RAPD-
PCR法によるテナガエビ属の種判別と多型性について

誌名	水産増殖
ISSN	03714217
著者名	Meruane,J. 高木,基裕 谷口,順彦
発行元	水産増殖談話会
巻/号	44巻3号
巻号補足	
掲載ページ	p. 299-305
発行年月	1996年9月

(水産増殖 44巻3号 299−305
SUISANZOSHOKU (1996-H8))

Species Identification and Genetic Variation of Three Species of *Macrobrachium* (Crustacea: Palaemonidae) by RAPD

Jaime MERUANE[1], Motohiro TAKAGI[2], and Nobuhiko TANIGUCHI[2],*

[1] *Faculty of Marine Sciences, Department of Aquaculture, Universidad Catôlica del Norte, P. O. Box 117-Coquimbo, Chile*
[2] *Faculty of Agriculture, Department of Cultural Fisheries, Kochi University, Monobe, Nankoku, Kochi 783, Japan*

Abstract

Species identification by RAPD-PCR was attempted using wild adults of "Minamitenagaebi" *Macrobrachium formosense* (Bate, 1868) and "Yamatotenagaebi" *M. japonicum* (De Haan, 1849) as well as farmed postlarvae and juveniles of "Onitenagaebi" *Macrobrachium rosenbergii* (De Man, 1879). Forty random 10-mer primers were screened in the preliminary experiments using three individuals from each species. Two primers (OPA-03, 17) were selected for further analysis. In the case of OPA-03, the banding patterns of *M. formosense* and *M. japonicum* were the same, but different from that of *M. rosenbergii*. The primer OPA-17 showed different banding patterns in the 3 species. Also, the banding pattern could be detected in preparation of single, whole specimens of postlarvae. These results suggested that RAPD is an useful genetic marker for species identification and population analysis in *Macrobrachium*.

Palaemonid prawns are an important part of the aquaculture industry in many areas of the world. Especially, *M. rosenbergii* "Giant Prawn of Asia" with a global farmed production of around 31.235 t during 1992[1]. Many other species of *Macrobrachium* such as *M. formosense* and *M. japonicum* have been cultured, mostly on a pilot-scale basis or have been the subject of aquaculture-related research[2-6]. Although most of the annual domestic yield of prawns comes from the wild harvest, increasing emphasis is being placed on pond culture. In general, commercial *M. rosenbergii* hatcheries rear larvae hatched from gravid females obtained from grow-out ponds. The latter are selected on the basis of their readiness to spawn. There is thus no direct control over mating and consequently no selective breeding.

Knowledge of the taxonomic identification and population structure from which the founder individuals are chosen is essential for maximum genetic improvement. By the 1960s, electrophoretic techniques for protein separation allowed geneticists to survey natural populations of Crustacea. Data from 97 crustacean species showed low levels of polymorphism[7].

In recent years, the RAPD-PCR (Random Amplified Polymorphic DNA-PCR) has been an excellent tool for species identification and population structure analy-

Received : January 30, 1996
Key words: *Macrobrachium*, RAPD-PCR, species identification, polymorphism
* Corresponding author.

sis. The RAPD-PCR is a convenient method which is relatively easy to perform in a short time, requires minuscule tissue samples, requires no prior sequence information, reveals large numbers of polymorphisms and uses a single short primer of arbitrary sequence to amplify DNA[10-15].

The objectives of this study were to identify three species of the Palaemonidae, *Macrobrachium rosenbergii, M. formosense* and *M. japonicum* by Random Amplified Polymorphic DNA (RAPD-PCR) and to assess their potential in species identification (molecular taxonomy), and population analysis.

Materials and Methods

Prawns samples and DNA preparation

Wild adults of *Macrobrachium formosense* (n = 11, mean body weight = 12.34 g) and *Macrobrachium japonicum* (n = 12, mean body weight = 13.73 g) were obtained from Shimanto river at Nishitosa, Kochi Prefecture. Fry and juvenile of *Macrobrachium rosenbergii* postlarvae specimens of 26 days after metamorphosis (n = 14, mean body weight = 0.029 g), and juveniles specimens of 3-month old (n = 15, mean body weight = 1.35 g) were obtained from a prawn farm at Ikazaki, Ehime Prefecture.

