST) databases are expanding for a number of nonmodel organisms, providing a wealth of primers for chain-termination sequencing (e.g. Salem *et al.* 2010). Abadía-Cardoso *et al.* (this issue) designed primers and screened 480 EST sequences from existing genomic databases to discover SNPs in *Oncorhynchus mykiss*; Clemento *et al.* (this issue) cross-amplified these same primers for discovery in Chinook salmon *O. tshawytscha*. Hansen *et al.* (this issue) used a combination of SNP mining, EST mining and cross-amplification to assemble a panel of 235 high-throughput SNP assays for *O. mykiss*.

The chain-termination approach can be especially effective as EST databases grow to include many candidate genes that may show elevated rates of variability because of selection. Hemmer-Hansen *et al.* (this issue) screened templates in Atlantic cod *Gadus morhua* that annotate to growth and reproduction genes and discovered 82 SNPs that can be used to scan natural populations for signatures of selection.

Next-generation sequencing

The newly mature next-generation sequencing technologies provide access to a wealth of sequence information on nonmodel organisms. Tautz *et al.* (2010) introduced 21 papers on this topic. One common theme was that for nonmodel organisms without a reference genome, a genome reduction step was required to acquire deep assemblies of redundant contigs required for SNP discovery (Slate *et al.* 2009).

Transcriptome sequencing is still one of the most common genome reduction approaches for nonmodel organisms despite challenges posed by differential gene expression among individuals. In this volume, Geraldes *et al.* (this issue) used Illumina GAII technology to map black cottonwood *Populus trichocarpa* transcriptome data to the completed genome (Table 1). The authors provide helpful recommendations to deal with intron–exon boundaries and alternative splicing. Working without a reference genome, Everett *et al.* (this issue) used both

Table 1 Overview of approaches for single-nucleotide polymorphism (SNP) discovery and their outcomes among the 22 manuscripts in this issue. Values are for general comparisons and do not reflect relative efficiencies. For example, SNP discovery was the endpoint of studies using Sanger sequencing, and validation was performed on all candidate SNPs. The three next-generation sequencing of SNP polymorphisms (NGS) studies focused on developing approaches to discovery—validation was only attempted on a small subset of candidates

Technology	Species	No. ascertainment individuals	Candidate SNPs	Validated SNPs	Studies
In silico	Steelhead trout	NA*	48	22	Hansen *et al.* 2011
Sanger random	Pacific salmon sp.	64	243	54	Campbell & Narum 2011
	Chinook salmon	24	228	117	Clemento *et al.* 2011
	Steelhead trout	22	506	139	Abadía-Cardoso *et al.* 2011
	Ringed seal	3	NS	768	Olsen *et al.* 2011
Sanger candidate gene	Atlantic cod	~35	82	30	Hemmer-Hansen *et al.* 2011
NGS†-Illumina	Rainbow and cutthroat trout	24	2923	NA	Hohenlohe *et al.* 2011
	Black cottonwood	20	561 302	38	Geraldes *et al.* 2011
NGS-SOLiD	Sockeye salmon	10	25 485	10	Everett *et al.* 2011

*Not applicable.
†Next-generation sequencing.

public EST databases and *de novo* assembly techniques to assemble SOLiD transcriptome reads to develop SNPs in sockeye salmon *O. nerka*. These authors provide recommendations on library construction and outline a framework for capitalizing on genomic resources from closely related species for SNP discovery.

One promising approach to genome reduction is to align redundant next-generation sequence reads adjacent to restriction sites in genomic DNA [restriction site associated DNA (RAD) markers: see Miller *et al.* 2007; Baird *et al.* 2008; reviewed in Ogden this issue;]. SNP discovery using RAD markers is performed on genomic DNA, ameliorating the problems of uneven gene expression that can hamper discovery using transcriptome sequencing. Hohenlohe *et al.* (this issue) use a RAD strategy to discover thousands of candidate SNPs spread relatively evenly across the *Oncorhynchus* genome.

