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APPLICATION OF DNA MICROARRAYS IN MODERN FISH-FARMING

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The paper is a review of the literature concerning the most advanced areas of applying the DNA microarrays method in modern fish farming. Particular attention is given to the efficiency of this approach in diagnostics of various stages of viral and bacterial fish diseases. DNA microarrays using is shown to deadly effect for studying of genetic changes in fish under the influence of temperature factors and aquatic pollutants (including feed excess). Various possibilities of DNA microarrays application of DNA microarrays for interspecies and intraspecies identification of individuals in the wild conditions and fish industry are discribed.

Key words: DNA microarrays, genotyping, molecular and genetic diagnostics.

Modern fish farming even more often demands the development of the innovative approaches allowing to conduct researches at the molecular and genetic level. Being the most important branch of a world aquaculture, it undergoes global changes in traditional technologies. Even more often at breeding and growing of fishes the methods considering ecological factors are used, methods of diagnostics and treatment of infectious diseases are developed. With the emergence of effective biotechnological approaches the traditional methods of selection based mainly on morphometric researches and visual control are often insufficient for selection of producers or maintenance of pedigree and population purity.

The most important problem of fish breeding is also quick and exact diagnosis of infectious diseases of fishes at early stages — many of diseases after emergence of obvious symptoms does not respond to treatment any more and bring to high death rate in populations. The most effective method in this case is genetic identification of the causative agent or its mediated definition based on nature of immune reaction in an organism of fishes.

With the advent of global markets hard economic standards (in particular it needs accurate product labeling) in fish farming in most developed countries-exporters gained extraordinary sharpness issue of fish species identification. Not less important for aquaculture is the problem of population genotyping — study the genetic structure of the fish features allows to maintain the purity of the breed and adjust the quality of individual stocks, avoiding the internal inbreeding, undesirable mutations, etc.

In today's environmental situation priority acquires the molecular genetic studies of the effect on fish external factors. In addition to the fairly well-known reactions to thermal fluctuations and widespread pollutants (cadmium, oil and so on), in recent years, genetic changes in fish caused by exposure to vegetable feed ingredients, which today are increasingly replaced the components of animal origin are studed intensively. One of the modern techniques for precise study of gene structure and molecular processes are DNA microarrays [1]. Traditional microchip is a rigid plate (Fig. 1) coated with a DNA fragment, the nucleotide sequences of which are known.

In the conditions of a modern ecological situation priority value is gained by molecular and genetic researches of influence on fishes of external factors. Besides enough known reactions to thermal fluctuations and widespread pollutants (cadmium, oil and so forth), the genetic transformations in an organism of fishes caused by influence of vegetable ingredients of fishfood, which today replace even more often the components of an animal origin with, are intensively studied in recent years.

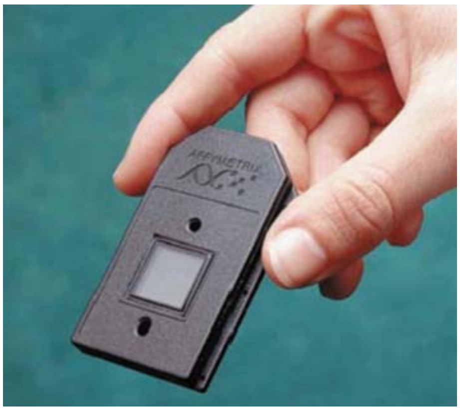

Fig. 1. **The outward of a DNA microchip brand Affymetrix GeneChip**
(picture from the site www.cfgbiotech.com)

The genome study is based on the expression characteristics of genes by hybridization of fluorescently labeled probes with the DNA chip [2]. Application of luminescent-labeled probes, unlike common in the early days of radioactive probes, has a number of significant advantages: increased sensitivity over a wide dynamic range, the ease of use, material availability and staining of samples with two different colors in a single experiment. The principle of DNA microarray by staining samples of the two colors is shown in Fig. 2.