DNA was extracted using a modified SDS-phenol chloroform method[16] from 100 % alcohol preserved samples. For *M. rosenbergii* fry total genomic DNA was isolated from each one total homogenized individual fry, and from the five pairs of pleopods for the juveniles. For *M. formosense* and *M. japonicum* DNA was isolated from the 2nd to 5th exopodites of pleopods.

Samples were incubated at 37 ℃ for 16h in a solution containing 100 mM EDTA, 10 mM Tris (pH 7.5), 1% SDS, and 1 μg/m*l* proteinase K. Then purified three times with phenol/chloroform/isoamyl alcohol (25 : 24 : 1) and then three times with chloroform/isoamyl alcohol (25 : 1). DNA was precipitated with 500 μl of 2-propanol and 50 μl of 3 M NaOAc. Precipitated DNA was washed with 70 % ethanol and resuspended in T E buffer.

PCR amplification

Amplification reactions were performed following a modification of the protocol reported by TAKAGI and TANIGUCHI, (1995). A reaction mixture of 100 μl contained; 1μg of template DNA, 2 μM of primer, 2.5 unit of Taq polymerase (Perkin Elmer Inc.), 0.2 mM DNTPS, 1 x reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$ 0.001% gelatin) (Perkin Elmer Inc.). Reactions were performed by a minicycler (MJ Research, Inc.), programmed for one cycle at 94℃ for 4 min., 35 cycles each 1 min. at 94℃, 1 min. at 36℃, 2 min. at 72℃, and one cycle at 72℃ for 5 min. Forty primers (OPA and OPB) were tested in a preliminary screening of all individuals of each species.

Following amplification, reaction products (15 μl) were run on a 5% polyacrylamide gel at 150 V/cm for 90 min using 1x TBE buffer. The gels were stained with ethidium bromide to visualize amplified fragments.

Results

Primer selection

To find a good primer for species identification and detection of polymorphisms, forty primers were screened using DNA extracts for 3 individuals of each species. Primers which produced many bands with poor resolution were discarded. Two primers were eventually selected from the amplification patterns (OPA-03, 17). These primers were then tested to find band patterns that allowed the separation of the three species and postlarvae of *M. rosenbergii*.

Species identification and polymorphism

Figure 1 shows the RAPD patterns of three species of *Macrobrachium* using the primer OPA-03. The patterns only distinguished two genotypes. All the samples of *M. rosenbergii* were Genotype I. All the samples of remaining two species of *Macrobrachium* were Genotype II. Table 1 shows the list of the number of genotypes detected. The postlarvae of *M. rosenbergii* had the same genotype (Genotype I) as the juveniles. Polymorphisms were not observed in the samples of the three species. The pattern produced by primer OPA-03 can be used to distinguish between *M. rosenbergii* and the other two species.

Figure 2 shows the RAPD patterns of three species of *Macrobrachium* using the primer OPA-17. Similarity

