National University of Ireland, Galway

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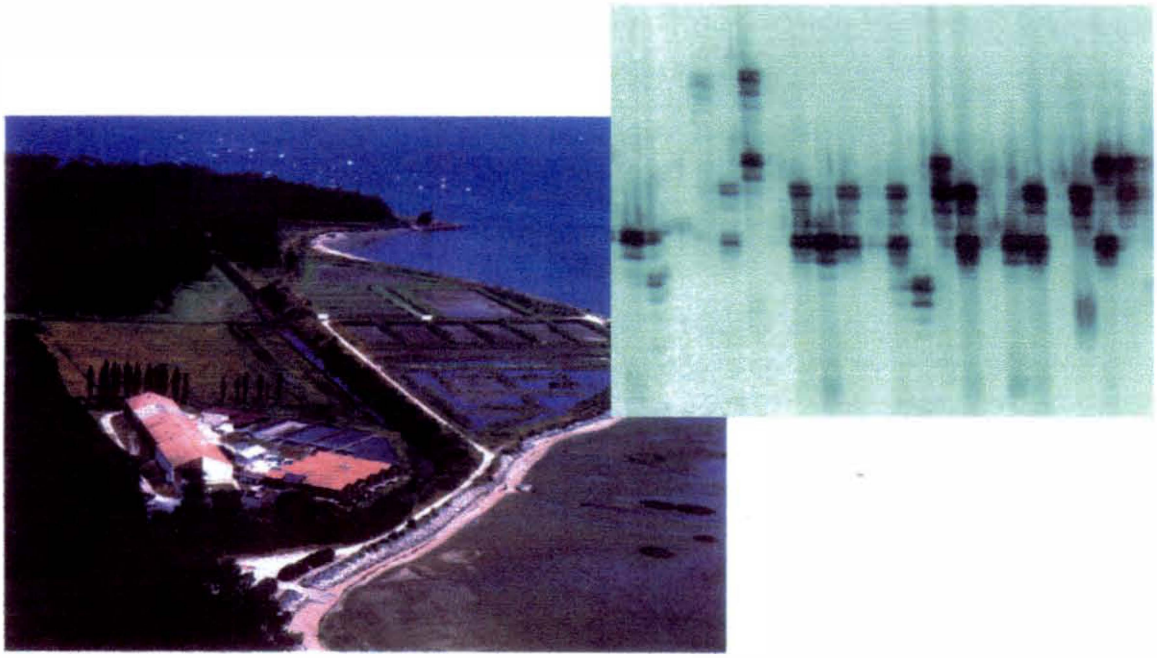
AquaTT report

Microsatellite Technique on Pacific Oyster: *Crassostrea gigas*



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IFREMER

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I. Introduction

The microsatellites markers are developed for somme years now (Tautz & Renz, 1984). The high degree of polymorphism displayed, the relatively even distribution in the genome, the ease with which they can be analysed by PCR method and the fact that the markers can be readily be adopted and used by others research group (PCR Primer Reaction), has made microsatellites the marker of choice in genetic linkage group studies of eukaryotic species.

Microsatellites were studied in different species in animals as well as in plants. For example many species of fish weres studied using microsatellites like *Salmo salar* (Slettan, 1993, 1997 ; O'Reilly, 1996), *Salmo trutta* (Estoup *et al*, 1993) and *Dicentrarchus labrax* (Garcia de Leon, 1995).

There are few microsatellites study on bivalves. Naciri (1995) published microsatellites results on flat oysters (*Ostrea edulis)*. McGoldrick (1997) identified 30 microsatellites in the Pacific oyster (*Crassostrea gigas*) but he has not used yet on oyster family. Three polymorphic loci in the Pacific oyster were published by Magoula (in press). Some study were done using 4 microsatellites to compare *C. gigas* and *C. angulata* (Huvet, 1997), microsatellites studies were used to evaluate the parental contribution in different cross in *C. gigas* (Boudry, in press ; Hervouet, 1998). These latter studies (Huvet, 1997 ; Boudry, in press ; Hervouet, 1998) were done in IFREMER La Tremblade Laboratory.

The aim of the training in IFREMER Laboratory in la Tremblade is to learn the microsatellites techniques on *Crassostrea gigas* . The microsatellites on *C. gigas* are used on routine now, there were developed in La Tremblade in co-operation with François Bonhomme from University of Montpellier (France). So four microsatellites markers were used and three others which has not been used in a population genetic study yet were tested. The individuals which were studied with microsatellites were also genotyped with allozymes in Galway (Ireland). These two techniques can be used on *Crassostrea gigas family* to built genetic map.

II. Presentation of the Host Laboratory : IFREMER -Ronce les Bains (17-France).

II.1 -Generality

IFREMER (The French Institute of Research for the Exploitation of the Sea) is a French government research agency, established in 1984 from the merging of CNEXO (Centre National pour l'Exploitation des Océans) and ISTPM (Institut Scientifique et Techniques des Pêches Maritimes). It is a public establishment, partly funded by the State carrying out academic, industrial and commercial activities involving 1700 engineers, researchers, technicians and administrators. Ifremer has 78 research laboratories distributed in 24 centres on the metropolitan coast and in the overseas territory (Figure 1). The IFREMER annual turnover is almost 1 billion FF.

