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What is known about genetic variation among Baltic Sea blue mussels and the promise of proteomics. A literature review.

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Abstract

The Baltic Sea is an evolutionary young sea that have developed a low salinity in its water from the fresh water that flows from the north and saltwater that flows from the south of the sea. The low salinity is too low for many marine organisms and too high for many freshwater organisms. Species like the blue mussel, which have adapted to the low salinity, may have developed different protein expression as a result. To study which protein that have been expressed in the organism proteome analysis is often used. 2-dimensional electrophoresis may be the only method that can do this kind of analysis.

Introduction

Baltic Sea

The Baltic Sea is 7500 years old and is thereby evolutionary young. The sea is the largest brackish sea after the Black Sea. The saltwater in the Baltic Sea is from the south and the freshwater from north (Rönnberg. C, 2004). The Baltic Sea surface water is known for the salinity gradient from north to south of 2 to 8. The salinity is too high for freshwater organisms and too low for marine organisms, which lower the diversity of species (Paavola. M, 2005).

The Baltic Sea has a low salinity which determines the possible distribution of marine species there. Saltwater flows to the Baltic Sea from the Danish Straits and freshwater inflow is mainly from northern and eastern parts of the Baltic Sea. Salinity decreases the further you get from the North sea thus a forming a stable salinity gradient. The saline water is even more diluted in the Gulf of Finland where the fresh water comes from east and the salt water comes from the west. Although salinity varies over time the gradient is stable. The variation occurs within seasons and even between years. Between 1976–1993 the Baltic Sea went through a period where less saline water entered the sea, leading to a salinity decrease in the area. Salinity fluctuates because of irregular inflow of high-salinity water from the North Sea and various meteorological factors. Many species in the Baltic Sea which have adapted to the low salinity are considerably different compared to the same species (conspecifics) in more saline marine environments. Because of osmotic stress due to low salinity many species are close to the limit of their physiological tolerance.

Baltic blue mussels are smaller than the blue mussels in the ocean because low salinity gives rise to osmotic stress The blue mussels live at the edge of their salinity in central Gulf of Finland and in the Åland area. The low salinity can affect the marine species because it affects reproductive success, growth and how long they live. The low temperature in the Baltic Sea has also reduced the annual growth of the blue mussels (Westerbom. M, 2002).

The blue mussel populations in the Baltic Sea and the North Sea show differences in morphology, allozyme frequencies and physiology. The differences have mostly been attributed to the two areas different environments with the difference in salinity the most important abiotic factor. Because of the genetic and physiological adaption to brackish water

differences is found between the blue mussels in the Baltic Sea and in marine waters (Tedengren. M , 1999).

Because of the differences between the Baltic Sea and the North Sea organisms have been adapted differently depending on their location. Many species like Baltic blue mussel, Mytilus trossulus, have established evolutionary lineages that differ genetically from the same species in other seas. The Baltic blue mussel does still change because its a relative new hybrid with DNA from *Mytilus trossulus* and *Mytilus edulis*

Three species of *Mytilus*, (*M. edulis, M. galloprovincialis and M. trossulus*) are spread in the southern and northern hemispheres. These three species are usually referred to the same species complex, the *Mytilus edulis* complex, because it is still not obvious if they are different species. Mytilus edulis is believed to be the ancestor from which *M. galloprovincialis and M. trossulus* have evolved (Gardeström J. 2008).

Mytilus edulis are distributed in the Southern and Northern hemispheres. The distribution and the interspecific hybridization make them an interesting model to study population dynamics and populations genetic in marine habitats because it describe their possibility for adaption (J.L. Lo´ pez, A. Marina, 2002).

Stress

Stress is a condition that disturbs a function in a biological system or a condition that decreases fitness. To be able to define and study stress it has to be compared to a normal state (Gardeström. J, 2008).

Organisms are exposed to many stressors and depending on their environment they can be affected differently. To compare how the same stress can affect organisms in different environments the survival rates of the blue mussel *Mytilus edulis* in the Baltic Sea have been compared with mussels from the marine North Sea. The comparison showed that there were differences between the blue mussel in the Baltic Sea and the North Sea. The Baltic blue mussel seems to be physiologically stressed as response to ambient conditions and they are more sensitive to anthropogenic stress compared to the blue mussels in North Sea.

Studies prove that blue mussel, *Mytilus edulis*, from the Baltic Sea are sensitive because of their physiological response and survival when they are exposed to toxic substances (Tedengren. M , 1999).