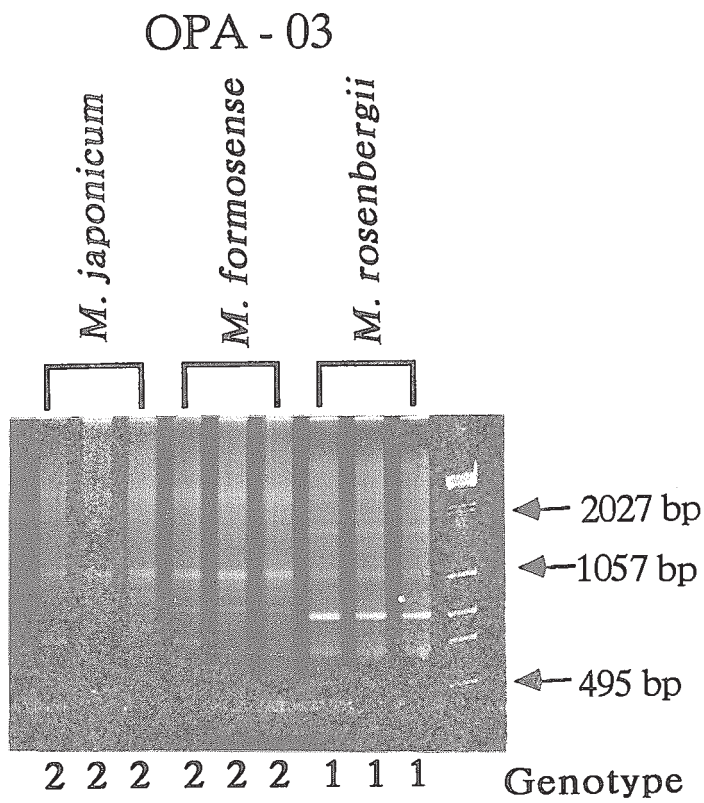

Fig. 1. Banding pattern of amplified DNA of three *Macrobrachium* species using OPA-03 primer. Counting from the right; lane 1, molecular size marker, lane 2-4, *M. rosenbergii*; lane 5-7, *M. formosense*; lane 8-10, *M. japonicum*.

Table 1. Number of polymorphic individuals estimated from RAPD-PCR fragments patterns detected with primer OPA-03

Species		Genotype	
		1	2
M. rosenbergii	Juvenile	15	0
	Postlarvae	8	0
M. formosense	Adult	0	11
M. japonicum	Adult	0	11

of band pattern tended to be high within species and low between species. Figure 3 shows the banding pattern of *M. formosense* using the same primer. This primer showed a high degree of polymorphisms and many genotypes appeared. Figure 4 shows the banding pattern of juvenile and postlarva *M. rosenbergii* using the same primer. The juvenile genotype was different for each individual but the postlarvae were mainly Genotype III.

The number of genotypes observed in each species with OPA-17 are listed in Table 2. Many different genotypes were detected: 10 genotypes were observed in *M. rosenbergii*, 5 in *M. formosense*, 8 in *M. japonicum*. Fewer genotypes were observed in the postlarvae of *M. rosenbergii*, and almost all of the individuals showed Genotype III. However, with primer OPA-17, there were no banding pattern shared by the three species.

Discussion

The results presented here show that the RAPD-PCR of OPA-17 can be used to identify three species of the genus *Macrobrachium*. OPA-17 could detect many genotypes, so this primer may be specially suitable for population analysis in *Macrobrachium*. The scarcity of polymorphism in *M. rosenbergii* postlarvae shows a decrease in genetic variability, suggesting that

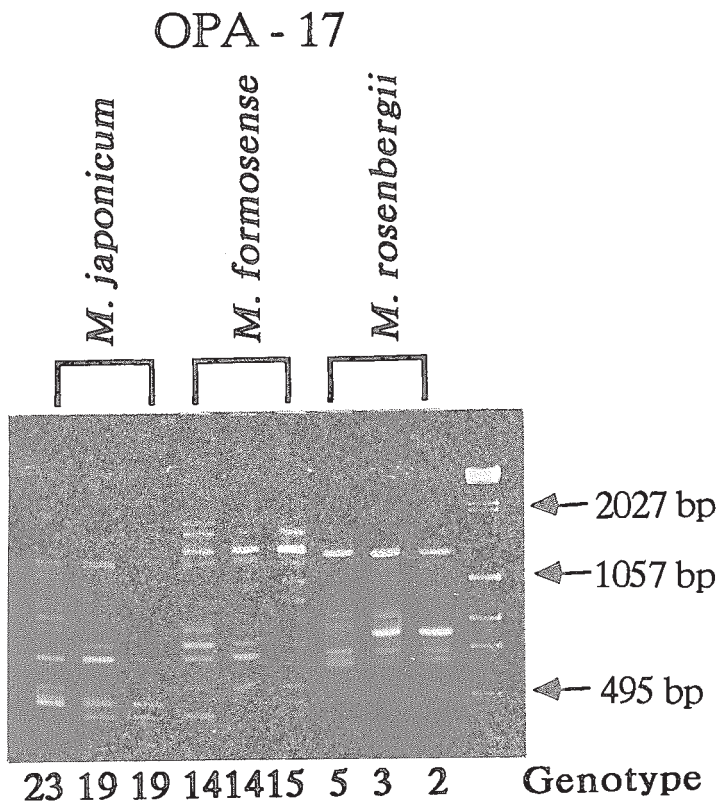

Fig. 2. Banding pattern of amplified DNA of three *Macrobrachium* species using OPA-17 primer. Counting from the right; lane 1, molecular size marker, lane 2-4, *M. rosenbergii*; lane 5-7, *M. formosense*; lane 8-10, *M. japonicum*.

