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Morphological Variation and Genetic Relationship among Populations of the Shortnecked Clam *Ruditapes philippinarum* Collected from Different Habitats

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The characteristics of the populations of shortnecked clam (*Ruditapes philipinarum*) originated from three different seed-production sites, Hadong, Kochang and Ulsan along the coast of Korea, were analysed by the morphological differences and the random amplified polymorphic DNA (RAPD) profiles. The morphology of the shell and survival rate for each population were also investigated 13-months after transplantation to a farming site in Shinsung. The morphology of the populations from Hadong and Kochang showed significant differences ($P<0.05$), but one year after transplantation to Shinsung, the morphology of these three populations was no longer significantly different ($P>0.05$). The template DNA for RAPD was efficiently extracted from the digestive diverticula of the clams. Up to 13 of amplified fragments were detected using arbitrary primers. Within the species of *R. philipinarum*, the genetic similarities ranged from 0.196 to 0.259. The populations from Hadong and Ulsan showed the highest similarity. The survival rates of the populations from Hadong (69.4%) and Ulsan (63.8%) were higher than that from Kochang (41.7%) 13-months after transplantation. From the RAPD analysis, it could be used as one of the primary criterion in determining which shellfish populations among various seed-production sites tend to be genetically similar and more adaptable and transplantable to a farming site.

Key words: genetic similarity, RAPD, *Ruditapes philippinarum*, shortnecked clam, transplantation

Introduction

The shortnecked clam (*Ruditapes philippinarum*) is an aquaculturable shellfish mainly distributed in an intertidal zone of Eastern-Asia including Korea, Japan and China. In Korea, it was recently reported that the aquacultural production of the shortnecked clam amounted to 54 kton; in other words, 86 million US dollars worth in 1992, composing between 7.2 and 18.5% of the total production of shellfish caught between 1980 to 1992 (The Fisheries Association of Korea, 1993).

In the practice of shellfish farming, one of the most important processes is to obtain good quality seed to be cultured. Despite the development of artificial seed production techniques, farmers still prefer to employ natural seed because of the low production cost in comparison with artificial seed. Shellfish farming using natural seed widely employs the method of dispersing seed: mostly transplanting seed from a seed-production site to a farming site or subsequently dispersing them again to the original seed-production site. However, the transplanted seed needs to be adapted to the new environment of the farming site. Recently, the massive death of whole stocks of the transplanted clam seed in the year following transplantation have been arisen in Korea. One possible cause of the mortality might be the differences in environmental factors between the seed-production site and farming site. So far, the seed to be

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transplanted have been chosen empirically without a decent criterion. In other species, Sephton (1991) reported that the mortality of mussel (*Mytilus edulis*) seed could be correlated with the geographic source of the seed. Thus, it is critical to investigate the morphological and genetic differences among those clams occurring in different habitats to establish primary criterion that helps farmers to choose the most transplantable seed.

In different habitats, morphological variation in this species is great (Choi, 1965) and the morphological variations under the different environments in shellfish appear to be related to genetic distances among them (Kohen, 1991; Yokogawa, 1997). It is, therefore, important to know which seed populations are morphologically closer to the clams that adapted to a farming site, and also how genetically distant they are.

In this study, the morphological variation of the seed from three different seed-production sites: Hadong, Ulsan and Kochang in Korea was investigated. Also, the genetic relationships among them were analysed by using the random amplified polymorphic DNA (RAPD) technique. The seed was then transplanted to a farming site, Shinsung in Kwangyang Bay, which is one of the largest aquaculture areas for clams in Korea. After cultivation, the morphological variations and the survival rate were also investigated in each population.

