REVIEW ARTICLE

Molecular genetics in aquaculture

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ABSTRACT

Great advances in molecular genetics have deeply changed the way of doing research in aquaculture, as it has already done in other fields. The molecular revolution started in the 1980's, thanks to the widespread use of restriction enzymes and Polymerase Chain Reaction technology, which makes it possible to easily detect the genetic variability directly at the DNA level.
In aquaculture, the molecular data are used for several purposes, which can be clustered into two main groups. The first one, focused on individuals, includes the sex identification and parentage assignment, while the second one, focused on populations, includes the wide area of the genetic characterization, aimed at solving taxonomic uncertainties, preserving genetic biodiversity and detecting genetic tags. For the future, the increase in the number of molecular markers and the construction of high density genetic maps, as well as the implementation of genomic resources (including genome sequencing), are expected to provide tools for the genetic improvement of aquaculture species through Marked Assisted Selection.
In this review the characteristics of different types of molecular markers, along with their applications to a variety of aquaculture issues are presented.

Key words: Aquaculture, Molecular markers, Practical applications.

RIASSUNTO

LA GENETICA MOLECOLARE IN ACQUACOLTURA

La genetica molecolare sta profondamente modificando il modo di fare ricerca nell'ambito dell'acquacoltura, così come è già avvenuto in altri settori. La rivoluzione molecolare è iniziata durante gli anni '80, grazie all'utilizzo degli enzimi di restrizione e della Reazione a Catena della Polimerasi, che permettono di studiare, in modo rapido e relativamente poco costoso, la variabilità genetica direttamente a livello del DNA. Nell'ambito dell'acquacoltura, i dati molecolari sono stati utilizzati per numerose applicazioni, che possono essere raggruppate in due categorie. Nella prima, riferita agli individui, rientrano il riconoscimento del sesso e la verifica di parentela, mentre nella seconda, riferita alle popolazioni, rientra il vasto settore della caratterizzazione genetica, finalizzata alla risoluzione di incertezze tassonomiche, alla tutela

della biodiversità e all'individuazione di etichette genetiche. I recenti progressi nell'identificazione di un numero sempre più elevato di marcatori, unitamente alla costruzione di mappe genetiche a maggiore densità, forniranno informazioni utili anche nell'ambito del miglioramento genetico. Questa rassegna illustra brevemente le caratteristiche dei diversi marcatori molecolari, soffermandosi più in dettaglio sulle loro applicazioni nel settore dell'acquacoltura.

Parole chiave: *Acquacoltura, Marcatori molecolari, Applicazioni pratiche.*

Introduction

Mendelian genetics deduced the principles of the heredity by observing the individual phenotypes resulting from appropriate breeding experiments. Its integration with information and methods made available by the progressive advances of molecular biology led to a new branch of genetics, called *molecular genetics*, whose aim is to investigate all aspects of the gene, such as its structure and functions. The molecular approach provided geneticists with very powerful tools, the *molecular markers*, which opened exciting perspectives of application in many research fields. The objective of this work is to review the characteristics and potential power of different types of genetic markers, with a major focus on their applications to a variety of aquaculture issues.

Molecular markers

A genetic marker can be defined as any trait which allows the identification of the genotype of an individual. Until the 1980's, the most used markers were proteins (allozymes), because amino acid differences in the polypeptide chain detected by electrophoresis reflect mutations in the coding gene. However, the protein markers have a reduced power in detecting the DNA variability both within and between populations (Valenta *et al*., 1977; Valenta *et al*., 1978; Šlechtová *et al*., 1995; Kohlmann and Kersten, 1998; Antunes *et al*., 1999), because some DNA mutations do not lead to amino acid substitutions, or the amino acid substitution does not always change the protein total electric charge.

