More free publications from Archimer

ICES STATUTORY MEETING 1998

Theme Session K
Use of Genetics in Aquaculture
CM 1998/K:7

The use of microsatellite markers for parentage analysis in the Pacific cupped oyster, Crassostrea gigas (Thunberg).

by

Boudry P.[1], Collet B.[1], Kotoulas G.[2], Magoulas A.[2], Hervouet V.[1], Bonhomme F.[3], Gérard A.[1]

[1] IFREMER, BP133, 17390 La Tremblade, FRANCE
[2] IBMC, P.O. Box 2214, 71003 Iraklion, GREECE
[3] LGP, 1 quai de la Daurade, 34200 Sète, FRANCE

Abstract: Highly polymorphic genetic markers can be useful tools for the analysis of parental contributions. The present paper reports 3 experiments using microsatellite markers to analyse parental contributions in controlled *in vitro* factorial crosses of the cupped oyster *Crassostrea gigas*. Parentage analysis was eased by the large polymorphism observed at the 3 loci studied. In a cross between 20 males and 20 females, a quarter of the progeny could be assigned to a given parent using a single microsatellite locus showing 28 alleles. In 3 crosses between 5 males and females, all the progeny could be assigned to a family by using 3 loci. In all cases, the combined genotyping at 2 loci allowed parentage to be determined unambiguously. Despite the balanced gametic contribution of each parent before fertilisation, unbalanced parental contributions are observed in the progeny, both at larval and juvenile stages. Evidence of gametic and zygotic competition is given to explain the observed unbalanced parental contributions.

Keywords: parental contributions, microsatellites, *Crassostrea gigas*.

Résumé: Les marqueurs génétiques très polymorphes sont des outils très utiles pour l'analyse des contributions parentales. Notre étude présente 3 expériences utilisant les marqueurs microsatellites pour l'analyse des contribution parentales dans des croisement contrôlés *in vitro* chez l'huître creuse *Crassostrea gigas*. L'analyse de parenté a été facilitée par le grand polymorphisme observé aux 4 locus étudiés. Dans un croisement entre 20 mâles et 20 femelles, le père ou la mère d'un quart des descendants ont pu être déterminés en utilisant un seul locus microsatellite présentant 28 allèles. Dans 3 croisements entre 5 mâles et 5 femelles, tous les descendants ont pu être assignés à une famille en utilisant 3 locus. Dans tous les cas, la combinaison des génotypes à 2 locus a permis l'identification des parents. Malgré des contributions gamétiques équilibrées entre parents avant la fécondation, des contributions parentales déséquilibrées sont observées dans les descendants, aux stades larvaires et juvéniles. Nous démontrons des effets de compétition gamétique et zygotique ce qui permet de comprendre les déséquilibres des contributions parentales observées.

Mots-clés: contributions parentales, microsatellites, *Crassostrea gigas*.

Introduction

Very high fecundity is a characteristic of many aquacultural species. It is therefore common practice for hatcheries to produce large amounts of offspring from a limited number of parents. The genetic variability present in these offspring is of course directly related to the number of parents used, but can be also reduced by unbalanced contributions of each parent, leading to very small effective population sizes. The genetic consequences of such practices are of concern, especially if some of these offspring are to be used as parents for the next generation. Inbreeding is likely to occur, leading to a decrease in performance (Kincaid, 1976; Bierne et al., 1998). Furthermore, a reduced genetic variability limits the possibilities of genetic improvement by selective breeding.

Individual tagging is impossible at early stages of life, so genetic management of stocks in aquaculture is commonly based on families, reared in separate structures. This practice can lead to environmental heterogeneity among families however and therefore bias genetic analyses. As replication is costly, alternative methods, based on DNA markers, have been proposed as a tool for pedigree identification of mass-reared aquacultural populations (Harris et al. 1991; Herbinger *et al.*, 1996a; Garcia de Leon *et al.* 1998) or for studies in the wild (Ferguson *et al*, 1995; Kellog *et al.*, 1995; Colbourne *et al.*, 1996; Herbinger *et al.*, 1996b).

In the wild, the effective size of a population (N_e) is almost always smaller than the actual number of individuals due to many reasons including overlapping generations, non random mating, unbalanced sex ratio and selection Hedgecock, 1994). One possible approach for the study of some of these factors is the examination of parental contributions from one generation to another, in wild or experimental populations.

