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REVIEW

DNA microarray technology and its application in fish biology and aquaculture

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Abstract Fishery is an important industry in China as well as in the rest of the world, and it provides a human food resource containing high-quality protein. Best practice in aquaculture requires a full understanding of the genomic controls and transcriptional profiles of cultured fish species. Improvements in aquaculture can be made by regulation of the expression of functional genes. Microarray technology is a powerful tool for rapid screening of genes or transcriptional profiles in a particular fish or for a particular economic character; for example, genes that are related to growth and disease control in the fish. This review provides a brief introduction to microarray technology and its methods and applications, together with a discussion of the achievements in fish biology that have resulted from this technology.

Keywords complementary DNA (cDNA) microarray, bioinformatics, transcription profiles, teleost fish, aquaculture

1 Introduction

Since the historical discovery of the DNA double helix in the last century, research on gene discovery and gene structures and functions has accelerated. The traditional approaches to molecular studies of a given effect, by analyzing a single gene or a few genes at a time, are important for investigation of gene function and regulation. However, the biological characteristics of a given cell or tissue, or even those of an organism, may be controlled by many molecular events and involve hundreds or thousands of genes (Fiehn, 2001; Fujimoto et al., 2007). DNA microarray analysis is a powerful tool that helps expand our knowledge of an organism's genome or transcriptome by measuring the expression levels of thousands of genes simultaneously in a particular cell or tissue (Douglas, 2006). This approach may reveal global patterns of gene expression and identify novel genes that are associated with phenotypic characteristics (Arcand et al., 2004; Gonzalez et al., 2004).

Recently, a number of studies have used microarray technology to determine gene profiles in different fish species. For example, a microarray consisting of 4512 complementary DNAs (cDNAs) was constructed to investigate the adaptive molecular responses of zebrafish to hypoxia during development (Ton et al., 2002, 2003). Linney et al. (2004a) analyzed 15 512 unique transcripts from wild-type *Danio rerio* and found that 23 muscle-specific genes were up-regulated as part of somite development. An Affymetrix microarray was also used to screen the genes that are involved in regeneration of the caudal fin (Schebesta et al., 2006). Apart from these examples, microarray analyses have been used to search for immune-related genes that are expressed following DNA vaccination or infection by fish pathogens (Byon et al., 2005, 2006; Kurobe et al., 2005; Lua et al., 2005) and to study environmental influences on gene expression (Williams et al., 2003; Koskinen et al., 2004; Lam et al., 2006).

Obviously, the use of DNA microarray technology in fish biology and aquaculture may have great significance and may be applied to discovery of novel genes, gene expression profiling from fish species of interest, and identification of the genomic responses to environmental stimulation in aquaculture. This review gives a brief introduction to the technology and its experimental design and data analysis and a discussion of recent progress in research using microarray technology in fish biology and aquaculture.

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2 Microarray platforms and general procedures

DNA microarrays for fish biology and aquaculture exist in two basic platforms: (1) High-density arrays, such as the Affymetrix Zebrafish Genchip (Catalog #900487) (Affymetrix, Santa Clara, CA, USA), have been commercially produced. In the Affymetrix, 15617 genes or expressed sequence tag (EST) fragments are spotted on the chip, which provides a "ready-to-use" chip for the expression analysis of a relatively large number of genes. (2) Customized cDNA microarrays using glass slides or nylon membranes spotted with polymerase chain reaction (PCR)-amplified cDNA fragments or synthetic oligonucleotides have been designed based on ESTs (Ju et al., 2007a, b). Customized microarrays have been successfully constructed for several fish species. They include a zebrafish cDNA microarray containing 4512 unique genes identified from zebrafish cDNA libraries (Ton et al., 2002), a European flounder (*Platichthys flesus*) cDNA microarray that contains 11060 clones spotted in duplicate (Cohen et al., 2007), and a chip for the African cichill fish, *Astatotilapia burtoni*, that contains about 4500 features (Renn et al., 2004). A salmonid microarray containing 16006 cDNAs has been used to study the transcriptome response to atrophy of fast-twitching muscles from gravid rainbow trout compared with those of sterile fish (Ewart et al., 2005; Salem et al., 2006). A microarray comprising 3514 cDNAs was constructed from a medaka EST library to elucidate the transcriptional responses associated with temperature shift from 25°C to 15°C in a medaka cell line (Hirayama et al., 2008). A custom-made microarray has also been developed for *Cyprinus carpio* (Moens et al., 2007). All of the above studies have provided the basis for the molecular and cellular analyses of complex traits in a wide range of fish species.