II.2 -IFREMER Activities

The common denominator of the IFREMER activities in the sea. These activities can be categorised as follows :

1)The fundamental research carried out in wide-ranging fields such as geology, physical oceanography, biology, chemistry and all others disciplines related to the sea. Numerous exchange programs and work are carried out with outside laboratories

2)Engineering and Technology : The objectives of these activities are mainly the development for use in the scientific fields as well as the promotion of new technology for our industrial partners.

3) The monitoring of aquacultural and fisheries resources and the environment in order to protect, control and improve the quality of the marine environment.

4) The management and operation of our oceanographic ships and underwater equipment.

Figure 1 : map of the Ifremer centres.

II.2.1 - Station of La Tremblade

The station is composed of 2 areas : Mus-de-Loup and Ronce-Les-Bains. The station is specialised in oysters farming and observation of the coastal environment .
In this station, 3 laboratories existed :

*Coastal environment Laboratory.

*Regional Shellfish farming Laboratory of Poitou Charentes.

*Genetic -Pathology Laboratory : GPL (Figure 2).

Figure 2: Station of Ifremer Ronce les Bains.

II.2.1.1 - Genetic -Pathology Laboratory (GPL)

The Genetic and Pathology Laboratory was created in 1994. A twenty people scientific staff are currently working in the laboratory. Beyond the development of specific studies in the fields of genetic or pathology, particular means to fight against shellfish infectious diseases leaded to the association of both geneticians and pathologist in one team.
The two aims of the GP laboratory are the Genetics and Pathology of bivalves :

*Health of the breeding population

-The cell's mechanism of resistance against Bonamiose in flat oysters (*Ostrea edulis*).

-Pathology of the herpes virus in cupped oysters *Crassostrea gigas.*

-Marteliosia : a disease of flat oysters (*Ostrea edulis*).

-Marteliosia : a disease of flat oysters (*Ostrea edulis*).

-Bacteriological study linked to the private hatcheries

*Genetic improvement.

-Research and use of genetic markers in marine species.

-Conservation of different strains of Pacific oyster (*Crassostrea gigas*).

-Studies of wild populations of *Crassostrea gigas.*

-Selection of strains of flat oysters for Bonamia-resistance.

-Selection of cupped oysters *(Crassostrea gigas*) on physiological criteria.

-Obtaining and testing of cupped oysters (*Crassostrea gigas*) triploids and tetraploïds.

III. Materiel & Methods

III.1 Biology of *Crassostrea gigas*

Crassostrea gigas is the Pacific oyster named by Thunberg in 1795.

III.1.1 -Historic

Historically, the demand for oysters in Europe has been enormous. The abundance of oysters shell in pre-historic kitchen middens throughout Europe testifies that their popularity is neither a passing fad nor a recent phenomenon.

Roman patricians, holidaying at Lake Lucrinus near Naples had their oysters requirements met by Sergius Orata (circa 100 B.C). Drawings and inscriptions on glass funeral jars dating from the first century BC indicates that the ancient Romans availabled of this spat to commence the artificial cultivation of oysters (Wilkins,1989). In 1860, the artificial cultivation using spat of *Ostrea edulis* started using the Pr Coste techniques. In 1874, 1706 parcs existed in the Bay of Arcachon (France) and 500 parcs in the Gulf of Morbihan (France). Oysters propagation by the French artificial cultivation technique became immensely successful in Holland and Belgium but it failed entirely in Germany and Britain.

After *Ostrea edulis* , *Crassotrea angulata*, Portuguese oyster was a major species for the shellfish industry in Europe until the 1970's. It had been introduced from Portugal into France in 1868, when some oysters were left in the Gironde estuary by a ship. They spread along the French Atlantic coast and became the basis of a large coastal industry, producing up to 100.000 tones per years in the 1950's. From 1967 to 1972, these Portuguese started to become affected by some diseases. A large mortality occurred, leading to the near disappearance of Portuguese oysters from French coasts by 1973 (Grizel and Heral, 1991). Large-scale introduction of a replacement species, the Pacific oyster *Crassostrea gigas* was decided. *Crassostrea gigas* represented 86 % of the world-wide oysters production (FAO data, 1995). *Crassostrea gigas* is the major specie produced over the world (Figure 3)

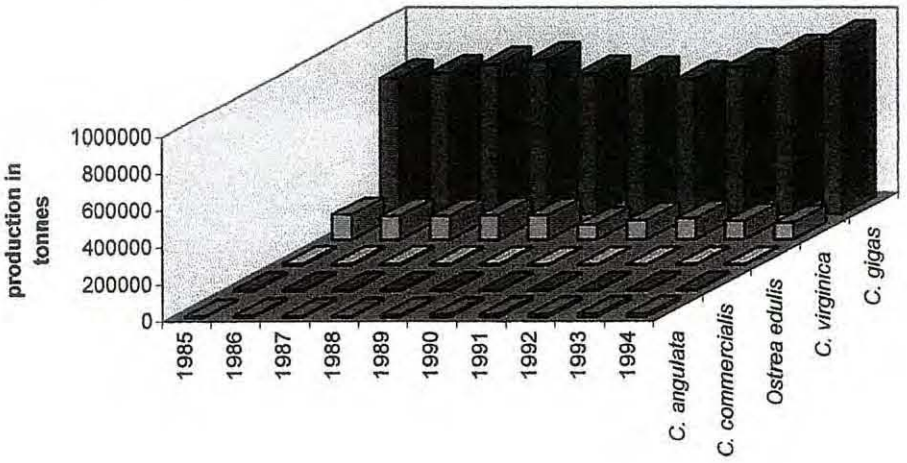

Figure 3 : Production in tonnes of different oysters species .