In this paper, we report some results concerning the analysis of parental contribution using microsatellite markers. The results of 3 different experiments are presented and some possible causes of the observed unbalanced parental contributions are identified.

Material and Methods

Parental oysters

Individuals were sampled from the French Atlantic coast where *Crassostrea gigas* was introduced in the early 1970s. For the first and the second experiment, mature oysters were chosen at random and crossed (see below). For each parent a fragment of gill was preserved in 100% ethanol for further DNA analyses. For the third experiment, 100 oysters were first analysed at 3 microsatellite loci (see below) and gill fragments were collected by biopsy. After the conditioning period, 10 individuals (5 males and 5 females) were selected in such a way that each chosen parent was heterozygote and carried alleles different from those of the other parents. This enabled the unambiguous identification of the 20 alleles present in the progeny in order to trace parentage, with no risk of null alleles.

Crosses

For each experiment, spermatozoids of each male were individually collected by stripping the gonad. The sperm was diluted with 1 µm filtered sea water, its concentration was estimated using Thoma slides coupled to an image processing system (Alcatel), and stored at 4 °C. Oocytes of each female were then collected using the same procedure and numbered using Malassez slides. For each female, oocytes were distributed in each fertilisation beaker

and fertilised separately by each male at a ratio of 100 spermatozoa per oocyte. Three different type of crosses were performed by in vitro fertilisation:

- First experiment: 20 males crossed with 20 females with separate fertilisation (20 x 20 = 400 separate crosses mixed together after 3h),
- Second experiment: 3 sets of 5 males and 5 females (3 sets of 5 x 5 = 25 separate crosses mixed together after 3h)
- Third experiment: 5 males and 5 females with two different types of crosses:
  - 5 males x 5 females = 25 separate crosses mixed together after 3h,
  - The gametes of the 5 males and the 5 females were first mixed within each gender and then mixed together.

Early embryonic development was examined using nuclear DNA staining with an epifluoresence microscope, as described in Gérard *et al.* (1994).

Larval rearing

Larvae were reared in GRP (Glass Reinforced Polyester) tanks filled with 1 µm filtered sea water (temperature 23 °C, salinity 28-30 ‰) and were fed *Isochrysis galbana* and *Extubocellulus criberiger*. The total amount of algae provided was 60 cells/µl. This concentration was in excess to avoid competition for food between larvae and therefore to maximise phenotypic variability. Every 48 hours, the larvae were collected by sieving and a sample was counted (Cell counter) and measured (Profile projector, Nikon). Density was progressively reduced by discarding a part of the population without any selective sieving.

Settlement

In the first experiment, Successive sieving operations were performed in order to separate "sieving groups". When the first pediveligera larvae appeared, the largest larvae (size greater than 280 µm) were retained by sieving and left to settle. The remaining larval population was kept in the larval rearing tanks. Successive sieving operations were performed in the same way. Each time, the largest individuals were transferred to the settlement raceways and the smaller individuals were returned to the larval rearing tanks. PVC boards were used as collectors. After settlement, the collectors were maintained vertically in 800 l tanks with *Skeletonema costatum* enriched sea water.

In the second and the third experiments, larvae were settled on cultch (Walne, 1974).

DNA extraction

Samples were preserved in 100 % ethanol. For large sized oysters, DNA extractions were performed by Phenol/Chloroform method. For larvae, because of the small amounts of tissue that were available, a Chelex-based extraction method was used (Bierne et al., 1998).

Microsatellite analysis

Four microsatellite loci were used to assess parentage. Three of these markers (Cg108, Cg49 and Cg44) are described by Magoulas et al. (in press). The forth one (Di10Cg) was developed more recently in the Laboratoire Génome et Populations (UPR 9060 CNRS, Université de Montpellier II, France). All have a dinucleotidic pattern.

Radioactive PCR amplifications were carried out in 15µl of a mixture containing 4µl of DNA, 1.5µl of TAQ buffer 10X, 1.5µl of dNTPs, 0.21µl of R-primer, 1µl of F-primer labelled with radioactive isotope, 1.8µl 1.2µl or 0.6µl of $Mgcl_2$ according to microsatellite

marker, 0.05μl of TAQ and qsp H_2O 15μl. Radioactive labelling was 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 0.51μl of H_2O. This mix was heated up to 37°C for 30 minutes and added to the PCR mix.