Due to its high efficiency and practical simplicity, this technique is widely used in the US and Europe. In Ukraine a similar experience in the experimental fish farming is virtually nonexistent. At the same time, many foreign studies show the high efficiency of DNA microarrays, used both independently and in combination with PCR or other methods to solve practical problems of world fisheries.

Diagnosis, prevention and treatment

The study of molecular genetic response of the body to the action of pathogens may carry important information about the correct treatment and prevention, and promotes the development of effective diagnostic approaches. DNA microarrays are successfully used for the study of gene expression in viral infectious diseases. Viral diseases are a major risk factor in the development of global aquaculture. In particular, a lot of experimental work using DNA microarray dedicated to comprehensive research of highly infectious salmon anemia virus (ISAV). With this method in 2008 it was analyzed the expression of genes of Atlantic salmon (*Salmo salar*), infected with the causative agent of infectious anemia [3] in order to establish the differences between individuals of the early, middle and late mortality. As a result, it was demonstrated a direct correlation of fish susceptibility to disease of viral pathogenicity and degree of activation of the innate immune response, which is not able to provide an adequate level of protection for an organism. In [4], based on analysis of transcriptomic responses of cells salmon ISAV, it was proposed a number of possible gene-markers of the disease, which was confirmed and expanded a few years later by other authors [5, 6]. During the study of infectious salmon anemia on the specifics of gene expression in the immune response, it was deternimed the existence of multiple viral strains of the pathogen [7], which significantly increased the efficiency in the future development of drugs for the treatment and prevention of ISAV. In recent years, a comparative analysis of gene expression of primary and repeated infectious anemia with infected individuals Atlantic salmon (*S. salar*) was done. These studies have confirmed the effectiveness of the vaccination quantity of fish with low virulence pathogens [8].

Along with ISAV, no problem at salmon farms is a disease called “cardiomiopathic syndrome” (CMS), caused by an RNA virus, structurally similar to members of the family *Totiviridae*. Molecular genetic studies of this disease is less ambitious than the study of infectious anemia, however, for CMS a number of immune marker was found as well, and it was even suggested a possible mechanism of the body’s reaction to the impact of fish virus [9–10]. Similar findings exist in respect of a virus infectious pancreatic necrosis trout, heart muscle inflammation of salmon and other fish diseases [11–13]. Important resultswere obtained in [14] where it was proposed 117 marker genes defined in the early immune response of Atlantic salmon to the most common viral diseases. Using DNA microarrays, the universal markers were prepared which enabled to

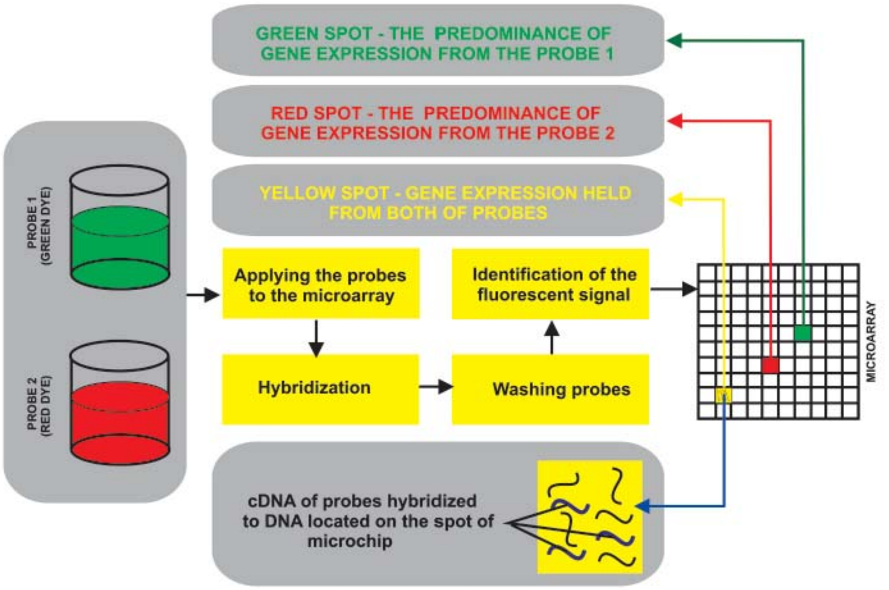

Fig. 2. **The principle of DNA microarray by staining samples of the two colors**