The advances in molecular genetics have made it possible to detect the genetic polymorphism directly at the DNA level, thanks to the widespread use of restriction endonucleases and Polymerase Chain Reaction (PCR) technology. Used alone or in association, they allow the identification of different types of DNA variability: base substitutions, commonly referred to as Single Nucleotide Polymorphism (*SNP*), insertion or deletion of nucleotides (*indel*) and Variable Number of Tandem Repeat (*VNTR*). The last group includes microsatellites (or Simple Sequence Repeats, *SSRs*), which consist of short sequences (mostly 2-4 base pairs) tandemly repeated up to tens or hundreds of times along the DNA strand (Levinson and Gutman, 1987; Tautz, 1989) and mostly located in non coding regions. Thousands of microsatellites have been found in farm animals; in fish, in particular, the presence of a microsatellite has been estimated every 10 *kb* (O'Connell and Wright, 1997), with a mutation rate at 10^{-2}-10^{-6} per locus per generation, which is much greater than that of non repetitive DNA (10^{-9}) (Weber and Wong, 1993), resulting in a very high level of polymorphism. Moreover, microsatellites are co-dominant and abundantly distributed throughout the genome. For their peculiar features, microsatellites are extensively used in a wide range of research fields and applications.

In aquaculture genetics, mitochondrial

DNA (mtDNA) is also quite popular, for its intrinsic and technical features: a relatively high mutation rate, haploid and maternal inheritance, which reduces the effective population size and thus increases the sensitivity to genetic drift, ease of isolation and manipulation (Avise, 1994; Moritz, 1994). The analysis of mtDNA has been generally carried out by PCR-RFLP and more recently by sequencing. Mitochondrial DNA complete sequences for some aquaculture species, such as common carp (*Cyprinus carpio*) (Chang and Huang, 1994), tench (*Tinca tinca*) (Saitoh *et al*., 2006) and red grouper (*Plectropomus leopardus*) (Zhu and Yue, 2008), are now available.

The last decade has seen a renewed interest in coding genes for studying the association of their variability with economically important traits. In this respect, comparative genomics can greatly accelerate the identification of effective markers, because many genes are very conservative, allowing the transmission of information between species, with reduction of time and costs.

Applications

Molecular markers can be used in a variety of aquaculture studies, at the individual or population level, and the choice of the markers to be used depends on both the aim of the research and on the marker characteristics. Some examples are briefly presented.

Gene mapping

Gene mapping provides fundamental information for genetic studies, including QTL identification, marker assisted selection and comparative genomics (Danzmann and Gharbi, 2001). In brief, a physical map defines, by *in situ* hybridization, the physical location of DNA segments on a chromosome, while a genetic map depicts the relative distance and the order of the loci along a chromosome on the basis of segregation analyses in reference populations or families: if two markers segregate together, they will locate very close on the chromosome, so they will define a *linkage group*, where the proportion of recombinants between the linked markers is used as a measure of the distance between them. The microsatellite markers represent the tool of choice for the construction of a primary framework map, which can be further enriched with SNP markers in coding genes (Gregory *et al*., 2004).

Although less developed than in other animal species, microsatellite and SNP-based linkage maps are now available for several aquaculture species (Table 1). As some studies have revealed extensive homology among vertebrates (Morizot, 1983; Woods *et al*., 2005), comparative evolutionary studies will greatly benefit from the increasing knowledge on the linkage groups arrangements in different species (Matsuoka *et al*., 2004; Gharbi *et al*., 2006).

Medium-term genome research will focus on the integration of genetic linkage and physical maps, which would significantly enhance the possibility to apply genome-based technologies to the genetic improvement (Somridhivej *et al*., 2008).

Sex identification

Sex identification is important in several biological sciences, such as genetics and conservation biology, but in fish it is often difficult because of the reduced sexual dimorphism and the frequent absence of heteromorphic sex chromosomes. Moreover, in fish species where one sex has better performances, monosex stocks have been developed, usually by sex reversal and family selection, with the need to discriminate between genetic and phenotypic sex (Devlin and Nagahama, 2002). Molecular genetics has proven to be very effective in solving the

Table 1. Genetic linkage maps in aquaculture species.