The basic principles and working procedures for the construction of fish DNA microarray biotechnology are described in Fig. 1. For the construction of a cDNA microarray, cDNA fragments or EST sequences for the fish species of interest must be prepared from a cDNA library or PCR-amplified products. Transcriptome sequences of many fish species are now available in the National Center for Biotechnology Information (NCBI) GenBank database (http://www.ncbi.nlm.nih.gov), and these collected EST sequences can be used to synthesize oligonucleotides and can be spotted onto array chips to produce cDNA microarrays. Currently, several fish species, such as *Danio rerio* (zebrafish), *Oncorhynchus mykiss* (rainbow trout), *Oryzias latipes* (Japanese medaka), *Salmo salar* (Atlantic salmon), *Takifugu rubripes* (pufferfish), and *Cyprinus carpio* (common carp), have at least 10000 ESTs that are available in the NCBI GenBank (reviewed by Ju et al., 2007b). Using these published DNA sequences or ESTs, various customized cDNA microarrays have been successfully constructed and have made significant contributions to the discovery of genes and molecular biology in fish (Pollack et al., 1999; Lo et al., 2003; Byon et al., 2005; Corredor-Adamez et al., 2005; Ju et al., 2007a).

Using commercially produced DNA microarrays, such as the Affymetrix Zebrafish Genchip, it is possible to make DNA probes from cRNA, or from cDNA fragments, and hybridize them to the chips. The images obtained are then scanned with an Affymetrix 418 array scanner, and all expression signals are analyzed using the R/Bioconductor software (http://www.bioconductor.org). The basic working procedures are outlined in Fig. 2. Although commercially available cDNA microarrays are limited in scope, some fish cDNA arrays can be heterogeneously hybridized to sequences from other species. For example, Renn et al.

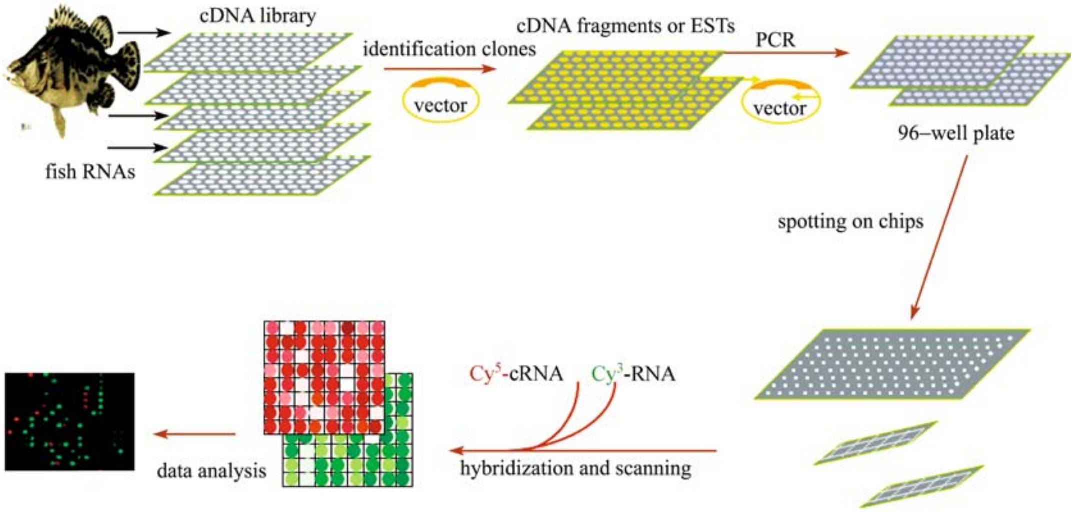

Fig. 1 Outline of the cDNA microarray technology. Fish total RNAs were isolated to construct a cDNA library, and PCR-amplified and purified DNA fragments were spotted on array chips. Complementary RNAs (cRNAs) were prepared from target samples that were hybridized to the array. The final microarray images are scanned and analyzed by various computer programs, and differentially expressed transcripts are identified.