study genetic changes in response to the virus not only for salmon but for other fish [15] as well. For example, carp fish, along with extensive genetic studies of viral diseases in model species (zebrafish and goldfish), DNA microarrays used in the study of immune response cells scaly carp (*Cyprinus carpio*) and koi (*C. carpio coi)* on the third herpesvirus type (CyHV-3), which can cause mass death of fish in the farms [16, 17]. Hybridization probes on microarrays DNA sequences was determined four types of micro-RNA encoded by the virus, which act as modulators infection process. Further analysis of gene expression by microarray and PCR allowed to set 76 genes, which together form the immune response of fish disease caused CyHV-3.

Similar studies of gene expression changes in fish infected with viral disease carried out for the Japanese flounder (*Paralichthys olivaceus*). In particular, it was found a mechanism of transformation of blood cells and fish liver of the species under the influence of the lymphocystis virus, shown the possible ways to control the development life cycle rhabdoviruses flounder by vaccinating animal protein and discovered a gene that plays a key role in the immune defense of plaice in the application of DNA vaccines viral haemorrhagic septicemia [18–20]. DNA microarrays are not less effective for the study of bacterial diseases. A powerful incentive for research in this area was the work Tsoi et al. [21], which in the absence of any practical use of special development with a human cDNA microarrays to differentiate healthy and diseased genes of pathogenic bacteria Aeromonas salmonicida liver of Atlantic salmon (Fig. 3).

Microchips with the sequences of the human genome proved to be quite useful for the early diagnosis of this disease in salmon described correlations fluorescent signal. The proven effectiveness of this method has led to a significant number of special microchips to fish. A year later the findings were presented that enabled to do simultaneous identification of up to 15 of the most common pathogens in global aquaculture [22]. There were also narrow-homologous chips for the study of *Aeromonas salmonicida*, the causative agent of furunculosis have salmon [23] presented the successful results of the analysis of genetic changes in the body of the Atlantic salmon afflicted with this disease. The studies were conducted using a microarray containing more than 4,000 amplicons. Similar experiments were carried out in recent years to *Aeromonas hydrophila*, causing severe damage to the external and mucous membranes blue (*Ictalurus furcatus*) and channel catfish (*Ictalurus punctatus*) [24–25]. For catfish representatives a number of studies of the immune response to Gram-negative bacteria exposure *Edwardsiella ictaluri*, causing intestinal septicemia in the body of fish was done [26–27].

The analysis of the expression characteristics gave number of genes responsible for the immune response to infection in the early stages and in the acute

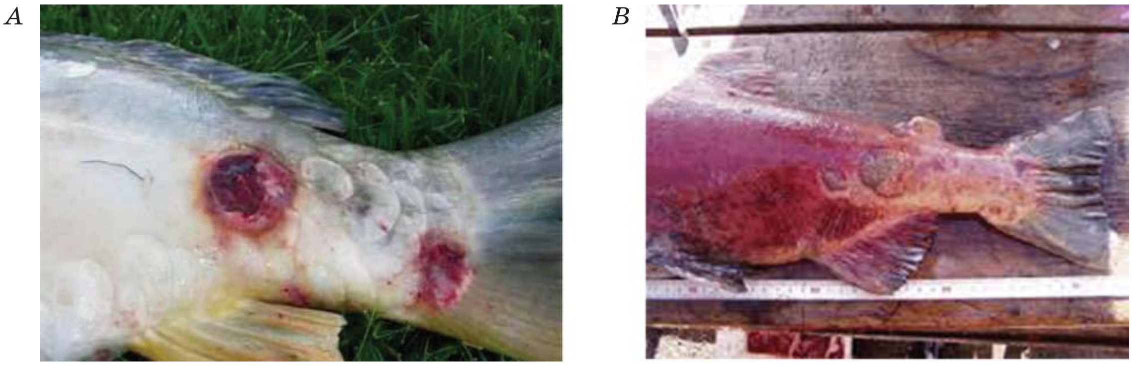

Fig. 3. **Furunculosis caused by the bacteria Aeromonas:**
A — an acute form;
B — scars on the body after recovery of sockeye salmon (picture from the site www.kamniro.ru)

phase. Similar studies using DNA microarrays were carried out for pathogen *Edwardsiella tarda* on the Japanese flounder (*Paralichthys olivaceus*) [28–29].

