

EDITORIAL



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High-throughput genotyping in estimating genetic resources and detecting pathogens in aquaculture

Aquatic genetic resources (AqGR) include DNA, tissues, gametes, embryos, and other early life stages, wild and farmed individuals, and communities of organisms of actual or potential value for food and aquaculture. Monitoring the AqGR at national, regional, and global levels would not only help to improve production traits, enhance disease resistance, and ensure the long-term sustainability of aquatic species but also provide valuable information on the state of rare or endangered aquatic species. While the importance of monitoring and reporting of AqGR is becoming more and more apparent among different stakeholders, efforts to date are still insufficient (FAO, 2022).

A common method to estimate the AqGR is genotyping, which is a process of determining the genotype at positions within the genome of an individual and comparing it to other individuals' sequences. It is often used to understand association between genotype and phenotype. Sequence variations like single-nucleotide polymorphisms (SNPs) or microsatellite loci are applied as markers in linkage and association studies to determine genes relevant to specific traits. SNPs are the most common sequence variant widely used in genome-wide association studies (GWAS). With more and more SNPs being discovered, SNP genotyping technologies have been greatly promoted and include low-throughput and high-throughput methods. Nowadays, demands of high-throughput SNP genotyping are increasing, especially for hybridization-based SNP arrays and various next-generation sequencing (NGS)-enabled genotyping methods, such as genotyping-by-sequencing (GBS).

Besides genotyping AqGR, related molecular methods have also been widely used in pathogen diagnosis in aquaculture. For farmers, early detection of pathogens can help to prevent spread of disease and minimize economic losses due to disease outbreaks. Rapid, pond-side methods allow for quick and efficient diagnosis of diseases, which can lead to more timely and effective treatment options. Furthermore, effective disease management can help to minimize the use of antibiotics and other treatments, thus reducing the risk of antimicrobial resistance and promoting more sustainable aquaculture practices.

Both good management of AqGR and disease control are key elements of sustainable aquaculture. Here, we summarize the methods used for genotyping AqGR and detecting disease by molecular diagnosis with an emphasis on high-throughput and onsite solutions.

1 | EARLY METHODS FOR GENOTYPING

The common laboratory procedure for genotyping involves sample collection, DNA extraction, PCR, and subsequent detection of genetic variation. For example, tissue samples are collected from fish, usually by taking a small piece of tissue such as a fin clip. DNA is extracted from the tissue sample using standard laboratory techniques, such as phenol-chloroform extraction or commercial DNA extraction kits. Then, specific molecular markers may need to be

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designed for detecting any genetic variation; an example is the mitochondrial cytochrome oxidase subunit 1 (COI) gene. Microsatellite markers can be used for genotyping, but SNP markers are now more commonly used for genotyping since more loci and greater information can be extracted from genome-wide SNP data. SNP genotyping can be performed using various methods, such as restriction fragment length polymorphism (RFLP), single-strand conformation polymorphism (SSCP), or sequencing. RFLP and SSCP are based on differences in the size and/or shape of the DNA fragments resulting from SNP variation, while sequencing provides direct information on the nucleotide sequences.

Compared with high-throughput genotyping, traditional methods are generally less efficient and time consuming, and may require a larger amount of starting material. Traditional genotyping methods may also have a higher error rate and be less sensitive to low-frequency variants. Additionally, traditional methods may not be able to genotype as many loci simultaneously as high-throughput methods, and this can limit their utility in certain applications such as genome-wide association studies.

2 | HIGH-THROUGHPUT METHODS

With the rapid development of next-generation sequencing (NGS), many high-throughput genotyping methods have been developed and are primarily divided into SNP arrays and genotyping by sequencing (GBS) methods (Scheben et al., 2017). The latter has various related methods, such as whole genome resequencing (WGR) and reduced representation sequencing (RRS), including sequence capture, restriction-site-associated DNA sequencing (RAD-seq), genotyping-in-thousands by sequencing (GT-seq), etc.

2.1 | SNP array

SNP array is a specific type of DNA microarray that contains custom-designed probes, including SNP positions. The basic principle of SNP array technology is to hybridize the probes to fragmented DNA, enabling determination of specific alleles of all SNPs on the array for a particular hybridized DNA sample (LaFramboise, 2009). As a mainstream genotyping approach, SNP arrays have been successfully applied to aquatic organisms, especially commercial species such as Nile tilapia, common carp, and rainbow trout (Joshi et al., 2018; Palti et al., 2015; Xu et al., 2014). Compared with NGS-based genotyping methods, SNP arrays do not need complicated library preparation steps or subsequent intensive bioinformatic processing (Clevenger et al., 2015), which is essential in DNA sequencing-based methods. Furthermore, using SNP arrays can avoid sequencing and alignment errors generated by sequencing and downstream bioinformatic data analysis (Wall et al., 2014). Compared with sequencing, SNP arrays also have higher repeatability and reproducibility due the simplicity of the procedure (Robledo et al., 2018). In addition, SNP array genotyping is considered to be low-cost if a corresponding species' SNP array is available.