All the anthropogenic stress in The Baltic Sea coming from things like industries and traffic which is combined with the low salinity describes the disturbance that occurs in the environment. That combination makes it difficult to understand what is caused by human activity and what is caused by natural stress (Olsson. B, 2004). Toxic exposure is often in low concentration and over a long time (Gardeström. J, 2008). But it certainly affects the Baltic Sea species and biodiversity.

Biodiversity and genetic diversity

The Baltic Sea Biodiversity contains biological organization, genetic resources, species, ecosystem and landscape and these are all depending on each other. Most studies have been on species diversity and not about genetic diversity. Genetic diversity is diversity in a population and between different populations. The sum of all the allelic variants in specific loci in a population is the genetic diversity in that locus (Gardeström. J, 2008).

Mutation is one of the things that can affect the genetic diversity because it introduces new gene variants. Other things that affect the genetic diversity are migration, genetic drift and selection. Migration increase genetic diversity. Genetic drift decreases genetic diversity in a population but increase genetic diversity between populations because it is a random change in gene frequencies in every generation. In small populations the random changes in allele frequency are larger. When a population is adapted there is selection. Populations with greater genetic diversity have a higher change to adapt to changes in the environment and survive (Lowe. A, 2004).

Molecular genetics in the study of Blue mussels in the Baltic Sea

The use of allozyme markers was spread in the 1970´s and has developed molecular genetics. It has enriched the genetic method. A microsatellite is a genetic marker that is repeated sequences of DNA that is not coding (non-coding) and is regularly dispersed in the genome. The repeating of microsatellites makes them more sensitive to mutations because the polymerase that adds nucleotides during DNA replication can easy go wrong when the same sequence repeats many times (Liu J.Z, 2004).

Molecular markers have been developed to describe genetic polymorphisms in mussels. To analyze genetic parts and anthropogenic changes it is useful to study informative markers like microsatellites (J.L. Lo´ pez, A. 2002).

Mytilus edulis in Baltic Sea was first designated as *M. trossulus* in 1988 based on allozyme data and analyses. Microsatellites have become one of the most used genetic markers during the last years (Varvio S, 1988). Studies that have been done on Baltic blue mussel have mostly been done by using allozymes and (or) mitochondrial DNA sequences. The introgression of mtDNA genes and nuclear markers from *M. edulis* prove that the Baltic lineage is not just from *M. trossulus* (Gardeström J, 2008).

Mytilus complex

Different techniques have been used to study the genetic variation and differentiation in the *Mytilus edulis* complex (M. edulis, M. galloprovincialis, and M. trossulus) (Miguel A. Varela, 2007). Many of the recent genetic studies on the *Mytilus edulis* complex have used allozymes or mitochondrial DNA sequences (J.L. Lo´ pez, A. 2002).

The *Mytilus* complex (*M. edulis*, *M. galloprovincialis*, and *M. trossulus*) have been studied by genetic markers since 1970 when the *M. trossulus* was discovered. To identify individuals within the *Mutilus* complex morphologic characters are usually used.

M. edulis and *M. trossulus* are 2 species living in the northwest Atlantic Ocean. (Gosling E. M. 1992). These species have been studied and identified by allozyme electrophoresis which showed that the populations were spread in almost all areas (Penney, R. W, 1999). Many *M. edulis* i found in the southern part of Nova Scotia and in the Bay of Fundy (Mallet. A. L, 1999).

Proteins and fitness

How well an individual handles stress can depend on the plasticity of the organisms physiological response. The physiological response can be related to the heterozogosity in the individual. Different sets of genes can produce different sets of proteins which give them many possibilities of response. Individuals with many different proteins have more possibilities to respond to the environment. That gives them better chances to survive and be selected (Mitton J. B, 1993).

Stressful conditions in the environment can cause changes in proteins. Proteins of the proteome will either be made in larger quantities, or in lesser quantities as a response to stress. Proteins may also be damaged and defect because of stress. Protein changes can identify changes in the environmental state of an organism.

Proteins are important for all organisms partly because they are needed in growth and reproduction. The biological function of a protein in an organism is depending on their tertiary structure. The tertiary structure can lose their function by heat or chemicals in their environment (Olsson. B, 2004).

To understand proteins and their expression the proteome needs to be studied. The proteome is all the expressed proteins. There are proteomes for an organism and for a specific cell. The proteome is usually studied to give a picture of the phenotype expressed at the moment.

One cells proteome consists of thousands of proteins. The very complex proteomes of cells can be studied with the help of two dimensional gel electrophoresis (Gardeström J, 2008).