After a denaturing step of 2 min at 94°, samples were processed through 30 cycles consisting of 1 min at 94°, 1 min at an optimal annealing temperature and 1 min 15 sec at 72°. The last elongation step was lengthened to 5 min. Then, 8 μl of loading buffer (25 ml formamide, 0.025g xylene cyanol, 0.025g bromophenol blue and 1 ml EDTA 0.5M) was added to each PCR product. The mixture was heated for 5 min at 94° and aliquots of 3.6 μl were electrophoresed on 8% denaturating polyacrylamide gels. Gels were run for 2-5 hours at 50W (voltage and intensity were set up non-restrictive conditions, 1900 V and 150 mA respectively), dried and exposed to film for 6-48 hr.

Results

First Experiment

In the first experiment, polymorphic microsatellite loci were used in order to identify parentage in the progeny of a factorial cross between 20 males and 20 females. The 400 bi-parental crosses were mixed together 3 hours after fertilisation. The first pediveligera and larvae larger than 280 μm appeared 17 days after fertilisation. The sieving operations were performed 17, 20, 23 and 26 days after fertilisation and led to the separation of 4 sieving groups, numbered 1, 2, 3 and 4 respectively.

Sampling was performed 45 days after fertilisation. The 40 parents and 197 spat were analysed using one locus (cg44). 28 alleles were observed among the 40 parents. Male and female parents were determined for 54 and 62 offspring respectively (Table 1) and 19 spat were unambiguously assigned to a single bi-parental family.

Table 1: Number of offspring unambiguously assigned to a single male or a single female parent in each sieving group of the "20 x 20" cross.

Males	Sieving group					Females	Sieving group				
(genotype)	1	2	3	4	Total	(genotype)	1	2	3	4	Total
B1m (16/16)		2			2	B1f (25/43)					
B2m (29/29)						B2f (2/47)					
B3m (37/37)						B3f (2/46)					
B4m (30/34)						B4f (24/42)	3	1		3	7
B5m (30/30)						B5f (32/34)	3	5	14	6	28
J1m (40/40)		1	1	4	6	J1f (30/38)	4	6	3	7	20
J2m (32/36)	1		2		3	J2f (29/47)					
J3m (26/26)		1	2	2	5	J3f (19/19)					
J4m (2/24)	3	2	3	6	14	J4f (40/37)		1	1	3	5
J5m (48/48)	2			2	4	J5f (39/39)					
M1m (28/28)			2	1	3	M1f (24/24)					
M2m (29/29						M2f (46/46)					
M3m (19/32)						M3f (14/39)				2	2
M4m (21/21)						M4f (34/34)					
M5m (34/41)						M5f (35/35)					
V1m (17/20)	1	1		2	4	V1f (29/29)					
V2m (32/39)						V2f (20/20)					
V3m (14/14)				2	2	V3f (20/20)					
V4m (34/37)						V4f (33/33)					
V5m (34/46)	4	5	1	1	11	V5f (33/33)					
Total	11	12	11	20	54	Total	10	13	18	21	62

Second experiment

In the second experiment, polymorphic microsatellite loci were used in order to identify parentage in 3 samples of 50 10-month old individuals, obtained by 3 "5 x 5" factorial crosses (A, B and C). The offspring of each bi-parental cross were mixed together after fertilisation, so the trace of parentage was lost. For the parentage identification, we utilised three loci , Cg108, Cg49 and Di10Cg, showing 32, 25 and 25 alleles respectively in the 30 parents. The results of the parentage analyses are summarised in Table 2.

In cross A, the parents were identified for 50/50 individuals. One animal out of the fifty analysed was proven not to be an offspring of any of the A cross, but an offspring of a family from the B cross. We classed this animal as a "contaminant". In this sample, a high frequency of a "null allele" for locus Di10Cg was observed in the progeny of a single female (A9), heterozygous at this locus. The assignment of these offspring to this mother was verified by the analysis based on the two other loci (Cg108 and Cg49). After parentage identification on the basis of these two loci had been done, we observed that the female J4 had produced 17 of the offspring in our sample.

In cross B, the parentage of all but one individual (49/50) has been unambiguously determined. One of these 49 animals with unambiguous parentage determination is a contaminant produced by the A cross.

In cross C, Parents have been identified for 47/50 individuals. No contaminants were found. For the three other specimens, the mother has been identified, but the father could be either of two possible males. These three animals were only typed for two loci.

