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Note

Isolation and use of microsatellite loci in *Melicertus kerathurus* (Crustacea, Penaeidae)

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Abstract – *Melicertus kerathurus* represents an economically important resource for fisheries and aquaculture. Seven microsatellite loci from 373 specimens of *M. kerathurus* collected in different parts of the Mediterranean and NE Atlantic were identified. Four of these microsatellites showed a moderate level of polymorphism, with 2 to 14 alleles per locus, whereas three had a monomorphic profile. Observed and expected heterozygosity ranged between 0.28 to0.64 and 0.28 to 0.65, respectively. Three microsatellite loci deviated from Hardy-Weinberg equilibrium in some populations, with a deficit of heterozygosity. Mean *Fst* values showed significant differentiation among sample sites analysed and indicate that these loci are useful for the study of genetic variation in this species. These results may supply functional information on the population genetic structure of *Melicertus kerathurus*, valuable for future sustainable management strategies.

Key words: Microsatellites / Genetic variation / PCR–RFLP / Penaeid shrimp / *Melicertus kerathurus* / Mediterranean Sea / Atlantic Ocean

1 Introduction

Melicertus kerathurus is a prawn widely distributed in the Mediterranean Sea and northeast Atlantic Ocean. It is a benthic species living on sandy-mud substrates, from a few metres to 90 m depth. The species is intensely exploited, mostly by trawlers and small-scale fisheries, and thus represents an economically important resource for fisheries: in 2006 the capture was 6.5 tons (FAO 2008). *M. kerathurus* has been considered for aquaculture since the beginning of 1970s (Lumare 1976), although interest in this species for aquaculture has decreased over the last two decades because of its low resistance to environmental culture conditions and to the progressive domestication of exotic species such as *Marsupenaeus (Penaeus) japonicus* (Lumare and Palmeggiano 1980). The life history of *M. kerathurus* is typical of some other penaeid species like those of the *Metapenaeus* and *Marsupenaeus* genera, encompassing a distinctive inshore planktonic larval phase, relatively sedentary juveniles and adults living in coastal waters, estuaries and lagoons (Holthuis 1980; Garcia and Le Reste 1981), and movement of adults to offshore waters during the reproductive season.

Previous studies based on protein electrophoresis have shown low levels of genetic variation in this species (Mattoccia et al. 1987; Zitari-Chatti et al. 2008), and these results seem in accordance with those reported for other penaeids (Benzie 2000 and references therein). Mattoccia et al. (1987), did not find any population differentiation, whereas Zitari-Chatti et al. (2008) observed two apparently genetically isolated stocks along the coast of Tunisia. Recently, using the cytochrome *c* oxidase I (COI) region of mtDNA, Pellerito et al. (2009) investigated eleven populations (nine from the Mediterranean Sea and two from the Atlantic Ocean) and found a significant level of population differentiation. Moreover, Zitari-Chatti et al. (2009), using cytochrome *c* oxidase I (COI) and 16S mitochondrial DNA genes, found restricted gene flow along the Siculo-Tunisian strait (Sicilian Channel). These results have posed new questions about the population structure of this species. Nuclear markers, such as microsatellites, can contribute to clarifying relationships among closely related populations – an important point that will be necessary for sustainable management.

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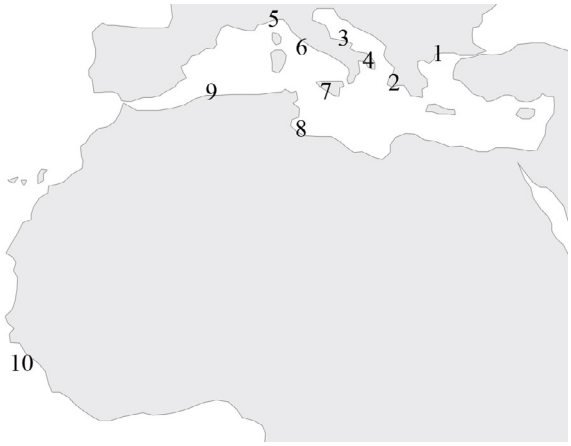

Fig. 1. Sample sites. *Mediterranean Sea*: 1-Kàvala (Greece); 2-Amvràkikos (Greece); 3-Aquatina (Italy); 4- Lesina (Italy) 5- Pisa (Italy); 6- Roma (Italy); 7- Selinunte (Italy); 8 –Sfax (Tunisia); 9-Annaba (Algeria); *Atlantic Ocean*: 10- Dakar (Senegal).

