Aquaculture Genome Technologies
Zhanjiang (John) Liu

Chapter 6
Single Nucleotide Polymorphism (SNP)

Zhanjiang Liu

Single nucleotide polymorphism (SNP) describes polymorphisms caused by point mutations that give rise to different alleles containing alternative bases at a given nucleotide position within a locus. Such sequence differences due to base substitutions have been well characterized since the beginning of DNA sequencing in 1977, but the ability to genotype SNPs rapidly in large numbers of samples was not possible until several major technological advances in the late 1990s. With the development of the TaqMan technology, gene chip technology, pyrosequencing, and MALDI-TOF, which is matrix-assisted laser desorption ionization-time of flight mass spectrometry (Haff et al. 1997, Tost et al. 2005), SNPs are again becoming a focal point in molecular marker development because they are the most abundant polymorphism in any organism (as shown in Table 6.1), adaptable to automation, and reveal hidden polymorphism not detected with other markers and methods. SNP markers are regarded by many as the projected markers of choice for the future. In this chapter, I will summarize methods for SNP discovery, review the traditional approaches for SNP genotyping and their principles, present several major platforms for SNP genotyping using recently developed technologies, and discuss the pros and cons of SNP markers for aquaculture genome research.

What Are SNP Markers and Why Are They the Future Markers of Choice?

SNP can be defined as base variation at any site of the genome among individuals, or an alternative base at a given DNA site (Figure 6.1). Single nucleotide polymorphisms are by no means new. They were noticed ever since DNA was sequenced back in 1977. They were not used as markers for two decades because of the lack of efficient genotyping technologies. The differences between alleles of SNPs are very small; the length is the same, the only difference is one base substitution, most often from A to G, or from C to T. Separation of such subtle differences requires special technologies. After two decades of genetic analysis, there are several types of very efficient DNA markers. As pointed out by Vignal and others (2002) in their review on SNP markers, in terms of genetic information provided, as simple biallelic codominant markers, SNPs can be considered to be a step backward when compared to the highly informative multiallelic microsatellites (Middleton et al. 2004, John et al. 2004, Lin et al. 2005, Ma et al. 2005, Thalamuthu et al. 2005). Why are SNPs regarded as the choice marker system of the future? The major reasons include the recent need for very high densities of genetic markers for the studies of multifactorial diseases (Schaid et al. 2004, Kim et al. 2005, Wilcox et al. 2005, Xiang et al. 2005) and the recent progress in polymorphism detection and genotyping techniques. Because

Table 6.1. Estimation of SNP rates in various organisms.

Organism	Genome or genes studied	π Value	One SNP per DNA	References
Human	Genome	0.0008	1,250 bp	Sachidanandam et al. 2001
Mouse	Genome	0.0096	104 bp	Abe et al. 2004
Bos taurus and B. indicus	Amyloid	0.019	52.6 bp	Konfortov et al. 1999
Bos taurus and B. indicus	Leptin	0.0026	384 bp	Konfortov et al. 1999
Bos taurus	Amyloid	0.0096	104 bp	Konfortov et al. 1999
Bos taurus	Leptin	0.0023	434 bp	Konfortov et al. 1999
Bos taurus	Cytokine	0.0022	443 bp	Heaton et al. 2001
Chickens	31 kb survey	0.0044	225 bp	Schmid et al. 2000
Chickens	ESTs	0.00047	2,119 bp	Kim et al. 2003
Channel and blue catfish	161 ESTs	0.013	76 bp	He et al. 2003

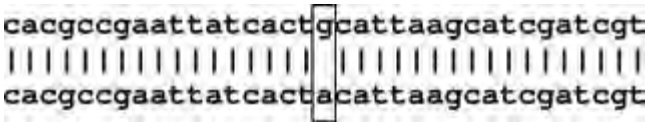

Figure 6.1. SNPs are alternative bases at a given site of DNA.

SNPs represent the ultimate reason for differences among individuals, and their analyses are most adaptable to automation, they are once again becoming the most popular molecular markers (Lai et al. 2001, Rafalski et al. 2002).