Two dimensional gel electrophoresis (2DE)

Proteome analysis includes measures the number of proteins and their presence. The proteomic study is used to characterize the network of cell regulation, which genomic DNA code or the mRNA that is expressed for the protein, gene product, can not do. To study which protein that has been expressed, how strong and if any posttranslational modifications are affected proteome analysis is performed. Two dimensional polyacrylamide gel electrophoresis is the only method that can do this analysis (J.L Lopez, 2006).

Two dimensional gel electrophoresis was first developed by O´Farrell in 1975. It is used to analyze and study protein expression. The method separates the proteins with isoelectric-focusing gel electrophoresis (IEF) and gel electrophoresis. The resulting protein patterns are then studied using computer programs (J.L. Lopez, 2002).

Isoelectric point, first demension

Proteins have acidic and basic groups and depending on their pH they become protonated or deprotanated. The sum of all positive and negative charges of a protein is that proteins net charge.

Proteins are first separated by pI, isoelectric point. This is at the pH where proteins don't have any net charge. The method is called isoelectric focusing, IEF. The charge of a protein depends on the ambient pH. At a specific pH, the proteins pI, the net charge is zero. This is usually in the pH range 3-12. Proteins have positive charge in solutions that have pH below their pI value. Proteins have negative charge in solution with pH over their pI.

IEF

Proteins are placed in a pH gradient and subjected to an electric field. The electric field makes the protein move towards the opposite charge. When the protein is uncharged it will stop the migration. On it's way the protein will either lose or pick up protons and the charge will decrease until the protein reaches the point where it's charge is zero, it's pI, and the ambient pH will be the equivalent. That stops the migration.

IPG strips

For best IEF result a stable pH gradient is important. IPG strips consist of pH gradients with acrylamido buffers that are derivatives of acrylamide and contains reactive double bonds and buffering groups. The acrylamido buffers are linked covalently in the gel and by using this method any gradient can be created

The largest strips are 17 cm and take longer time to run then those which are 11 cm and 7 cm, but give a more accurate result.

Power in IEF

The electrical conductivity in the gel changes as the IEF is running. It changes with time. In the beginning the current will be high because many charge carriers are present in the gel. The current will decrease while proteins move towards their pl.

pH gradient, strip length and electric field affect the sensitivity of the IEF run. In high voltage thin gels are better because they can dissipate heat more than thick gels.

The time that is needed depends on the programmed volt-hours on the pH range of the IPG strip. If there is more than one sort of strips running at the same time the electric condition will be individually.

Second dimension

During the second separation the proteins are separated by molecular mass, Mr or MW.

Second dimension gel grade the proteins after size depending on how dodecyl sulfate coat the protein. Gradient gels give proteins with wide range of protein mass to be analyzed. Gradient gels also decreases pore size in the gradient. Homogeneous gels is used for protein within a protein mass range.

The IPG strips are placed on the top of the agarose gel. The gel has to be molten so that the strips will get in close contact with it.

Staining

There are many stains. All stains interact differently depending on which protein it is. There are no stains that stain all protein in the gel proportionally to their mass. For better analysis the gels should be stained with different stains. Coomassie Blue-stain gel can be used with silver or a fluorescently stained gel with silver or colloidal Coommassie. It does not seem to make any differences in which order as long as gels are washed between the stains with high-purity water.

To stain the proteins in the polyacrylamide gel Coomassie Brilliant Blue R-250 is most common. It is sensitive and stain the protein linearily.

SYPRO Ruby Fluorescent Staining is a gel endpoint stain with background staining. It can stain glycoprotein, lipoproteins, ptoteins with low MW and metalloproteins that can not be stained enough by other stains. SYPRO Ruby does not stain nucleic acids.

There are two methods of silver staining for 2-D analysis. They have similar sensitivities for protein but have different chemistries. The proteins in the gel are first fixed with alcohol and acetic acid, and then they are oxidized in potassium dichromate in dilute nitric acid. Before they are treated with silver nitrate solution they are washed with water. The silver ions bind to the oxidized proteins and by treatment with alkaline formaldehyde the metallic silver will reduce. When the staining has been achieved the development of the color can be stopped with acetic acid. Because the oxidative step changes the protein mass this staining method is not used for mass spectroscopic analysis.

Silver Stain Plus stain has only one staining and development step. The proteins are fixed with methanol, glycerol and acetic acid and washed with water. The gels are then put in silveramine complex bound to colloidal tungstosilicic acid. By ions exchange the silver ions goes from the tungstolicic acid to the proteins in the gel. The silver ions are reduced to metallic silver by formaldehyde which makes the protein spots. The reaction can be stopped with acetic acid. This staining is usually used in proteomics when the proteins are identified by mass spectrometric analysis.