III.1.2 -Systematic

Crassostrea gigas belongs to the Mollusc Branch, Bivalves Class, Fillibranche Order, and Crassostreidae Family (Grassé, 1960) (Table 1)

Table 1:Systematic

Branch	MOLLUSC
Class	BIVALVIA /LAMELLIBRANCHIA
Order	FILLIBRANCHE
SubOrder	ANISOMYRIA
Super Family	OESTREIOIDEA
Family	CRASSOSTREIDAE
Genus	CRASSOSTREA
Species	*gigas*

III.1.3 -Geographic Repartition

Crassostrea gigas is represented in the Pacific Ocean, Okhostsk (Vladisvostock), Sakhalin Island, Japan, Korea, Taiwan. *Crassostrea gigas* was introduced in different part of the world for oyster farming, but reproduction is the wild was successful like in the Pacific coast of the North America, the Cap (South Africa), Tasmania (Australia), Europe, New Zealand (Smith, 1986). The different areas are represented in the figure 4.

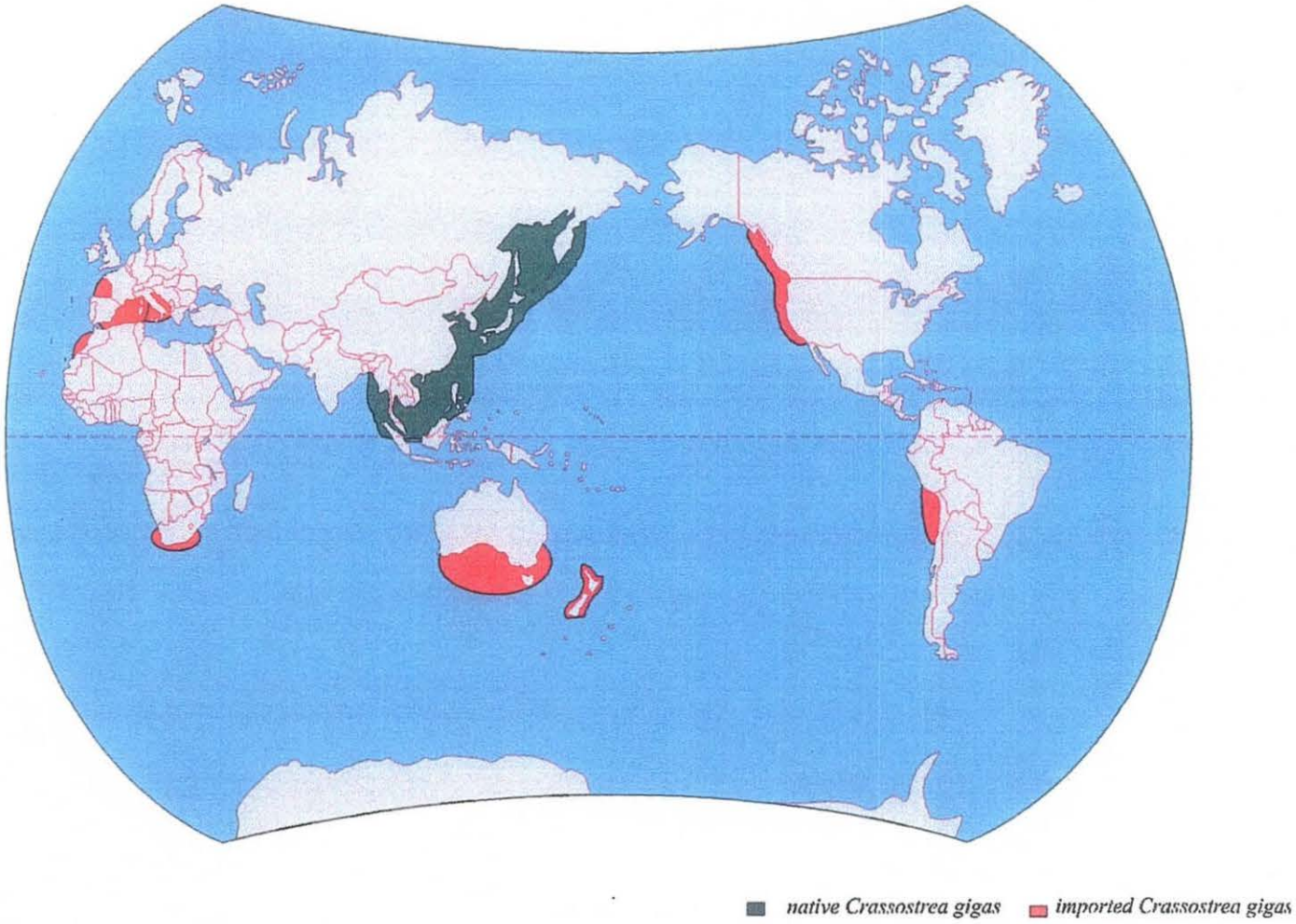

Figure 4 : Geographic repartition of *Crassostrea gigas*.