Materials and Methods

Materials and Site

The shortnecked clams (*R. philippinarum*) were collected at three environmentally different seed-production sites: Hadong, Kochang and Ulsan in Korea. The seed was cultured at an experimental farming site in plots measuring 1 m×2 m by triplicate at the intertidal zone at Shinsung in Kwangyang Bay, Chunranamdo, Korea (Fig. 1). The survival rates were calculated 13-months after transplantation. At Shinsung, there is atmosphere exposure for 2~3 hours at ebb tide and submersion to a depth of about 3 m at high tide. The environmental characteristics of the farming site and each seed-production site are given in Table 1. Two similar species, *Tapes variegata* and *Protothaca jedoensis*, also commonly called shortnecked clams by local farmers, were used as the negative control for the RAPD analysis.

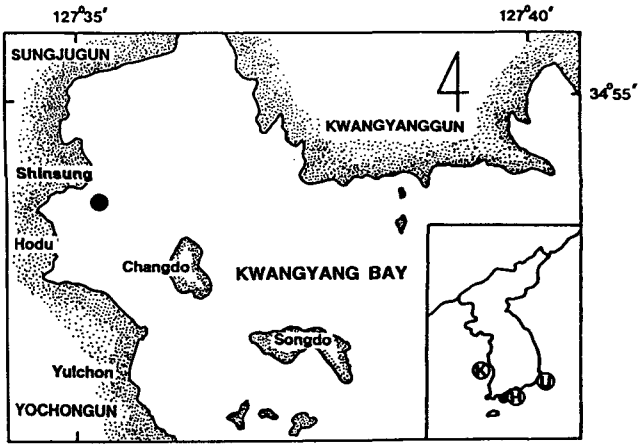

Fig. 1. Map of three seed-production sites and a farming site. A symbol "●" indicates a farming site of the shortnecked clams *R. philippinarum* for 13-months following their transplantation. Letters of H, K and U indicate seed-production sites of Hadong, Kochang and Ulsan, respectively.

Table 1. Environmental characteristics of three seed-production sites of Hadong, Kochang and Ulsan, and a farming site of Shinsung.

	Hadong	Kochang	Ulsan	Shinsung
Temperature (℃)	7/23	4/27	10/24	7/23
pH	8.1/7.7	8.0/7.6	8.1/7.2	8.1/7.7
DO (mg/ℓ)	12.1/6.7	10.1/7.2	9.2/8.3	12.1/6.7
SS (mg/ℓ)	7.8/8.5	10.2/10.0	9.6/5.3	7.8/8.5
Salinity (‰)	29.3/34.7	26.2/31.9	33.1/32.0	29.3/34.7

All data were represented as February/August from The Ministry of Environment (1994). DO, dissolved oxygen. SS, suspended solid.

Morphological Characteristics

The morphological differences among the various populations of shortnecked clams were determined by comparing the slopes of the relative growth for each population. The relative growth was calculated by using data including shell length, shell height, shell width, total weight and flesh weight (n=100 for each population). The relative growth 13-months after transplantation was also analysed for each population. To compare the morphological difference between the slopes of the relative growth from different populations, slope tests ($P<0.05$) followed by Scheffe's multiple comparison were performed (Zar, 1984).

