# A molecular tool to enhance salt tolerance in an organism

## Field of the invention

The present invention relates to a molecular tool to enhance salt tolerance in an organism. More specifically, the invention concerns enhancing salt tolerance in species important in international aquaculture. The invention concerns transferrin linked genetic markers (microsatellite-transferrin haplotypes) in an organism, wherein the genetic markers are segregating with salt tolerance, and it also concerns functional mutants (SNPs) in the transferrin gene, which coincide with the marker haplotypes, and hence are regarded as functional mutants tightly linked with salt tolerance.

## Background of the invention

Tilapias belong to a genus of fishes within the cichlid family and are becoming the world's leading aquaculture species, with the Nile tilapia (*Oreochromis niloticus*) at the forefront (Bentsen et al., 1998; Kocher, 2002; Roderick, 1999; Trewavas, 1983).

Tilapias are easily raised and harvested, may be fed a diet of abundant algae and zooplankton. The commercial most important species and strains are predominantly found in fresh water. Since the availability of freshwater is severely limited in many countries, the exploitation of brackish water areas and facilities, typically abandoned coastal shrimp farms, may present an opportunity to expand the tilapia aquaculture industry with only modest investments (Yi et al., 2002). However, such development presupposes a tilapia that tolerates saltwater without reduction in growth performance and health. Increased knowledge of genes involved in saltwater tolerance will facilitate selection for this trait.

The genus *Oreochromis* tolerates brackish water, but with a great variation among the species. *Oreochromis mossambicus* and *O. aureus* (blue tilapia) show a higher degree of salt tolerance than the Nile tilapia (Avella et al., 1993; Cataldi et al., 1988; Doudet, 1992), strongly indicating that salt tolerance is controlled by genetic factors.

Transferrin is an iron binding glycoprotein. It is involved in several biological functions in a wide range of organisms first of all as an iron transporter. Transferrin has an important role in the immune system, since the binding of iron limits the availability of iron for replicating pathogens (Cnaani et al., 2002; Stafford and Belosevic, 2003). Transferrin (TF) is expressed primarily in liver and transported around in plasma supplying most body-tissues with iron, but is also expressed in several other organs (Briggs et al., 1999). Experiments show that the gene is expressed in the brain in Atlantic cod (*Gadus morhua*) in contrast to salmon and other vertebrates where brain expression was not detected (Denovan-Wright et al., 1996). Transferrin belongs to a gene family including ovotransferrin (OTF) and lactotransferrin (LTF). OTF is encoded by the avian transferrin gene and is expressed in eggs and LTF is secreted into milk by the mammary gland preventing proliferation of invading microbes. These variants of transferrin are believed to be produced locally (in mammals and avian) not readily reached by transferrin in plasma (Briggs et al., 1999; Lambert et al., 2005).

All the members in the transferrin-family consist of a single polypeptide chain that folds into two globular halves referred to as the amino- and carboxy-lobes. In TF, OTF and LTF, each lobe coordinate iron binding involving four amino-acid ligands (2 Tyr, 1 His and 1 Asp) with two additional ligands providing a synergistic anion (Thr and Arg) (Lambert et al., 2005). It is suggested that these two lobes originates from a gene fusion and gene duplication of transferrin, since there are similarities in size, nucleotide segments and amino acid composition between the respective exons in the two lobes of the gene (Park et al., 1985).

Our efforts to identify genes associated with salt tolerance included differential expression analysis and a candidate gene strategy. We established a hypothesis that transferrin could be a candidate gene associated with salt water tolerance. Therefore, in the present study, we focused on the expression of transferrin in a saltwater experiment and the structure of the Nile tilapia transferrin gene, notably by revealing the genomic sequence. Just fragments of the sequence from other tilapia species (*O. aureus* and *O. mossambicus)* was available (Cnaani et al., 2002) until the present study. To verify the effect of the transferrin gene, we have performed a functional study; a haplotype segregation analysis among survivors and non-survivors after a salt water challenge, based on genotyping of polymorphic markers closely linked to transferrin and SNPs detected within the gene, and finally a differential expression study of transferrin in the same fish.

## Detailed description of the invention

The inventors of the present invention have shown that transferrin is involved in saltwater tolerance. An expression study on tilapias exposed to rising salinity for several days have been performed. Transferrin showed a significant up-regulation when analyzed by real-time PCR.

Provided here are also the transferrin marker haplotype of salt tolerant tilapia and a set of functional mutants (SNPs) in the transferrin gene that coincide with the marker haplotypes and hence are regarded as functional mutants tightly associated with salt tolerance in this species.

A haplotype analysis among survivors and non-survivors after the salt water challenge has been performed, based on genotyping of polymorphic markers closely linked to transferrin and SNPs detected within the gene. Fish from two families in a saltwater experiment, including survivors in salinity above 30ppt and those dying at a low salt-concentration, were genotyped with the closely linked markers TF-A and TF-B. The genotyping of the two markers returned two haplotypes, and the results showed a significant segregation distortion (Table 1). The salt tolerant fish had a majority of haplotype 2 and the

less salt tolerant fish a majority of haplotype 1. The segregation distortion of the two markers, TF-A and TF-B, closely linked to the transferrin gene, strongly indicated that transferrin could be involved in salt water tolerance. Consequently, parents and offspring in the salt water challenge experiment were checked (=sequenced) to look for potential functional mutations (SNPs) that showed a segregation distortion in the offspring. These studies showed that a haplotype with several SNPs co-segregated with the microsatellites TF-A and TF-B. One of these haplotypes, haplotype 2, was strongly associated with increased salt tolerance.

14 of the SNPs detected within the transferrin gene, resulted in amino acid changes and strongly imply that these changes in the transferrin protein in some Nile tilapias are functionally associated with improved saltwater tolerance.

The two transferrin-linked microsatellite-markers, TF-A and TF-B, return the haplotype variants, 287-184 and 289-188, along with the accompanying expressed mutations (SNPs) in transferrin, which display differential effect on salt tolerance in the experiment of the invention, may be used to customize individuals to various environments defined by variation in salinity. This can be achieved by selective breeding, through other genetic enhancement regimes or it might be obtained through procedures influencing the expression of either transferrin or other genes linked with transferrin. The potential for genetic gains by employing the markers in a marker assisted selection is evident from the fact that the survival is 63 % in the group with the transferrin haplotype 2 and only 38% in tilapia with haplotype 1. The survival attributable to haplotype 2 in this population of fish is 11% (overall survival 49%, survival in haplotype 1 fish= 38%; 49%-38%=11%). By the use of systematic selection, it should be possible to further increase the frequency of haplotype 2 slowly to 100%, and it is expected that this could directly increase survival by 14% (0,63-0,49=14%). Furthermore the invention might also discover new transferrin haplotypes with an even higher survival.