Computer analysis

Before 2-D gels can be analyzed with computer analysis it must be digitized. Usually camera system, densitometer, phosphor images or fluorescence scanner is used.

GS-800 calibrated imaging densitometer is made for analysis of gels. It gives good imaging to resolve close spots. The 2-D gel can be scanned with red, green, and blue color.

To digitize 2-D gels that is stained with fluorescent stain or radioactive compounds a specific imaging system needs to be used. Molecular Imager FX Pro Plus is a flexible instrument. Gels that have radiolabled protein can be seen better with Kodak phosphor screen. Proteomic fluorescent stains and SYPRO Ruby protein gel and SYPRO Orange protein gel can be imaged with single color. Multicolor fluorescence can be imaged directly by laser excitation.

Computer-Assisted Image Analysis of 2-D Electrophoretic Gels

In a computer program the spots can be analyzed between two different gels from every group. The protein spots are depending on molecular mass and isoelectric point.

Among other things computer-assisted image can save a lot of image data and rapidly analyze experimental information.

PDQuest software from Bio-Rad is commonly used. Gel analysis of digitized gel image includes spot detection, spot quantitation, gel comparison and statistical analysis.

Spot Detection and Spot Quantitation

Image data need to be corrected and the gel background handled before the software can detect the protein spots in the 2-D gel. The hit rate of the spot is depending on the quality of 2-D gels.

Comparison

Gel analysis software find small differences in the individual spot position within the gel series. PDQuest software compares the 2-D gel to a selected 2-D gel series as a reference. Proteins that are not in the references are added manually.

Landmarks and identical spots need to be manually identified before software can be used.

Data Analysis

With PDQuest software all the gels are seen as one unit in the experiment. References images of gels can be used to compare from different experiments. Each spot will have a number and identical spots have identical numbers (Bio-Rad).

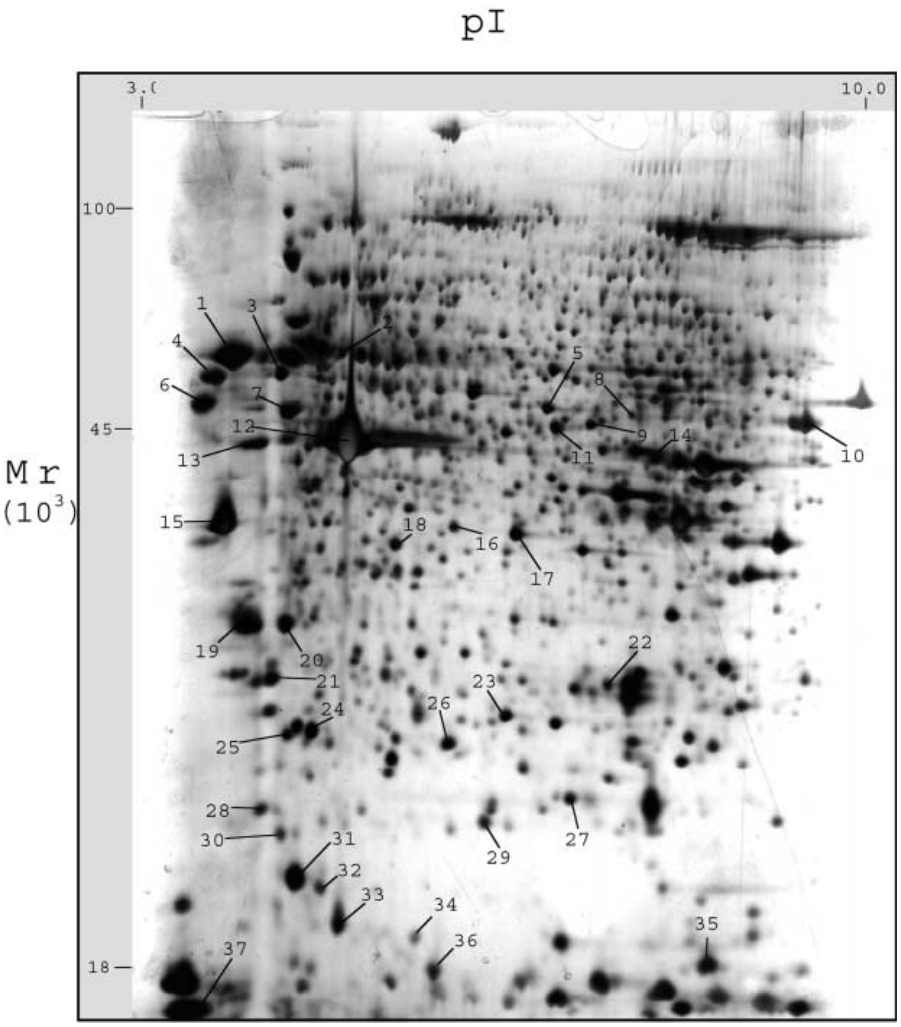

Fig 1. Figure showing a typical silver stained 2 dimensional gel. The acidic pl is on the left and the basic pl on the right. Low Mr is in the bottom and high Mr on the top (J.L. Lo´ pez, A, 2002).

