

Deliverable 19: Implementation and Development of Genetic Markers

by

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Abstract

For redfish species identification, a total of six markers was elaborated; at least four microsatellites, a combination of eight AFLP primers or a total of six markers that can be used.

In addition, we have elaborated 16-24 markers that can be used to identify some of the species pairs. Those are 27 isozymes, two haemoglobins, one eye lens protein, 7-10 microsatellites, one sequencing 7S rDNA ITS, 1 RFLP 7S rDNA ITS, one SSCP cyt-b and one SSCP control region and ND3 gene sequencing.

For stock structure and stock origin analyses of *S. mentella* and *S. marinus*, at least 24 isozymes, 12 haemoglobin, 56 microsatellites, one combination of eight AFLP primers and one RAPD or a total 10-13 suitable markers was identified. However, for 45 isozyme loci, 47 microsatellite loci, one ND3 gene, one SSCP of cyt-b and one SSCP of the control region, so for a total of 12-14 loci, more samples need to be screened to determine their usefulness for identification of stock structure and origin. For population differentiation, future work will be concentrated on microsatellites, isozymes, haemoglobins (partly) and AFLPs.

1. Introduction

Cooperative research to reveal the species composition and the genetic stock structure of North Atlantic species of the Genus *Sebastes* has been ongoing for the past few years by Iceland, Norway and Canada. In the recent EU-REDFISH project, emphases have been laid on the deep-sea and oceanic types *S. mentella* in the Irminger Sea and adjacent waters as well as *S. marinus*, while the other species will be studied for comparison of species divergence. From the previous published and unpublished work by the participants of this project, some information on the species and stock discrimination has become available based on haemoglobin, isozyme and other protein as well as dinucleotide microsatellite markers. However, to obtain more complete information about the structure of the Genus *Sebastes* in the North Atlantic and to make use of recent development in molecular biology, a significant part of the sub-workpackage 1.3 has been to identify and implement new markers that have not been screened in redfish before. In the present report, the results of this work will be presented.

Consequently, during the first year of the project, analytical methods had to be developed and evaluated in respect of their suitability to differentiate species and stocks of North Atlantic redfish. A set of reference samples, hereafter called "CORE" samples, was obtained from A. K. Daniélsdóttir (partner 3) and T. Johansen (partner 5), and used to get first insight into the applicability of the new techniques before applying them to a large number of samples. The CORE samples consisted 12 individual samples of each of six species/main stocks of redfish (*Sebastes*). These samples were drawn from deep-sea and oceanic *S. mentella*, ordinary and giant *S. marinus*, *S. viviparus* and *S. fasciatus*, totally 72 specimens (Table 1). The CORE samples were analysed by all partners, and partner 1 in addition analysed *Helicolenus dactyloperus* as an out-group for comparison. The samples were screened for 35 isozyme loci, two haemoglobin loci, eye lens proteins, 12 DNA microsatellite loci, 107 AFLP loci, four DNA RAPD loci, two rDNA ribosomal introns, two rDNA ITS loci, one mtDNA ND3 locus, one mtDNA cyt-b locus and one mtDNA control region.

The methods used are mentioned in the body of the report. The preliminary results are not treated here in detail, because the low sample size for the CORE samples does not allow any statistically secure conclusions for population structuring.

Dendrograms were calculated from the data of isozyme, microsatellite, RAPD, ND3 gene sequence and AFLP analyses and showed that the species appeared at separate main branches and the stocks clustered together within the main branches. This was consistent with the genetic differentiation values (p and F_{ST}), and will be more closely dealt with in the annual report.

In the following, short descriptions are made for the markers identified for proteins, nuclear DNA and mitochondrial DNA separately.

Table 1: Redfish genetic "CORE" sample exchange 2000.

Survey (Nation)	Month	Area	Type of sample	N	Species
BTH1-1997 (IS)	May	Iceland	1)	12	<i>S. mentella</i>
TH1-1996 (IS)	Oct.	Irminger Sea	1)	12	<i>S. mentella (oceanic)</i>
BTH1-1997 (IS)	May	Iceland	1)	12	<i>S. marinus</i>
TJ1-1996 (IS)	Aug.	Reykjanes Ridge	1)	12	<i>S. marinus ("Giant")</i>
TJB1-1996 (IS)	Nov.	Iceland	1)	12	<i>S. viviparus</i>
FCAP- (NO)		Flemish Cap	Tissue EtOH	13	<i>S. fasciatus</i>
FCAP-2000 (E)	July	Flemish Cap	2)	15	<i>S. fasciatus</i>

1) Blood & muscle in EtOH (to partner 1). Frozen eye lenses (to partner 1). Frozen liver, muscle and red blood cells (to partner 5)
 2) Gills in EtOH (to partner 1). Frozen eye lenses (to partner 1). Frozen liver, muscle and red blood cells (to partners 3 and 5).