The potential for using the information from the invention to change an individual's salt tolerance through regulatory pathways can be predicted from the possibility that regulatory elements and potential polymorphisms of such (not described in this invention) might be linked or pathway wise associated with the described haplotypes of the invention and hence be used as alternative tool for improvements or customization, still developed with basis in the present invention.

Any transferrin gene variation, linked or unlinked with the haplotype of the invention, which is associated with salt tolerance and has been revealed with assistance of this invention may be used to achieve the goal of this invention; a novel molecular tool to enhance salt tolerance.

The invention shows that tilapia with different transferrin genotypes and different transferrin haplotypes have different survival in saltwater. The invention further suggests using transferrin haplotyping as a tool for marker assisted selection to improve saltwater resistance in fish. The invention can therefore be used to investigate and compare individuals with different transferrin variants, both in tilapia and other fish species, with the purpose to detect new transferrin haplotypes, not yet detected, to obtain an increase in salt water resistance using alternative transferrin haplotypes than the one described. Any sequence tagged site (anonymous or expressed tag) of the vertebrate genome or in tilapia, linked with transferrin, with the haplotype of the invention or with any other polymorphism detected with basis in the invention and associated with salt tolerance may be used to achieve the goal of this invention; a novel molecular tool to enhance salt tolerance. Such polymorphic tags or sites associated with salt tolerance might be tightly or loosely linked with the genomic polymorphic structures of the invention. There are examples that fairly loose linkages can be useful to change genetic makeup in desired direction. One of the most well-known examples of this is the change in the frequency of the halothane sensitivity locus (later discovered as the Ryanodin receptor gene) using haplotypes of markers linked to the locus (Doize et al.,

1990). The invention can therefore be used to develop genetic tests based on linked loci in stead of the invention per se.

Any sequence tagged site/polymorphic genomic tag (anonymous or expressed tag) of the vertebrate genome or in tilapia, not linked with transferrin, nor with the haplotype of the invention, but which is associated with salt tolerance and which has been revealed through the assistance of the invention may be used to achieve the goal of this invention; a novel molecular tool to enhance salt tolerance. This can be obtained using our invention, to establish materials with the same transferrin haplotypes or to statistically control for transferrin variation, as a means to detect other genetic factors influencing on salt water resistance or performance in salt water.

Any expression study using transferrin-expression or expression of other genes closely linked to transferrin can be used to indirectly identify the transferrin haplotype and thereby to provide information about saltwater resistance associated with the invention. The study of linked or unlinked regulatory genes like promotors, enhancers or regulatory elements on the same or other chromosomes, can directly or indirectly be used to find other genes, polymorphisms or expression patterns that are associated with the invention.

The invention describes a method to identify new genes also associated with saltwater tolerance. A gradually increase in the saltwater content will increase the saltwater tolerance of some fish or a different species. A differential expression study can then be carried out in comparison with fish or a different species in freshwater to identify new genes.

The improvements or customizations related to salt tolerance that might be achieved based on the invention may not be restricted to fish/animal or plant breeding or improvements but might also be applicable to human pharmacology and well-being. The saltwater expression study shows that transferrin expression in an organism may easily be changed and that this can improve health/survival and adaptation to shifting and various environmental conditions. For instance will selection of pigs/piglets with increased/optimal transferrin-expression result in increased/changed transferrin-expression in the

intestine to adjust transferrin/iron level and improve resistance to infectious diseases. Or selection of cattle with increased/optimal transferrin-expression may result in increased/changed transferrin-expression in the mammary glands to improve resistance to mammary gland infection.

Indirect adjustment/optimization of transferrin expression may also be possible through breeding of tilapia, salmon, fish, animals and plants for the purpose of systematic changing the optimal level of transferrin genotypes or expression level for the purpose of improving health/resistance, survival, performance, longevity.

The production of animals or products/consumables (milk, meat, plants, extracts or other items) through selection of animals with optimal transferrin expression (resulting in optimal concentration in products) for the purpose of marketing this as health product, for increased survival, health, welfare for human, fish, animal, plants.

## Examples

### *Example 1. Biological material*

A saltwater tolerance experiment was performed on a Nile tilapia population. Salt concentration was gradually increased from 0-30ppt over three days in the containers keeping full-sibs from five different families. We collected 23 surviving offspring at the final salt concentration to compare with 24 non-survivors from one family (family no.1) in addition to 37 survivors and 40 non-survivors from a second family (family no.2). Fin clips were stored in 96% ethanol.

DNA was extracted with the MagAttract DNA M48 kit (Qiagen) as recommended. Fin fragments were dissolved in lysis buffer and proteinase K over night at 37°C. The automated isolation was completed on a BioRobot M96 Workstation (Qiagen) and DNA was eluted by volumes of 50µl.

Total-RNA from Nile tilapia gills and brains, preserved in RNAlater® (Ambion) at -20°C, was isolated with RNeasy Midi Kit (Qiagen) after standard protocol. The organs were added the appropriate amount of RLT-buffer (lysis) and homogenized by a roller in sterile plastic bags (Seaward, Stomacher) and additional homogenizing was carried out by passing the lysate through a sterile 19G syringe. RNA was eluted twice with 150µl RNase-free water and precipitated with ethanol after standard procedure to increase the concentrations. The concentrations were measured on a Lambda EZ 201 (Perkin Elmer) and the organs pooled. The total RNA was then treated with DNA-*free*™ (Ambion), as recommended by the protocol, to remove contaminating DNA from the extracted RNA.

***Example 2. Sequencing of transferrin***

Based on published transferrin sequences of the tilapia species *O. aureus* and *O. mossambicus* of exon 7, 9 and 10 (acc.no AJ318861 and AJ312311), primers were constructed for forward and reverse gene-walking. Gene-walking was performed using the DNA Walking SpeedUp™ Kit (Seegene, Inc.). The gene-walking process consists of several steps of PCRs using a set of universal PCR primers provided with the kit in combination with our TF-specific designed PCR-primers in both directions.

The temperature profile and reaction setup were followed as recommended in the protocol with. The products were tested on 1% agarose gel and purified with ExoSAP-IT before the selected samples were sequenced after standard protocol with BigDye® Terminator (v3.1) Cycle Sequencing Kit (Applied Biosystems) with 0.25µl 10µM Universal primer (Seegene, Inc.) and 0.5µl 5µM of the respectively nested and 2.nested designed primers, on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems).

As a supplement to the gene-walking we used RACE-technology and RT-PCR on total-RNA to amplify cDNA sequence data.