SNP arrays do have some drawbacks. One of the disadvantages is the tedious nature and upfront cost of SNP array development. Prior genetic information is needed for designing SNP probes, which often depend on large-scale resequencing. SNP arrays can only be applied to genotype known SNP loci (Wang et al., 2014), and selecting SNP markers can be a time-consuming process. During the design of probes, several factors, including SNP depth, SNP types, and SNP frequency, need to be considered, and this mainly depends on the criteria of platform. Ascertainment bias, caused by small sample size or unrepresentative sampling, can also be an obstacle for SNP arrays (Heslot et al., 2013). If sample sizes are small, some rare alleles will not be captured (Gravel et al., 2011). The species with a strong population structure may also suffer from ascertainment bias, which presents a major issue when aquaculture strains for a certain species are highly variable. Thus, the utility of an SNP array may vary widely depending on the relationship of the strain to the population used for SNP discovery (Robledo et al., 2019).

2.2 | Whole genome resequencing (WGR)

Whole genome resequencing is a powerful tool for querying entire genomes to identify differences between genotypes of individuals or targeted populations. In the process of WGR, millions of short reads generated from NGS are aligned to a reference genome, and variants between different individuals are identified. In the last decade, WGR has been used in many ways, including selective breeding as well as genomic and evolutionary biology studies (Xiao-kai et al., 2018). Undoubtedly, WGR provides the most comprehensive genetic variation information compared with other genotyping methods. However, some limitations exist for WGR. First, prior genetic information of a species of interest is needed for WGR analysis, and this is usually achieved by *de novo* genome sequencing and assembly. Second, WGR may generate redundant data if the fragments of interest are only parts of the genome. Third, data quality of WGR is highly dependent on accuracy of the reference genome and the sequencing depth and coverage of the data (Nielsen et al., 2011). Deep sequence coverage of overlapping reads can significantly reduce errors in SNP calling. Typically, >30X coverage of WGR per sample is needed to identify the sample-specific variation. The coverage should be >100–200X for some rare allele detection (Wang et al., 2016). The requirement of high coverage leads to an unaffordable cost. Such an expensive sequencing cost restricts the application of high-coverage WGS-based genotyping (Heffelfinger et al., 2014).

Many technologies were developed based on the WGR for lower cost and higher accuracy. Low-coverage WGR (LC-WGR) is a type of WGR with low coverage ranging from 5X to 1X (Nielsen et al., 2011). LC-WGS reduces the cost and improves the ability to multiplex samples in a single sequencing run. Low accuracy, poor ability to distinguish SNPs, and inherent error of LC-WGR are unavoidable when a reference genome and sufficient samples are absent. Another advanced WGR method called Pool-seq obtains the whole resequencing data from pooled DNA of individuals per population to high coverage (Schlötterer et al., 2014). Several limitations are found in Pool-seq, including the absence of individual genotypes, unreliable allele frequency estimation, and difficulty in detecting rare and low-frequency variants (Fuentes-Pardo & Ruzzante 2017). In summary, WGR is a good alternative when the sample size is moderate or funding is not a concern. The cost of WGR is dropping with development of better sequencing techniques, and WGR has great potential to be widely applied to genetic resource surveys.

2.3 | RAD-seq

RAD-seq is a strategy that enables sequencing a fraction of a genome, generating 0.1% up to 10% of a selected genome. (Baird et al., 2008). It combines a reduction in genome complexity using restriction enzymes (REs) with high-throughput sequencing. In the following decades, many new technologies have been developed based on RAD-seq (You et al., 2020), including RRL (reduced representation libraries), ddRAD (double-digest RAD), and 2b-RAD (type IIB restriction enzymes RAD). RAD-Seq and related techniques are cost-effective, which is why they are some of the most popular genomic approaches for high-throughput SNP discovery and genotyping. Since its first description in 2008, RAD-seq has been used in aquaculture in many ways, including the construction of genetic maps, comparative genomics, and detecting SNP resources for future SNP array development (Houston et al., 2014; Kakioka et al., 2013; Recknagel et al., 2013).