III.1.4 -Anatomy

This is a large oyster and it can attain a length of at least 12 inches (figure 5). The shape is very irregular and depends on the type of bottom on which it is grown, as well as the degree of crowding. The external surface may be either quite smooth or highly fluted. The upper flat right valve is smaller than the lower cupped left valve (Quayle, 1988).

Figure 5 : Anatomy of *Crassostrea gigas*

III.1.5 -Reproduction and life cycle.

The sexes in *Crassostrea gigas* are separate. There are male and female but some hermaphrodites occur occasionally. The sex of individuals changes from year to year, the change taking place in winter. The environmental conditions (temperature, salinity...) have a considerable influence on the determination of sex in oysters (Heral, 1986) .

Gamete production and maturation occur during the spring time. When the gametes are mature, the genitors spawn the gametes into the sea where they meet each other at random. The numbers of eggs and sperm produced in a Pacific oyster is related to the size of the oyster and to its state of nutrition. The numbers of eggs produced by an average market size oyster has been estimated at 20 to 100 millions (Marteil, 1976) ; the numbers of sperm is very much greater.

III.1.5.1 -Larval development

Immediately after fertilisation which must occur within 10-15 hours after spawning , rapid cell divisions and development take place. In about 24 hours, two tiny shells may be seen. Later a swimming organ called the velum is formed. The larva is now said to be in the straight-hinged stage (Figure 6) . The size is about 70µ.

Figure 6 :The straight-hinged larva of *Crassostrea gigas*.

At 10 days, when the size is approximately 150µ, a hook appears, called the Umbo. (Figure 7).

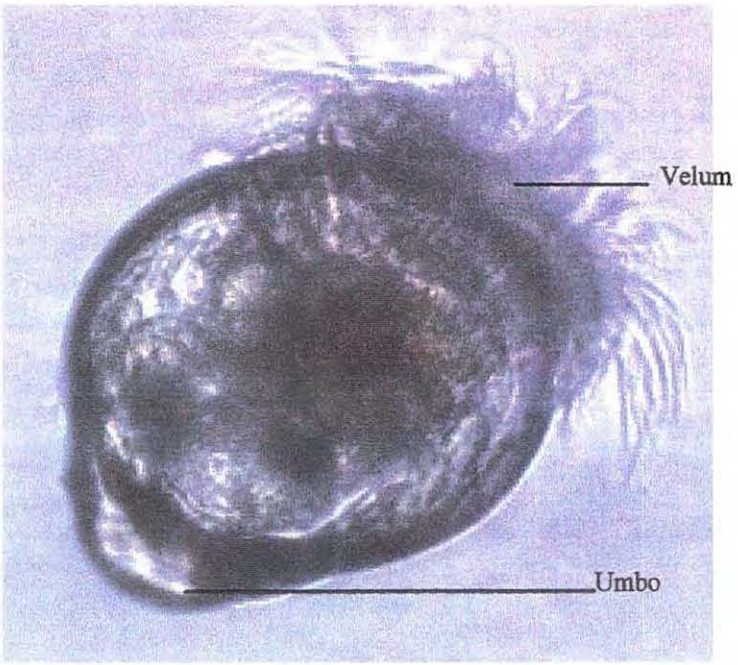

Figure 7: A 10 days larva.

When the young oysters reaches a length of 0.30mm, they are ready to become attached and the process is called setting or spatting. The larva is carried along by the current with the velum active and the foot protuded until it strikes a solid object. When a right spot is found, a rocking motion of the shells forces out a small drop of cement from the gland in the foot. After the foot and the velum disappear

III.2 - Molecular techniques : Microsatellites

III.2.1 -Generality

Microsatellites is a tandem repeat motifs of 1 to 5 bases pairs. These tandemly repeated blocks of DNA are dispersed throughout the genome of most, if not all, eukaryotic organism (O'Reilly and Wright, 1995).

Microsatellites locus shows a large polymorphism due to the numbers of repetitions of the pattern (Litt & Luty, 1989 ; Weber and May, 1989). The repeated sequences are usually between sequences which are little variable called flanking region (figure 8). The definition of primers in this flanking region can amplify specifically a microsatellites sequence by PCR (Polymerase Chain Reaction, Saiki et al, 1998). The size relatively low of amplified fragments allows a good separation of the amplified fragments.

The microsatellites are characterised by a high mutation rate which give new alleles. Mechanisms of mutation are likely linked to unequal crossing over (Brook et al ,1992) or are due of the polymerase slipping replication., following by no reparation (slipped strand mispaing ou SSM ; Levinson & Gutman, 1987 ; Strand & al, 1994). All theses mechanisms are not really known.

The microsatellites are very variable, codominant and can be studied quickly with PCR on routine. So they are useful for genetic study.