The RACE-technology was carried out as recommended by the GeneRacer™ RACE Ready cDNA Kit Manual (Invitrogen). Gene specific

primers (GSP), forward, reverse and nested, were constructed based on existing sequence, based on the protocol's recommendations. All steps were performed as recommended by the protocol. The PCR-products were purified by ExoSAP-IT (Amersham Biosciences) and then sequenced after standard protocol with BigDye® Terminator (v3.1) Cycle Sequencing Kit (Applied Biosystems) and the respective PCR primers, reverse and forward, on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems).

The two-step protocol of Ready-to-Go-RT-PCR Beads (Amersham Biosciences) was performed as recommended. The PCR-products were purified by ExoSAP-IT (Amersham Biosciences) and then sequenced as described above.

New primers were constructed to run PCR across the introns, based on the obtained exon sequences. PCR conditions were as follows: 2µl 10x PCR buffer including 15mM $MgCl_2$ (Applied Biosystems), 2µl dNTP (2mM), 0.5µl $MgCl_2$ (25mM), ~75 ng DNA, 1µl PCR primer, forward and reverse (5pmol/µl), 0.2µl AmpliTaq® DNA Polymerase (5U/µl, Applied Biosystems) and water to a total volume of 20µl. Annealing temperature was 58°C for 1 minute and the PCR was performed with 35 cycles. The PCR-products were sequenced by the method described above. Then DNA and cDNA sequences were aligned by Sequencher 4.1.4 (Gene Codes Co.) to reveal the exon-intron boundaries. Alignments were also performed by blastn (Basic Local Alignment Search Tool for nucleotides) and by bl2seq (aligning of two sequences) at NCBI. The transferrin sequence of Japanese medaka (*Oryzias latipes*) (acc. no.: D64033) was used as reference sequence.

The complete DNA sequence of the revealed transferrin gene in Nile tilapia, with detailed information of exon and intron boundaries, will also be available in GenBank (acc. no.: DQ272465) after filing of the patent application. The gene consists of 7010 base pairs, with 17 exons separated by 16 introns, including parts of the 5' and 3'UTR regions. (SEQ ID NO:1) The complete coding domain sequence (CDS) is composed by 2085bp (SEQ ID NO:2).

The Nile tilapia transferrin protein consists of 694 amino acids. A protein-protein BLAST (blastp) gave highest similarity match with the transferrin protein sequence of medaka with identity score of 77% followed by red and black sea bream and brown trout with 73, 72 and 67% identity, respectively. A detailed overview of this protein comparison is given in **Figure 1** which shows several highly conserved regions and some regions with diverging amino acids among all species. Also observed is an insertion of three amino acids in the protein sequence of tilapia only.

The iron and anion binding residues in the two lobes of the protein is found conserved as described by (Lambert et al., 2005) in the five fish species compared, except for one residue where tilapia has an aspartic acid instead of the conserved histidine in the N-lobe of the protein.

***Example 3. Genotyping of microsatellites closely linked to transferrin***

Microsatellites linked to transferrin were identified by screening an available tilapia pooled BAC-library (Katagiri et al., 2001) for a BAC clone containing the gene, by PCR amplification with primers designed from available sequence of the gene (Cnaani et al., 2002). A Clone BAC DNA kit (Princeton Separations) was used to isolate the BAC clone following the recommended protocol. The clone was then fragmented by a suitable restriction enzyme and the DNA fragments, of 500-1000bp, were purified by a StrataPrep® Gel Extraction Kit (Stratagene).

The BAC DNA fragments were ligated to pUC19 Plasmid DNA vectors (Sigma-Aldrich Co) and transformed into XL10-Gold® Ultracompetent Cells (Stratagene) following the given instructions. Hybridization techniques were carried out by traditional methods (Sambrook, 1989) using Colony/Plaque Screen™ nylon membranes (NEF 990A, Perkin Elmer NEN) for colony-lift. $gt_{10}$ and $ct_{10}$ -probes were end-labeled at 37°C for 40 min, 95°C for 10 min and 15 min on ice, in a solution of 4µl $gt_{10}$-probe (1µg/µl, MGW), 4µl $ct_{10}$-probe (1µg/µl, MGW), 6µl T4 Polynucleotide Kinase 10U/µl (NEB), 12µl T4

Polynucleotide Kinase Reaction Buffer 10X (NEB), 50µl [$\gamma^{32}$P] ATP 500µCi, (Amersham Biosciences) and 44µl $H_2O$.

Labeled probes were added to the pre-hybridization solution, and the filters were hybridized overnight in accordance with standard methods. The filters were washed and then covered in Saran Wrap and exposed to Hyperfilm MP (Amersham Biosciences) overnight at -70°C before the films were developed. Positive colonies were identified and a secondary screening was performed as described for the first screening steps.

The positive clones were picked and DNA plasmids were purified with QIAprep Spin Miniprep Kit (QIAGEN) as recommend by the protocol. The vector inserts were sequenced to find the potential microsatellites, after the standard protocol for the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), with M13 primers, on ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems).

To verify that the picked BAC clone included the correct gene the isolated BAC-clone was amplified by PCR with the same primer pair used for finding the specific clone in the BAC library. The PCR product was then sequenced as described above, now with the respective PCR primers. The results were subsequently run by BLAST and that way verifying that the sequence matched the correct gene (Rengmark et al., 2006).

Two microsatellite markers were identified in the BAC-clone containing the transferrin gene. TF-A consisted of an repeated sequence of $(GT)_{10}$ (SEQ ID NO:3) and TF-B of repeated sequence of $(GT)_{14}$ (SEQ ID NO:4).

The two microsatellites closely linked to transferrin, TF-A and TF-B, were genotyped on the surviving and non-surviving tilapias from family 1 and 2 in the saltwater tolerance experiment. PCR conditions were as reported above, but with an annealing temperature of 55°C for 45 seconds and 32 cycles. The PCR primers used to genotype marker TF-A were SEQ ID NO:5 and 6 and for marker TF-B: SEQ ID NO:7 and 8. The samples were run on an ABI PRISM® 3100 and the results were analyzed by GeneMapper v 3.0 (Applied Biosystems).

## Haplotypes of salt tolerant tilapia

The two loci were inherited as one haplotype block. Haplotype 1 consisted of the alleles 287 and 184 and haplotype 2 of the alleles 289 and 188, represented by marker TF-A and TF-B respectively. The parents tested were heterozygous for both markers, except the sire in family 2, which was homozygous for both loci with the respective alleles 287 and 184.
The haplotypes and $\chi_2$-distribution are presented in Table 1. We observed that salt tolerant fish showed a tendency of possessing haplotype 2 and the less salt tolerant fish had a majority of haplotype 1. The segregation distortion was significant at $p<0.025$ and 0.05.