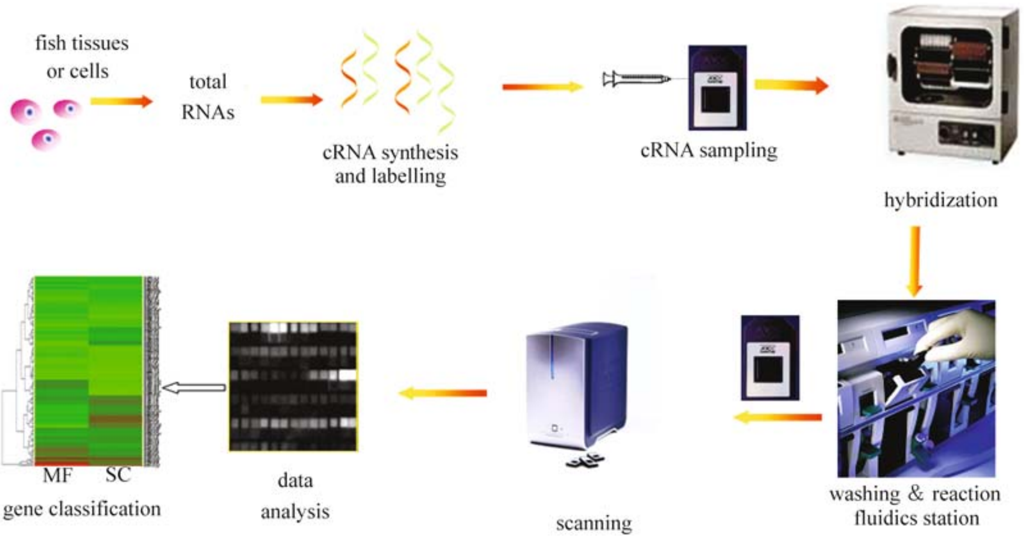

Fig. 2 Use of commercially available cDNA microarray for gene discovery and transcriptome profiling. RNAs are isolated from the fish tissues of interest and labeled, hybridized to array chips. The obtained image was scanned, and data were analyzed for differentially expressed gene profiling. MF: Madarin fish; SC: Siliver carp.

(2004) applied a cDNA microarray from an African cichlid fish, *Astatotilapia burtoni*, to analyze the transcription profile of eight different fish species; Ju et al. (2007a) used Japanese medaka (*Oryzias latipes*) cDNAs to screen several related human genes; and Lam and Gong (2006) used a zebrafish cDNA microarray to screen for genes that are involved in human liver cancer, thus providing additional confidence in the approach that uses the zebrafish cDNA microarray. Such applications indicate that use of the zebrafish cDNA microarray is a valid way to examine the genes of other fish (and mammalian) species. In our laboratory, we have applied an Affymetrix zebrafish cDNA microarray to characterize transcriptional profiles in the muscle tissues of the Chinese mandarin fish, *Siniperca chuatsi*, and the silver carp, *Hypophthalmicthys molitrix*. Using this method, 375 genes have been identified in the muscle tissues of both the Chinese mandarin fish and the silver carp. The number of genes identified in the muscle tissue of these fish appears to be a reasonable representation of the transcription profile for muscle tissues. Using this approach, gene expression profiles of the muscle tissues have been established for both fish species, and genes that may be related to the differences in muscle structure have been identified.