The aim of the present study was to identify microsatellite loci in *M. kerathurus* and to further explore the genetic variation of this species across its geographical distribution range. Microsatellites are nuclear sequences that usually present a high level of variability. They are generally non-coding and their pattern of differentiation can be explained by migration and genetic drift, ignoring the role of selection. Because of larger diploid effective size, compared with mtDNA, which is transmitted by females only, microsatellites are *a priori* less sensitive to bottlenecks or founder effects (Hare 2001) than the mtDNA markers. The information provided by these two types of markers is therefore complementary.

2 Materials and methods

Microsatellite isolation

Total DNA was extracted from pleopod muscle of 373 *M. kerathurus* individuals collected across ten regions, nine from the Mediterranean Sea and one from the NE Atlantic (Fig. 1), using the DNAeasy tissue kit (QIAGEN). According to Zane et al. (2002), the DNA was exposed twice to multiple enzymatic digestion (Hamilton et al. 1999) with Rsa I/Hae III and Rsa I/Dra I for three hours. After size fractionation with agarose-gel electrophoresis, 500-1000 base pair (bp) fragments were excised and purified with QIAquick gel extraction kit (QIAGEN).

The fragments were ligated to 10 pmol of specific adaptors in a 10 μl volume reaction containing a high concentration T4 DNA ligase (New England Biolabs) and 0.1 μl bovine serum albumin (BSA), overnight at room temperature; they were then purified by ethanol precipitation. After denaturation, the fragments were hybridised for 20 min at 56 °C to simple-sequence oligonucleotide probe, constituted by a mix of dinucleotide and tri/tetra-nucleotide 5'-biotinylated repeats $(GT)_{18}$ $(CA)_{14}$ $(GA)_{14}$ $(GATA)_7$ $(CAT)_9$, bound to streptavidin coated beads (Kijas et al. 1994; Kandpal et al. 1999) and separated magnetically from the supernatant using a PolyATtract isolation system (Promega). After several washes with different concentrations of SSC solutions (four washes with 200 μl SSC 2X, four washes with 200 μl SSC 1X at 30 °C), the fragments complementary to the microsatellite oligo probes were recovered by elution with NaOH 0.15 M, neutralised with TE 10X and AcOH 1.25 M and purified with Qiaquick purification kit (QIAGEN). A 25 μl linker –primed PCR was conducted, with 4 pmol of adaptor-specific primers, an initial cycle of 95 °C for 10 min, 60 °C for 2 min and 72 °C for 1 min 30 s, followed by 30 cycles of denaturation at 94 °CC for 30 s, annealing temperature for 30 s and 72 °C for 1 min 30 s and a final elongation step at 72 °C for 10 min. The purified 500–1000 bp fragments obtained by electrophoresis size fractioning, were then cloned into vectors using a TOPO TA Cloning kit (Invitrogen). The positive clones (ampicillin selection) were picked and resuspended in LB medium overnight. A 50 μl PCR screening with standard primers M-13 Forward and M13 Reverse was carried out to analyse the insert length in the positive transformants. The plasmids containing an insert over 400 bp were isolated and sequenced on an ABIPrism 310 automatic sequencer (Applied Biosystems).

On the basis of the sequence repeat quality and their flanking regions, seven primer pairs were designed using Oligo Analyzer 1.0.3 (Table 1).

Polymerase chain reaction (PCR) conditions

The polymerase chain reaction (PCR) was performed in a total volume of 10 μl constituted by 10 ng DNA template, 1.5 to 3 pmol of each primer (one labelled with 6-FAM or Hex fluorescent dye), 1 μl 10X Buffer, 2.5 mM $MgCl_2$, and 0.05 unit Taq DNA polymerase (AmpliTaq DNA Polymerase Applied Biosystems). The reaction profile had an initial step of denaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C for 1 min, 1 min at annealing temperature for each primer set and 72 °C for 1 min, with a final step of elongation at 72 °C for 7 min. The amplified fragments were analysed by electrophoresis on an ABIPrism 310 automatic sequencer (Applied Biosystems) and fluorograms were processed with Genescan 3.7 fragment analysis software (Applied Biosystems).