The spectrum of use of DNA microarrays in diagnostics and finding ways to improve the immunity of fish is not limited to the study of viral and bacterial pathogens — this method can effectively analyze the impact of ecto and ekzo parasites [30, 31] to study mutations and solve many other problems in this area [32] .

Effect of feed ingredients and pollutants

In the modern fish farming new feed and feeding techniques is constantly use aiming to improve product quality and its cost reduction. However, in practice the innovative additives or feeding programs could provoke side effects associated with changes in immunoresistance, foreign product standards, etc. The method of DNA microarrays are widely used in the study of molecular-genetic effects of new substances on the ontogeny of fish.

In order to improve the economic efficiency in the early 21st century World fishing companies began actively replace actively replace fish meal and live foods in the diets of predatory fish with vegetable counterparts. In 2008, a study was conducted concerning gene expression of liver of Atlantic salmon (*Salmo salar*) species who consumed aqua feed with the addition of rapeseed, soybean and linseed oil [33]. It has been shown that such a change does not significantly affect the growth of the fish, which was confirmed later [34] However, while there was a significant increase in the biosynthesis of 18 fatty acids while reducing the concentration of substances necessary for the formation of NADPH, which adversely affected the well-being and the total energy of fish.

A similar effect of deficiency of unsaturated fatty acids is also described and gene expression in liver samples Japanese flounder (*Paralichthys olivaceus*) replacing in its diet on animal fat and olive oil, linseed [35]. The authors of [36] suppose that substitution of animal fats for vegetable counterparts is the probable cause of obesity Atlantic salmon. It turned out to be equally dangerous the use of soybean meal, which began to replace bone meal in the diet of salmonids since 2008. Thus, using the method of DNA microarrays have shown the presence of specific immune responses of the body, side effects that lead to a breach of the intestinal epithelium, and the appearance of enteritis due to which there was not only a sharp decrease in the vital functions of fish, but also the deterioration in the quality of the final product [37].

Investigation of the effect of feed additives in the fish using DNA microarray technique is useful to consider the example of tetradecylthioacetic acid (TTA). This material is added to the diet of Atlantic salmon in most modern households. The main function of TTA — increase the metabolism of excess fat, that enter the body composition of fish feeds for rapid growth and might lead to obesity. The use of DNA microarrays has allowed to determine the mechanism of TTA: this supplement is a stimulator of the immune system. With this preparation the body sees an excess concentration of lipids as an inflammatory process, activating in response the metabolic reaction of fatty acid oxidation and glycolysis [38]. In addition, based on the analysis of gene expression it was found TTA capacity to stimulate overall growth in the heart of salmon and increase its contractile ability [39].

Using cDNA microarrays interesting results were obtained regarding the impact of the lack of food in the course of molecular processes in fish (there are a number of methods of feeding, which provides various intervals for periods of starvation). It was shown [40] that prolonged fasting brings to a body energy gap for formation of full immune response — such fish harder resistant to pathogens. The authors recommend the fish farmers to return the system to full power fish diet at the slightest threat of mass disease. Also, there was an attempt to use DNA microarrays with fragments of the genome of *Danio rerio* to determine characteristics of other members of the carp starvation [41]. However, instead of this problem, the authors have identified the unique characteristics of expression of genes in response to a reduction in food for various species, including squamous carp.

Based on the DNA microarray technique it was done, a series of studies on the effects of transgenic food fish, in particular the effect of growth hormone (GH), all of which the fish grows more than usual in the shortest time (Fig. 4).