2. Isozymes and other proteins:

2.1 Isozymes. None of the isozyme loci could be used on their own to unambiguously and diagnostically differentiate between all four species, but for each species pair, there were 27 loci that could be used on their own. At least four loci for *S. mentella* and four for *S. marinus* could be used for stock differentiation. An extensive material will be analysed for species discrimination by starch (partner 5) and polyacrylamide (partner 3) gel electrophoresis for 27 isozyme loci for each species pair, totally 11 isozyme loci: ADH-1*, EST-2*, EST-4*, EST-5*, IDHP-1*(L), MEP-1*, MDH-1*, MDH-2*, MDH-3*, PEP-B* and PGM-2*. *S. mentella* stocks will be analysed for 4(9) isozyme loci: ADH-1*, (EST-2*, EST-4*, EST-5*), IDHP-1*(L), (MDH-3*), MEP-1*, (PEP-B*) and PGM-2*. *S. marinus* stocks will be analysed for 2-(6) isozyme loci: ADH-1*, (EST-2*, EST-4*, EST-5*), IDHP-1*(L) and (PEP-B*).

2.2 Haemoglobins. Haemoglobins are analysed by both agar gel electrophoresis and isoelectric focusing (IEF, partners 3 and 5). Haemoglobin patterns consist of multiple strong and weak bands in all *Sebastes* species, and at least two loci are involved. The electrophoretic mobility of the strong bands distinguishes clearly between *S. mentella* and the other species, and is also different for giant *S. marinus* compared to ordinary *S. marinus*. Thus this trait can be used to discriminate between some of the species pairs (*S. marinus* vs. *S. mentella*, *S. mentella* vs. *S. viviparus*, *S. mentella* vs. *S. fasciatus* and ordinary *S. marinus* vs. giant *S. marinus*), although not for all species taken together (some reservation has to be taken with respect to *S. fasciatus* and *S. mentella* in the Northwest Atlantic because previous analyses have revealed patterns which may be interpreted as hybrids in the Gulf of St. Lawrence, as well as intraspecies variation in Greenland waters are observed for *S. marinus*). The minor haemoglobin bands display both intra- and interspecies differentiation (clearest by IEF), but these observations have to be supported by further studies, focusing on repeatability, before their potentialities may be finally decided.

2.3 Isoelectric focusing of eye lens proteins (partner 1). Coomassie staining of IEF polyacrylamide gels revealed the presence of more than 30 bands for each individual sample. The protein patterns of *S. marinus* and *S. mentella* were identical, whereas *S. viviparus* could be diagnostically distinguished from the former two species (*S. fasciatus* not analysed). The eye lens proteins could not be used to discriminate between all four species, but could be used for all species pairs but *S. mentella* and *S. marinus*. IEF of eye lens proteins was not suited for differentiation of stocks of *S. marinus* and *S. mentella*.

3 Nuclear DNA

3.1 Microsatellites (partner 3): Microsatellite loci are analysed by polyacrylamide gel electrophoresis in an ABI377 sequencer. Seven dinucleotide, four tetranucleotide and one pentanucleotide loci were selected for screening of the

CORE samples and partly also a larger material. All these loci contain a high number of alleles, which partly are shared by two or more species, partly are private for one or another of the species. Of the microsatellite loci, four could be used on their own to identify statistically all four species, and for each species pair 710 loci could be used on their own, depending on which species pair. At least 5-6 loci could be used for stock differentiation. All four species can be identified by each of the following four loci: Seb9*, Seb31*, Seb46* and Sme5*. *S. mentella* stocks can be analysed for each of the following 6-(10) microsatellite loci: Seb9*, Seb25*, Seb31*, Seb45*, Seb46*, (Smar1*), (Sme5*), (Sme10*), Sal1* and (Sal3*). *S. marinus* stocks can be analysed by the following 5-(12) microsatellite loci: (Seb9*), Seb25*, Seb31*, (Seb33*), Seb37*, (Seb45*), (Seb46*), (Smar1*), Sme5*, (Sme10*), Sal1* and (Sal3*). Parentheses indicate loci, which need further analyses before final conclusion can be drawn.