Compared with SNP arrays, RAD-seq allows SNP discovery and genotyping without a reference genome (Davey & Blaxter, 2010; Willing et al., 2011). Thus, this method is especially suitable for non-model organisms. Another advantage of RAD-seq is its high level of control. The REs or fragment size selection can be customized and flexible for specific studies, which is also the reason why so many related technologies have been developed. In addition, data availability of RAD-seq is relatively high. The paired-end data from RAD-Seq allows identification and removal of putative PCR duplicates, which can greatly improve the efficiency of Illumina sequencing data (Schweyen et al., 2014). Finally, RAD-seq, as with other reduced-representation sequencing methods, can provide researchers

with a greater depth of coverage per locus and the potential to sequence large numbers of samples in a cost-effective way (Andrews et al., 2016).

RAD-seq has several drawbacks. RAD-seq can only capture SNPs adjacent to enzyme cut sites. RAD-seq also shares some sources of sequencing and genotyping errors with all next-generation sequencing methods. Moreover, RAD-seq has some unique errors and bias, including allele dropout, null alleles, and variance in depth of coverage among loci (Andrews et al., 2016). The impact of those errors is mainly caused by the library preparation protocol and statistical analysis and can lead to lower repeatability and reproducibility of RAD-seq when compared to SNP arrays. Another main drawback of RAD-seq is caused by the shearing step in NGS library preparation, which often occurs due to sonication and can result in the presence of random and variable DNA fragments. It may impede the efficiency of RAD-seq (Davey et al., 2013).

2.4 | Genotyping-in-thousands by sequencing (GT-seq)

GT-seq is a method of next-generation sequencing of multiplexed PCR products and is used to generate genotypes (Campbell et al., 2015). GT-seq enables thousands of individual samples to be sequenced in a single Illumina HiSeq lane, which greatly improves the efficiency of large-scale genomics research, especially for population genetic studies. For example, GT-seq has been used to monitor and manage several commercial aquaculture species, including Atlantic salmon, Coho salmon, and walleye (Aykanat et al., 2016; Beacham et al., 2018; Bootsma et al., 2020). In addition, GT-seq allows for the generation of genotypes for thousands of individuals, which leads to the application of GT-seq in large-sample genotyping.

Besides its ability to genotype thousands of samples at the same time, the cost of GT-seq is relatively low, about \$3.5 per sample (from extracting DNA to NGS library preparation). Unlike SNP arrays, GT-seq is flexible and can target any SNP in the genome, including SNPs associated with phenotypic variation. A higher read depth and more reliable genotyping are observed in GT-seq compared with previous genotyping methods (McKinney et al., 2018). GT-seq, like SNP array, requires prior genomic knowledge for selection of SNPs. The development of GT-seq markers can be costly if no related genomic information is reported. In addition, the design of primers in GT-seq is time-consuming. Thousands of indexes need to be inserted into the primer for identify individuals or populations. Moreover, the primers of GT-seq are often designed around conserved regions of the genome. The absence of suitable priming sequences can lead to missing data or nonspecific amplification. Finally, each GT-seq marker is only suitable for individual species or sibling species.

2.5 | Hyper-seq

Another multiplex-PCR-based “genome simplification” method is Hyper-seq (Zou & Xia, 2022), which seems to be cost-efficient, flexible, and high-throughput genotyping approach. It was designed based on the observation that exons are more frequently found in GC-rich regions, and introns and promoters are frequently found in AT-rich regions. One of the Hyper-seq-G primers is composed of 6-bp barcodes, 10-fixed bases, 4 GC-rich core bases (CCGG), and overhangs (NNN), whereas the other set of Hyper-seq-G primers contains the AATT core sequence. Thus, the amplified products are supposed to cover both exon and intron regions. The density of the makers can be decreased by using less degenerate 3' overhangs or increased by combining different sets of fixed bases. The genome simplification, barcoding, and library prep steps can be done in one PCR, which significantly lowers the cost to ~\$10 per Gbp of data. Another advantage of Hyper-seq is that high-quality DNA is not required to apply this method. While Hyper-seq is a novel method and mainly applied in plant genomics research, its utility awaits for testing in different taxonomic groups.

3 | DETECTION OF PATHOGENS IN AQUACULTURE

Intensive aquaculture with high stocking density can lead to outbreaks of disease. The best way to control aquatic disease is by prevention, but detection of a problem and timely treatment can be equally important. A rapid, pond-side pathogen detection method would be essential and aid in the control of disease in aquaculture.