Data analysis

Microsatellite polymorphism was measured as the mean number of alleles per locus, and observed and expected heterozygosity were then determined. Hardy-Weinberg (HW) equilibrium and genetic differentiation (*F-statistics*) between sample sites were computed using GENETIX 4.02 (Belkhir et al. 2001) and GENEPOP version 3.4 (Raymond and Rousset 2004). Sequential Bonferroni corrections for multiple comparisons were applied. To score null alleles and errors, the Micro-checker program (http://microchecker.hull.ac.uk) was used (Oosterhout et al. 2004).

3 Results

More than 600 clones containing potential dinucleotide microsatellite loci were obtained. Out of the 80 clones

Table 1. Locus name, repeat pattern, primer sequence and annealing temperature (T_a) for *M. kerathurus*.

Locus	Repeat unit	Primer sequence 5'	T_a (°C)
Plasm2	(GT)n	AGCAGTTTGTGACAGGTGTG TTGTGTAGCATTTAGGTTG	50
Plasm7	(GA)n	TCGGACTGGTAGGTAGAG AAGGCGGGAAATTGGCACGA	47
Plasm9	(CT)n	CGAGCTGGGTCCAAATCAT GTACCAAGGGACTAGCCA	50
Plasm15	$(GGAT)_n$	TAGAGTTTTGTCTGTTTCCC TCCTTCGTCCACCCATTTAT	52
Plasm29	$(CCT)_3CTTTT$	CGCTCTCTTATTCTACTTCTT CGTCGCAAGTGATTAAGGGC	50
Plasm51	(CG)n(TG)n(CA)n	GACCTCATGGCCTGACG ATGCGGATGCGTGCTGC	50
Plasm52	(TG)n	ACGGACTTCAGTGGCTGGAC CGCAACGGCAAGGAACT	50

Table 2. Polymorphic microsatellite loci in *M. kerathurus*. Total number of individuals assayed (*N*), number of alleles per locus (A), total number of alleles for each locus across all populations (N_a); observed (H_o) and expected (H_e) heterozygosity. HW deviation after sequential Bonferroni procedure (in bold) (*$p < 0.05$).

Locus	Size range (mm)		Site location										Tot.sample size
			Egeo	Ionio	Aquatina	Lesina	Pisa	Roma	Selinunte	Sfax	Annaba	Senegal	
Plasm2	180-200	*N*	40	33	29	31	23	40	47	39	44	40	366
		A	**2**	**2**	**3**	**3**	**2**	**2**	**3**	**4**	**3**	**4**	
		Na											4
		H_o	0.351	0.303	0.586	0.452	0.435	0.412	0.362	0.538	0.486	0.401	
		H_e	0.461	0.339	0.491	0.503	0.464	0.426	0.481	0.469	0.583	0.394	
Plasm7	108-290	*N*	32	24	30	26	16	38	42	38	40	37	323
		A	**14**	**5**	**4**	**4**	**10**	**12**	**3**	**5**	**3**	**9**	
		Na											20
		H_o	**0.344***	0.583	**0.567***	**0.192***	0.503	**0.263***	**0.524***	**0.395***	**0.525***	**0.351***	
		H_e	0.534	0.642	0.817	0.742	0.756	0.781	0.815	0.772	0.805	0.836	
Plasm15	30-350	*N*	35	33	28	25	22	34	37	31	29	38	312
		A	**4**	**4**	**10**	**8**	**7**	**11**	**14**	**10**	**9**	**12**	
		Na											21
		H_o	**0.457***	**0.636***	0.643	0.28	**0.591***	**0.412***	0.73	**0.098***	**0.207***	**0.395***	
		H_e	0.696	0.839	0.554	0.261	0.726	0.639	0.762	0.213	0.39	0.638	
Plasm52	64-400	*N*	35	36	30	24	21	38	37	22	20	39	302
		A	**13**	**11**	**5**	**6**	**11**	**11**	**10**	**5**	**7**	**5**	
		Na											24
		H_o	**0.684***	0.589	0.567	0.208	**0.952***	**0.658***	**0.730***	**0.091***	0.103	0.615	
		H_e	0.861	0.638	0.445	0.233	0.752	0.769	0.496	0.255	0.099	0.504	
	mean	H_o	**0.459***	0.541	0.592	**0.283***	0.619	**0.645***	**0.630***	**0.421***	**0.463***	**0.442***	
		H_e	0.629	0.599	0.566	0.427	0.658	0.433	0.586	0.28	0.305	0.585	