RAPD Assay

The RAPD technique was used to analyse the genetic similarity among the clams. The template DNAs were extracted from 50 mg of the gonad, digestive diverticula, marginal mantle membrane, siphon, foot, intestine, gill, labial palp, posterior retractor muscle and anterior retractor muscle using the procedure of Jackson et al. (1991). The dissected tissue was incubated at 37℃ for 1 h with 0.6 mℓ of lysis buffer (100 mM NaCl, 10 mM Tris-Cl, 25 mM EDTA, 0.5% SDS, pH 8.0) and proteinase K (0.1 mg/mℓ). DNA was extracted using phenol/chloroform and precipitated by ethanol. The DNA was then resuspended in 50 μℓ of distilled water and quantified with a fluorometer (Hoefer, Model TKO 100). PCR amplifications were carried out using the DNA thermal cycler (Perkin-Elmer Cetus) with arbitrary primers of 10-base oligonucleotides (Operon Technologies, Inc). A 25 μℓ of PCR reaction mixture contains 1 μℓ of template DNA (0.46 ng), 1 μℓ of 2.5 mM dNTPs, 2.5 μℓ of PCR buffer (500 mM KCl, 100 mM Tris-Cl, pH 8.3, 0.01 % gelatin), 2 μℓ of 25 mM $MgCl_2$, 1 μℓ of 12.5% Tween 20, 0.2 μℓ of *Taq* polymerase (5 U/μℓ), and 1 μℓ of each primer (0.5 nM/μℓ). The thermal cycler was programmed for 45 cycles of 5 sec of denaturation at 94℃, 2 min of annealing at 36℃, 2 min of extension at 72℃, including an initial incubation at 94℃ for 5 min and a final cycle of 10 min of full extension at 72℃ (Yu and Pauls, 1992). Each 10 μℓ of amplification product added with 0.1 vol of loading dye (0.25% BPB, 20% Ficoll 400) was electrophoresed on a 2% agarose gel containing 0.5 μg/mℓ EtBr. Electrophoresis was then run for 1 h at 100 V in 0.5 x TAE buffer (20 mM Tris-acetate, 1 mM EDTA, pH 8.0). A similarity matrix was prepared based on the presence or absence of individual prominent bands generated by each primer set, and all pairwise comparisons were calculated using Jaccard's equation (Sneath and Sokal, 1973), $Jij = Cij/(ni + nj - Cij)$, where Cij is the number of common bands for a pair of samples, and ni and nj are the numbers of bands in i and j members of the pair, respectively. Using Jaccard's equation to calculate the similarity coefficients, values of 1 and 0 indicate an identical match and complete dissimilarity, respectively. The genetic distances among the populations and relative species were constructed based on the similarity matrix data by applying cluster analysis (Nei, 1987).

Results

Morphological Difference

The shape of the shells among the populations of the shortnecked clams produced at different sites showed significant differences ($P<0.05$). The populations from Hadong and Kochang had higher shell heights than those from Ulsan, while their shell lengths were the same. The population from Kochang had a wider shell width than that from Ulsan, while their shell lengths were the same (Fig. 2). The relative growth of the total weight (Y) versus the shell length (X) showed a curvilinear regression as follows: Hadong $Y=0.19\times3.06$; Ulsan $Y=0.23\times2.79$; Kochang $Y=0.26\times2.80$. The slopes of these regressions were significantly different from each other ($P<0.05$), with the exception of the relationship between Ulsan and Kochang. The relative growth of the body weight (Y) versus the shell length (X) also showed a curvilinear regression as follows: Hadong $Y=0.23\times2.40$; Ulsan $Y=0.06\times3.19$; Kochang $Y=0.18\times2.56$. There were no differences in the slopes for the relative growth of the body weight versus the shell length between three different populations (Fig. 3). However, 13-months after transplantation to Shinsung, the shapes of the shells were no longer significantly different regardless of the origin of the population ($P>0.05$) (Fig. 4).