3.2 Amplified fragment length polymorphism (AFLP, partner 1): The AFLP method is a relatively new molecular tool combining the reliability and reproducibility of RFLP (restriction fragment length polymorphism) analyses with the advantage of RAPD (randomly amplified polymorphic DNA) analyses of screening a high number of loci in a single PCR reaction (Liu *et al.* 1998). AFLP reactions and electrophoresis conditions were optimised for use in redfish according to a protocol by Trautner (1999) and eight selected primer combinations were tested for the CORE samples.

Out of the eight primer combinations 107 polymorphic sites could be scored. Polymorphic sites were chosen according to the 95% criterion. The eight primer combinations together could be used to differentiate between all four species, stock pairs and stocks of *S. marinus*. However, no species specific AFLP marker has been observed yet and the calculated differences between species are due to different allele frequencies.

AFLP analyses show high potential in discriminating stocks within redfish species due to the high number of polymorphic loci.

3.3 ITS and IGS introns (rDNA RFLP's and sequencing):

3.3.1 ITS intron of 7S rDNA (partner 1): A number of PCR systems, proposed for amplifying ITS introns, were tested in respect of amplicon production and occurrence of unspecific bands. The second intron of the 7S ribosomal protein gene yielded sufficient amount of amplicon to be used for sequencing and RFLP. Sequencing 551bp of the second intron of the S7 ribosomal protein gene of seven individual samples from each species and stocks gave indication of some species separation. The dendrogram of the sequences of the intron demonstrates that *S. mentella* is clearly separated from the other species, which on the other hand are closely related. More samples need to be analysed to test the usefulness of the sequencing for species and stock separation. For RFLP of the 7S ribosomal gene ITS intron, two enzymes, Rsa I and Tru 9I, were used. Six different fragment patterns were found. By means of RFLP, it was only possible to differentiate between *S. mentella* from the other three redfish species tested. Differentiation between *S. marinus* and *S. mentella* was possible by using only one of the enzymes, and six hybrids were identified. *S. viviparus* and *S. fasciatus* gave the same pattern as *S. marinus*, whereas *H. dactylopterus* gave several specific patterns. RFLP analysis of this intron will be continued by using more restriction enzymes until month 24, along with screening of samples.

3.3.2 ITS1 (between 18S and 5.8S rDNA, partner 5): Comparison of ITS1 460 bp sequences between the four species only resulted in minor differences between them. ITS1 showed only minor differences in sequences between *S. marinus* and *S. mentella* and will be omitted from further analysis.

3.3.3 ITS2 (between 5.8S and 28S rDNA, partner 5): Comparison of ITS2 460 bp sequences between the four species resulted in band length heterogeneity of individuals of all the species. The PCR product had to be cloned (TA-cloning kit) before being able to sequence the ITS2 region. The giant *S. marinus* and *S. mentella* showed one base pair each in difference from the other redfish species. ITS2 did not show species or stock structuring and will be omitted from further analysis.

3.3.4 IGS1&2 (partner 5): The Inter genic spacers (IGS) are located between the clusters of rDNA genes. It has been suggested that this area could include possible species separation areas for *S. mentella* and *S. fasciatus*. We would like to test this out by making new primers. Development of primers to identify IGS1&2 rDNA will continue until month 24 of the project along with the screening of stocks.

3.3.5 Haemoglobin (partner 5): Development of primers for identifying haemoglobin genes was tried, but proved to be difficult, probably because more than one gene seems to be present for haemoglobin. Development of primers will continue until month 24 of the project along with the screening of stocks.

3.3.6 RAP (partner 5): The PCR based random amplification of polymorphic DNA (RAPD) was based on 80 different 10 base-pair primers of three individuals of each of the four species. Only one primer out of four that were selected for use in routine analysis, OPA20, could be used to distinguish between species. Previous results show that Norwegian *S. marinus* clusters together with Giants and samples of *S. viviparus* from Iceland and Norway cluster together in a dendrogram. RAPD seems not to be a suitable marker to identify larvae of *S. marinus* and *S. mentella* as a combination of primers is needed for that purpose. RAPD gives indication of species structure, stock origin and stock structure of redfish. RAPD may be applied for studies on stock structure of *S. mentella*.