Some attention must be given to the effects of ascertainment bias when SNPs are discovered from a small number of individuals subjected to next-generation sequencing. Ascertainment bias results from the selection of loci from an unrepresentative sample of individuals that are used to infer various aspects of population structure and genetic variability across a broader part of the species range than represented by the original discovery panel. Bradbury *et al.* (this issue) examine the influence of ascertainment bias and its potential impact on assignment of Atlantic cod *Gadus morhua* individuals to populations ranging widely in origin. Seeb *et al.* (this issue) describe ascertainment bias in a species-wide data set for chum salmon, and Helyar *et al.* (this issue) provide a critique of considerations specifically associated with the application and population genetic analysis of SNPs in nonmodel taxa, including statistical implications of ascertainment bias.

SNP genotyping

Low-density arrays and high-throughput genotyping

Most of the application papers in this issue use one of the genotyping strategies reviewed in either Seeb *et al.* (2009) or Garvin *et al.* (2010) (see also Table 2). Many used the low-density array based upon the 5′ nuclease chemistry that can facilitate the analysis of thousands of individuals for panels of 96 SNPs (e.g. Hess *et al.* this issue). Two exceptions were Karlsson (this issue) and Hohenlohe *et al.* (this issue). Both authors had access to thousands of SNPs on their respective platforms, but the goals of both were to preferentially select a smaller set of diagnostic SNPs for their applications that could be analysed more effectively on low-density platforms. Although next-generation genotyping promises to become a powerful force for population genomics, low-density arrays will continue to be sufficient for many applications (see Fig. 1).

Next-generation genotyping

The recent advances in RAD sequencing (Baird *et al.* 2008) foreshadow a future in which genotyping is based on next-generation sequencing. The limiting step in nonmodel organisms has been the availability of genetic markers. The RAD approach allows for simultaneous discovery and genotyping of thousands of SNPs throughout the genome and requires no prior development of geno-

Table 2 Overview of approaches for single-nucleotide polymorphism (SNP) genotyping and their outcomes among the 22 manuscripts in this issue

Technology	Species	No. individuals	No. SNPs	Studies
7K Atlantic salmon SNP-chip	Atlantic salmon	1309	4514	Karlsson *et al*. 2011
5′ nuclease endpoint PCR	Sockeye salmon	539	80	Hauser *et al*. 2011
	Chinook salmon	4014	92	Hess *et al*. 2011
	Chum salmon	10 458	60	Seeb *et al*. 2011
	Sockeye salmon	942	45	Smith *et al*. 2011
	Chinook salmon	23 269	45	Templin *et al*. 2011
MALDI-TOF Mass Spectrometry	Atlantic salmon	227	320	Freamo *et al*. 2011
Multiplex hybridization array	Atlantic cod	279	1641	Bradbury *et al*. 2011
Real-time PCR	Sperm whale	287	36	Mesnick *et al*. 2011

mic resources. For example, Emerson *et al*. (2010) used RAD sequencing to reveal previously unresolved genetic structure in the pitcher plant mosquito *Wyeomyia smithii*. They used six individuals from 21 populations and two lanes of an Illumina sequencer. Their goal was to identify nucleotide positions that were fixed or nearly fixed within populations and variable among populations. This allowed them to develop consensus sequences for each population that could be used in phylogenetic analysis. In this volume, Hohenlohe *et al*. (this issue) used RAD sequencing in a study of introduced rainbow trout *O. mykiss* and native westslope cutthroat trout *O. clarki lewisi* for SNP discovery. They identified 2923 candidate species-specific SNPs from a single Illumina sequencing lane containing barcoded DNA from 24 individuals. Their particular goal was to design 5′ nuclease assays for high-throughput, low-cost hybrid identification using 50–100 loci. However, as laboratory and statistical techniques are refined and costs reduced, using results directly from next-generation sequencing will likely become more common for applications where large numbers of loci are desired for a moderate number of individuals. This approach will provide a tremendous amount of genomic information for fine-scale resolution of population structure and phylogeography as well as wildlife forensics (Ogden this issue).

Comparisons of genotyping with SNPs and microsatellites

Often there is a need to compare the relative power of techniques as new approaches emerge to replace commonly accepted standards (Hauser *et al*. this issue; Hess *et al*. this issue; see Fig. 1).