**Table 1. $\chi_2$-distribution of the two haplotypes in two QTL-families shows that salt tolerant individuals possess haplotype 2.**

| | QTL- family 1* | | | | QTL-family 2 ** | | |
|---|---|---|---|---|---|---|---|
| | Salt tolerant | Early dead | Total | | Salt tolerant | Early dead | Total |
| Haplotype 2 | 26 | 15 | 41 | Haplotype 2 | 24 | 14 | 38 |
| Haplotype 1 | 20 | 33 | 53 | Haplotype 1 | 50 | 66 | 116 |
| Total | 46 | 48 | 94 | Total | 74 | 80 | 154 |
| Individuals | 23 | 24 | | Individuals | 37 | 40 | |
| Degrees of freedom: 1 | | | | Degrees of freedom: 1 | | | |
| Chi-square = 6.10 | | | | Chi-square = 4.61 | | | |
| *p* is less than or equal to 0.025 | | | | *p* is less than or equal to 0.05 | | | |
| The distribution is significant | | | | The distribution is significant | | | |

*** Family 1: Both parents with heterozygous haplotype 1/2**

****Family 2: One parent with heterozygous haplotype 1/2, the other with homozygous haplotype 1/1**

*Example 4. Identifying SNPs in the transferrin gene*

Additional primer pairs were designed to find potential SNPs in the expressed part of the gene. Standard PCRs were first performed on the family 2-parents, then sequenced by the MegaBACE™ 1000 DNA Analysis Systems (Amersham Biosciences) using the DYEnamic™ ET Dye Terminator Kit (Amersham Biosciences). Reaction conditions were as follows: 4µl ET reagent premix, 4.5µl $H_2O$, 1µl PCR-product and 0.5µl primer (5µM) with the following step repeated 28 times: 95°C, 15 seconds, 58°C, 10 seconds, 60°C, 1 minute. The post reaction clean-up was performed as recommended by the protocol with ethanol and 7.5M ammonium acetate. SNPs were identified by aligning and comparing the sequence data by Sequencher 4.1.4 (Gene Codes Co.). If SNPs were detected, the two offspring groups in the family (salt-water survivors and non-survivors) were then sequenced over the determined SNPs to define their genotypes.

**SNPs detected in the transferrin gene**

25 heterozygous SNPs were identified in the transferrin gene, 15 of these were located in exon 7, 8, 14, 15 and 16. Fourteen of these resulted in an amino acid change (Table 2).

We observed that all the SNPs, genotyped on offspring from family 2, segregated as two haplotype blocks that also include the two microsatellites. The haplotype distribution is shown in detail in Table 1 and the linkage distortion strongly implies that the individuals possessing haplotype 2 are more salt tolerant. A complete overview of the haplotypes is given in Table 3. The iron and anion binding residues are all conserved except for one (Figure 1). The histidine residue in the N-lobe codes for an aspartic acid (GAC) in Nile tilapia (position 258). All the other fish species have His in this position (CAC or CAT). Lambert et al. (2005) reports that this His-position is the most variant residue in the N-lobe and Asp in this position is also found in different insects like fruit fly and termite. Interestingly, this position in exon 7 contains a SNP

(see Table 2) in the neighbouring amino acid just upstream for the Asp resulting in an amino acid change from alanine to glycine in the surviving group of fish.

**Table 2. SNPs detected in the transferrin gene. Listed are location in gene, base pair number (correspond with sequence in GenBank, acc. no.; DQ272465), genotype in wild type and SNP, amino acid change if any and the amino acid position (see also Figure 1 where these positions are underscored).**

| | Gene position: | Bp no.: | Wild type: | SNP: | Amino acid change: | Amino acid no.: |
|---|---|---|---|---|---|---|
| 1 | ex7 | 2968 | CC | GC | Ala (A) → Gly (G) | 357 |
| 2 | ex8-1 | 3190 | GG | GA | Ala (A) → Thr (T) | 306 |
| 3 | ex8-2 | 3224 | GG | GA | Arg (R) → Lys (K) | 317 |
| 4 | ex8-3 | 3229 | TT | GT | Leu (L) → Val (V) | 319 |
| 5 | intr8 | 3330 | AA | GA | - | - |
| 6 | ex14 | 5368 | GG | GA | Val (V) → Ile (I) | 546 |
| 7 | intr14-1 | 5437 | GG | GA | - | - |
| 8 | intr14-2 | 5462-3 | GG/TT | GT/CA | - | - |
| 9 | intr14-3 | 5467 | CC | CT | - | - |
| 10 | ex15-1 | 5521 | GG | GT | no change | 568 |
| 11 | ex15-2 | 5598 | CC | CT | Ala (A) → Val (V) | 594 |
| 12 | intr15-1 | 5689 | GG | GT | - | - |
| | intr15-2 | 6530 | CC | CT | - | - |
| 13 | ex16-1 | 6549 | CC | CT | Ala (A) → Thr (T) | 618 |
| 14 | ex16-2 | 6562-4 | GG/CC/TT | GA/CG/TC | Ala (A) → Ser (S) | 623 |
| 15 | ex16-3 | 6659 | AA | GA | Glu (E) → Gly (G) | 655 |
| 16 | ex16-4 | 6685-8 | GG/AA/AA/GG | GA/AT/AT/GT | Glu (E) Ala (A) → Ile (I) Ser (S) | 664-5 |
| 17 | ex16-5 | 6697-8 | GG/AA/TT | GA/AC/TC/ | Asp (D) → Thr (T) | 668 |
| 18 | ex16-6 | 6729 | TT | TA | Asp (D) → Glu (E) | 678 |
| 19 | ex16-7 | 6733 | GG | GA | Ala (A) → Thr (T) | 680 |
| 20 | ex16-8 | 6736 | TT | CT | Ser (S) → Pro (P) | 681 |
| 21 | intr16-1 | 6769 | TT | CT | - | - |
| 22 | intr16-2 | 6777 | AA | TA | - | - |
| 23 | intr16-3 | 6780 | AA | CA | - | - |
| 24 | intr16-4 | 6785 | AA | GA | - | - |
| 25 | intr16-5 | 6848 | CC | CA | - | - |

**Table 3. Overview of transferrin haplotypes 1 and 2 across the two genetically linked microsatellite markers (TF-A and TF-B) and the 25 SNPs (within the gene) in salt tolerant Nile tilapia.**

| | TF-A | TF-B | ex7 | ex8 | | | intr8 | ex14 | intr14 | | | ex15 | intr15 | ex16 | | | | | | | | | intr16 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 287 | 184 | C | G | G | T | A | G | G | T | C | G | C | C | GCT | A | GAAG | GAT | T | G | T | T | A | A | A | C |
| 2 | 289 | 188 | G | A | A | G | G | A | C | A | T | T | T | T | AGC | G | ATTT | ACC | A | A | C | C | T | C | G | A |

*Example 5. Expression study on transferrin*

A parallel saltwater experiment was carried out for the expression study. A total of 200 Nile tilapias of four different families were used in this experiment. The average body length was 10cm and there was an approximately equal distribution of the sexes. Twenty-five fish from each family were pooled in two tanks with identical physical environments. The salt concentration in one of the tanks was gradually increased every second day from 0-32ppt during a period of ten days, and the fish were kept for another five days in this saline condition. All the fish were then killed and dissected. Brains and gills were directly transferred to and preserved in RNA*later*® (Ambion) at -20°C.