Discussion

There are many environmental and biological factors that can affect the blue mussels distribution and abundance in a certain area. In general the salinity is the major factor that can affect the size and the distribution of the blue mussel. But in more local areas, where salinity does not differ, wave exposure is the most affecting factor on the blue mussel because it affects the food access, predation effects and accumulation of sediment (Westerbom. M, 2008).

The physiology of the blue mussel and its genetic diversity are depending on each other. The Baltic Sea is a stressful environmental because of it's brackish nature. Mussels from northern Baltic Proper have lower growth rate and are smaller than those in the south. Mussels from north have to spend more energy at dealing with osmotic stress and therefore have less resources available to the handling of additional stress (Prevodnik. A, 2007). The stressful environment probably have an impact on the mussels reproduction and growth which can reduce the population size and increase the impact of drift (Gardeström J, 2008; Westerbom M, 2008).

Maybe the most important reason for the smaller size of the blue mussel living in the Baltic Sea is the lower salinity. If a creature adapted to water with higher salinity, lives in water with lower salinity, a smaller size will make it easier to maintain the proper intracellular environment. A smaller mussel has a larger area/volume quota than a large one. This facilitates transport in the smaller mussel since a larger area supports a smaller volume. Although, salinity probably is the abiotic factor that affect blue mussels the most also biotic factors, like predators and other competing species, affect the blue mussels population structure (Westerbom M, 2008).

The known differences in physiological response and stress tolerance that have been shown between blue mussel from northern and southern populations, do not correspond to a genetic difference in their microsatellite loci (Gardeström J, 2008). This demonstrates the importance of combining the use of molecular markers like ISSR to analyze the genetic variability with physiological investigations in the Baltic blue mussel because the physiological response and genetic variation is not always comparable. Repeated sequences on the makers show high genetic variation in the *Mytilus* complex. In larger populations fixation and loss of allele caused by genetic drift is less than in smaller populations. Because of the high mutation frequency it is useful to use microsatellites as genetic makers (Miguel A, 2007). However, the populations in the Baltic could not be separated by this method.

Can the southern and northern Baltic blue mussels be still be different genetically? The observed difference in phenotype is caused either by phenotypic plasticity, that is different expressions of proteins, or to different genes being selected. These proteins or genes can be involved in physiological adaptations to the different concentrations of saline.

Phenotypic plasticity is when the same genotype produce different responses in different surroundings. One sort of phenotypic plasticity is different gene expression depending on which genes that are activated in different environments.

Differences in gene expression can be seen as different proteins being expressed under different stressful conditions. But it could also be because of genetic differences in the studied loci between the mussels in north and south. It has been found that there is genetic variation in a locus called Lap under high selected pressure in blue mussels in a salinity gradient. The different Lap alleles that where favored in high and low saline water have different effects on the mussels osmoregulation. It has been shown that there are differences in allele frequencies in the Lap locus between areas in the Baltic Proper that have different salinity in the same range as the northern and southern areas (Gardeström J, 2008).

By using 2-dimensional gel electrophoresis different gene expressions from the same genome can be detected and also post-translational modifications. And this quickly and rather simply.

The 2-dimensional gel electrophoresis separates the protein by their Isoelectric point and molecular mass which makes the identification useful. 2-dimensional electrophoresis is good method to identify proteins and their expression in different conditions, between species and cell types (J.L. Lo´ pez, A, 2002).

It has been shown that mussels adapt to changes in the environment result in different protein expression profiles (J.L. Lopez, E, 2006). A study using this method on Baltic blue mussels could possibly detect phenotypic differences between northern and southern populations. The phenotype depend on genotype and environmental differences.

Another way to study this problem could be by reciprocal transfer of mussels between northern and southern parts of the Baltic which result in genetic variation. If mussels adopt the physiological responses of their new environment then there probably are no genetic population differences. Whereas, if they retain their physiological responses typical of their site of origin there would be a reson to consider the populations genetically different.

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