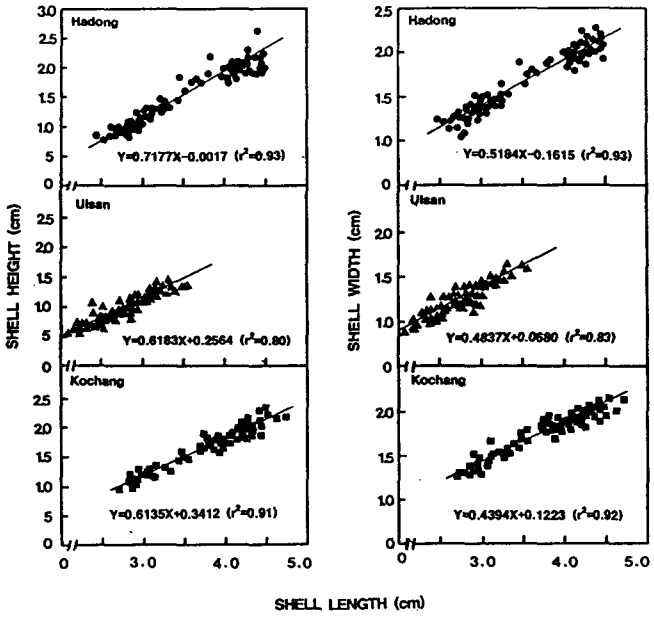

Fig. 2. Regressions of shell height and shell width versus shell length for populations of *R. philippinarum* originating from Hadong, Kochang and Ulsan.

Genetic Similarity

The extracted DNA from each clam's digestive diverticula was chosen as the PCR template after comparing the DNA extraction yield of digestive diverticula with those of the gonad, marginal mantle membrane, siphon, foot, intestine, gill, labial palp, posterior retractor muscle, and anterior retractor muscle. When the electrophoretic separation of PCR products was obtained using 1 $\mu\ell$ of the total DNA extract (46~0.46 ng) in a 25 $\mu\ell$ reaction volume with a primer OPA 03 (AGTCAGCCAC), the prominent reaction products from 4.6 ng and 0.46 ng of the extracted DNA were represented by single and five bands (Fig. 5). Thus, the extracted DNAs of all samples were adjusted to 0.46 ng in a 25 $\mu\ell$ reaction volume, and applied to the PCR amplification as the template DNA. A representative polymorphic pattern was obtained by the RAPD using the DNAs of shortnecked clams from the three different seed-production sites and two related species, *T. variegata* and *P. jedonesis*. Fig. 6 displays a representative polymorphic DNA bands obtained

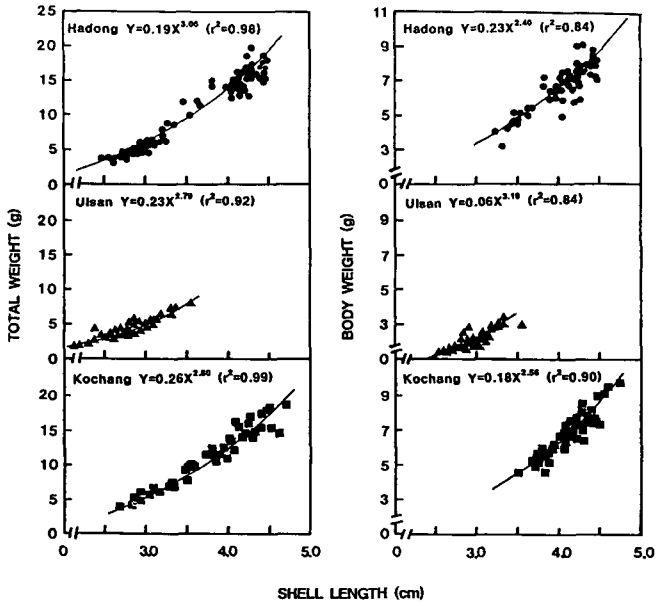

Fig. 3. Regressions of total weight and body weight versus shell length for populations of *R. philippinarum* originating from Hadong, Kochang and Ulsan.