SNP Discovery

In spite of its increasing popularity as the choice of markers for the future, SNP discovery is a daunting task because as stated in its definition, single nucleotide polymorphism discovery depends on sequencing. Several approaches have been used for the discovery of SNPs in humans and animals. Earlier efforts used approaches such as single-strand conformation polymorphism (SSCP) analysis (Gonen et al. 1999), heteroduplex analysis (Sorrentino et al. 1992), and direct DNA sequencing. However, several recently developed approaches provide greater efficiencies.

The first option and the simplest is to conduct direct sequencing of genomic polymerase chain reaction (PCR) products obtained from different individuals. However, two factors really limit the use of this strategy. First, this approach requires the use of locus-specific PCR primers, and when large numbers of loci are involved, this approach is costly. Second, accurate sequencing of PCR products for the discovery of SNPs can be a great challenge. Dealing with sequencing ambiguities while attempting to identify

SNPs is not an easy job. Sequencing artifact with double peaks cannot be distinguished from true heterozygotes. In addition, for many aquaculture species, sequence information is limited.

The second strategy involves data mining from Expressed Sequence Tag (EST) projects, if EST libraries were constructed using multiple individuals. This approach is realistic because EST resources already exist, or are to be developed for the majority of important aquaculture species. This approach is advantageous in that the SNPs are coming from genes and many of them can come from coding regions and, therefore, the SNPs discovered are Type I markers. Also the coding region SNPs would allow analysis of association of SNPs with traits for the discovery of the "causing SNPs" for the traits (Bader 2001, Marnellos et al. 2003, Halldorsson et al. 2004, Stram 2004). However, this approach has major limitations. Because of evolutionary restraint on mutations in coding regions, SNP rates are generally much lower in coding regions than in noncoding regions. In addition, in some rare cases, sequence variation in ESTs may not represent SNPs in the genome due to RNA editing.

The third approach involves data mining from genome sequencing projects. Sequence comparisons in overlapped bacterial artificial chromosome (BAC) clone regions can be used for the discovery of SNPs. This approach depends heavily on genetic background of the DNA used for the construction of BAC libraries. Obviously, only SNPs that reside within the overlapping BAC regions can be discovered. More importantly, this approach is not applicable for species without a whole-genome sequencing project, which is the case currently for almost all aquaculture species.

The fourth approach is called reduced representation shotgun sequencing (RRS) (Altshuler et al. 2000). This approach is based on the fact that genomic segments of the same origin with the same size will migrate to the same position in gel electrophoresis. In this approach, DNA from multiple individuals (and in the case of humans, many individuals from all ethnic groups) is mixed together, cleaved with a restriction enzyme, and separated on agarose gels. A subset of the genomic digest contained within a slice of the gel is cloned and subjected to sequencing. A 2–5 fold shotgun sequencing is conducted to generate overlapping sequences, allowing sequence alignment and SNP discovery.

Traditional Approaches for SNP Genotyping

Unlike microsatellites, for which genotyping is standard with PCR amplification and sizing, many approaches have been considered for SNP genotyping. Traditional methods available for SNP genotyping include direct sequencing, single base sequencing (reviewed by Cotton 1993), allele-specific oligonucleotide (ASO) (Malmgren et al. 1996), heteroduplex analysis, denaturing gradient gel electrophoresis (DGGE) (Cariello et al. 1988), SSCP assays (Suzuki et al. 1990), and ligation chain reaction (LCR) (Kalin et al. 1992). Each approach has its advantages and limitations, but all are useful for SNP genotyping, especially in small laboratories limited by budget and labor constraints. Large-scale analysis of SNP markers, however, depends on the availability of expensive, cutting-edge equipment. Obviously, direct sequencing is the most accurate way of SNP genotyping, but the cost, efforts, and time requirement made it impractical.

Single-base Sequencing (Primer Extension Typing, PET)

Sequence only one base or analyze the primer extension product under conditions only one base is allowed to be extended. In this procedure, the sequencing primer is designed with its last base ends one base ahead of the SNP sites. The primer is extended only one base by using dideoxynucleotide triphosphates (ddNTP). The primer extension product is then analyzed on a sequencing gel.

Allele-specific Oligo Hybridization (ASO)

This approach uses the principle of reverse Southern blot hybridization. Oligo primers with SNPs are synthesized and immobilized on a solid support. Genomic DNA is then amplified and used to hybridize to the oligos. Hybridization is conducted in strictly controlled temperature regimes so that the perfectly matched oligo hybridizes, but the oligo with a single mismatch does not.