Species	Reference
Zebrafish *(Danio rerio)*	Knapik *et al.*, 1998; Shimoda *et al.*, 1999; Woods *et al.*, 2005
Nile tilapia *(Oreochromis niloticus)*	Kocher *et al.*, 1998; Agresti *et al.*, 2000
Medaka *(Oryzias latipes)*	Naruse *et al.*, 2000
Channel catfish *(Ictalurus punctatus)*	Waldbieser *et al.*, 2001; Liu *et al.*, 2003
Rainbow trout *(Onchorhynchus mykiss)*	Young *et al.*, 1998; Nichols *et al.*, 2003; Rexroad *et al.*, 2008
Arctic charr *(Salvelinus alpinus)*	Woram *et al.*, 2004
Atlantic salmon *(Salmo salar)*	Moen *et al.*, 2004, 2008; Gilbey *et al.*, 2004
Pacific oyster *(Crassostrea gigas)*	Li and Guo, 2004
European sea bass *(Dicentrarchus labrax)*	Christiakov *et al.*, 2005
Brown trout *(Salmo trutta)*	Gharbi *et al.*, 2006
Gilthead sea bream *(Sparus aurata)*	Franch *et al.*, 2006
Atlantic halibut *(Hippoglossus hippoglossus)*	Reid *et al.*, 2007
European flat oyster *(Ostrea edulis)*	Lallias *et al.*, 2007
Bighead carp *(Aristichthys nobilis)*	Liao *et al.*, 2007
Silver carp *(Hypophthamichthys molitrix)*	Liao *et al.*, 2007
Coho salmon *(Oncorhynchus kisutch)*	McClelland and Naish, 2008

problem through the possibility of detecting sex-linked markers in different species, including Atlantic salmon (Devlin *et al.*, 1991; Du *et al.*, 1993; Clifton and Rodriguez, 1997), African catfish (Kovacs *et al.*, 2000), rainbow trout (Iturra *et al*., 2001; Felip *et al*., 2005), tongue sole (Chen *et al*., 2008).

Individual identification and parentage assignment

In many fields, such as conservation genetics or selection, the unambiguous identification of the individuals and reliable genealogical records are required for the management programmes. Unfortunately, these data are difficult to obtain in fish populations, because physical tags are impossible to apply in juveniles and, when possible, such as in farmed mussels, they are lost in 40-90% of the cases (MacAvoy *et al*., 2008). The alternative to keep different families in separate ponds is expensive and limits the number of animals available for selection.

Several studies have demonstrated the ability to identify a unique genetic profile for each individual and to establish parentage in fish using highly polymorphic markers, especially microsatellites. Investigations on rainbow trout (*Onchorhynchus mykiss*) (Herbinger *et al.,* 1995), Atlantic salmon (*Salmo salar*) (Norris *et al.*, 2000) and the New Zealand mussel *Perna canaliculus* (MacAvoy *et al.,* 2008) showed that appropriate sets of microsatellites allow a success rate of 95-99.9% in parentage assignment. The disadvantage of using molecular mark-

ers is the relatively high cost, which can be limited by choosing the minimum number of markers compatible with the maximum level of accuracy and developing multiplex systems able to co-amplify the markers used, as already done for many aquaculture species (O'Reilly *et al*., 1996; Fishback *et al*., 1999; Porta *et al*., 2006; Johnson *et al*., 2007).

Population genetics

The genetic characterization of the individuals leads to the possibility of describing a population by means of *allele frequencies*. In recent decades, the molecular markers have been extensively used to define the genetic structure of many aquaculture populations, which represents the fundamental step for the definition of the *taxonomic status*, that is for species, subspecies, breeds and strains identification. From a practical point of view, once a *status* has been assigned to each population, the information can be used for either understanding their role in determining the whole variability of the species, or detecting cases of crossbreeding and/or hybridization.

For the identification of species, separated by large genetic distances, almost all markers can be used. On the contrary, markers with a very high resolution power are needed for breeds and strains identification because the genetic distances between the phylogenetic units are often quite small. The microsatellite variability made it possible to distinguish European and American populations of Atlantic salmon (McConnell *et al*., 1995), as well as seven populations of masu salmon living in the Atsuta river (Kitanishi *et al*., 2009), while significant differences between two adjacent Canadian populations were revealed by mtDNA (Tessier *et al.,* 1995). David *et al*. (2001) successfully applied AFLP markers to distinguish nine common carp populations, showing that the Amur carp was the most different, so that one marker was sufficient to recognize it, while two or more markers were necessary to distinguish the other populations.