Brains and gills from each treatment were pooled in separate containers and total-RNA was extracted as recommended by the RNeasy Maxi Kit (Qiagen). Equal amounts of the brain and gill isolations were mixed together to give a salt and freshwater pool respectively. The intention of organ pooling was to reveal a total expression of the salt tolerance metabolism.
Transferrin was analyzed by RT-PCR on an ABI PRISM® 7700 Sequence Detection System (Applied Biosystems). Total-RNA was treated with DNA-*free*™ (Ambion) to remove contaminating DNA, as recommended by the protocol. A two-step RT-PCR was performed with a TaqMan® Gold RT-PCR Kit (Applied Biosystems) with PCR reagents and cycling conditions as recommended. This two-step RT-PCR includes the addition of AmpErase UNG, which can prevent carryover contamination from PCR products. Specific TaqMan® primers and probes were constructed in terms of the protocol. Several incremental dilutions were done in advance to test the best $MgCl_2$, primer and probe concentrations for optimal RT-PCR conditions. Standard deviations for each sample were measured based on three parallels runs.

Successful RT-PCRs were run on pools of total-RNA from tilapia brain and gills, in addition to gills only. This states that transferrin is expressed in

gills and indicates expression also in brain, which up to this point has not been detected in other vertebrates than Atlantic cod (Denovan-Wright et al., 1996). Transferrin showed a significant up-regulation when analyzed by RT-PCR.

## List of sequences

**SEQ ID NO:1**

The complete DNA sequence of the transferrin gene in Nile tilapia, with detailed information of exon and intron boundaries and amino acid information is given in GenBank acc.no.: DQ272465

```
   1 tgatccggag accccaccgc ccaacatgaa gcctctcctc ctagtgacgt ctcttggtga
  61 tccggagacc ccaccgccca acatgaagcc tctcctccta gtggcgctgc ttggactgct
 121 cggtaaaagc ttttcagccc cgcactacga agtaggcaaa gagttcgctg cacccggctc
 181 tcagaaggtg aaatggtgcg tcaagtcaga ccaagaattg cgtaaatgtt cagatctcgc
 241 cgctgcgatt ccttgcgtca tctgtgtgaa aaaagaaaac accctagagt gcattgttgc
 301 cattaaggtg agcgcttttt acgtccagag caccaaatta accacaaatg ctcaataaca
 361 gactttgacc tgggcaattg ttataaacgt aacawttctg ataarcgtat aartgtaact
 421 gaagtgcttc acaggtgctc tgcttattct gaaatgttac ttaagtaaaa ataagtgaat
 481 gtaatgactt cattttattg tttttttct tcactttctt cgagtaggtt catactcgaa
 541 ctactactaa aacgtatcta aagtaaagat aaattaactg tatctgctgt ggagtgttct
 601 agaaaaattg agtagagtta aaaatattaa aaaaaattaa atccgaacca aatgcttttt
 661 taactatttc aattgcatct aaatattcaa tttaatttgg ttacataaat acaatttaaa
 721 tgttacattt aatggaagct tgtcaaataa aatgtatttg attggcatgg attcagttta
 781 taccaagcac attttccttg attattattt agctcattaa agaaacattt gattttatat
 841 atttcagctt tactcacttt actcagaatk attacatcat gtttgstgag gatgaataat
 901 ttttttaaa ccctttaag yatttaatgt ggggaaaaaa ctaccaatgc cttcaactca
 961 ttcagtgaaa ttctctgtaa aaatgacttg acctcagtca tacaacaaat actctcgggc
1021 aggctggtga agcagatgcc attactgtgg acggtgggga catttacact gctggcttga
1081 acaactacga cctccatccc atcattgctg aggactacgg caccggtatg tccactcatc
1141 agctgtcatt agtagcctaa tgaaataagc tcaagcaaga acatgtttac cgttttatgt
1201 gaatgtgatt tagctgtttc aataatatgt atccattttc atcattcatt cagcttcgga
1261 cacctgctac tacgctgttg ctgtggcaaa gaagaatact ggatttggct tcagagatct
1321 caggggggaag aaatcatgcc acactggttt ggggaaatct gcaggctgga acattcccat
1381 aggatctctg gtgtccatga atgttattga gtggggtggc attgaagaca aacctttgga
1441 ggaaggtgag ggactttttt ttctggtgag ctcataacat ttgacagctt tagtgcatgt
1501 atggtcgaag gacgataaga tcagaccttt ttatgagnaa ctggacgcct gagaaacaat
1561 taaacacact tatttgcttt actgacgaga atttattgat gggatcaaaa ctacaggaaa
1621 agtagaaaca gacagggaag gggctaccta gcttcttttt tattagtcat ttatttaacc
1681 aggtaaaaaa atcctcattg agattaaaaa tctctttcca agagagacct tgcttctgcc
1741 cagggattat aaaatcctgt gcctgtcagc acttcttatc acttataaca cttccttttg
1801 ttaaatataa ttaactgaag ttcaaaaact agggcgtttt agactagtta ggccctgtta
1861 gacagattga acaaatcaat atccatccat cttcttctaa ccaaccaccg gacacgacag
1921 gggctggagg cttgcctctc agtgctgaga ggggggaaag gtcgccagcc atgtactcac
```