Single-strand Conformation Polymorphism (SSCP)

SSCP relies on the fact that within a short DNA segment (usually no more than 300 base pairs [bp]), a single base change in the sequence can cause major changes in single-stranded conformation that is a reflection of the secondary structure of single-stranded DNA upon hairpin formation and minor base pairings (Figure 6.2). In the procedure, double-stranded DNA is first generated by PCR, followed by denaturation and formation of single-stranded structures by self-annealing under relative diluted concentrations that favors formation of single-stranded conformation over annealing between the two strands. The SSCP is then analyzed on nondenaturing gels.

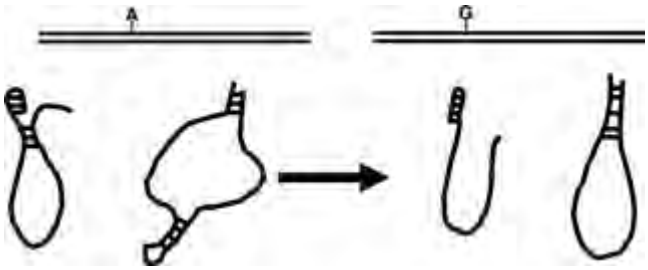

Figure 6.2. Schematic presentation of conformational changes due to single base substitutions. Note the conformations drawn are by no means a reflection of a real situation, but just an illustration of major conformation change. A single nucleotide change by base substitutions can lead to major changes in conformation of the single-stranded DNA, allowing allelic variations of SNPs to be differentiated by nondenaturing gel electrophoresis.

Heteroduplex Analysis

This approach relies on the fact that heteroduplexes run slower in gels than the homoduplexes. Upon amplification of a specific locus, the PCR products are subjected to heteroduplex analysis. In this procedure, the PCR products are first heat-denatured, and then allowed to re-anneal among strands. Three annealing products will be formed: two homoduplex representing the two genotypes with the SNP and one heteroduplex annealed between the molecules with SNP (Figure 6.3). Two types of heteroduplexes are common. Mutations involving deletions often lead to the formation of a bulge type of heteroduplex that can be readily differentiated on agarose gels, while base substitutions often lead to the formation of a bulb type of heteroduplex that requires a special gel mix to differentiate the heteroduplex from homoduplex (Bhattacharyya and Lilley 1989).

Denaturing Gradient Gel Electrophoresis (DGGE)

The principle of this technique is to separate DNA strands, based on their actual base composition, or the ratio of GC to AT base pairs that make up a particular segment of DNA. This is accomplished by exposing the DNA to a gradient of denaturant at

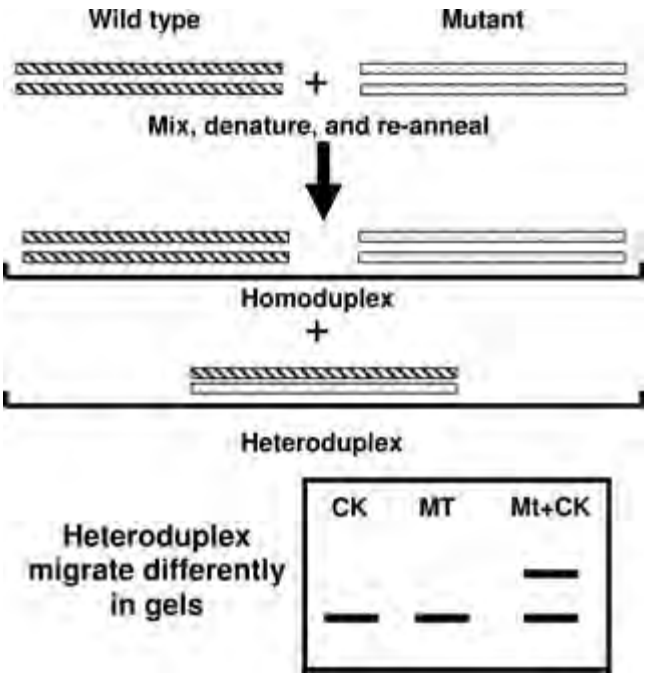

Figure 6.3. The principles of heteroduplex analysis.

elevated temperatures within a polyacrylamide gel. As the DNA sample progresses through the gel, from a low denaturant concentration to a higher one, it starts to melt at varying points. This is akin to the DNA "unzipping." The higher the GC content of the sample, the harder it is to melt. Thus, the DNA sample is able to progress further into the gel before stopping. Samples with lower GC content melt more rapidly in comparison. Therefore, they progress more slowly within the gel, thus becoming separated from the other faster-moving strands of DNA.