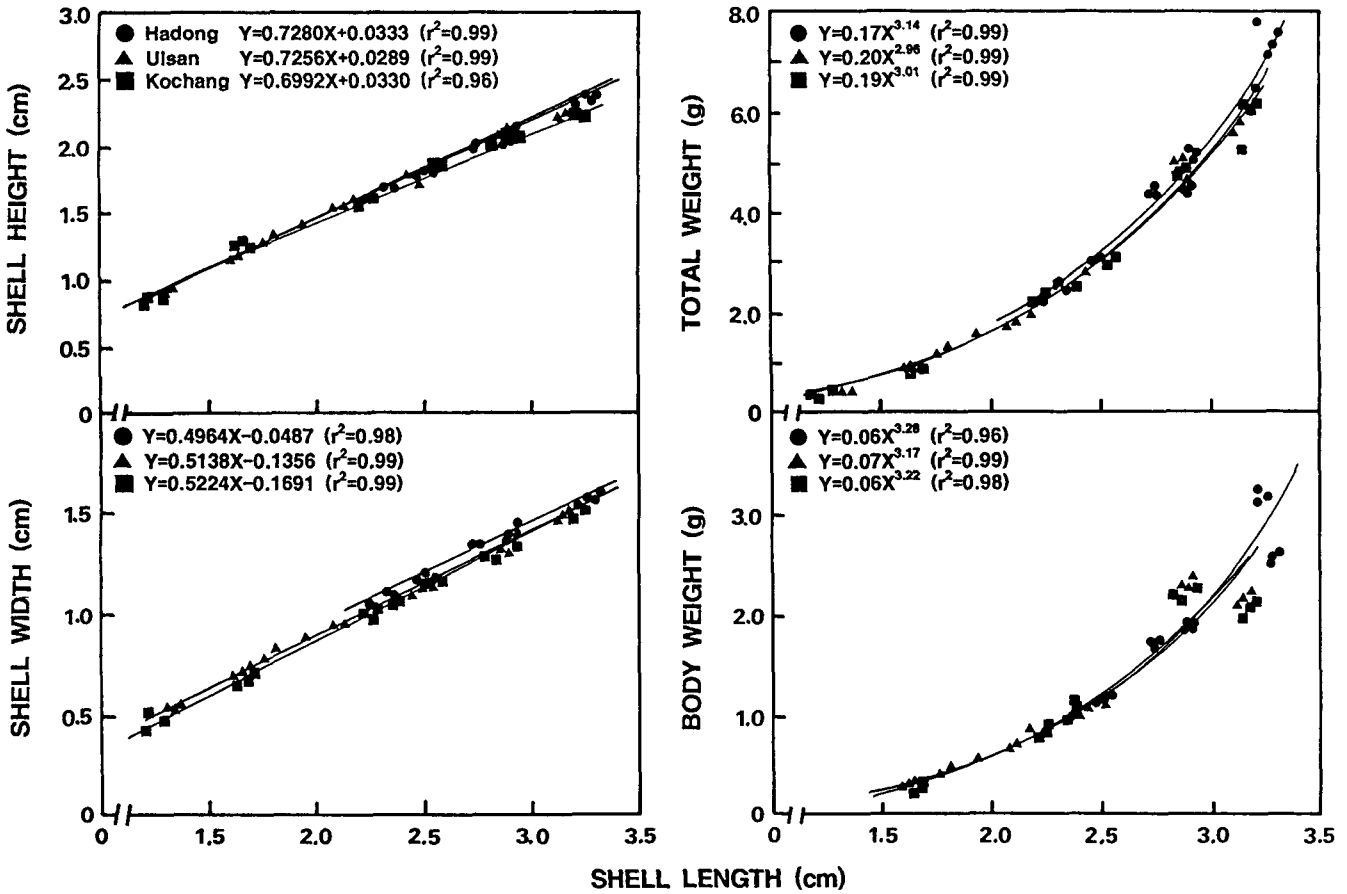

Fig. 4 Regressions of shell height, shell width, total weight and body weight versus shell length for populations of *R. philippinarum* from three different seed-production sites of Hadong, Kochang and Ulsan collected 13-months after transplantation to Shinsung.

from the PCR with the primer OPA 10 (GTGATCGCAG). Depending on the primers and populations, the polymorphic DNA bands ranged in size approximately from 200 to 1000 bp, and the numbers of bands were 1 to 13. The total number of amplified products using 62 primers in,