3.1 | Conventional diagnosis methods

The conventional methods of pathogens diagnosis rely on observations of characteristic clinic and histological signs (Frans et al., 2008) and subsequent isolation and identification of the causative pathogen from samples of moribund fish. The basic steps include culturing of the etiological agent using various methods and analysis of morphological, phenotypic, or biochemical characteristics of the presumed pathogen. The accuracy and reliability of these techniques can be highly dependent on the expertise of those involved, and culturing potential disease-causing organisms can be time-consuming and tedious. For most aquatic viruses or other pathogens like nervous necrosis virus (NNV), their isolation using cell lines has been limited or is not reliable (Doan et al., 2017). Less than one percent of the microbes in an environmental sample can be cultured (Rappé & Giovannoni, 2003). Another conventional diagnosis approach that has been used in some cases is based on serological techniques such as the enzyme-linked immunosorbent assay (ELISA; Clark & Adams, 1977). Such antigen-based techniques can be real-time and cost-effective, and the process is rapid with no need of sophisticated instruments. Usually, a diagnosis can be completed within several hours. Due to those features, there are some pond-side detection methods and commercial test kits developed for aquaculture, such as Infectious Salmon Anemia Virus (ISAV) Rapid Kit (Aquatic Diagnostics Ltd.), Bacterial Kidney Disease (BKD) ELISA Kit, and Salmonid Rickettsial Septicemia (SRS) ELISA Kit (Ango Ltd.).

Antigen-based approaches also have limitations. First, the sensitivity and specificity of ELISA can sometimes be unsatisfactory (Adkison et al., 2005). Second, the detection reagents in antigen test kits are usually monoclonal or polyclonal antibodies, which must be tested thoroughly to ensure false positive or false negative results are not obtained when multiple pathogens may be in a sample. Second, antigen-based methods can only detect single pathogen at a time. Thus, several different antigen test kits are required for multiple antigens, and antigen-based method may not be suitable when the causative agent is unknown.

3.2 | Metabarcoding and ddPCR

Compared with morphological methods, molecular techniques can avoid problems in investigating organisms for which culture medium, cell lines (for viruses), or detection methods are not available. The molecular techniques can also be more efficient and accurate. Two popular molecular techniques for pathogen detection are metabarcoding using 16 s rRNA gene and droplet digital PCR (ddPCR) (Testerman et al., 2022). The first method, 16 s rRNA gene sequencing, combines pathogen detection with microbial community profiling for addressing immediate and long-term fish health concerns, while ddPCR can produce rapid and sensitive detection that can be used in management and rapid clinic responses. Although NGS-based approaches have been developed for several years, diagnostic metagenomics is still an unconventional method in fish disease diagnostics (Testerman et al., 2022). Some novel molecular methods have been developed for pond-side diagnosis, such as loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA) (Bohara et al., 2022; Notomi et al., 2000; Piepenburg et al., 2006). For example, loop-mediated isothermal amplification (LAMP) is used to rapidly and efficiently amplify DNA or RNA through a strand displacement DNA polymerase and a set of four to six specially designed primers that recognize multiple regions of the target DNA or RNA. A new study (Song et al., 2018) combines bioluminescent assay in real-time and loop-mediated isothermal amplification (BART-LAMP) technology with smartphone-based detection, which

connects a PCR reaction plate or a LAMP reaction tube to the camera of a smartphone and an application that displays and analyzes the reaction results. At this point, this technology has only been applied for human disease, but there is potential to develop this technology for pathogen detection in aquaculture.

Development of high-throughput genotyping approaches and high-throughput pathogen detection methods is important and has application for sustainable aquaculture. High-throughput genotyping allows for accurate and efficient identification and selection of superior breeding stock. High-throughput pathogen detection methods can be ideal for screening populations and allow rapid diagnosis of disease and timely treatment or implantation of prevention strategies. Rapid pathogen detection may also assist in the production of specific pathogen-free seeds (SPF-seed), leading to increased production and profitability in aquaculture. Such molecular methods for pathogen detection should, however, be fully validated and optimized and confirmed using gold standard methods when used for regulating fish movement or for satisfying import/export requirements related to disease. Ultimately, the methods described here will reduce economic losses and potential environmental impacts for aquaculture. Continuous research and development are essential in these areas and will ensure long-term success of the aquaculture industry. The Journal of the World Aquaculture Society is one of the best platforms for sharing new knowledge and novel inventions in these areas that may help sustainable aquaculture.

DATA AVAILABILITY STATEMENT

There are no data used for this editorial.

Chenhong Li 

Junlong Jiang

College of Fisheries and Life Science, Shanghai Ocean University, Shanghai, China

Correspondence

Chenhong Li, Shanghai Ocean University.

Email: chli@shou.edu.cn

ORCID

Chenhong Li  <https://orcid.org/0000-0003-3075-1756>

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