Ligation-mediated PCR

DNA ligases catalyze covalent joining of two DNA strands on the DNA template at a nick junction. The strict requirement of a base pair complementarity at the nick junction has been exploited for development of ligase-based technologies aimed at detection of sequence variations. After discovery of thermostable ligases, methods employing amplification of the diagnostic signal through repeated cycles of denaturation, annealing, and ligation have been developed analogous to PCR. The test is usually performed by designing two oligonucleotides specific for each allele and labeled differently on one side of SNP, and one common oligonucleotide on the other. Detection of the alleles can be performed directly in the microtiter plate wells by colorimetric approaches (Tobe et al. 1996), or by gel separations.

Recently Developed SNP Genotyping Approaches and Platforms

Several options are available for efficient genotyping using state-of-the-art equipment. Particularly popular are methods involving MALDI-TOF mass spectrometry (Ross et al. 1998, Storm et al. 2003), pyrosequencing (Ahmadian et al. 2000, Alderborn et al. 2000, He et al. 2003), *TaqMan* allelic discrimination (Li et al. 2004), real-time quantitative PCR (Nurmi et al. 2001), and the use of microarray or gene chips (Hacia et al. 1999). Mass spectrometry and microarray technologies require a large investment in equipment. The equipment for pyrosequencing and quantitative PCR is generally less than $100,000, and should be more affordable for laboratories working in the area of aquaculture genetics. Another consideration is the expense of genotyping in relation to sample sizes. Microarray (gene chip) technology and quantitative PCR are particularly useful in medical and clinical settings where large numbers of samples (thousands of individuals per locus) are involved and that can justify the cost involved in the development of the gene chips and hybridization probes. Mass spectrometry and pyrosequencing are relatively cost-effective (after acquisition of the equipment) when working with relatively small sample sizes (e.g., hundreds of individuals per locus), as is most likely the case in aquaculture research.

In addition to these major platforms, several recently developed SNP genotyping systems are particularly adaptable for situations involved in aquaculture genomics. The SnapShot SNP detection system and the invader assay are especially attractive because of their ease to automation. The principles of several SNP genotyping systems are described below.

TaqMan Technology

TaqMan technology integrates a PCR-based assay with laser scanning technology to excite fluorescent dyes present in the specially designed *TaqMan* probes. Its use in SNP genotyping is based on allele-specific hybridization. Briefly, the method is based on the 5′-3′ exonuclease activity of the *Taq* DNA polymerase that results in cleavage of the *TaqMan* probes, allowing fluorescence to be emitted during PCR; the intensity of fluorescence is then measured by a Sequence Detection System. The *TaqMan* probe is located between the two PCR primers and has a melting temperature 10°C higher than that of the primers; binding of the *TaqMan* probe prior to the primers is crucial because without it PCR products would be formed without generation of fluorescence intensity and thus without being detected. The *TaqMan* probe has two fluorescent tags attached to it. One is a reporter dye, such as 6-carboxyfluorescein (FAM), which has its emission spectra quenched due to the spatial proximity of a second fluorescent dye, 6-carboxy-tetramethyl-rhodamine (TAMRA). Degradation of the *TaqMan* probe, by the *Taq* DNA polymerase, frees the reporter dye from the quenching activity of TAMRA and thus the fluorescent activity increases with an increase in cleavage of the probe, which is proportional to the amount of PCR product formed (Figure 6.4). For SNP detection, two *TaqMan* probes are designed with one for each allele, and they are labeled with different fluorescent dye (e.g., VIC dye is linked to the 5' end of the Allele 1 probe. FAM dye is linked to the 5′ end of the Allele 2 probe). *Taq* DNA polymerase cleaves only probes that are hybridized to the target. Mismatches between a probe and target reduce the efficiency of probe hybridization. Furthermore, DNA polymerase is more likely to displace a mismatched probe without cleaving it, which does not produce a fluorescent signal.