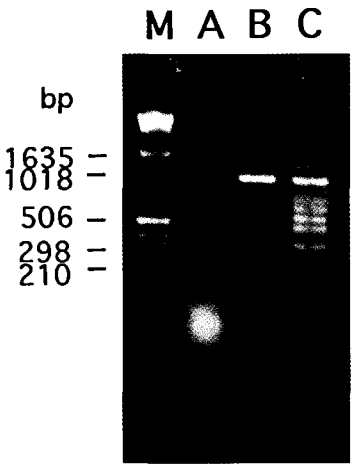

Fig. 5. PCR amplification using the extracted DNA from the digestive diverticula of *R. philippinarum*. The DNA was tested for its ability to function as a template for the PCR amplification with a primer OPA 03 (AGTCAGCCAC). Lane M, size marker of 1 kb DNA ladder from BRL/Gibco. Lane A, template DNA of 46 ng/$\mu\ell$. Lane B, template DNA of 4.6 ng/$\mu\ell$. Lane C, template DNA of 0.46 ng/$\mu\ell$.

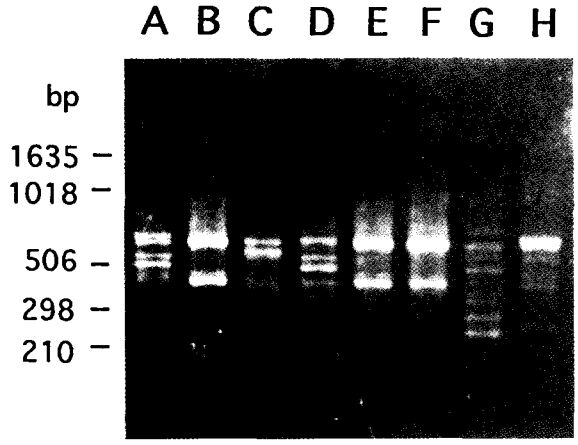

Fig. 6. Representative polymorphic DNA patterns obtained by the RAPD using DNAs of *R. philippinarum* (A-F) from three seed-production sites and related species of *P. jedoensis* (G) and *T. variegata* (H). Lanes A and D, from Hadong. Lanes B and E, from Ulsan. Lanes C and F, from Kochang.

Hadong, Ulsan, Kochang of *R. philippinarum and P. jedonesis* and *T. variegata* were 153, 114, 109, 123 and 117, respectively (data not presented). Hadong and Ulsan origin showed the closest genetic similarity (0.259) compared to the others (Ulsan and Kochang: 0.212; Hadong and Kochang: 0.196). The genetic similarities among the shortnecked clams were shown in Fig. 7. The group of *R. philippinarum* had relatively low degree of similarity with two local species of *T. variegata* and *P. jedonesis*, as evidenced by the similarity coefficients of 0.104 and 0.098 by cluster analysis, respectively.

Survival Rate after Transplantation

The survival rate of the clams transplanted and cultivated for 13-months at a farming site in Shinsung showed significant differences between clams of Kochang origin and clams of Hadong and Ulsan origin. The survival rate for the population originating from Kochang was the lowest (41.7%) compared with those of Hadong (69.4%) and Ulsan (63.8%).