3 Target preparation, microarray screening, and data analysis

3.1 Target preparation

In the use of cDNA microarray technology for fish biology and aquaculture, several important issues should be addressed. These include the identification of functional genes for growth and metabolism in commercially important fish species, the effect of environmental stimuli on gene expression, and the discovery of genes that act against diseases, such as viral infections. First, before beginning such experiments, the selection of fish samples and treatments must be considered. Each target fish sample should have suitable controls. For example, to identify differential transcription profiling in fish following infection by viruses or bacteria, uninfected samples should be used as parallel controls.

Second, for successful microarray hybridization, a high quality of RNA extraction is required. Use of TRIzol^R Reagent (Invitrogen Life Technologies), following the manufacturer's standard protocol for total RNA preparation, is applicable, but the obtained RNAs should be further purified on an RNeasy column (Qiagen). All the RNA sample handlings require an RNAse-free environment and lower temperatures. In the standard protocol, purified RNAs (about 5 μg) from the fish samples of interest were reverse transcribed to single-stranded cDNA using a label star Array kit (Qiagen), and then the purified cDNAs or cRNAs were labeled with Cy^3 or Cy^5-dCTP using a CyScribe first-strand cDNA labeling kit, following the manufacturer's protocol (Byon et al., 2005, 2006). At present, there are four dyes that have been tested for cDNA labeling, namely, Alesxa 488, Alexa 594, Cy^3, and Cy^5, and the three dyes, Alexa 594, Cy^3, and Cy^5, have been found to be efficient for use in oligonucleotide arrays (Xiang and Chen, 2000). Methods for hybridization of labeled cDNA or cRNA to cDNA microarrays vary among different reports (Rise et al., 2004; Douglas, 2006; Martyniuk et al., 2007; Moens et al., 2007). In our work, purified labeled cRNAs from both Chinese mandarin fish and silver carp were first reacted with the hybridization mixture, which contained 0.5 mg/mL cRNAs (30 μL), Oligo B_2 (50 μL), 9.3 mg/mL fish sperm DNA (3.3 μL), 50 mg/mL bovine serum albumin (3 μL), 2×hybridization buffer (150 μL), dimethyl sulfoxide (30 μL), and RNAse-free water (63.7 μL) in a total reaction volume of 300 μL. The hybridization mixture was added to the microarray and incubated at 45°C for 16 h with gentle shaking at 60 r/min. After hybridization, the hybridization mixture was removed, and the array was rinsed three times with a rinsing buffer. The above procedure produced positive results in our studies, but efficient protocols for cDNA hybridization should be developed for different samples.

3.2 Microarray screening and data analysis

Microarray chips are usually scanned using an Affymetrix 418 array scanner. The images and data files obtained may be uploaded to software programs for image and statistical analyses. Image analysis is the first important step in obtaining reliable data from microarray experiments. All expression signals may be analyzed using the R/Bioconductor software (http://www.bioconductor.org). Data normalization is also important in order to remove or minimize non-biological effects that may occur during performance of the experiment, such as variations in scanning parameters, dye selection, and hybridization conditions (Xiang and Chen, 2000). Background correction and normalization may be performed using the Robust Multi-array Average, known as gcRMA, in R/Bioconductor (Bolstad et al., 2003; Wu et al., 2005; Ranheim et al., 2008). Other methods for normalization have been frequently used, such as rimmed mean and global mean (Yang et al., 2001), local mean (Colantuoni et al., 2002), locally weighted scatter plot mean (Berger et al., 2004), and Bayesian analysis (Zhang et al., 2005). For example, we applied Affymetrix zebrafish cDNA microarrays in the analysis of transcriptional profiling in four fish species. Four Affymetrix microarrays containing 15617 genes each were analyzed using the standard package R/Bioconductor (http://www.bioconductor.org). gcRMA, with the help of probe sequence information, was used for background correction and normalization of the microarray (Xiang and Chen, 2000; Bolstad et al., 2003; Ranheim et al., 2008). Fig. 3A shows the preliminary plots and data analyses before the background correction and data normalization, and Fig. 3B illustrates the distribution of gene expression after background correction and data normalization.