The Invader Assay

The invader assay enables simultaneous detection of two different alleles (e.g., Ryan et al. 1999, Kwiatkowski et al. 1999, Cooksey et al. 2000, Hsu et al. 2001). Two oligonucleotide probes (an allele-specific primary probe and an invader probe) hybridize in tandem to the target DNA to form a specific overlapping structure (Figure 6.5). The 5′-portion of the primary probe contains a 5′-Flap that is noncomplementary to the target DNA and therefore cannot hybridize to the target sequence. The 3′-end of the invader probe overlaps the primary probe by a single base at the SNP site. A cleavage enzyme (Flap endonuclease I) recognizes the overlapping structure and cleaves the 5′-Flap on the primary probe at the base of the overlap releasing it as a target specific product. If the probe does not hybridize perfectly at the site of interest, no overlapping structure is formed, no cleavage occurs, and the target-specific product is not released.

The invader assay consists of two primary probes and two invader probes, with each set of primary probes and invader probes specific to Allele 1 or Allele 2, respectively, generating two target-specific products.

The target specific 5′-Flap oligos are involved in a secondary reaction for quantification of the fluorescent signals. The released target-specific 5′-Flap oligos act as invader probes on a fluorescent resonance energy transfer (FRET) cassette leading to the formation of an overlapping structure that is recognized by the cleavage enzyme. When the

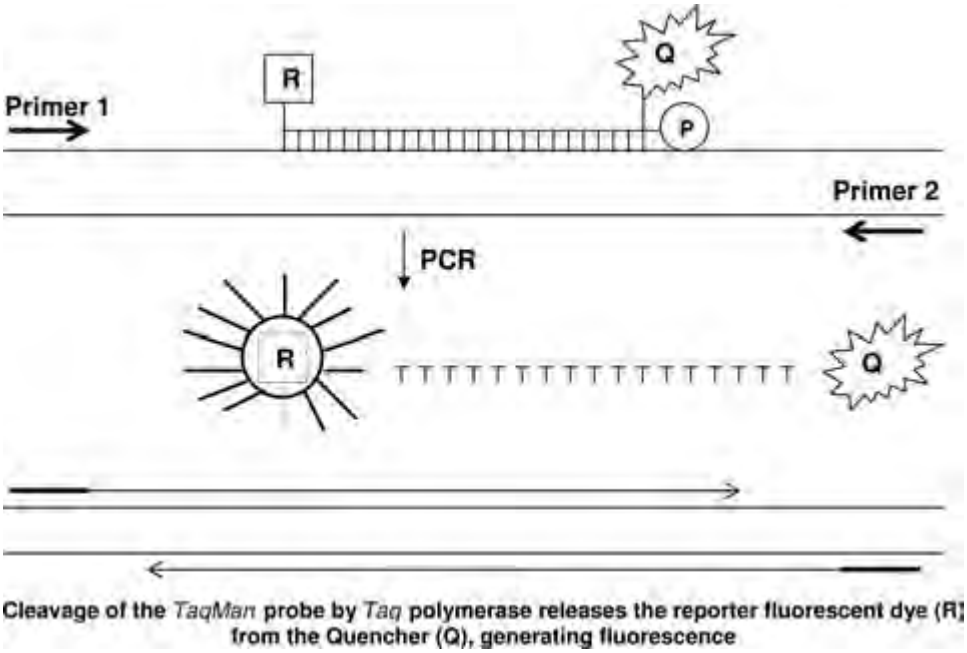

Figure 6.4. The *TaqMan* 5′-3′ nuclease assay. PCR primers 1 and 2 and a *TaqMan* probe, labeled with a reporter dye, FAM, (R) and a quencher dye, TAMRA, (Q), bind to the DNA template. The 3′ phosphate group (P) prevents extension of the *TaqMan* probe. The presence of the enzyme, *Taq* polymerase, enables extension of the primer which displaces the *TaqMan* probe. The displaced probe is cleaved by *Taq* DNA polymerase resulting in an increase in relative fluorescence of the reporter.

FRET cassette is cleaved, a fluorophore is released from a quencher, generating a fluorescence signal. There are two signal fluorophores attached to two different FRET cassettes that are spectrally distinct and specific to either allele of the biallelic system. The ratios of the two fluorescent signals then allow a genotype to be assigned.