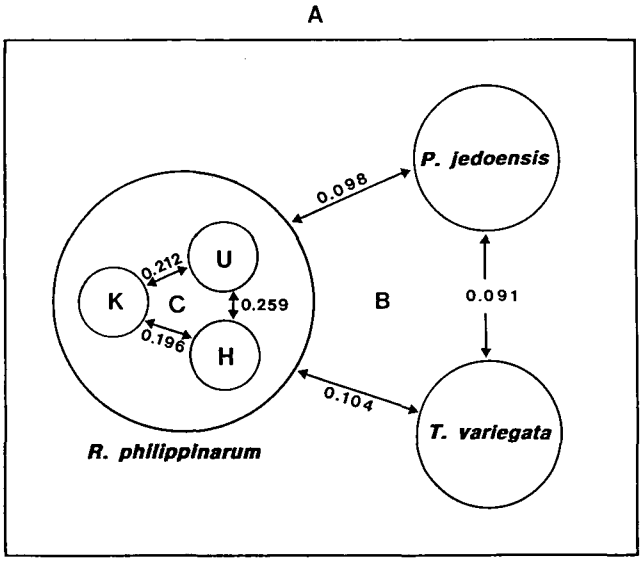

Fig. 7. Genetic similarities among the shortnecked clams and related species. Letter of H is represented as *R. philippinarum* from Hadong. Letter of K is *R. philippinarum* from Kochang. Letter of U is *R. philippinarum* from Ulsan. Letter of A is represented as the level of shellfish group. Letter of B is represented as the level of interspecies. Letter of C is represented as the level of populations habitated in different regions.

Discussion

The results in this study suggest that there are morphological and genetic differences amongst clam seed originating from different habitats and that these differences are likely to affect the survival rate of the clams when transplanted. The morphological differences observed between the populations of Hadong and Kochang might have resulted from the different environmental conditions (water temperature, the amount of suspended solids, salinity present in these areas). The morphological variation in the shortnecked clam has been found to be great in different local habitational environments (Choi, 1965). However, 13-months after transplantation from seed sites to a farming site, there were no longer any differences in the shapes of the three transplanted populations. Stirling and Okumus (1994) reported that two different populations of mussels *Mytilus edulis* L., with significant morphological differences from each other, became indistinguishable from those of the recipient populations one year after reciprocal transfer. These results suggest that the shape of shellfish is a function of environmental factors. In the present study, clams of Ulsan and Kochang origins showed relatively less differences in morphology than that of Hadong origin. However, the lowest survival rate occurred in clams of Kochang origin, while clams of Hadong and Ulsan origins showed relatively high survival. Given these results, the morphological difference alone can not be an absolute criterion to choose transplantable clam seed. Thus, it would be meaningful to look into genetic similarities at a DNA level between the populations from different seed-production sites.

The RAPD technique has been successfully subjected to fish (Bardakci and Skibinski, 1994), bivalves (Patwary et al., 1994) and lobster (Harding, et al., 1997) to study intra or interspecific differences at a DNA level. In the present study, the RAPD analysis showed low genetic similarities between *R. philippinarum* and *T. variegata*, and *P. jedoensis* (0.104 and 0.098), when compared with the similarities among the populations of *R. philippinarum* from three different habitats (0.196 to 0.259). These genetic similarities were consistent with the differences of survival rates in *R. philippinarum* populations. Clams in Kochang showed relatively lower similarities to clams in Hadong (0.196) and Ulsan (0.212) than similarity between clams in Hadong and Ulsan (0.259). Interestingly, the survival rate of 13-months after transplantation for the group originating from Kochang was the lowest at 41.7% as compared with those from Hadong at 69.4% and Ulsan at 63.8%. These consistent differences of Kochang population to others are likely to be related to environmental factors of habitats. As shown in Table 1, Kochang has different environments compared to the other habitats, Hadong and Ulsan, and a farming site. Gartner-Kepkey et al. (1980, 1983) described genetic differences between samples taken through embayments, as well as between different geographic areas, in some mussel populations in Nova Scotia, Canada. The differences were attributed to habitat-specific differences in temperature and salinity. The difference observed in the population of clams from Kochang may be related to having the greatest fluctuations in ambient water temperature and a lower salinity. These results support the hypothesis that mortality is correlated with the geographic source of seed (Sephton, 1991).

Taken together, it can be suggested that a RAPD analysis for the genetic similarity is recommendable in choosing a decent population as seed, which would be genetically similar and capable of being transplanted, whether alone or in combination with various seed-production sites. As one of the practical applications in population identification, the RAPD assay is relatively rapid and independent of gene expression in different environments (Goodwin and Annis, 1991). In conclusion, identifying populations by RAPD assay provides a very useful primary test in selecting adequate seed for transplantation to a certain farming area and should be equally beneficial for classifying local isolates of the shellfish, *R. philippinarum*.

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