Fig. 4. **Atlantic salmon (*Salmo salar*), the modified GH chinook salmon (*Oncorhynchus tshawytscha*)**
(picture from the site http://www.odditycentral.com/)

First the substance is viewed solely as a stimulant. However, in the context of rainbow trout (*Oncorhynchus mykiss*) it has been shown that the GR does not only modulate the growth control genes but also is a stimulant of the immune system start process and metabolism [42]. Subsequently, the therapeutic effect of GH was confirmed — when introduced into the body of the zebrafish, a sharp increase in regenerative was observed abilities (in this case, control was made by restoration of the otoliths in the inner ear) [43]. Conditions for the efficient use of use of GR for food fish continue to refine. So, using the microarray, it was found that the shortage of food in transgenic animals must be constantly artificially eliminate the deficit of hemoglobin, which is spent on the metabolism of a number of other processes [44].

In recent years, on the basis of the results of 39 microchips using it has been found that in order to increase transgenesis efficiency and safety of such treatments it is reasonable to use uterine sterile herd consisting of triploid not able to produce offspring [45].

Environmental and ecotoxicological studies

Water pollution varies and abiotic factors significantly affect the final product quality fishery and economic effect. The study of molecular and genetic changes in fish, caused by toxins, radioactive rays and temperature fluctuations repeatedly investigated, including a method using a DNA microarray. Influence of permanent water pollution from industrial activities in the hereditary characteristics of fish is obvious.New studies of the problem at the molecular genetic level was conducted with the advent of DNA microarray technique. The first works in this area were mainly related to studying the influence of individual water contaminants. In particular, in 2006 it has been found the so-called biomarkers of oxidant stress in the body flounder (*Platichthys flesus*) in response to the presence in the environment of a significant amount of cadmium, a prototype of heavy metals which, are often located in industrial waste [46]. Based on the obtained results it was created the specific cDNA chips enabled to determine effectively general fish condition and cadmium concentration in its body [47].

Using microarray techniques, it was studed genetic changes for salmonids affected by M4 syndrome — disease, wich if fillowed by early mass mortality of fish and reproductive disorders. The results of cDNA microarrays application confirmed with high reliability that this pathology is a consequence of the impact of organochlorine compounds in water [48, 49]. The authors assert the universality of the obtained genetic responses to organic chlorine not only for the most fish, and vertebrates in general. For carp (*Cyprinus carpio)* similar studies were conducted as well. Thereat the molecular-genetically modified organisms were considered under the influence of perfluorooctane sulfonate, which is used in the global industry for about 50 years and accumulated a considerable amount in the water bodies [50]. Authors on the basis of DNA microarray prove conclusively that these compounds, although not cause high mortality rates, but require a large expenditure of energy metabolism of the organism, which significantly affects the growth rates, reproductive capacity and other economically important fishery indicators. Using DNA microarray method it was studed the effect of another common pollutants — heavy oil on the body of Japanese flounder (*Paralichthys olivaceus)* [51]. The result identified a number of specific biomarkers for heavy oil and other polycyclic hydrocarbons, and also shows the presence of the genes of the immune system changes that reduce the overall resistance to the action of pathogens.

Studying the influence of aquatic pollutants on the body of the fish using DNA microarrays, an emphasis was put on the transformations of molecular systems under the influence of wastewater. Through the example of carp it was shown that the average composition of wastewater substantially disrupt the energy balance of the body with the subsequent changes in the synthesis of digestive enzymes. Moreover, flows affect the mechanisms of lipid and carbohydrate metabolism, which leads to deterioration of the general condition of the fish at all levels — from growth inhibition to reproductive properties suppression [52]. Recent long-term studies of the effect of different concentrations of wastewater on immature chinook salmon (*Oncorhynchus tshawytscha*) also showed a high probability of genetically irreversible age-related conditions: impaired glucose transport system and inhibiting the action of insulin-like growth factor [53]. Especially practical importance for fish farming are the results of studies of genetically caused changes under the influence on the body fluids of fish with significant concentrations of uneaten food containing herbal ingredients [54].