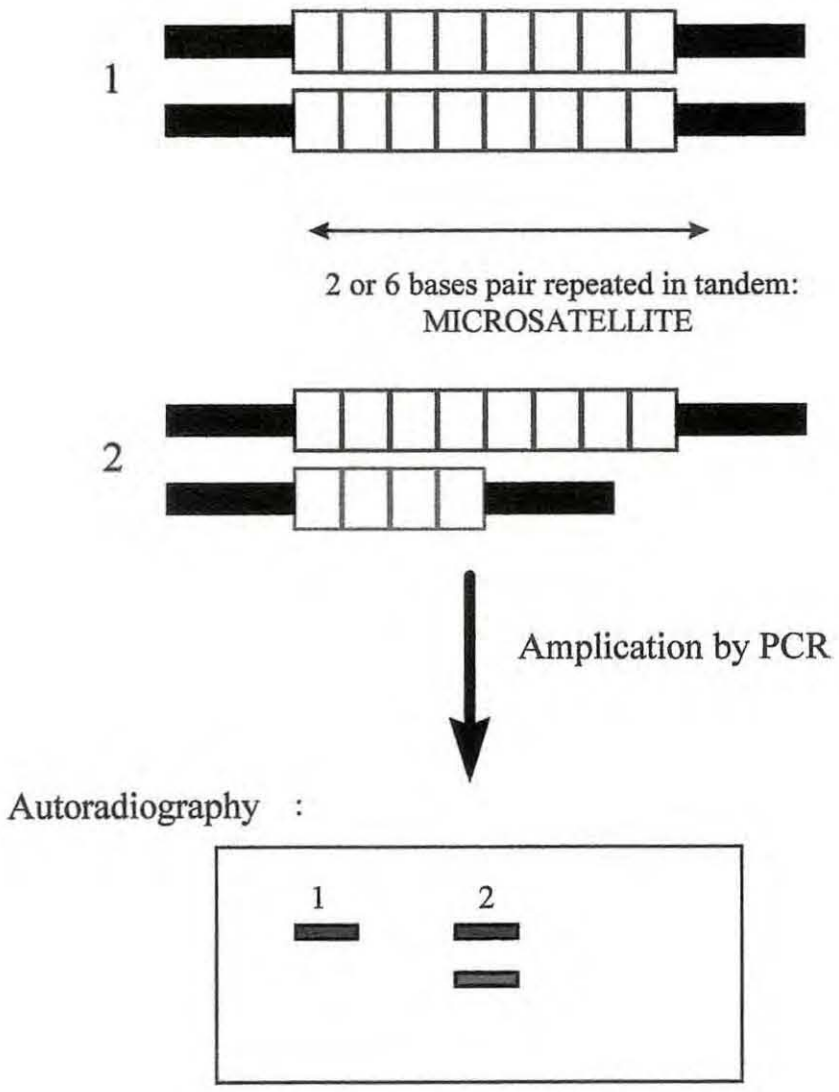

Figure 8: schematic description of microsatellites sequence (modified from Sophie Launey) ; « FR : Flanking Region ».

III.2.2 -Techniques

III.2.2.1 - Extraction

Gills of *Crassostrea gigas* were taken from individuals of 18 month years old.

DNA extraction from gill oysters using the phenol-chloroform techniques :

* In 1.5 ml eppendorf tube put a piece of gill mix with 400 µl of extraction buffer and 5 µl of Proteinase K.

Extraction Buffer :
-10mM Tris HCl pH 8.0
-0.1 M EDTA pH 8.0
-0.5 % SDS

*Incubate at 50°C overnight in a double boiler .

*Add 500 µl Phenol and 500 µl SEVAQ (chloroform + isoamylalcohol).

*Mix gently.

*Centrifuge for 10 minutes at 10 000 rpm at 4°C.

*Remove the upper layer and put in 400 µl of 100% ethanol -20°C.

*Mix 1H at -20°C or -80°C.

*Centrifuge for 10 minutes at 10 000 rpm.

*Wash with 7O% Ethanol -20°C.

*Centrifuge 10 minutes.

*Dry by opening the eppendorf for 4 hours.

*Dissolve the DNA pellet with 150 µl or 300 µl of TE .

III.2.2.2 -PCR : Polymerase chain reaction.

The most widely used method for the enzymatic amplification of specific sequences of DNA has been the polymerase chain reaction. The PCR method permits the selective amplification by a factor of 10^6 or more, of a specific segments of DNA in a complex mixture of sequences. The specificity is derived from information about the nucleotide sequence at the boundaries of

the segment to be amplified. With this information, pairs of oligonucleotide primers are constructed, each with sequence complementary with one of the ends. The target DNA is combined with huge molar excess of these primers, all four desoxynucleoside triphosphates, and a heat-resistant DNA polymerase (*Taq*) from *Thermus aquaticus*. This mixture is heated to denature the target DNA than cooled to allow the primers to hybridise (anneal) with the complementary sequences. The DNA polymerase synthesises new DNA from the primer/template complexes formed during the annealing step. The primers hybridise to the opposite strands of the target sequence and are oriented so that both newly synthesised. Strands extend across the interval to be amplified. New synthesised DNA, when denatured, also functions as template for subsequent DNA synthesis. Consequently, each iteration of the denaturation, annealing and extension steps, in theory, doubles the amount of DNA that lies between the primers, affording a exponential accumulation of DNA (Shattuck-Eidens *et al*, 1992).