Table 2: Number of offspring per family in the 3 "5 x 5" crosses and χ^2 heterogeneity tests.

CROSS A	Males					Total	Heterogeneity test between	
Females	A1	A2	A3	A4	A5		males	females
A6	1	2	3	2	3	11		
A7	1	2	3	6	8	20		
A8	0	0	0	0	0	0	NS	***
A9	4	3	4	1	5	17		
A10	0	0	0	0	1	1		
Total	6	7	10	9	17	49		

CROSS B	Males					Total	Heterogeneity test between	
Females	B1	B2	B3	B4	B5		males	females
B6	1	6	0	0	0	7		
B7	0	1	2	0	0	3		
B8	0	1	0	0	0	1	***	***
B9	1	6	1	1	1	10		
B10	5	17	0	4	1	27		
Total	7	31	3	5	2	48		

CROSS C	Males					Total	Heterogeneity test between	
Females	C1	C2	C3	C4	C5		males	females
C6	0	0	2	0	1	3		
C7	0	2	12	0	2	16		
C8	0	0	3	0	0	3	***	**
C9	1	0	11	0	2	14		
C10	2	1	7	0	1	11		
Total	3	3	35	0	6	47		

***: P <0.001, ** : P<0.01, NS: not significant

The observed numbers of offspring per parent (male or female) are significantly different between parents in the 3 crosses (except among males in the cross A).

We compared the early embryonic development, recorded 3 hours after fertilization, and the parental contributions in the spat using the Spearman's rank correlation coefficient method. None of the coefficients are significant but the coefficients are rather high for females in the cross A.

Table 3: Comparison between early embryonic development and parental contributions in the 3 "5 x 5" crosses.

Cross	Males		Females	
	Early embryonic development (%)	Parental contribution (%)	Early embryonic development (%)	Parental contribution (%)
A	77,2	12.2	79,6	06.1
	74,8	14.3	97,4	16.3
	75,8	20.4	26,6	00.0
	80,8	18.4	97,0	10.2
	80,6	34.7	88,6	02.0
Spearman's rank correlation coefficient	$r_s = 0.3$ ($p = 0.54$)		$r_s = 0.9$ ($p = 0.07$)	
B	87,8	14 .6	70,4	14.6
	88,0	64.6	86,8	06.3
	87,4	06.3	75,6	02.1
	64,4	10.4	83,4	20.8
	84,8	04.2	96,2	56.3
Spearman's rank correlation coefficient	$r_s = 0.7$ ($p = 0.16$)		$r_s = 0.5$ ($p = 0.31$)	
C	94,8	06.4	84,4	06.4
	75,8	06.4	96,0	34.0
	91,0	74.5	96,2	06.4
	89,2	00.0	74,2	29.8
	75,4	12.8	75,4	23.4
Spearman's rank correlation coefficient	$r_s = -0.05$ ($p = 0.84$)		$r_s = -0.31$ ($p = 0.68$)	

Third experiment

In the third experiment, microsatellite analysis of 6-day and 18-day old larvae was performed on a single locus (Di10Cg). Parentage analysis was made easier as the 10 parental oysters involved were initially chosen to carry different alleles and to be heterozygote (i.e. 20 alleles segregating in the progeny).

Gametic competition during in vitro fertilisation was assessed by comparing two crosses :

- Cross D: 5 males x 5 females = 25 separate crosses mixed together after 3h,
- Cross D': gametes of the 5 males and the 5 females were first mixed within each gender and then mixed together.

The male contributions were found to be significantly different between the two crosses ($p=0.026$) but the female contributions were not ($p=0.51$).

Change of parental contributions over time during the larval stage was assessed by comparing larvae sampled 6 and 18 days after fertilisation in cross D. Male contributions are significantly different between these two samples ($p=0.012$) but female contributions are not ($p=0.93$).

Table 4: Male and Female contributions in the crosses D (25 separate crosses) and D' (pooled gametes) at day 6 (D and D') and day 18 (D).