Following background correction and data normalization, cluster analysis is required to extract gene expression patterns and to define the relationships between gene expression profiles across different experiments and data points. Using cluster analysis, similar expression patterns or structures in data from different samples can be identified (Eisen et al., 1998). Three methods are generally used for microarray data clustering: they are hierarchical clustering, self-organizing maps, and principal components analysis (Ju et al., 2007b). Statistical analysis, including calculation of *P*-values, fold changes, and gene expression patterns, is then necessary to provide biological insights from the microarray data. Finally, the potential functions of the identified differentially expressed genes are screened in GenBank.

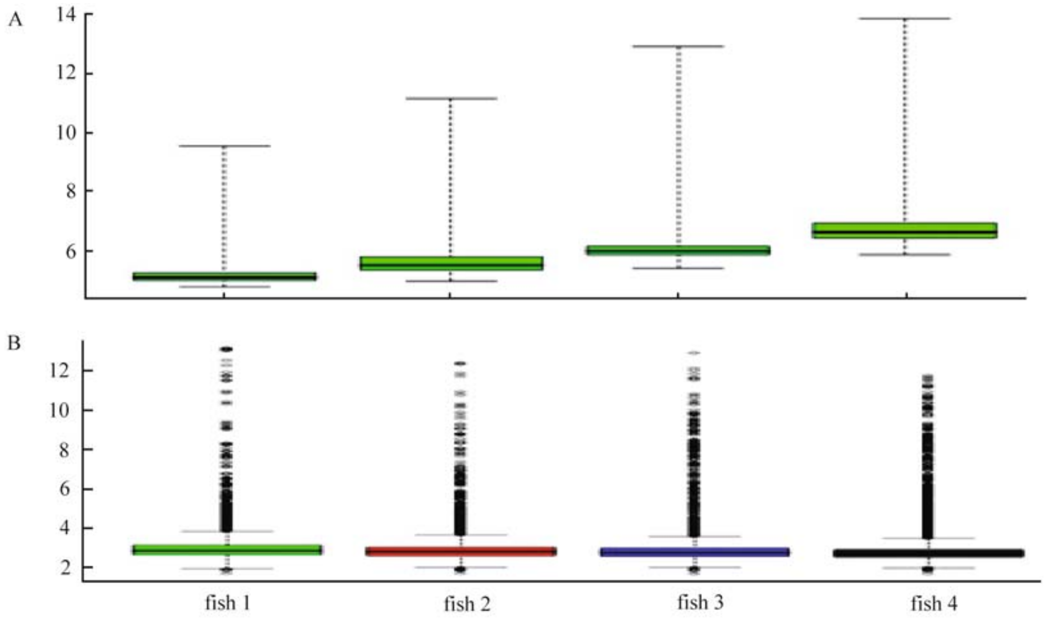

Fig. 3 Microarray data analysis before (A) and after (B) the background correction and data normalization

4 Application and progress of microarray technology in fish biology

4.1 Use of microarrays for fish gene expression profiling and discovery

Screening of global gene expression profiles and discovery of differential gene expression have been used for identification of genes in tissue-specific or development-specific profiling in various fish species. Zebrafish has provided a model for the application of microarray technology. By construction of a zebrafish cDNA microarray, gene expression patterns and profiling in zebrafish during development have been well defined at five time points. This work has revealed that some functional genes, such as those for actin, myosin heavy and light chains, and parvalbumin, are expressed in close correlation with the muscle development and growth of the fish (Ton et al., 2002). Linney et al. (2004a) analyzed 15512 unique transcripts from wild-type *Danio rerio* and detected 420 up-regulated and 386 down-regulated genes during development at 12 hours past fertilization (hpf). In addition, 23 muscle-specific genes were found to be up-regulated during the initial 24 hpf, corresponding to the development and differentiation of the somites (Linney et al., 2004a). During larval development in sea bass, 6626 distinct genes are expressed at 10 different time points between 7 and 43 days post hatching (dph), and the differentially expressed genes ($n = 485$) are generally up-regulated in two periods: between 7 and 23 dph and between 25 and 43 dph (Darias et al., 2008).