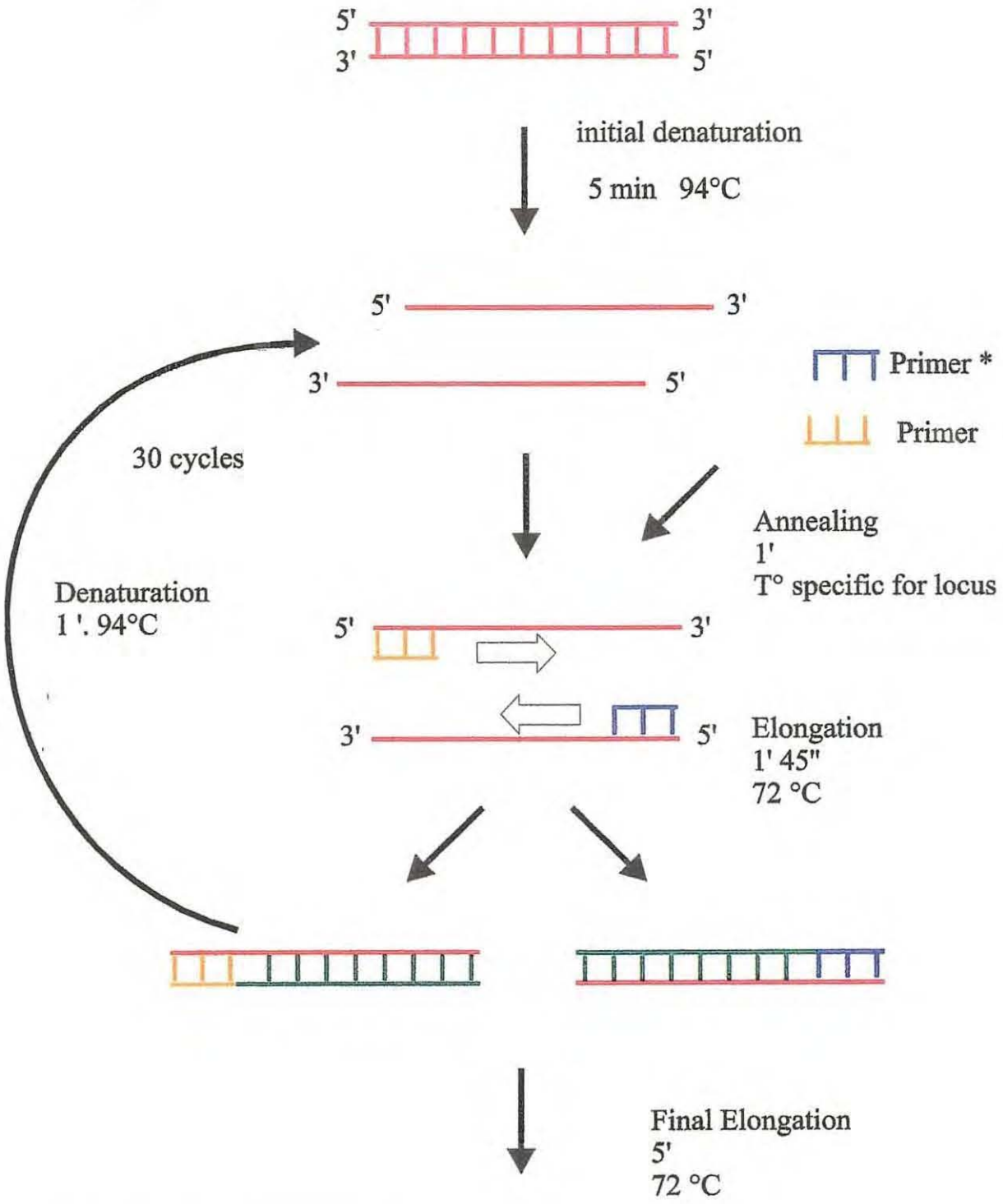

Figure 9 : Schematic representation of PCR

Radioactive PCR amplifications were carried out in 15µl of a mixture containing 4 µl of DNA, 1,5 µl of TAQ buffer 10X, 1.5 µl of dNTPs, 0.21 µl of R primers, 1µl of F- primer labelled with radioactive isotope, 1.8 µl or 1.2µl or 0.6µl of MgCL2 according to the microsatellites markers (Annexe 1), 0.05 µl of Taq and qsq H2O 15µl. Radioactive labelling is made with γ33P and carried out in a mixture of 1µl containing 0.23µl of F-primer, 0.1µl of kinase buffer 10X, 0.06µl of kinase, 0.1µl of γ33P and 0.51 µl of H2O. This mix is heated up to 37°C and added to PCR mix.

Table 2: Microsatellites markers (MT : melting temperature). The 3 microsatellites : CG 44, CG 49, CG 108 (Magoulas, in press)

LOCUS (code genbank)	Repeated Sequence	Primers	MT	Size of cloned fragment
CG 44 (Y12085)	$(CA)_{13}$	S : GAA GAA TGT CAT AGA TTG ATG G AS : CAT GCC TGT TTA CCA GTA TTC	53°C	391 bp
CG 49 (Y12086)	$(CA)_{35}$	S : CAT CAG GGG TAA ATT AAA GTA AGC AS : CCA CAG ACG ATT TCA TAT ATC CTG	53°C	194bp
CG 108 (Y12087)	$(CA)_{18}$	S : ATA TGT AAT GAT TAC GAA ACT AS : GTA TGA GAT TTG GTT CCA C	55°C	228bp
L10	$(AG)_{26}$	confidential	55°C	330bp

The PCR engine used is a PTC-100^{TM} Programmable Thermal Controler from MJ Research, INC (Figure 10).After a denaturing step of 2 min at 94°C, samples were processed through 30 cycles consisting of 1 min at 94°C, 1 min at an optimal annealing temperature and 1 min 15 sec at 72°C. The last elongation step was lengthened to 5 min.