CROSS D Day 6	Females					Contribution (%)	χ^2 heterogeneity test between	
Males	D1	D2	D3	D4	D5		males	females
D6	18	10	8	9	25	0.257		
D7	9	13	11	8	19	0.221		
D8	8	8	3	10	14	0.158	NS	NS
D9	11	17	4	5	21	0.213		
D10	10	13	7	6	5	0.151		
Contribution (%)	0.205	0.224	0.121	0.139	0.308	1		

CROSS D' Day 6	Females					Contribution (%)	χ^2 heterogeneity test between	
Males	D1	D2	D3	D4	D5		males	females
D6	4	1	3	0	26	0.137		
D7	7	8	5	5	21	0.185		
D8	4	14	2	9	11	0.161	*	**
D9	19	21	20	4	31	0.382		
D10	5	9	6	6	8	0.137		
Contribution (%)	0.157	0.213	0.145	0.096	0.390	1		

CROSS D Day 18	Females					Contribution (%)	χ^2 heterogeneity test between	
Males	D1	D2	D3	D4	D5		males	females
D6	25	20	17	19	56	0.343		
D7	4	12	4	5	16	0.103		
D8	8	11	10	8	17	0.135	**	NS
D9	20	22	17	6	22	0.218		(p = 0.06)
D10	19	17	12	17	15	0.201		
Total	0.19	0.205	0.150	0.138	0.316	1		

*: $p < 0.05$, ** $p < 0.01$, NS: not significant

Discussion

Most papers dealing with parentage analysis report results concerning the following question : "Who are the parents of that offspring ?".

In some cases, a probabilistic approach is developed (e.g. Alford *et al.*, 1994) as it is not possible to unambiguously distinguish all possible parents, due to limited polymorphism in the potential parents. Such results can be of great use in mass selection breeding programs, in order to minimise inbreeding. In other cases, polymorphism is high enough to differentiate all possible parents and therefore assign each offspring unambiguously to its parents.

When parentage analysis is possible, genetic study of quantitative characters can be performed on the progeny reared in a common environment (e.g. Batargias et al. in Magoulas, 1998). These studies also enable the quantification of the contribution of each parent to the following generation, and subsequently makes possible the identification of the factors that influence these contributions.

Parentage analysis

When parents are chosen randomly, parentage analysis in the progeny is mostly dependent on polymorphism and the number of loci scored. If, at a given locus, an allele is carried by a single potential parent, then it can be used directly to assign any offspring carrying that allele to that parent. So the rarer an allele is, the more informative it is. In our

first experiment, a quarter of the studied progeny could be assigned to a given parent using only one locus, showing 28 alleles over the 40 parents. In our second experiment, 3 loci were scored and 100 % of the progeny could be assigned to one of the 25 full-sib families. In our three crosses, only 4, 18 and 32 % of the studied progeny required the combination of 2 loci to determine parentage. This efficiency is mostly due to the high polymorphism of the markers used: 11 to 18 alleles per locus segregating in each "5 x 5" cross, and more than 50 alleles per locus in the wild (Huvet *et al.*, in prep).

Why unbalanced parental contributions ?

Our results clearly demonstrate that parental contribution in the studied progenies are unbalanced, despite the care taken to balance gametic contributions between males and between females at the fertilisation stage. Three main factors can be distinguished to explain such unbalanced contributions.

The quality of the gametes can be different between parents when crosses are performed. These differences could be due to differences in the timing of maturation between parents (some oysters being ready to spawn earlier than others despite the common pre-conditioning and conditioning periods) or other environmentally induced differences. These differences are commonly known as "maternal effects" (and similarly paternal effects could also exist). In order to assess the quality of the gametes, we have compared early embryonic developments (3h after fertilisation) with parental contributions recorded (second experiment). No significant relationships were found with parental contribution, but this might be due to the limited number of parents involved in these crosses. Nevertheless, it can be noted that one of the female parents which had a very low mean early embryonic development percentage (26.6 %) has a parental contribution equal to 0 (Table 3). Pre-fertilisation methods should be developed in order to assess the potential importance of gamete quality in unbalanced parental contributions.

Non random mating can also explain unbalanced parental contributions. In the case of controlled in vitro fertilisation (with balanced pre-fertilisation gametic contribution), gametic competition can occur. In our third experiment, we compared parental contribution between each gender with and without gametic competition (crosses D' and D respectively). Our results show that male contributions are significantly different but that female contributions are not. As male contributions are not significantly unbalanced in cross D but they do in cross D', this is likely to be due to competition between spermatozoa in cross D'.