Microarray technology is also a functional tool for investigating tissue or organ development of fish at the transcriptional level. For example, Nishidate et al. (2007) constructed a cDNA microarray and identified 140 transcripts that were up-regulated during regeneration of fins in the medaka fish. Among the genes, 22 were involved either in the differentiation of cartilages or in the development of the basal wound epidermis, or blastema. Katogi et al. (2004) identified six candidate genes that were associated with blastema formation. During the development of the retinal pigment epithelium (RPE) in zebrafish, 8810 genes were significantly expressed in the RPE at 52 hpf, of which 1443 potentially had biologically meaningful expression levels. Furthermore, 78 and 988 probe sets were found to be significantly over-expressed or under-expressed in the RPE, respectively (Leung et al., 2007). In addition, heart regeneration in zebrafish is associated with sequential up-regulation of genes that are associated with wound healing and growth factors, which suggests that the plate-derived growth factor (PDGF) signaling is required (Lien et al., 2006).

4.2 Use of microarrays for identification of genes in fish that are related to immunity or disease resistance

Bacterial and viral infections are associated with important diseases of fish. Analysis of the expression pattern of immunity-related genes not only provides insight into the molecular mechanisms underlying the fish immune system, but also assists in the development of effective vaccines for use in aquacultural practice (Adams and Thompson, 2006). Microarray technology has made it possible to clarify the immune system in detail, and several pioneer works have applied the techniques to this system (Tsoi et al., 2003; Rise et al., 2004; Byon et al., 2005; Ewart et al., 2005; Martin et al., 2006). For example, the *Mycobacterium marnum*–zebrafish infection model has been used for analysis of a host transcriptome response to mycobacterial infection. Three types of array were used, namely, the MWG and Sigma zebrafish oligonucleotide arrays and the Affymetrix zebrafish chip, and it was found that 66 unregulated and 93 down-regulated genes consistently showed an altered expression in each of the three microarrays (Meijer et al., 2005).

Red sea bream iridovirus (RSIV) is an infectious pathogen that is responsible for causing serious diseases in aquatic animals, including fish, and the RSIV disease causes mass mortalities and huge economic loss in aquaculture. Lua et al. (2005) developed a DNA microarray for RSIV to monitor the temporal kinetic transcription program of the viral genes during *in vitro* infection. They demonstrated that the major capsid protein (MCP) is a structural protein that comprises up to 45% of the total protein expression, and the proteins are assembled into RSIV virions about 2 days after infection.

Byon et al. (2005) constructed a cDNA chip that contains approximately 900 different cDNA clones, including more than 200 immune-related genes. These researchers immunized juvenile Japanese flounder with a recombinant plasmid expressing the G-protein of viral haemorrhagic septicaemia virus (VHSV) and analyzed gene expression with cDNA microarrays. Using this method, the genes that are responsible for a strong protective non-specific immune response and a specific immune response were elucidated (Byon et al., 2005, 2006). Tsoi et al. (2003) used a human cDNA microarray to identify differentially expressed genes in Atlantic salmon liver during infection with *Aeromonas salmonician*. These examples show that the use of microarray technology is an efficient technique for screening and identification of genes that are related to the immune system.

4.3 Use of microarrays for identification of genes that are responsive to environmental variation

Fish offers important advantages for defining the organism–environment interface and the genomic responses to natural stressors. Microarray technology serves as a valuable tool in toxicogenomics, with which environmental adaptation in fish has been evaluated (Ju et al., 2002, 2007a; Ton et al., 2003; Linney et al., 2004b; Moens et al., 2007). Brown et al. (2008) have developed a microarray for transcriptomic analysis of chemical responses in populations of *Gasterosteus aculeatus* under laboratory and field conditions, and a full set of genes that are responsive to pollutants has been identified. Finne et al. (2007) applied a cDNA microarray platform in ecotoxicological screening of single chemicals and environmental samples that are relevant to the aquatic environment. The work was performed to validate biomarker gene responses of *in vitro* cultured rainbow trout (*Oncorhynchus mykiss*) hepatocytes that were exposed to model chemicals and to investigate the effects of mixture toxicity in a synthetic mixture. Chemicals used for 24-h exposure to single chemicals and mixtures were 10 nmol/L 17alpha-ethinylestradiol (EE2), 0.75 nmol/L 2,3,7,8-tetrachloro di-benzodioxin (TCDD), 100 mol/L paraquat (PQ), and 0.75 mol/L 4-nitroquinoline-1-oxide (NQO). The results revealed that exposure to this mixture led to an average loss of approximately 60% of the transcriptomic signature found during exposure to a single chemical. Larkin et al. (2002) used microarray techniques for detection and distribution of estrogenic compounds in sheepshead minnow, and several estrogen-responsive genes were identified. A similar investigation led to the identification of a transcriptional fingerprint of estrogen exposure in live rainbow trout (Benninghoff and Williams, 2008).