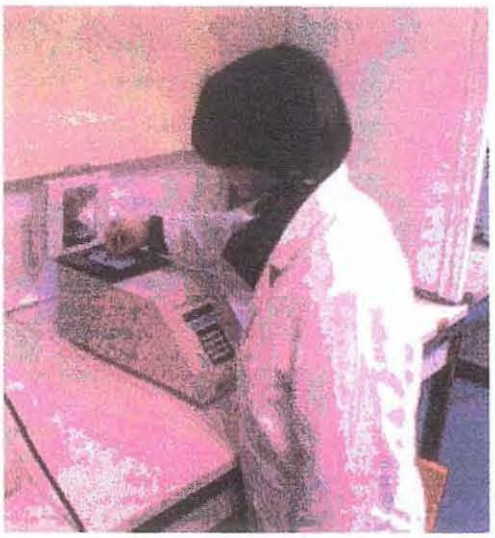

Figure 10 : PCR system

Then, 8µl of loading buffer (25 ml formatted, 0.025g xylem cyan, 0.025 g bromophenol blue and 1 ml EDTA 0.5M) was added in each PCR product. The mixture was heated for 5 min at 94°C .

III.2.2.3 -Gels

Aliquots of PCR products of 3.6µl were electrophoresed on 8% denaturing polyacrylamide gels (Figure). Gels were run for 2-5 hours at 50W (voltage and intensity were set non-restrictive conditions, respectively 1900V and 150 mA), dried and exposed to film for 6-48 hr (Figure 11)

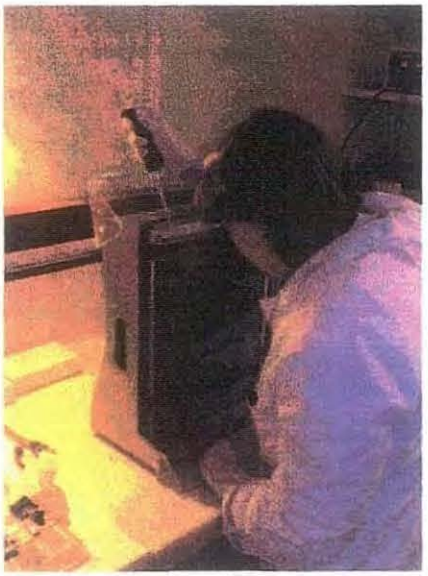

Figure 11 : Aliquots of PCR products were electrophoresed on 8 % denaturing polyacrylamide gels.

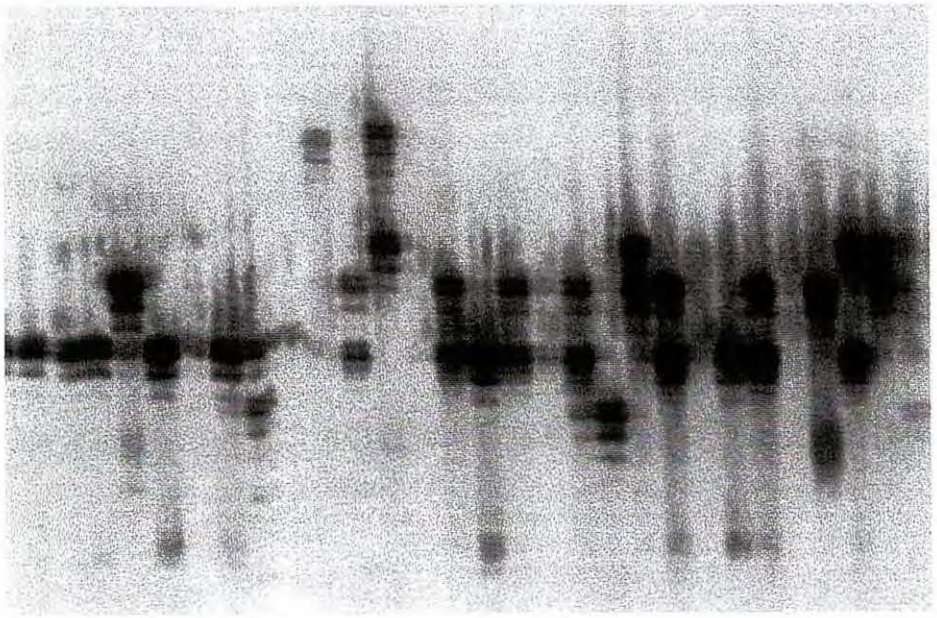

Figure 12 : Autoradiography of the CG44 locus

IV. Results

One hundred individuals of Crassostrea gigas from a full sib family were analysed for 7 microsatellites loci. Only the loci 10S/AS, CG44, CG108 and CG 49 gave some results which can be tested. Three others loci which are not used on routine L8, L16, L48 were used to know if they can be used in family study. Only L16 seems to give readible results, the L8 and L48 need to be tested with other PCR conditions.

Table 3: Genotypes frequencies and the Mendelian segregation probability.
P= the probability of the χ 2 test of the observed genotypes versus expected genotypes under a Mendelian segregation.