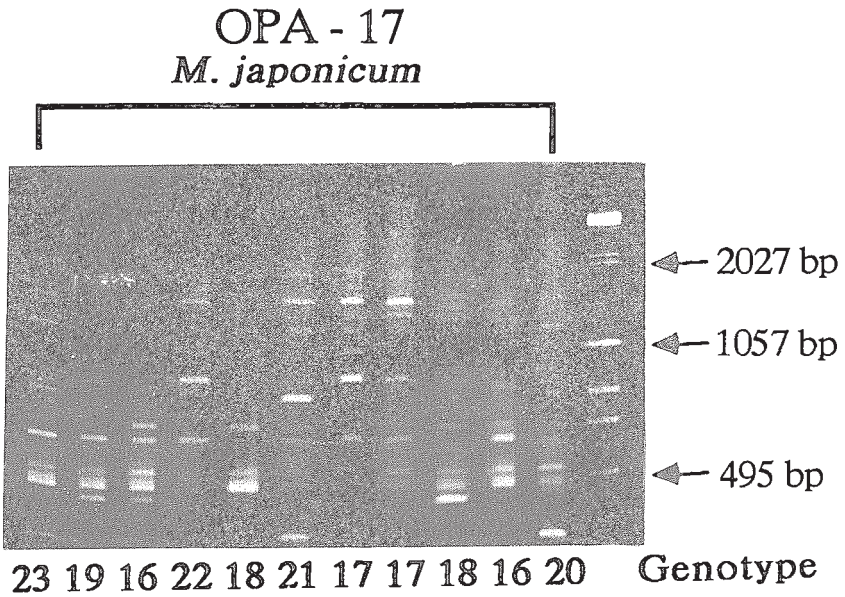

Fig. 3. Banding pattern of amplified DNA of *M. japonicum* using OPA-17 primer. Counting from the right; lane 1, molecular size marker, lane 2-12, *M. japonicum*.

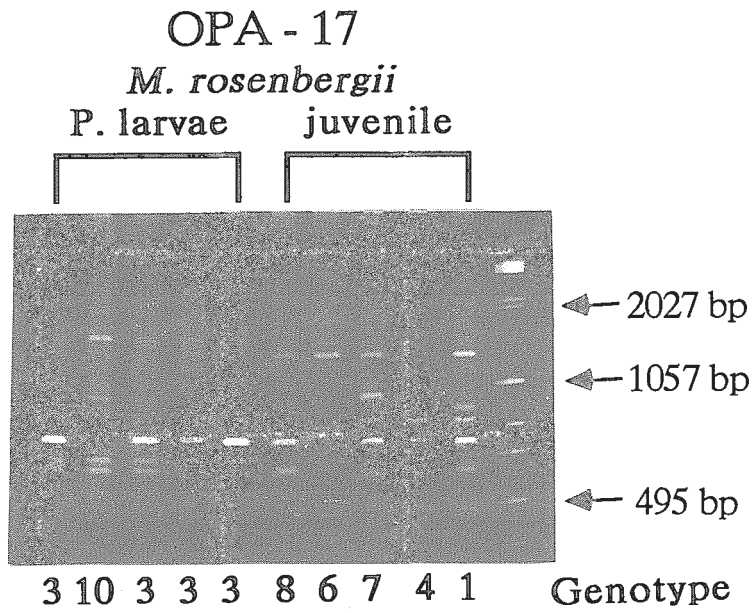

Fig. 4. Banding pattern of amplified DNA in juvenile and postlarvae of *M. rosenbergii* using OPA-17 primer. Counting from the right; lane 1, molecular size marker, lane 2-6, juvenile; lane 7-11, postlarvae.