1981 attcgtacat atggtcagtt tagaactgac aattaaccta acatgaaaga aactggtgta
2041 tccagtatcc catggatgca aactctgcac acacaggata atatgaaact tctaaatgtt
2101 aaaaatgcat atgggaactt taatatttgt ctttaaaggc agacagtggt gacactctgg
2161 ctcttctgtc agtgttttgg ttttcttttc taaattatga ctgtggcctt gtttgatttt
2221 gttatgcagc ggtgagcacc ttcttccatg ccagctgtgc ccctggggca gcaagaggca
2281 gcaagctgtg tgaactgtgc aagggagact gctccaggtc ccagagggag ccttactatg
2341 actacaatgg agcctttcag tcagtgtcca ctttttaatg cttctcatta gtgaacataa
2401 acttctacac atttagccga acctttttatc tgtaataata tgacttttgt aacaggtgtt
2461 tggccgaggg agctggagat gtggcttttg tgaagcatct cactgtgcca ggtcagtatc
2521 acaacaacct ttatccattc gaacatataa agaagcactg catcatagca ttgtcaggtt
2581 ttattcatca aaaacgagcc tcaccgagat taaaaatttc attttgaaaa gcgactggga
2641 caagaaggga cctgatgaac atgcatgaca tacaaataca actgttgcac actgcacaag
2701 tcaaggacaa gtcaagcaga atttcaaagc aaatttcaga gcaatcacat tgaccaagag
2761 tgtcattttc tcaaatgatt aaatggtctt aaacaccctg aaacttctac aaatatggga
2821 aagaataact gtgaaaaaca ctgatgtaaa acctgtgtca tgtcacactt ccagaatcgg
2881 ataagccaat gtacgagcta ctgtgcaagg acaacaccag agcacctatt gacaactata
2941 acacctgctt cctggccaga gtgccagctg acgctgttat tactcgcaag gatccacagc
3001 tggccgactt tatctgggag acccttgacc gagttcagac tgaccacgta ggaagtccac
3061 tgaataacat ctcatttcaa cgtattctga ttctttgtgt ttcagcagcc tcattgcttt
3121 cctaatgaat catctcactt gctgcctttt cagagcttta acctcttctc atctgaagcc
3181 tacgcgcccg ccaagaacct gatgttcaaa gattcaacag tgaggctctt gagggtgccc
3241 ccaaacacag actccttcct gtatctgggt gctaactaca tgagcatcgt ccattccctt
3301 aaaaaaggta aagaacacaa gcaagacaga cagaaacaat tgttagtcag gtgtcggtct
3361 ggcttatgtc taagtctttt tcccgtctct agagcaggca tcagacgttg catcccctgc
3421 cattaggtgg tgtgctgtgg gccatgctga gactgctaag tgtgacacgt ggagcatcaa
3481 cagtgtgtct gatgacaccg cctccattga atgccagagc gcccctacag ttgaagattg
3541 cctgaagaag attatggtaa actcagtagg aaatatcgaa atctgttact ttattatttt
3601 ttgactccac tctgtgacgt tcatctctgc tttgaatcgc cccgcagcgt aaagaggctg
3661 acgcagtcgc cgtggatgga ggtcaggtgt ttacggctgg aaagtgtggc ctggttcctg
3721 tcatggtgga gcagtatgac caaggtagat aacaagaaga agaaataaca cacacacaat
3781 cctgtgtcgt atttcacctt ttctatatga gtaatatttg ttcattttgt cttctctcgc
3841 agagatgtgc ggcaactcta atggtagtaa actttcatct ttttcctcct ctttttatga
3901 tctggacaaa ttttttaaaa agcctcttga agtgcatcat tttaattgta acacagcttc
3961 gatgacctgt tgtgctgatg acaagatttg tattttatat ttgtatcctt ggactctacc
4021 actctactac tacaactaca actataattt atatgaacaa ccagttcaag gaatccttat
4081 ttattcatta gtaacccctt agttcatcct ctgaaacaaa cctctgcgga agctttgaac
4141 aacaggtgga tggagcttgt cttcattata tattaaggat atagtctctt tatggcttta
4201 caaacaacca agatttttaa aaactacaaa aaaattccaa attgagcatt tacataaatc
4261 tggagaccta gcatttagct cattggcatt gcttgtacat tgtctaaatt tttagtggct
4321 ccggttttgt aagtatccac gattttaatg atagattacc tttaaaaagt ggattaattt
4381 taagcagccg taaacagagt agatttatat gaacgtctct ctccagttga atcatctgaa
4441 atgtctactt cccgtatcca ctctcagccc cagcctcctc atactatgct gttgctgtgg
4501 taaagaaggg ttcaggggtg acctgggaga atctgaaggg caagaagtct tgccacacag
4561 gcatcggcag aactgctggc tggaacatcc ccatgggtct aatctacaac agagaacatg
4621 actgtgactt cagtgagtga ctgcgagctt cttccttctt ttaccaacag aaagaaattt
4681 aaagccaccc acagaaatca tacaactccc ctttttctaa aaaaaaagtt tgtttagatg
4741 actcatgtga ttaatctata tttaaacaaa agccacaatc ttccattaac cttgacaaaa
4801 gagaggtttg ctgattgttg agctctttct gcatctcttc ctctcctcag ctaagttctt
4861 cagtagcggc tgtgcccccg gagcagagcc cacctctccg ttctgttctc tgtgtgtcgg
4921 cagcggtaaa gctgtgggag acgaggccaa atgcaaagcc agcgctgatg agaagtacta
4981 cggttatgct ggagccttta ggtacagcaa gamacagaat ggcatcgcat acaagtttgt