10S/AS	Number	Frequency	P
21/30	25	0.316	0
24/30	39	0.494	
21/37	10	0.127	
24/37	5	0.063	
Total	79		
49	**Number**	**Frequency**	**P**
02/04	16	0.552	0.3616
02/02	4	0.138	
04/04	9	0.31	
Total	29		
108	**Number**	**Frequency**	**P**
01/04	21	0.5	0.0109
01/05	6	0.015	
04/05	13	0.325	
01/01	8	0.19	
Total	48		
44	**Number**	**Frequency**	**P**
03/04	3	0.136	0.0305
01/02	6	0.273	
02/03	11	0.05	
01/04	2	0.091	
Total	22		

Some difficulties occurred during the training. Firstly, different biochemical had to be tested to find why we could not get good gels. It took a long time before we found that it was the

primers sent by EUROGENETEC which was faultly. Morever, the radioactivity was not available for 3 weeks so we had to waited before doing PCR and gels.
To sum up, many reactions had to be done before getting autoradiography "readible". So many mistakes can be done, so time is necessary to learn the techniques and to use it properly.

Anyway, four loci were used. Only some individuals were genotypes for the 44 locus because it was the last done, and the migration are longer that the other loci (5 h vs 2.30h) , so only one gel can be done per day.

V. Conclusion

Using microsatellites techniques require some training. But this technique is very useful in population genetics and it is often use in genetic study. The microsatellites and allozymes technique can allowed to build genetic map because they can produced many polymorphic markers. So the next step will be to create a F2 of *Crassostrea gigas* and to use the both technique to built genetic map.

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ANNEXES

I-Buffer

TBE X10
*162 g TRIS
*27.5g Boric Acid
*50 ml EDTA

Acrylamide solution 6% :
*150 ml Acrylogel
*100ml TBE X10
*420 g Urea
*qsq 1000ml H_2O

II-Microsatellites

The reaction are for one individual.

***CG49**

Marquage Mix		MixPCR	
Primer F	.23µl	Taq buffer10X	1.5µl
Kinase Buffer 10X	0.1µl	$MgCl_2$	1.2µl
Kinase	0.06µl	dNTPs	1.5µl
33P	0.1µl	Primer R	0.21 µl
H_2O	0.51µl	Polymerase Taq	0.05µl
Total	1 µl	H_2O	5.54µl
		DNA	4µl
		Marquage mix	1µl
		Total	15µl

*CG108

Marquage Mix		MixPCR	
Primer F	.23µl	Taq buffer10X	1.5µl
Kinase Buffer 10X	0.1µl	MgCl2	0.6µl
Kinase	0.06µl	dNTPs	1.5µl
33P	0.1µl	Primer R	0.21 µl
H_2O	0.51µl	Polymerase Taq	0.05µl
Total	1 µl	H_2O	6.14µl
		DNA	4µl
		Marquage mix	1µl
		Total	15µl

*CG44

Marquage Mix		MixPCR	
Primer F	.23µl	Taq buffer10X	1.5µl
Kinase Buffer 10X	0.1µl	MgCl2	1.8µl
Kinase	0.06µl	dNTPs	1.5µl
33P	0.1µl	Primer R	0.21 µl
H_2O	0.51µl	Polymerase Taq	0.05µl
Total	1 µl	H_2O	4.94µl
		DNA	4µl
		Marquage mix	1µl
		Total	15µl

***L10**

Marquage Mix		MixPCR	
Primer F	0.27µl	Taq buffer10X	1µl
Kinase Buffer 10X	0.14µl	MgCl2	0.4µl
Kinase	0.07µl	dNTPs	0.37µl
33P	0.68µl	Primer R	0.4 µl
H_2O	0.20µl	Polymerase Taq	0.07µl
Total	1.35 µl	H_2O	5.61µl
		DNA	2µl
		Marquage mix	1.35µl
		Total	15µl

***L8**

Marquage Mix		MixPCR	
Primer F	0.27µl	Taq buffer10X	1µl
Kinase Buffer 10X	0.14µl	MgCl2	0.6µl
Kinase	0.07µl	dNTPs	0.37µl
33P	0.68µl	Primer R	0.4 µl
H_2O	0.20µl	Polymerase Taq	0.07µl
Total	1.35 µl	H_2O	5.41µl
		DNA	2µl
		Marquage mix	1.35µl
		Total	15µl

Annualing Temperature : 51°C

*L16

Marquage Mix		MixPCR	
Primer F	0.27µl	Taq buffer10X	1µl
Kinase Buffer 10X	0.14µl	MgCl2	0.5µl
Kinase	0.07µl	dNTPs	0.37µl
33P	0.68µl	Primer R	0.4 µl
H_2O	0.20µl	Polymerase Taq	0.07µl
Total	1.35 µl	H_2O	5.51µl
		DNA	2µl
		Marquage mix	1.35µl
		Total	15µl

Annualing Temperature : 55°C

*L48

Marquage Mix		MixPCR	
Primer F	0.27µl	Taq buffer10X	1µl
Kinase Buffer 10X	0.14µl	MgCl2	0.6µl
Kinase	0.07µl	dNTPs	0.37µl
33P	0.68µl	Primer R	0.4 µl
H_2O	0.20µl	Polymerase Taq	0.07µl
Total	1.35 µl	H_2O	5.41µl
		DNA	2µl
		Marquage mix	1.35µl
		Total	15µl

Annualing Temperature : 53°C