MALDI-TOF Mass Spectrometry Platform

SNP genotyping using MALDI-TOF mass spectrometry involves PCR of the SNP region, annealing of a primer immediately ahead of the mutation spot, primer extension using dideoxy nucleotides, and mass spectrometric analysis based on molecular size of the primer extension products (Lawrence et al. 1997). It is one of the most efficient SNP genotyping methods and can perform 60,000 genotypes a day.

DNA Chip Platform for SNP Genotyping

Genotyping of SNP using the DNA chip technology can be viewed as the very high density of allele-specific oligo analysis as discussed above. A DNA chip is a small

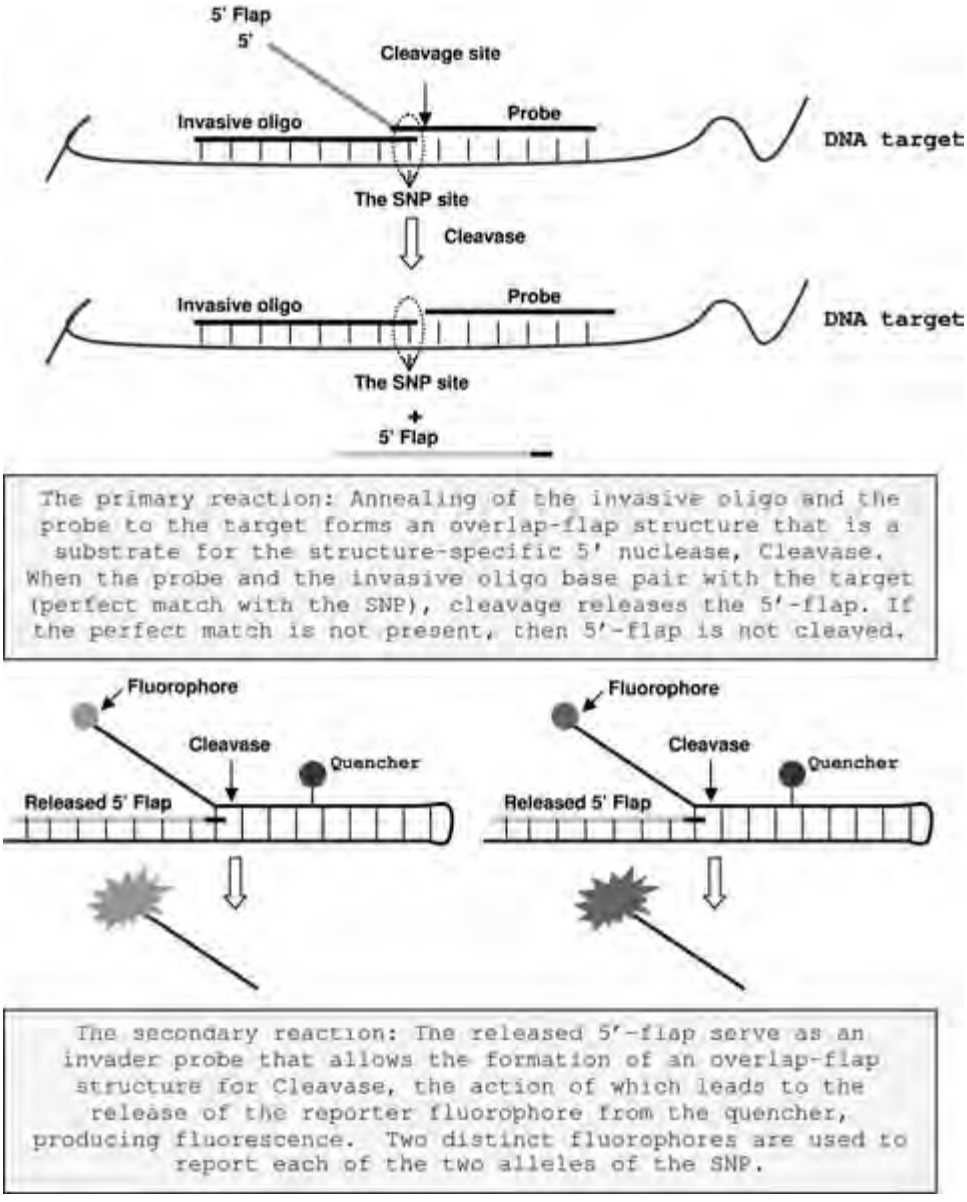

Figure 6.5. A schematic presentation of the invader assay of SNPs. (Also see color plate.)