Genotyping of nonmodel organisms was dominated by microsatellite analyses for over a decade after the introduction of automated DNA sequencers that facilitated fragment analysis. The dominance of microsatellites persisted, in spite of their limitations (Morin *et al*. 2010), in part because successful cross-amplification of primers greatly accelerated discovery in sister species or genera. The length-slippage modes of mutation (Dieringer & Schlötterer 2003) nearly ensured that microsatellite loci amplified from one species would be polymorphic in others (e.g. Chen & Dorn 2010; Hendrix *et al*. 2010). However, as fragment polymorphisms, microsatellites suffer from reproducibility among laboratories, and the resulting complications in comparing genotypes provided momentum to establish public databases with SNPs (Stokstad 2010). Nevertheless, the uncertainty about the number of SNPs needed to replace a certain microsatellite set lingers and is discussed in this issue for parentage analysis (Hauser *et al*. this issue) and population assignment (Hess *et al*. this issue). These issues will likely become less relevant in the near future.

With increasing throughput of sequencing and SNP genotyping, many current benefits of microsatellites are likely to disappear. A panel of several hundred SNPs will always be more powerful than a dozen microsatellites for standard population genetics applications relying on multilocus estimators of differentiation (Coates *et al*. 2009), migration, population (Hess *et al*. this issue) or parentage assignment (Hauser *et al*. this issue), especially given the higher reproducibility and lower genotyping error of SNPs. However, in single-locus tests such as outlier tests for selection, multi-allelic microsatellites may provide higher power (Foll & Gaggiotti 2008). By combining biallelic SNPs into multiallelic haplotypes, higher single-locus power for specific applications may be possible for SNPs (Smith & Seeb 2008; Jones *et al*. 2009; Morin *et al*. 2009). It is therefore likely that microsatellites will eventually be confined to very specific applications in specific species and that SNP genotyping (using either high-throughput or next-generation sequencing approaches) will become the 'workhorse' of molecular ecology, opening additional avenues to investigate local adaptation and evolutionary change and their interactions with population demography.

SNP applications

Many papers in this volume document the application of SNPs to the study of local adaptation, one of the most promising aspects of the emerging technologies. These approaches allow simultaneous estimation of neutral (genome wide) processes along with the identification of specific genomic regions or SNPs influenced by recent selection.

In the candidate gene approach, specific SNPs appear as outliers relative to the patterns observed at neutral markers (e.g. Hemmer-Hansen *et al.* this issue). Outliers are typically identified using one or several of the F_{ST} outlier detection methods (e.g. Beaumont & Nichols 1996; Foll & Gaggiotti 2008; Excoffier *et al.* 2009; see review in Helyar *et al.* this issue). For example, two F_{ST} outlier SNPs were identified in chum salmon *O. keta* (Seeb *et al.* this issue); both showed variation associated with latitude across the Pacific Rim. Karlsson (this issue) screened 7000 SNPs and identified a panel of 60 loci that collectively were diagnostic in identifying individual Atlantic salmon *Salmo salar* as being farmed or wild. Although not specifically identified as outliers, these SNPs had significantly higher F_{ST} values when separated into wild and farmed groups relative to allocation of the same individuals into two random groups. The authors hypothesize that the differences observed at the 60 SNPs between the wild and farmed groups arose during domestication.

Genome scans using anonymous markers can also be used to detect selection signatures and local adaptation. Hohenlohe *et al.* (2010) used RAD sequencing to identify and genotype 45 000 SNPs across the genome in five populations of three-spined sticklebacks *Gasterosteus aculeatus*. The density of markers allowed population genetic statistics such as F_{ST} to be visualized as a pseudo-continuous distribution along the chromosomes. They found genomic regions exhibiting signatures of both balancing and divergent selection.

A comparative sequence approach to detecting selection was used by Brieuc & Naish (this issue). They compared synonymous (d_S) and nonsynonymous (d_N) substitutions from published sequences of Pacific salmon. This approach, widely used in model organisms, is hampered by the lack of reference genomes in nonmodel organisms. However, Brieuc & Naish (this issue) describe several steps to streamline this approach.

Non-neutral markers can be useful for individual and compositional assignment. For example, major histocompatibility complex (MHC) loci have long been recognized as particularly valuable for mixture analysis in sockeye salmon (Beacham *et al.* 2004; Ackerman *et al.* this issue). The use of non-neutral SNPs in population assignment was explored by Freamo *et al.* (this issue). They investigated whether non-neutral SNPs could be used to assign Atlantic salmon more accurately than existing neutral markers. Using four F_{ST} outlier detection methods, they identified and found outlier loci that were powerful for distinguishing groups of conservation concern.