4. Mitochondrial DNA

4.1 Mitochondrial ND3 gene sequencing (partner 1):

The ND3 gene could be used to differentiate between all four species and species pairs, but more samples need to be screened to see if they are also useful for stock discrimination. New redfish specific ND3 primers were designed by cloning the ND3 gene of the four species into plasmid vectors. Eleven different haplotypes were identified: Three in *S. mentella* (one haplotype is appearing just in the oceanic sample, one just in the deep-sea sample and one is appearing in both), four in *S. marinus* (one haplotype only in ordinary *S. marinus*, two just in the giant *S. marinus* - off which one is found also in oceanic *S. mentella* - and one in both giant and ordinary *S. marinus*), three in *S. viviparus* and two in *S. fasciatus*.

4.2 SSCP & RFLP of mitochondrial DNA genes (partner 1):

Two methods were used for the screening of samples: PCR-SSCP (PCR based single strand conformation polymorphism) and PCR-RFLP.

4.2.1 Cytochrome b gene. The cytochrome b gene contains variable, as well as more conservative parts, which may be used for differentiation of fish on various phylogenetic levels. The whole cytb gene of *S. marinus*, *S. mentella* and *S. viviparus* has been amplified by PCR and sequenced to identify regions for screening of samples. The similarity index was larger than 99.5 % for *S. marinus* and *S. mentella*, and 98.4 % between the former species and *S. viviparus*. In general, the cytb gene is less variable than the control region. However, despite of the very low level of divergence of the DNA sequences, a region of the cytb gene could be identified and used for SSCP, located between position 283 and 410. A short (126/128 bp) fragment was amplified and analysed by SSCP. Three different patterns of ssDNA were obtained, tentatively designated as SSCP patterns for *S. mentella*, *S. marinus* and *S. viviparus*. All CORE and some other redfish samples were analysed by this SSCP technique. *S. fasciatus* showed a pattern nearly identical to that of *S. marinus*, therefore it was impossible to distinguish between these two species. The reliability of the SSCP was tested by sequencing the 126/128 bp fragment. The results of SSCP were confirmed by sequencing. Five variable positions were identified, No.: 3, 24, 48, 57 and 63. *S. marinus*, *S. viviparus* and *S. fasciatus* are characterised by a "T" in position 48, whereas *S. mentella* has a "C" in 48. From SSCP patterns, as well from the results of sequencing, the conclusion has to be drawn that the identification based on morphological characters of *S. marinus* and *S. mentella* often disagrees with the results of mtDNA cytb gene analysis.

Differentiation between the deep-sea and oceanic type of *S. mentella*, as well as between ordinary and giant type of *S. marinus*, was not possible. A region of the first part of the cytb gene was selected for PCR-SSCP analysis due to the variation of sequences of three of the species. The SSCP analysis of the various redfish species/stocks resulted in three different patterns: Pattern No. 1 was characteristic for deep-sea and oceanic *S. mentella* and giant *S. marinus*, No. 2 for ordinary *S. marinus* and *S. fasciatus* and No. 3 was found for *S. viviparus*. Variation within a set of samples from the same stock was observed but larger samples sizes need to be screened before concluding about its usefulness for stock discrimination.

RFLP analysis of the cytb gene: Due to the great similarity of the cytb gene sequences, RFLP is not suited in this case for differentiation of redfish species and stocks.

4.2.2 Mitochondrial control region: Sequencing of a fragment of about 530 bp showed that the region was characterised by a very high content of A and T (about 70 %). The similarity between species and stocks varied between 96 and 99%. The mitochondrial control region showed less variability than expected.

SSCP analysis of 187 bp of the mtDNA control region: Construction of primers for PCR-SSCP led to a PCR assay producing an amplicon of 187 bp, which gave six different patterns of single-stranded DNA. Sequencing of five specimens of each species or stock has been performed to test this approach for differentiation of redfish species. Further work on SSCP and sequencing is necessary for determining the usefulness for differentiation of redfish species and stocks.

RFLP analysis of the mtDNA control region (Tru 9 I, Rsa I and Nla III restriction sites): Due to the unbalanced base composition, only a few restriction endonucleases could be used for RFLP. Patterns of digestion fragments were obtained, but they were found to be unsuited for differentiation of redfish species and stocks.

Literature:

Liu, Z., A. Nichols, P. Li and R.A. Dunham, 1998. Inheritance and usefulness of AFLP markers in channel catfish (*Ictalurus punctatus*), blue catfish (*I. furcatus*), and their F1, F2, and backcross hybrids. *Mol-Gen-Genet.* 258(3):260-268.

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