Table 2. Number of polymorphic individuals estimated from RAPD-PCR fragments pattern detected with primer OPA-17

Species		Genoptype											
		1	2	3	4	5	6	7	8	9	10	11	12
M. rosenbergii	Juvenile	5	3	2	1	1	1	1	1	0	0	0	0
	Postlarvae	0	0	11	0	0	0	0	0	2	1	0	0
M. formosense	Adult	0	0	0	0	0	0	0	0	0	0	3	2
M. japonicum	Adult	0	0	0	0	0	0	0	0	0	0	0	0

(continued)

Species		Genoptype										
		13	14	15	16	17	18	19	20	21	22	23
M. rosenbergii	Juvenile	0	0	0	0	0	0	0	0	0	0	0
	Postlarvae	0	0	0	0	0	0	0	0	0	0	0
M. formosense	Adult	2	2	1	0	0	0	0	0	0	0	0
M. japonicum	Adult	0	0	0	2	2	2	2	1	1	1	1

a bottle neck effect had happened during the artificial propagation of cultured stocks.

On the other hand, the primer OPA-03 detected the same genotype between *M. formosense* and *M. japonicum*. The indicates that OPA-03 may be used to separate the two groups of species; *Macrobrachium rosenbergii* exported to Japan from South East of Asia, and *Macrobrachium japonicum* and *M. formosense* which are native species. This result suggested a closer relationship between *M. formosense* and *M. japonicum* than *M. rosenbergii*. After testing 40 primers, two primers were selected on the basis of good resolution at the species level. The number of ten-mers is virtually unlimited. The degree of taxonomic resolution of the technique

may therefore be improved by testing more primers.

To be useful for taxonomic purposes, RAPD-PCR has to be uncomplicated and reproducible. This technique is simple, rapid (3.5 hr depending on the thermal cycler) and safe because it does not involve the use of radioactive isotopes. One notable feature of PCR in relation to other genetic studies, is that it only requires micrograms amounts of DNA. In Crustacea, this makes it possible to analyze as little material as a single larva, postlarva or juvenile, which is useful in the studies of larval ecology or in breeding programs. Besides, pleopods can be taken without sacrifice of the animal, meaning that individuals can be easily typed for characteristics of interest without prejudice to their continued use in culture or breeding programs. Furthermore, samples can be stored in freezer (> -20 ℃) for a long period of time, or can be kept in 100 % alcohol before the extraction of DNA.

Reproducibility of results is the most critical point for the taxonomic application of the technique. The number and, to a lesser extent, the intensity of bands clearly vary from one experiment to another. In addition to these, a variable number of less intense bands appear in every reaction. As a result, several replications with the same primer must be made to determine which bands are reproducible in their entirety. It is also essential to work under the same laboratory conditions (PCR, electrophoresis etc.).

We consider that the proper use of such primers provides excellent sensitive markers for species identification and estimation of genetic variability in *Macrobrachium*.

Acknowledgments

The authors are grateful to Mr. H. Tanaka, Ikazaki prawn center for supplying prawn samples. We wish to express our sincere thanks to Mr. B. Gonzales for critical reading of the manuscript.

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RAPD-PCR 法によるテナガエビ属の種判別と多型性について

Jaime Meruane[1)]・高木基裕[2)]・谷口順彦[2)]

([1)]Univ. Catolica del Norte, [2)]高知大学農学部)

腹肢より抽出したDNAを40種のランダムプライマー（OPAとOPB）を用いてRAPD-PCRを行い，テナガエビ属3種ヤマトテナガエビ，ミナミテナガエビおよびオニテナガエビの種判別を行うとともに，RAPDの多型性について検討した。プライマーOPA-03を用いたところ，ヤマトテナガエビとミナミテナガエビは同じ遺伝子型を示したがオニテナガエビとは容易に識別することができた。一方，OPA-17を用いたところ，3種共に多型性を示した。また，Postlarvaeにおいても増幅断片を検出することができた。以上のように，RAPD-PCR法が幼生期を含むテナガエビ属の種判別および集団分析における多型的遺伝マーカーとして応用できる可能性が示された。