piece of silicon glass (approximately 1 cm^2) to which a large number of synthetic, single-stranded DNA oligonucleotides ("oligos") have been chemically bonded. Oligos function as DNA probes. They anneal selectively only to those DNA molecules whose nucleotide sequences are exactly complementary—T pairs with A, and G with C. Therefore, they can be used to identify the presence of specific DNA sequences in a heterogeneous mixture of genes. DNA chips can be used to look for DNA sequences that differ by SNPs. To determine which alleles are present, genomic DNA

from an individual is isolated, fragmented, tagged with a fluorescent dye, and applied to the chip. The genomic DNA fragments anneal only to those oligos to which they are perfectly complementary.

The Beadarray Platform (The GoldenGate Assay)

Very recently, a Beadarray platform was developed by Illumina (Fan et al. 2003). As this platform provides the greatest flexibility, has the highest troughput, and is one of the most economical platforms (pennies per genotype), the Illumina's GoldenGate assay has become the most popular large-scale SNP assay. The allele discrimination at each SNP locus is achieved by using three oligos (P1, P2, and P3, each is tailed at 5′ with sequence A, B, and C, respectively, serving as universal primers for PCR), of which P1 and P2 are allele-specific and are Cy3- and Cy5-labeled. P3 is locus-specific designed several bases downstream from the SNP site. If the template DNA is homozygous, either P1 or P2 will be extended to meet P3; if the template is heterozygous, both P1 and p2 will be extended to meet P3, allowing ligation to happen. Upon ligation, the artificial, allele-specific template is created for PCR using universal primers. P3 contains a unique address sequence that targets a particular bead type with a complementary sequence to the address sequence. After downstream-processing, the single-stranded, dye-labeled DNAs are hybridized to their complement bead type through their unique address sequences. After hybridization, the BeadArray Reader is used to analyze flourescence signal on the Sentrix Array Matrix or Beadchip, which is in turn analyzed using software for automated genotype clustering and calling. Most recently, an even higher throughput system called iSelect Infinium Custom Genotyping was launched by Illumina, allowing tens of millions of genotypes to be determined simultaneously. Although the equipment performing the GoldenGate and iSelect assays are expensive, genotyping services are available. Interested readers are referred to the company's web site (http://www.illumina.com/). Undoubtedly, such efficient systems will have a tremendous impact on aquaculture genome research as well as on medical genomics.

Inheritance of SNP Markers

Theoretically, a SNP within a locus can produce as many as four alleles, each containing one of four bases at the SNP site—A, T, C, and G. Practically, however, most SNPs are usually restricted to one of two alleles (quite often either the two pyrimidines C/T or the two purines A/G) and have been regarded as biallelic. They are inherited as codominant markers in a Mendelian fashion. Obviously, their polymorphic information content (PIC) is not as high as multiallele microsatellites, but this shortcoming is balanced by their great abundance.

Conclusion

With so many approaches for SNP discovery and genotyping, it is not easy to determine the best approach. It all depends on the situation and objectives. Clearly, the genome of a large number of aquaculture species will not be sequenced and therefore, SNP

discovery in aquaculture species likely will come in the form of data mining using ESTs and BAC end sequences, although limited efforts using targeted PCR or reduced RSS are possible.

SNPs can be genotyped with a wide range of techniques and instrumentations, from small-scale, low-budget to expensive high-throughput systems. For SNP genotyping, the greatest determinants of the genotyping platform depend on the availability of equipment. Given the availability of the equipment, considerations can be made based on budget, number of markers, number of individuals, and the requirement for robustness. In spite of its current low levels of application in aquaculture genome research, SNP markers should gain in popularity as more and more sequence information becomes available in aquaculture species. Equally important, once the genetic linkage maps are well constructed, genome scans for quantitative trait loci (QTL) are expected to follow, in order to study traits important to aquaculture, which then depends on the use of well-defined association analysis. Because SNP markers are great markers for the analysis of trait-genotype associations, their application to aquaculture will become essential. It may be the case that a few laboratories working in aquaculture genomics will be able to map a large number of SNPs to genetic maps, saving the trouble for most other laboratories that can concentrate on studies involving biology and aquaculture traits.

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