sequenced, 52 (65%) contained microsatellite sequences. Dinucleotide repeats were the most abundant, accounting for 68%, whereas tri and tetranucleotides represented 18% and 14%, respectively.

On the basis of amplification results, three loci (*Plasm9*, *Plasm29* and *Plasm51*) had monomorphic profiles, while four showed a moderate level of polymorphism: (*Plasm2*, *Plasm7*, *Plasm15*, *Plasm52*; Table 2).

Allele size ranged from 30 to 400 bp and the number of alleles per locus ranged from 2 to 14 across the 10 populations, with a mean number across loci ranging between 5.25 and 9.25 (Lesina and Rome, respectively; Table 2). This number of alleles per locus is very close to that obtained by Velles-Jimenez et al. (2005) in *Litopenaeus vannamei* (2–13), but is lower than that observed in other penaeids (Benzie 2000; Brooker et al. 2000; Xu et al. 2001). The observed and expected mean heterozygosity values ranged from 0.45 to 0.57 and were comparable to values reported for microsatellite loci in others penaeid species (Tassanakajon et al. 1998; Benzie 2000; Brooker et al. 2000; Ball and Chapman 2003). The data showed a heterozygote deficit in many of the sampled sites and there was a significant departure from the Hardy-Weinberg equilibrium in three of the four loci analysed (Table 2), including after Bonferroni corrections (mean $Fis = 0.21$; $p < 0.05$); only the locus *Plasm2* was in Hardy-Weinberg equilibrium. The departure from HW equilibrium has also been observed in other crustacean species and seems to be a natural characteristic of many marine prawn species (Benzie 2000; Valles-Jimenez et al. 2005). In principle, departure from genetic equilibrium could be explained by a number of different mechanisms such

Table 3. Pairwise *Fst* values between sample sites. (*$p < 0.05$).

	Amvrakikos	Egeo	Aquatina	Lesina	Pisa	Roma	Selinunte	Sfax	Annaba	Dakar
Ionio										
Egeo	0.303*									
Aquatina	0.180*	0.006								
Lesina	0.177*	0.001	–0.010							
Pisa	0.152*	0.012	–0.013	–0.011						
Roma	0.260*	–0.018	–0.002	0.004	0.008					
Selinunte	0.167*	0.007	–0.009	–0.011	–0.014	0.008				
Sfax	0.235*	–0.008	–0.009	–0.002	0.004	–0.010	0.003			
Annaba	0.060*	0.096*	0.031	0.024	0.020	0.083*	0.025*	0.063*		
Dakar	0.318*	–0.015	0.021	0.024	0.040	–0.005	0.033	0.001	0.121*	

as inbreeding, null alleles, Wahlund effect or selective forces acting against heterozygotes. Inbreeding can be excluded because it would lead to a deficit visible across all loci, i.e. all loci should be correlated (Castric et al. 2002), and no linkage disequilibrium was observed in any of the loci we examined (at 95% confidence levels). We can thus say that the loci segregate independently. The Micro-checker programme identified null alleles in only two loci, *Plasm 15* and *Plasm 52*, with a proportion over 10% (0.14 and 0.16, respectively) and there was no significant evidence for scoring error due to stuttering.