Hypoxia is important in both biomedical and environmental contexts and requires rapid adaptations in metabolism. Fish live and survive in environments with low and variable levels of oxygen, and the survival mechanisms induced in response to environmental variation have been studied. During this response, several changes may occur in physiology, genomics, and gene expression (Ton et al., 2003; van der Meer et al., 2005; Ju et al., 2007a). Ton et al. (2004) used a zebrafish cDNA microarray to examine the expression of more than 4500 genes in zebrafish embryos that were exposed to 24 h of hypoxia during development. The work revealed that hypoxia resulted in changes in the gene expression profile of the zebrafish embryos and that these changes could be reversed by exposure to a normoxic (20.8% O_2) environment. Ju et al. (2007a) developed a microarray containing 8046 medaka unigenes and measured gene expression changes in the brain, gills, and liver of fish exposed to hypoxia. They found that 501 genes in the brain, 442 in the gills, and 715 in the liver were differentially expressed in medaka exposed to hypoxia. Two biological pathways, namely, ubiquitin-proteasome and phosphatidylinositol signaling, were significantly dysregulated in medaka upon exposure to hypoxia. In long-term adaptive responses to hypoxia in adult zebrafish, use of the microarray identified 367 differentially expressed genes, of which 117 showed hypoxia-induced and 250 hypoxia-reduced expressions. A novel adaptive mechanism to hypoxia, i.e., the induction of genes for lysosomal lipid trafficking and degradation, was suggested as a result of these findings (van der Meer et al., 2005).

In addition, a change in water temperature is another factor that affects fish growth and physiology, and exposure to different water temperatures may cause changes in the pattern of gene expression (Gracey, 2007; Kassahn et al., 2007; Hirayama et al., 2008).

4.4 Use of fish DNA microarrays to study genes in other organisms, or heterogeneous microarray hybridization

With the limited availability of custom-made and commercially produced cDNA microarrays for fish, it is advantageous to exploit the possibility of hetero-hybridization of one array to other species. Cohen et al. (2007) utilized a cDNA microarray for European flounder (*P. flesus*) containing 11060 clones to cross-hybridize with genes of several other fish species. The work revealed that cross-species cDNA microarray hybridization in fish, at a suborder level and closer, is a useful tool for gene expression profiling. Renn et al. (2004) applied a cDNA microarray from an African cichlid fish, *Astatotilapia burtoni*, to analyze the transcription profile of eight different fish species. Ju et al.(2007a) used Japanese medaka (*Oryzias latipes*) cDNAs to screen several related human genes, and Lam et al. (2006) used a zebrafish cDNA microarray to screen for genes that are involved in human liver cancer, thus providing additional confidence in the use of the zebrafish cDNA microarray. Such applications indicate that use of the zebrafish cDNA microarray is a valid way to examine other fish (and mammalian) species.

Because cDNA microarrays are not available for the Chinese mandarin fish and the silver carp, the Affymetrix zebrafish cDNA microarray has been used in our laboratory. Although the Chinese mandarin fish is far removed from the zebrafish in an evolutionary sense, the results of this research showed that the zebrafish cDNA microarray is suitable for analysis of mRNA populations in the Chinese mandarin fish. As presented in Fig. 4, the cDNAs from both Chinese mandarin fish and silver carp hybridized successfully to the Affymetrix Zebrafish Genchip. Three hundred seventy-five genes were identified in the muscle tissues of both the Chinese mandarin fish and the silver carp. The number of genes identified in the muscle tissue of each fish appears to be a reasonable representation of the transcription profile for a single muscle tissue. The microarray data were also validated by quantitative real-time PCR. However, the possibility cannot be excluded that certain genes in both fish species were not detected.