Molecular markers are widely used in conservation genetics as well, to indirectly estimate the inbreeding level by measuring the heterozygosity degree, whose changes during time reveal population size fluctuations and possible bottlenecks occurred in the past (Gross *et al*., 2007). The main purpose of these studies is to provide tools for preserving the existing genetic variability, which is fundamental for the survival of the species, because it allows individuals to face changes in the environmental conditions. Focusing on farmed animals, the genetic diversity represents the possibility of both adapting to new rearing conditions/food sources/diseases, and providing improved products in answer to new requirements. Nevertheless, any selection programme leads to a reduction of the genetic diversity, which should be limited as much as possible in order to avoid the negative effects, known as inbreeding depression (Pante *et al.*, 2001). From this point of view, molecular information can be used to maximise the genetic diversity when assembling a founder population in order to ensure maximum long-term genetic response from the breeding programmes (Hayes *et al*., 2006).

Fortunately, most of aquaculture species have a great advantage compared to other species: as the domestication process started only recently, wild populations still exist and can represent the source of genetic diversity in the future. In this context, molecular genetics plays a basic role because molecular markers are able to detect differences between wild and cultured populations and to reveal processes which determined the observed differences. Mitochondrial DNA is especially employed for this purpose in several aquaculture species. For example, Hansen *et al*. (1997) used mtDNA polymor-

phism to detect differences between brown trout cultured strains and wild populations from three river basins, revealing a greater reduction of genetic variability in hatchery strains, mainly due to the small numbers of founder females. Also data on microsatellite markers showed that farmed strains were genetically quite similar, while clearly separated from wild populations in rainbow trout (Gross *et al*., 2007) and tench (Kohlmann *et al*., 2007). The ability to discriminate wild and cultured populations can be also exploited to identify escaped domesticated animals, as demonstrated for Chinook salmon reared in marine netpens (Withler *et al*., 2007).

The genetic characterization offers the possibility to trace back to the origin of a processed product (*traceability*). Species-specific, strain-specific or population-specific markers can be used as *genetic tags,* which make it possible to detect the original *taxon* and secure the consumer's rights to be informed about the purchased product. For example, the polymorphism of the mitochondrial *Cyt b* gene made it possible to discriminate between 23 species, including European eel (*Anguilla anguilla*), Atlantic salmon (*Salmo salar*), Atlantic cod (*Gadus morhua*), bass (*Dicentrarchus labrax*), sea bream (*Sparus aurata*), tuna (*Thunnus thynnus*) and plaice (*Pleuronectes platessa*) (Wolf *et al.,* 2000). Recently a species database of fish, molluscs and crustaceans has been created with the aim to identify species of origin of seafood products by previously defined AFLP patterns (Maldini *et al*., 2006).

A strictly related application is in forensic science to detect cases of fraud, for example in caviar trade (Wuertz *et al*., 2007), or illegal poaching of threatened species. Primmer *et al.* (2000) reported a funny case of fraud that occurred during a fishing competition in Finland: the microsatellite genotyping, together with software able to assign an individual to its population, provided a highly significant power for excluding the possibility of a suspected fish originating from the competition lake. At the end, the offender confessed to purchasing it in a local fish shop!

Selection

Selection is aimed at modifying the genetic structure of a breed in order to obtain animals with superior performances for the traits of interest. The classical approach is to estimate the breeding value of the individuals on the basis of phenotypic values, to select the ones with the best genetic performances and to mate them within appropriate breeding schemes.

Although the basic concepts are the same, the selection strategies used in most farm animals do not directly apply to fish species for their biological and breeding characteristics. On one hand, the extremely high reproductive capacity and the external fertilization of fish offer a great flexibility in the implementation of selection programmes with a high precision in the estimates and permits the use of higher intensities of selection. On the other hand, the peculiar breeding management has some practical limitations, such as the difficulty in obtaining accurate genealogical and phenotypic data, or the influence of the competition between individuals in the same pond, which induces a distortion in the estimates of the genetic parameters, with negative effects on the selection response (Moav and Wohlfart, 1974).