The mean *Fst* value (0.18; $p < 0.05$) showed higher population substructure than the allozymes used by Mattoccia et al. (1987), but was in accordance with the results found by Zitari-Chatti et al. (2008), also using allozymes. The apparent incongruity between these papers could result from the fact that different allozyme loci were scored, or from the number of sample sites or individuals analysed. Our data agree with the results found previously by Pellerito et al. (2009) and Zitari-Chatti et al. (2009) using mtDNA, where a moderate but significant level of differentiation was recorded among the analysed sites. Moreover, pairwise *Fst* estimates among populations confirmed the conclusion of Pellerito et al. (2009) (Table 3), according to which the Amvrakikos sample is significantly different from all other samples and that a relationship exists between genetic and geographic distances among all sites. The Gulf of Amvrakikos in particular was submitted to a strong degradation in the environmental quality during the last 20 years; an intensive fisheries effort contributed to reducing the population size (Conides et al. 2006). Despite these pressures, heterozygosity and allelic counts were not obviously reduced in this sample (Table 2). Thus, another explanation should be sought. All the other *Fst* pairwise values were either null (inside the western Mediterranean) or very moderate (e.g. between Annaba and the rest of the sites).

4 Discussion

The moderate allelic variation was clearly revealed by these four easily-identifiable microsatellite loci, compared with previous studies that used allozymes and mtDNA. These results confirm the value of these markers for studying genetic variation over the wide geographical range covered by this species. The moderate number of alleles can actually be considered an advantage since, according to Hedrick (1999), moderate allelic variation in microsatellite loci with good resolution ensures optimal sensitivity to variation in a differentiation parameter like *Fst*. The data obtained by analysing the four microsatellites in one Atlantic and nine Mediterranean populations lead us to reject the hypothesis of panmixia and are in accordance with the results obtained previously by Pellerito et al. (2009) using the cytochrome *c* oxidase I (COI) mtDNA region. Nevertheless, differences between mtDNA and nuclear markers are expected because of their different effective sizes (lower in mtDNA), due to their mode of transmission. Historical events such as bottlenecks or founder events affect the genetic diversity of mtDNA more strongly than nuclear loci, which could be the case in the Amvrakikos sample.

These markers could be useful in the development of more effective management strategies for a sustainable use of this important resource for fisheries and aquaculture, although further sampling will be needed to explain the evolutionary history of the species.

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References

Ball A.O., Chapman R.W., 2003, Population genetic analysis of white shrimp, *Litopenaeus setiferus,* using microsatellite genetic markers**.** Mol. Ecol. 12, 2319–2330.

Belkhir K., Borsa P., Chikhi L., Raufaste N., Bonhomme, F., 2001, GENETIX 4.02, logiciel sous Windows ™ pour la génétique des populations. Laboratoire Génome, populations, interactions. CNRS UMR 5000, Univ. Montpellier II, Montpellier.

Benzie J.A.H., 2000, Population genetic structure in penaeid prawns. Aquac. Res. 31, 95–119.

Brooker A.L., Benzie J.A.H., Blair D., Versini J.J., 2000, Population structure of the giant tiger prawn, *Penaeus monodon*, in Australian waters using microsatellite markers. Mar. Biol. 136, 149–157.

Castric V., Bernatchez L., Belkhiri K., Bonhomme F., 2002, Heterozygote deficiencies in small lacustrine populations of

brook charr *Salvelinus fontinalis* Mitchill (Pisces, Salmonidae): a test of alternative hypotesis. Heredity 89, 27–35.

Conides A., Glamuzina B., Jug-Dujakovic J., Papaconstantinou C., Kapiris K., 2006, Age, growth and mortality of the kamarote shrimp, *Melicertus kerathurus* (Forskal, 1775), in the east Ionian Sea (Western Greece). Crustaceana 79, 33–52.

FAO 2008, FAO Yearbook. Fishery and aquaculture statistics 2006, pp. 1–80.

Garcia S., Le Reste L., 1981, Cycles vitaux, dynamique, exploitation et aménagement des stocks de crevettes pénaeides côtières. FAO Doc. Tech. Pêches 203, pp. 1–210.

Hamilton M.B., Pincus E.L., Di Fiore A., Fleischer R.C., 1999, Unversal linker and ligation procedures for construction of genomic DNA libraries enriched for microsatellites. Biotechniques 27, 500–507.