5041 gtataaggag aaagttagca aagagacagg aagcactgga aaacaacaga accagctctt
5101 ctsaatsttg gcagctactg ttatgttttt tttcttatat atttcatttg taaacagcca
5161 agaatgtgtc aagagctgaa gcatgttttc ctcctaaaac caggactttt taatcttttt
5221 ttaaattgtt gtgaacttta gcaatgatga ggtgtaattt ttaattggtt agccatttac
5281 ccccaacttc actctttgtc tccaaagtca tgatgtatgg catatcatta caggtgtctg
5341 gttgagggtg gtggtgatgt tgccttcgtt aaacacacaa ctgtgacaga aaacagcgat
5401 ggtaagagga ctgcactgat ctgcactacc tgctccgatg acattacaac agcttctaaa
5461 gtttactcca cctctcttta ccgaaggtaa cggtccagac tgggctcgta atctgagatc
5521 gtctgattac cagctcatct gccctgggaa gggaccagtg gagatcagtg attatgccac
5581 ttgtaacttg gctcaggcgc ctgcacacgc tgtggtgact cgcccagaaa gccacagtaa
5641 ggtggtccgc atccttcagg aacagcaggt ttgtgataaa gtctaagaga gcyacttgaa
5701 atgaaataag agsttaaaat aaaattgttg ttgtttcaaa gcactgaatt cctagtgcaa
5761 aaatctkaca tcrmggttca acttttctag ctgacacaga tgctgagtct gaaatagaga
5821 agcatcctgt cattacagaa gctgcataca gtttgtttgg cattctgctt tgagcacaac
5881 aatgaatttt taacagattt tccaaatagt tgccaattaa ctctgacaac tttaacattg
5941 tccctttgta ggggctacat ctaagactct acaggcctca gttaacatgt taaatgttaa
6001 agcttatgac agcagaatta gaaaaagact gagcaaatgt agcttgtttg gaaagaaaaa
6061 gccgcttctc tctacagaga acatggcagc acagcttttg tttgcagagt tgcatctgaa
6121 caaaccaaga gacttctgga acaatgttcg tctggcctta atgtacagga ccacgtttgt
6181 tgaaaatcaa acatagcata ttcgcacaaa cacctcatag aaactggtgg tggaggggtg
6241 ttgatttggg cttcttttac agcaacgtgg cacaaacatt tcaagttact gctcccaaag
6301 ttgattctac ttgctactga atcatggagt gtacttagtt tgtcacaact gcatagagtc
6361 ctgtgaaaac tttctttttg atatgactgt acttgcactt gatctcaagt ggggggacct
6421 tggcaattca aaaaccaata gtgacttctg tttttctaca ttctgccggt ttcaatgagt
6481 aaagtaacca gtgtaataga aatctaaaaa ttaaataact gcttcaaacc taaattttgt
6541 tcataggcca gatttggaaa cgctgggact gatccttcat tcagaatgtt ccagtcagag
6601 aatggaaaga accttctctt ccaagactcc actaagtgtc tccaggaggt accagaggaa
6661 agaacctatg agcagttttt gggagaagct tacatggatg ccatgaaggc actgagacat
6721 tgcagcgata ctgcttcagg tttgtactac tgcagaaatt acttagcatt taaatgataa
6781 ttttatacca tcttgtacca gataaaataa cattcatgct cgatattctc caattgtttt
6841 tgtttgcctc tcttcccaac agatctggaa aagtcttgca cattccatgc ctgccagcaa
6901 ggctaatgga gatgtagaca acacagtgat tcagccatct tacccactta cttcctgcac
6961 ttacccaaac atagtgtttc tcctcttctg atcttactat agactataga

## SEQ ID NO:2

Amino acid sequence of transferrin:

/translation="MKPLLLVALLGLLGKEFAAPGSQKVKWCVKSDQELRKCSDLAAA
IPCVICVKKENTLECIVAIKAGEADAITVDGGDIYTAGLNNYDLHPIIAEDYGTASDT
CYYAVAVAKKNTGFGFRDLRGKKSCHTGLGKSAGWNIPIGSLVSMNVIEWGGIEDKPL
EEAVSTFFHASCAPGAARGSKLCELCKGDCSRSQREPYYDYNGAFQCLAEGAGDVAFV
KHLTVPESDKPMYELLCKDNTRAPIDNYNTCFLARVPADAVITRKDPQLADFIWETLD
RVQTDHSFNLFSSEAYAPAKNLMFKDSTVRLLRVPPNTDSFLYLGANYMSIVHSLKKE
QASDVASPAIRWCAVGHAETAKCDTWSINSVSDDTASIECQSAPTVEDCLKKIMRKEA
DAVAVDGGQVFTAGKCGLVPVMVEQYDQEMCGNSNAPASSYYAVAVVKKGSGVTWENL
KGKKSCHTGIGRTAGWNIPMGLIYNREHDCDFTKFFSSGCAPGAEPTSPFCSLCVGSG
KAVGDEAKCKASADEKYYGYAGAFRCLVEGGGDVAFVKHTTVTENSDGNGPDWARNLR
SSDYQLICPGKGPVEISDYATCNLAQAPAHAVVTRPESHSKVVRILQEQQARFGNAGT

DPSFRMFQSENGKNLLFQDSTKCLQEVPEERTYEQFLGEAYMDAMKALRHCSDTASDL
EKSCTFHACQQG"

**SEQ ID NO:3**

**Sequence of microsatellite marker TF-A:**

TCCGCTATTCAAAGTGAGTCATTATCTGGAACAACACTAAGCTGATATCTT
TAACTCTTTTACGGTGTTTTAAAAAAGCGCATTTGAGTACTTACATTTCCA
TCACAGCTGGGACATGATGTTGCTGAGTAGACTGTGATTAGCCTCTTGTTG
CAGAAAACAATGACGTTAGCTTAATTTTATTTTACTGCATCGCGTTTGATT
GGGAATATGATCATTTCTGTTTATTTAATGTTAATCTGTTTAATATCTTGAA
ATAAGCACTAATGTATCTGATATTAATTTTAGTTGTTTAACAAAGCAGTAG
TATGGGAATATAGTACAGTACTGAGCGAAAGTCTCAAGCCATTCCTCATA
TACATACATACATAC**GTGTGTGTGTGTGTGTGTGTG**CAAGCATTCATGG
AAATAAAGTATATACCATTCCCATTGTCTTTAGAGTCACTGTCCTGGTTGA
AAATGAAGCCGATGCCAATGGAATGCTTTCCAGGTGGTATTGCATGGTAG
ATCAATGGATCAGTCCCTTCACCACTGGCTGAAATG

**SEQ ID NO:4**

**Sequence of microsatellite marker TF-B:**

TAGAGCGAGTTAATTAGAAGCGCAGTTACACATGTGGCTGTTATTATCGT
AGGTGCTAATTAGCAGCATTTGTCAGATGTAAAAATAAAAAAAATTAAAA
AGGGGGGTGGGGATGGCAATCAGGAA**GTGTGTGTGTGTGTGTGTGTGT**
**GTGTGTG**CGCGCACGCTTAAAAGCACCTCACGTAATTAACTGCTCCAGAG
A

**SEQ ID NO:5**

PCR forward primer for microsatellite marker TF-A:

TTTTACTGCATCGCGTTTGA

**SEQ ID NO:6**

PCR reverse primer for microsatellite marker TF-A:

ATCGGCTTCATTTTCAACCA

**SEQ ID NO:7**

PCR forward primer for microsatellite marker TF-B:

GCGAGTTAATTAGAAGCGCAGT

**SEQ ID NO:8**

PCR reverse primer for microsatellite marker TF-B

TGGAGCAGTTAATTACGTGAGG

## References

Avella, M., Berhaut, J., Bornancin, M., 1993. Journal of Fish Biology 42, 243-254.

Bentsen, H.B., Eknath, A.E., Palada-de Vera, M.S., Danting, J.C., Bolivar, H.L., Reyes, R.A., Dionisio, E.E., Longalong, F.M., Circa, A.V., Tayamen, M.M., Gjerde, B., 1998. Aquaculture 160, 145-173.

Briggs, D.A., Sharp, D.J., Miller, D., Gosden, R.G., 1999.. Molecular human reproduction 5, 1107-1114.