Apart from these differences, the traits objectives of selection in aquaculture are similar to those of other species (including growth, carcass composition and quality) and the genetic improvement realized so far mainly depends on the application of traditional methods, involving selection, crossing and hybridation (Wohlfarth, 1993; Bakos and Gorda, 1995; Hulata, 1995; Hulata, 2001). However, the progress in molecular genetics provides perspectives for implementing the

marker assisted selection (MAS), aimed at choosing the genetically superior individuals using molecular information.

To perform MAS, markers tightly linked to the loci responsible for quantitative traits (QTL) or major genes directly involved in the phenotypic expression should be found. In the first case, a QTL can be identified and localized due to the co-segregation with a molecular marker; in this respect the microsatellites are very helpful, being highly polymorphic and widely distributed throughout the genome. In the second case, candidate genes possibly responsible for quantitative traits are chosen, based on previous knowledge of their position and/or function, and the statistical associations between their SNPs and the phenotypic expression of the trait of interest are investigated. The identification of genetic markers related to traits objective of selection would have a great impact on the selection response, mainly for traits with low heritability, such as reproduction or disease resistance. In fact, if a given allele were associated to a given trait, it would be sufficient to select the individuals carrying that allele in order to improve the associated trait. If so, the selection process would be greatly simplified, because simple Mendelian traits would be concerned, instead of complex quantitative traits.

Up to now, the QTL analysis in fish is in its infancy because the genetic mapping is not so advanced as in other species, even if medium-density linkage maps are available at least for the principal aquaculture species, as mentioned above. The first data on QTLs date back to the late 1990's, when Jackson *et al.* (1998) identified two markers associated with the upper temperature tolerance in rainbow trout. Later on, other QTLs were reported in different aquaculture species (Table 2).

In aquaculture many efforts are devoted to the search of markers for disease resistance, due to both the enormous economical implications and the difficulties of the classical selection, which requires the genetic evaluation of the individuals through exposure to the virus. Ozaki *et al.* (2001) first identified, in rainbow trout, two markers associated with the infectious pancreatic necrosis (IPN), a highly contagious disease against which the presently available vaccines offer only a partial protection. More recently, a marker for resistance to lymphocystis disease in Japanese flounder has been identified (Fuji *et al.*, 2007); the potential of MAS for improving the disease resistance is demonstrated by the fact that no affected fish were observed in an experimental population selected for the allele associated to the lymphocystis resistence, compared to about 5% of the control population.

Concerning major genes, the available data are still limited (Table 3). However, aquaculture species have the great advantage that the economically important traits are similar to those included in the selection programmes of many farm animals, so that the huge amount of information already available for other vertebrates can be exploited to find homologous genes in fish, where the research in this field is less advanced. For example, growth rate, which is one of the main objectives of selection in most fish species, has been thoroughly investigated for many years in other animals, where the genes associated to the somatotropic axis have been identified, mapped and sequenced. These results have accelerated the research in fish, leading to the identification in different species of *GH* (*Growth Hormone*), *GHR* (*Growth Hormone Receptor*), *GHRH* (*Growth Hormone Releasing Hormone*), *IGF-I* (*Insulin-like Growth Factor I*) genes (De-Santis and Jerry, 2007). The identification of polymorphic sites in these genes is the further step towards investigations on the possible associations with production

Table 2. Examples of QTL in aquaculture species.