The third main reason for unbalanced parental contribution is zygotic competition and differential viability between families. In this case, parental contributions might change over time as mortalities occur. In our third experiment we show that parental contributions are significantly different between day 6 and day 18. This demonstrates that some selection occurred during the larval stage, favouring the progeny of some parents over others. The analysis of samples at later stages (namely after settlement) should bring new insights about selective processes before and after metamorphosis.

Acknowledgements

This research was supported by the EU contract FAIR PL. 95.421. We thank J.-P. Baud, S. Heurtebise, C. Ledu, M. Nourry and P. Phélipot for technical assistance in hatchery and nursery work. We thank H.R. McCombie and S. Hubert for helpful comments on this manuscript.

References

Alford R.L., Hammond H.A., Coto I., Caskey C.T. (1994). Rapid and efficient resolution of parentage by amplification of short tandem repeats. American Journal of Human Genetics 55:190-195.

Bierne N., Launey S., Naciri-Graven Y., Bonhomme F. Early effect of inbreeding as revealed by microsatellite analyses on *Ostrea edulis* larvae. Genetics 148:1893-

Colbourne J.K., Neff B.D., Wright J.M., Gross M.R. (1996). DNA fingerprinting of bluegill sunfish (*Lepomis macrochirus*) using (GT)n microsatellites and its potential for assessment of mating success. Canadian Journal of Fisheries and Aquatic Sciences 53: 342-349.

Ferguson, A., Taggart J.B., Prodohl P.A., McMeel O., Thompson C., Stone C., McGinnity P., Hynes R.A. (1995). The application of molecular markers to the study and conservation of fish populations, with special reference to *Salmo*. Journal of Fish Biology 47(Suppl. A): 103-126.

Harris A.S., Bieger S., Doyle R.W., Wright, J.M. (1991). DNA fingerprinting of tilapia, *Oreochromis niloticus* , and its application to aquaculture genetics. Aquaculture 92(2-3): 157-163.

Garcia de Leon F.G., Canonne M., Quillet E., Bonhomme F., Chatain B. (1998). The application of microsatellite markers to breeding programmes in the sea bass, Dicentrarchus labrax. Aquaculture 159(3-4): 303-316.

Gérard A., Naciri Y., Peignon J.M., Ledu C., Phelipot P. (1994) Optimization of triploid induction by the use of 6-DMZAP for the oyster Crassostrea gigas (Thunberg). Aquaculture and Fisheries Management 25: 709-719.

Hedgecock, D. (1994). Does variance in reproductive success limit effective population sizes of marine organisms? In Beaumont A.R. (ed.) Genetics and evolution of aquatic organisms. London: Chapman and Hall, pp. 122-134.

Herbinger C.M., Doyle R.W., Pitman E.R., Paquet D., Mesa K.A., Morris D.B., Wright, J.M., Cook D. (1996a). DNA fingerprint based analysis of paternal and maternal effects on offspring growth and survival in communally reared rainbow trout. Aquaculture 137(1-4): 245-256.

Herbinger CM, Doyle R.W, Taggart C.T., Lochmann S.E., Brooker A.L., Wright J.M., Cook D. (1996b). Family relationships and effective population size in a natural cohort of cod larvae. Canadian journal of Fish and Aquatic Science54 (Suppl. 1) 11-18.

Kellog K.A., Markert J.A., Stauffer J.R., Kocher T.D. (1995). Microsatellite variation demonstrate multiple paternity in lekking cichlid fish from lake Malawi, Africa. Proceedings of the Royal Society, London 260B: 79-84.

Kincaid H.L. (1976). Inbreeding in rainbow trout (*Salmo gairdneri*). Journal of the Fisheries Research Board, Canada 33: 2420-2426.

Magoulas A. (1998). Application of molecular markers to aquaculture and broodstock management with special emphasis on microsatellite DNA. Proceeding of the Seminar Genetics and Breeding of Mediterranean Aquaculture Species. Network on Technology of Aquaculture in the Mediterranean (TECAM). Zaragossa, Spain, April 28-30, 1997. pp 153-168.

Magoulas A., Gjetvaj B., Terzoglou V., Zouros E. Three polymorphic microsatellites in the Japanese oyster, Crassostrea gigas (Thunberg). Animal Genetics, in press.

Walne, P.R., (ed) 1974. Culture of bivalve molluscs: fifty years experience at Conwy, Whitefriars Press Ltd, London.