Data sharing

With wide-ranging or highly migratory organisms, evaluating population structure or monitoring migrations at multiple life history stages can be challenging and beyond the scope of a single research group. Scientists are increasingly forming large international consortia to develop shared databases to address these types of questions. SNPs are becoming the marker of choice because they avoid the well-documented problems associated with standardization of microsatellite alleles across laboratories in different nations and continents (Seeb *et al.* 2007; Morin *et al.* 2010).

FishPopTrace is an international project funded by the European Union (EU) to generate panels of SNP markers for geographic assignment of four commercially important marine species (Atlantic cod *Gadus morhua*, European hake *Merluccius merluccius*, common sole *Solea solea* and Atlantic herring *Clupea harengus*; Martinsohn & Ogden 2009). Fifteen research groups from the EU, Norway and Russia are collaborating to discover and validate SNPs and to create a standard set of operating procedures for use by each (http://fishpoptrace.jrc.ec.europa.eu/). Their plan incorporates both the ability to modify the number of SNPs in relation to the level of population differentiation and associated geographic scale, and the inclusion of SNPs under selection that collectively allows unprecedented levels of population assignment in commercial fish. Moreover, the essentially binary nature of SNP variation facilitates their forensic validation and use in global data sets to fight illegal fishing and promote consumer protection.

The Pacific salmon research community has a long history of shared international databases working through international treaty organizations including the North Pacific Anadromous Fish Commission (NPAFC; http://npafc.org/) and the Pacific Salmon Commission (PSC; http://www.psc.org/) with the original collaborations dating from allozyme studies in the 1980s (Waples & Smouse 1990). In this volume, Seeb *et al.* (this issue) review the *PacSNP* collaboration for chum salmon among North American and Asian researchers. Collaborative efforts for Chinook salmon are also included: Clemento *et al.* (this issue) present over 100 new 5′ nuclease assays for SNPs in Chinook salmon, and Templin *et al.* (this issue) present a wealth of SNP data from Alaskan Chinook salmon populations, long the missing component for a Pacific Rim-wide database for this species. Both of these studies build on earlier collaborative efforts to build

shared SNP databases (Smith *et al.* 2007; Narum *et al.* 2008). The existence of a shared sockeye salmon database (Habicht *et al.* 2010) emphasizes the importance of these resources to the research community; the study of Smith *et al.* (this issue) that examined historical harvest trends from archived salmon scales would not have been possible without access to this comprehensive database.

Finally, nowhere is the need for cooperative and collaborative studies more apparent than in the investigation of population structure in globally dispersed and highly mobile marine species. Mesnick *et al.* (this issue), as part of a large international collaboration for which samples were collected over a 35-year period, document the population structure of sperm whales *Physeter macrocephalus* in the eastern and central North Pacific using SNPs, mtDNA and microsatellites. The slow accrual of samples covering the global distribution of this species makes it an ideal case for use of SNPs: SNP scores of A, C, T or G remain constant through time, as genotyping platforms become obsolete or evolve, while the scores of length fragment polymorphisms do not. The challenges the group faces in understanding global and regional population structure in sperm whales are significant, including the analysis of samples collected over many decades and the future need to combine data collected by researchers with access to samples in different oceanic regions. Mesnick *et al.* (this issue) show that SNPs have sufficient power to detect population structure even when genetic differentiation is low, and SNPs appear to offer the best foundation for this group's collaborations.

Conclusions

This issue on the application of SNPs to nonmodel species not only demonstrates the utility and range of genomic approaches and resources available, but importantly introduces the application and collation of international data sets to elucidate the dynamics of demographic and adaptive processes. In addition to the necessary mining of factors driving the distribution and abundance of taxa in the wild, the ability to bring together high-throughput genotyping with robust comparative data sets that can be readily augmented will promote their use in conservation and management. The long-held proxy in conservation genetics that assumes rough correspondence between neutral and adaptive variation can be tested empirically across diverse taxa where traditional genomic resources are scant. Studies that encompass the temporal and spatial dynamics of SNP variation facilitate the traditional approach of ecological genetics (Clarke 1975): the ultimate demonstration of fitness consequences of patterns of genetic diversity in the wild.

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Conflict of interest

The authors have no conflict of interest to declare and note that the sponsors of the issue had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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