Cataldi, E., Crosetti, D., Conte, G., D'Ovidio, D., Cataudella, S., 1988. Journal of Fish Biology 32, 191-196.

Cnaani, A., Ron, M., Lee, B.Y., Hulata, G., Kocher, T.D., Seroussi, E., 2002. Mapping the transferrin gene in tilapia. Animal Genetics 33, 78-80.

Denovan-Wright, E.M., Ramsey, N.B., McCormick, C.J., Lazier, C.B., Wright, J.M., 1996. Part B, Biochemistry & molecular biology 113, 269-273.

Doize, F., Roux, I., Martineau-Doize, B., DeRoth, L., 1990. Can J Vet. Res. 54, 397-399.

Doudet, T., 1992. Aquaculture 102, 275-288.

Katagiri, T., Asakawa, S., Minagawa, S., Shimizu, N., Hirono, I., Aoki, T., 2001. Animal Genetics 32, 200-204.

Kocher, T.D., 2002. Tilapia Genomics and Applications. Available at http://nps.ars.usda.gov/static/arsoibiotecws2001/contributions/Kocher.htm.

Lambert, L.A., Perri, H., Halbrooks, P.J., Mason, A.B., 2005. Part B, Biochemistry & molecular biology 142, 129-141.

Park, I., Schaeffer, E., Sidoli, A., Baralle, F.E., Cohen, G.N., Zakin, M.M., 1985. Proceedings of the National Academy of Sciences of the United States of America 82, 3149-3153.

Rengmark,A.H., Slettan,A., Lee,W.J., Lie,Ø., Lingaas,F. 2006. Identification and mapping of genes associated with salt tolerance of tilapia . (UnPub)

Roderick, E., 1999. Seafood International 46-50.

Sambrook, J., 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, New York.

Stafford, J.L., Belosevic, M., 2003. Developmental and comparative immunology 27, 539-554.

Trewavas, E., 1983. Tilapiine fishes: of the genera Sarotherodon, Oreochromis and Danakilia / Ethelwynn Trewavas. British Museum (Natural History), London.

Yi, Y., Lin, C.K., Diana, J.S., 2002. Nineteenth Annual Technical Report. Culture of Red Tilapia in Brackishwater Ponds. Available at http://pdacrsp.oregonstate.edu/pubs/technical/19tch/9NS4.pdf.

## Claims

1. Use of transferrin haplotyping as a tool for marker-assisted selection to improve saltwater resistance in fish.

2. Use as claimed in claim 1, wherein the fish is tilapia.

3. Use of microsatellite-transferrin haplotypes 1 and 2 according to Table 3 for the development of tools or criteria in a selection or management regime to customize an organism to environments defined by the level of salinity.

4. Use of transferrin SNP polymorphisms according to Table 2 and 3 for the development of tools or criteria in a selection or management regime to customize an organism to environments defined by the level of salinity.

5. The use of claims 3 or 4, wherein said organism is an aquatic species, vertebrate or invertebrate.

6. The use of claims 3 or 4, wherein said organism is a terrestrial species, vertebrate or invertebrate.

7. The use of claims 3 or 4, wherein said organism is a vertebrate, terrestrial or aquatic.

8. The use of claims 3 or 4, wherein said organism is a teleost fish species.

9. The use of claims 3 or 4, wherein said organism is a species belonging to the cichlid family.

10. The use of claims 3 or 4, wherein said organism is a species belonging to the Oreochromis genus.

11. The use of claims 3 or 4, wherein said organism is Oreochromis niloticus (Nile tilapia).

12. Use of transferrin-linked microsatellite markers TF-A (SEQ ID NO: 3) and TF-B (SEQ ID NO: 4) to customize individuals to various environments defined by variation in salinity.

13. Use of SNPs according to Table 2 or Table 3 to customize individuals to various environments defined by variation in salinity.

14. Use as claimed in claim 12 or claim 13, wherein the customizing the individuals is by selective breeding, through other genetic enhancement regimes or through procedures influencing the expression of either transferrin or other genes linked with transferrin.

15. A PCR primer wherein the nucleotide sequence of the PCR primer comprises or consists of the sequence given in any one of SEQ ID NOs: 5, 6, 7 or 8.

16. A nucleic acid molecule wherein the nucleotide sequence of the nucleic acid molecule comprises or consists of the nucleotide sequence given in SEQ ID NO: 1.

17. A nucleic acid molecule wherein the nucleotide sequence of the nucleic acid molecule comprises or consists of the nucleotide sequence given in SEQ ID NO: 3 or SEQ ID NO: 4.

18. A polypeptide wherein the amino acid sequence of the polypeptide comprises or consists of the amino acid sequence given in SEQ ID NO: 2.

19. A method of producing an animal or production of animals or products/consumables through selection of animals with optimal transferrin expression.

20. Method of detecting in an organism at least one genetic marker that is genetically linked and co-segregating with the transferrin gene, said method comprising analyzing the transferrin gene informative region in the organism's genome for the presence of the genetic marker.

21. The method of claim 20, wherein the genetic marker is TF-A and/or TF-B according to SEQ ID NO: 3 and SEQ ID NO: 4, respectively.

22. The method of claim 20, said method further comprising using as a probe any of SEQ ID NO: 5-8, or a probe with 80% or more sequence identity with any of these nucleic acid sequences, for detecting the presence of the genetic marker.

23. Method of detecting in an organism at least one polymorphic gene that is genetically linked and co-segregating with the transferrin gene, wherein polymorphisms result in a quantitative variation in expression, where each mean level of expression is associated with a specific genotype of this gene, wherein the method can be used to indirectly define and/or select for the linked transferrin haplotype through expression analysis of the linked gene.

24. Use of information about regulatory elements for transferrin expression to manipulate expression of the transferrin gene.

25. Use according to claim 24, wherein the regulatory elements for transferrin expression are unlinked or linked regulatory elements.

26. Drug or feed additive which can adjust transferrin-expression, in a way that improves saltwater resistance.

27. Environmental factor that affect and increase transferrin expression, which in turn improves saltwater resistance

28. Method of selecting individuals with high tolerance for saltwater, said method comprising measuring levels of transferrin expression in the individuals, for commercial use or similar.

29. A method for obtaining an indication of the salt tolerance of a fish, the method comprising determining the presence or absence in a sample of nucleic acid which has been obtained from the fish of one or more SNPs as defined in Table 2 and 3 in the fish transferrin gene, wherein the presence of one or more SNPs of Haplotype 2 as defined in Table 3 is indicative of a fish having an increased salt tolerance compared to a fish having one or more SNPs of Haplotype 1 as defined in Table 3.