5 Conclusions and perspectives

DNA microarray analysis is a powerful tool for analysis of an organism’s genome or transcriptome by measuring the expression levels of thousands of genes simultaneously in

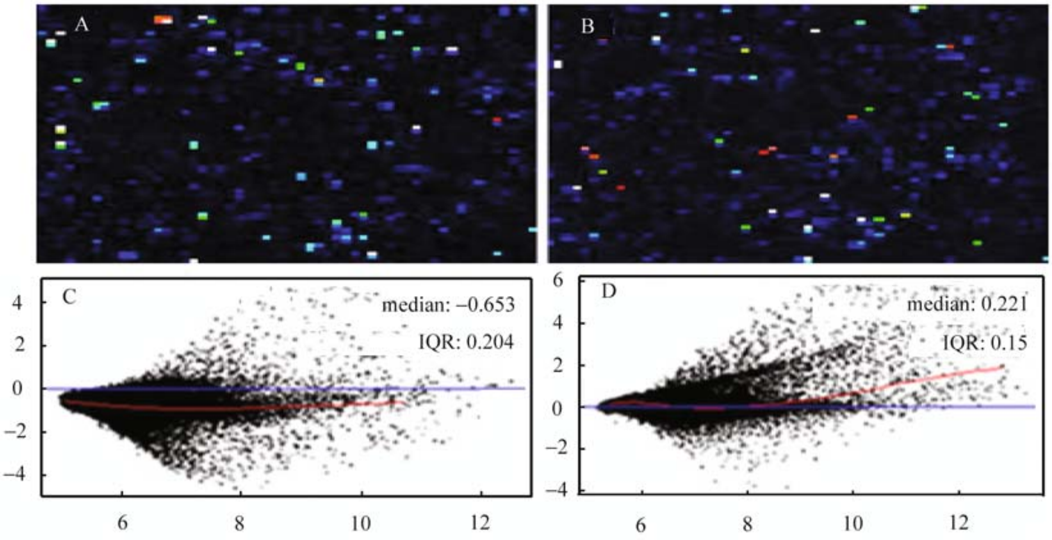

Fig. 4 Microarray hybridization image. Biotin-labeled cDNAs from the mandarin fish (A) and silver carp (B) hybridized with Zebrafish cDNA chip. Color dots in white, red, blue, and dark blue represent the hybridization intensities from the highest to the lowest. C and D indicate gene distribution plots (*vs*. pseudo-median reference chip).

a particular cell or tissue. Its applications to fish biology and aquaculture exist on two basic platforms: commercially produced high-density arrays, such as the Affymetrix Zebrafish Genchip, and customized cDNA microarrays using glass slides or nylon membranes spotted with PCR-amplified cDNA fragments or synthetic oligonucleotides based on ESTs. With these platforms, great progress has been achieved in the understanding of fish genomics and molecular biology (Douglas, 2006; Ju et al., 2007b; Kochzius et al., 2008). Because of the limited number of available microarrays and the cost and time required to construct new arrays, we should exploit the possibility of cross-species, or heterogeneous hybridization of microarrays among aquatic species. Several researchers have reported the feasibility of this approach (Oostlander et al., 2004; Renn et al., 2004; van der Ven, 2005; Kassahn et al., 2007).

The application of DNA microarrays to fish in China is still at an early stage. There are many commercially important fish, such as the four major cultured fish, namely, black carp, grass carp, silver carp, and bighead carp, which make a great contribution to the aquaculture of the country. However, their genomics, transcriptional profiles, and functional genes are under-researched. Fish scientists and biologists should work together to exploit microarray technology and bioinformatics in research of the genomics of commercially important fish in order to have a better understanding of the biology of fish and make further achievement in aquaculture.

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