Hare M.P., 2001, Prospects for nuclear gene phylogeography. Trends Ecol. Evol. 16,700–706.

Hedrick P.W., 1999, Perspective: highly variable loci and their interpretation in evolution and conservation. Evolution 53, 313–318.

Holthius L.B., 1980, FAO species catalogue. Shrimp and prawns of the world. An annotated catalogue of species of interest to fisheries. FAO Fish Synopsis, 1, 125.

Kandpal R.P., Kandpal G., Weissman S.M., 1994, Construction of libraries enriched for sequence repeats and jumping clones, and hybridisation selection for region –specific markers. Proc. Nat. Acad. USA 91, 88–92.

Kijas J.M., Fowler J.C., Garbett C.A., Thomas M.R., 1994, Enrichment of microsatellites from the Citrus genome using biotinylated oligonucleotide sequences bound to streptavidin – coated magnetic particles. Biotechniques 16, 656 –662.

Lumare F., 1976, Research on the reproduction and culture of shrimp *Penaeus kerathurus* in Italy. General Fisheries Council for the Mediterranean. Stud.Rev. 55, 35–48.

Lumare F., Palmegiano G.B., 1980, Acclimatazione di *Penaeus japonicus* nella laguna di Lesina (Italia Sud Orientale). Rivi. Ital. Pisc. Ittiopatol. 15, 53–58 (in Italian).

Mattoccia M., La Rosa G., De Mattheis E., Coboldi-Sbordoni M., Sbordoni V., 1987, Patterns of genetic variabilità in Mediterranean populations of *Penaeus kerathurus* (Crustacea Decapoda). In: K. Tiews (Ed.), Selection, Hybridization and Genetic Engineering in Aquaculture. Heenemann-Verlag, Berlin, pp. 131–142.

Oosterhout C.V., Hutchinson W.F., Wills D.P., Shiply P., 2004, Micro-checker: software for identifying and correcting genotyping errors in microsatellite data. Mol. Ecol. Notes 4, 535–538.

Pellerito R., Arculeo M., Bonhomme F., 2009, Recent expansion of Northeast Atlantic and Mediterranean populations of *Melicertus* (*Penaeus*) *kerathurus* (Crustacea: Decapoda). Fish. Sci. 75,1089–1095.

Raymond M., Rousset F., 2004, GENEPOP version 3.4: population genetics software for exact test and ecumuenicism, Available at: http://kimura.univ-montp2.fr/~rousset/Genepop.htm.

Tassanakajon A., Pongsomboon S., Jarayabhand P., Klibunga S., Boonsaeng V.V., 1998, Structure in wild populations of black tiger shrimp (*Penaeus monodon*) using randomly amplified polymorphic DNA analysis. J. Mar. Biotechnol. 6, 249–254.

Valles-Jimenez R., Cruz P., Perez-Enriquez R., 2005, Population genetic structure of Pacific withe shrimp (*Litopenaeus vannamei*) from Mexico to Panama : Microsatellite DNA variation. Mar. Biotechnol. 6, 475–484.

Xu Z., Primavera J.H., de la Pena L.D., Pettit P., Belak J., Alcivar-Warren A., 2001, Genetic diversity of wild and cultured black tiger shrimp (*Penaeus monodon*) in the Philippines using microsatellites. Aquaculture 199, 13–40.

Zane L, Bargelloni L., Patarnello T., 2002, Stategies for microsatellites isolation: a review. Mol. Ecol. 11, 1–16.

Zitari-Chatti R., Chatti N., Elouaer A., Said K., 2008, Genetic variation and population structure of the camarote prawn *Penaeus kerathurus* (Forskal) from the eastern and western Mediterranean coasts in Tunisia. Aquac. Res. 39, 70–76.

Zitari-Chatti R., Chatti N., Fulgione D., Chiazza I., Aprea G., Elouaer, A., Said K., Capriglione T., 2009, Mitochondrial DNA variation in the camarote prawn *Penaeus (Melicertus) kerathurus* across a transition zone in the Mediterranean Sea. Genetica 3, 439–447.