Species	Trait	Reference
Rainbow trout *(Oncorhyncus mykiss)*	Upper temperature tolerance	Jackson *et al.*, 1998
	Spawning time	Sakamoto *et al.*, 1999
	IPNV resistance	Ozaki *et al.*, 2001
	Embryonic development rate	Robison *et al.*, 2001
	Sex	Iturra *et al.*, 2001
	Early maturation	Haidle *et al.*, 2007
	Cortisol level	Drew *et al.*, 2007
Tilapia *(Oreochromis hybrid)*	Stress/immune response	Cnaani *et al.*, 2004
	Body colour	Lee *et al.*, 2005
Coho salmon *(Oncorhynchus kisutch)*	Flesh colour	Araneda *et al.*, 2005
	Spawning date	Araneda *et al.*, 2007
Atlantic salmon *(Salmo salar)*	IPNV resistance	Houston *et al.*, 2007
	Body lipid percentage	Derayat *et al.*, 2007
Sea bass *(Dicentrarchus labrax)*	Morphometric traits	Chatziplis *et al.*, 2007
Japanese flounder *(Paralichthys olivaceus)*	Lymphocystis disease	Fuji *et al.*, 2007
Eastern oyster *(Crassostrea virginica)*	Disease resistance	Yu and Guo, 2006

Table 3. Examples of candidate genes in fish species.

Species	Gene	Trait	Reference
Zebrafish *(Danio rerio)*	*MYO*	Growth	Acosta *et al.*, 2005
Rainbow trout *(Oncorhyncus mykiss)*	*Clock*	Spawning time	Leder *et al.*, 2006
Asian seabass *(Lates calcarifer)*	*PVALB1*	Body weight/ length	Xu *et al.*, 2006
Grouper *(Epinephelus coioides)*	*Epinecidin-1*	Antimicrobial activity	Yin *et al.*, 2006
Tilapia *(Nile tilapia)*	*ACTB, ATP2B1, HBB, POMC*	Water salt tolerance	Rengmark *et al.*, 2007
Masu salmon *(Oncorhyncus masou)*	*MELO*	HUFA biosynthesis	Alimuddin *et al.*, 2008

traits. Another promising candidate gene is *Myostatin*, whose variability is responsible for the double muscled phenotype in cattle. Recently, Acosta *et al* (2005) reported that the inactivation of the *Myostatin* gene in zebrafish resulted in an increased weight gain (+45% compared to the control), as observed in mice (McPherron *et al.*, 1997). Therefore,

the polymorphism of the gene could be associated to growth differences also in fish.

Transgenesis

The knowledge of molecular genetics gives a basic contribution to the genetic engineering, which in fish is more advanced compared to other animals, for the simpler manipulation, due to the external fertilization and embryogenesis (Maclean, 2003).

Following the milestone experiment of Palmiter *et al.* (1982) in mice, the main application in aquaculture concerned the growth rate, with the successful transfer of *GH* gene from mammals and more recently from fish. The results were impressive, with growth rate four times higher in salmon (Devlin *et al.*, 1994) and 2.5 - 4 times in tilapia (Rahman *et al.*, 2001). However, some results showed that the growth enhancement is relatively low in species selected for growth over centuries, as they had less 'capacity' for extra growth (Devlin *et al.*, 2001). Therefore, the existence of biological limitations could reduce the usefulness of the transgenesis. The consumer acceptance of the transgenic fish, probably related to the perceived risk, and possible adverse environmental impacts are also to be taken into account (Maclean, 2003).

Considerable work in the field of transgenesis concerns the pathogen resistance and freeze resistance, but, even if transgenic fish have been produced, the research remains at a preliminary stage (Maclean, 2003). The development of ornamental fish expressing naturally fluorescent proteins in the skeletal muscle (Gong *et al.*, 2003) and the construction of models for human diseases (Kari *et al*., 2007) demonstrate the wide range of application of the transgenic technology.

Conclusions

For the last twenty years the genetic research in aquaculture has been exponentially increasing thanks to the widespread use of molecular technologies together with the possibility to exploit the impressive amount of data available for other species.

Up to now the knowledge on molecular genetics has been mainly applied to the genetic characterization of the populations, covering a variety of aspects, with special emphasis on diversity analysis and conservation. For the future considerable progress can be expected from gene mapping thanks to the efforts presently devoted to both the enrichment of the genetic maps and to the integration of genetic linkage and physical maps, which is essential for the understanding of genes responsible for performance traits, including growth and disease resistance. With the increasing global demand for aquaculture products and the early stage of selection for most aquatic species, molecular genetics is expected to play a major role in the management of breeding programmes aimed at developing improved strains for the most economically important species.

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