Pesticides and aromatic hydrocarbons have a significant impact on expression characteristics of genes (suppression of the synthesis of unsaturated fatty acids, slowdown decomposition of vitamin D_3, etc.), and the products of heat treatment of seeds and other plant-derived components, essentially breaking the molecular genetic regulatory processes .

Noteworthy practice of fish farming is also the use of microarrays to study the effects of residues of pharmaceuticals in water for healthy fish. In [55] for acetaminophen, carbamazepine and atenolol — active substances most common analgesics, anticonvulsants and beta-blockers show a sharp increase in gene expression directed at decomposition of these substances. The powerful energy consumption of such a process with a high probability will affect the collection of body weight of fish and growth factors.

The thermal vibrations of the aquatic environment are a common phenomenon in the fishing industry. Changes in temperature stimulates the launch adaptation processes, side effects that may impair the features of growth and development. Using DNA microarrays it was done a series of studies concerning fish reaction to heat in water with the position indicators of gene expression. Over the years, it was determined acclimatization and the role of the immune protein Wap65 from channel catfish (*Ictalurus punctatus*) [56], as well as gene TFII, responsible for the thermal adaptation of Arctic char (*Salvelinus alpinus*) [57]. The results of heat stress were studied on the example of wild sockeye salmon (*Oncorhynchus nerka*) and pink salmon (*Oncorhynchus gorbuscha*) [58]: at a temperature of 13–14 °C it were observed significant violations of the spawning migration, and upon reaching 19 °C fixed mass mortality of the experimental fish. It was revealed 29 unique genes responsible for denaturation and protein synthesis, oxidation processes, especially ion transport and metabolism at an elevated temperature.

Unlike the comprehensive research of thermal stress, the molecular genetic basis of cold adaptation of fish is much less studied. In 2002 microarray application enabled to find out a number of genes channel catfish, whose expression changes abruptly due to temperature lowering [59]. However, the authors acknowledged that in fact different stages of cold adaptation involve far greater molecular genetic structures, the determination of which requires a lot of effort and time. In recent years, it was published a comparative study of acclimatization to cold in transgenic and wild zebrafish from the standpoint of the energy needs of many immune-genetic responses to changes in temperature [60]. This revealed additional specific biomarkers, but in general they were devoted rather to transgenic organisms than to the study of acclimatization process and potential recommendations for practical fish farming.

Species and intraspecific identification of fish

The role of accurate and rapid identification of the species of fish on the farm is difficult to overestimate: the individual breeding lines, isolated populations as a result of mutation, inbreeding, and other factors vary considerably in the phenotype, which significantly complicates the identification of the group species visually or by morphometric analysis. However, international standards require the clear labeling of the species in international trade in fishery products. Equally important problem is the development of accurate intraspecific identification methods for fish farming, which would control the playback of desired traits in prompt and certification rocks.

One of the first successful attempts to apply the method of DNA microarrays in the field of genotyping was to develop a set of oligonucleotide sequences of mitochondrial DNA to identify the haploid genotype of chum salmon (*Oncorhynchus keta*) [61]. The proposed method made it possible to determine up to 30 haplotype of the species just in 4 hours. Subsequently, the authors applied this method for the regular monitoring of the gene pool of chum salmon in the Bering Sea and North Pacific Ocean [62]. In parallel with these studies it was obtained the original microchip to establish cost-effective features (in particular — disease resistance) for channel catfish (*Ictalurus punctatus*) [63]. As a result of this innovative structure it was found about 138 species-specific sequences that form the level and nature of the different immune responses.

Along with the works devoted to the species identification, there were described successful attempts of intraspecific genotyping using DNA microarrays. Thus, in 2006 the genomes of dwarf and normal ecotypes lake whitefish (*Coregonus clupeaformis Mitchill*) were analyzed [64]. Scientists have established a clear set of genes taking part in phenotype identification and estimation of energy level. Additional studies in this area have been conducted with European flounder (*Platichthys flesus*) — it has been shown that the level of gene expression is largely dependent on the nature of the fish to adapt to specific conditions [65]. Thus it was identified the interpopulation genes determinants responsible for biosynthesis of various mechanisms, osmoregulation and the overall resistance to external stress.

Interesting results were obtained on the basis of large-scale studies of genomes of different species of salmon [66]: the authors, taking the main object of the Atlantic salmon (*Salmo salar*), convincingly demonstrated its high affinity (86,2–94,6%) and smelt (Osmerus eperlanus), grayling (*Thymallus thymallus*), trout (*Oncorhynchus mykiss),* whitefish (*Coregonus lavaretus*) and other representatives of the family. On the basis of large amounts of practical information on the structure of the genome of the industrially valuable and the most common in nature sea fish, Kochzius M. and staff proposed an effective method for identification of the species [67]. Studying the genes of mitochondrial DNA, the authors have created a microchip containing 68 oligonucleotide probes for hybridization options whereby it is possible to distinguish the genotypes of 30 species of bony fishes.

Problems of accurate labeling of fish products in recent years has also become a way to solve the genetic identification. Thus the US scientists developed a DNA microarray that that enables to classify a specimen catfish to one of the 7 species authorized for industrial trade in the country [68]. Innovation challenge in identifying the species of fish was the issue of the possibility of genotyping in the early stages of development. One of the most successful research in this direction is a comparative description of the transcriptome of eggs of Atlantic salmon (*Salmo salar*) and Atlantic cod (*Gadus morhua*) [69]. The authors were able to establish the specific features of both species, which will continue to carry out early identification of these fish.

Certainly, the use of DNA microarrays in fish culture is not limited to sectors considered. In particular, the current developments provides an opportunity to speak about the prospects of this technique for monitoring the cultivation of young and age of transformation, regulation of sexual aspects (including feminization), and overall improvement in product quality fisheries. The study of fish genomic structure, diagnosis of diseases, study of the influence of external factors, effective genotyping at different levels are the urgent issues of modern fish farming, which answer could be obtained using DNA microarrays. However, fisheries and livestock production as a whole is not the only sector in which the use of this method would be justified: this technique is a powerful tool for solution of general biological issues and urgent problems of modern medicine. Such a wide range of functional application of DNA microarrays, along with a significant number of practical results obtained in various fields of science suggests its prospects.

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ЗАСТОСУВАННЯ ДНК-МІКРОЧИПІВ У СУЧАСНОМУ РИБНИЦТВІ

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Подано огляд даних літератури, що стосуються найбільш перспективних сфер застосування методу ДНК-мікрочипів у сучасному рибництві. Особливу увагу приділено ефективності цього підходу під час діагностики різних стадій вірусних та бактеріальних захворювань риб. Показано ефективність застосування ДНК-мікрочипів у дослідженнях генетичних змін в організмі риб під впливом температурних чинників та забруднювачів водного середовища (зокрема надлишків кормів). Описано різні можливості використання ДНК-мікрочипів для міжвидової та внутрішньовидової ідентифікації особин у природі та в умовах рибних господарств.

Ключові слова: ДНК-мікрочипи, генотипування, молекулярно-генетична діагностика.

ПРИМЕНЕНИЕ ДНК-МИКРОЧИПОВ В СОВРЕМЕННОМ РЫБОВОДСТВЕ

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Представлен обзор данных литературы, касающихся наиболее перспективных сфер применения метода ДНК-микрочипов в современном рыбоводстве. Особое внимание уделено эффективности данного подхода при диагностике различных стадий вирусных и бактериальных заболеваний рыб. Показана эффективность использования ДНК-микрочипов в исследованиях генетических изменений в организме рыб под воздействием температурных факторов и загрязнителей водной среды (в том числе излишков кормов). Описаны различные возможности применения ДНК-микрочипов для межвидовой и внутривидовой идентификации особей в природе и в условиях рыбных хозяйств.

Ключевые слова: ДНК-микрочипы, генотипирование, молекулярно-генетическая диагностика.