GENETIC DIVERSITY AND STOCK STRUCTURE OF HANASAKI CRAB *PARALITHODES BREVIPES*

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Taniguchi, N. Genetic diversity and stock structure of Hanasaki crab *Paralithodes brevipes* [Text] / **N. Taniguchi, M. Ikeda** // Water life biology, resources status and condition of inhabitation in Sakhalin-Kuril region and adjoining water areas : Transactions of Sakhalin Scientific Research Institute of Fisheries and Oceanography. – Yuzhno-Sakhalinsk : SakhNIRO, 2011. – Vol. 12. – P. 191–202.

Tabl. – 9, fig. – 4, ref. – 2.

1. OBJECTIVE

The catch of Hanasaki crab, *Paralithodes brevipes*, in the coast of Hokkaido has been decreasing. To ensure resource sustainability and to avoid population crashes, the scientific data are required for fisheries management. A proper knowledge of the genetic structure and diversity of Hanasaki crab populations is an important task to aid fishery managers to define fishery stocks and to analyze data and models in terms of the geographical and ecological limits of the populations. We have to answer the questions such as "Have the populations of Hanasaki crab on the coast of Hokkaido (especially, around the Nemuro Peninsula) already reached to genetically threatened level?" and "Are the populations on the coast of Nemuro Peninsula genetically connected with other populations in Russia?" to contribute developing the effective international strategy for sustainable use and conservation of Hanasaki crab. The aims of this study can be summarized as follow:

1) Developing of the DNA markers (nuclear microsatellite and mitochondrial DNA) to evaluate genetic variability and population structure of Hanasaki crab;

2) Identification of the genetically differentiated stocks and to estimate the level of genetic differentiation among them;

3) Developing of new DNA extraction method without damaging the crabs, and without affecting their commercial value for future genetic assessment.

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2. RESULTS

1) Development of nuclear microsatellite and mitochondrial DNA markers

(1) Microsatellite DNA

The microsatellite DNA (simple sequence repeats of 1-6 base pairs of DNA) always exists in nuclear DNA of organisms. However, in order to develop the primer sets for PCR (Polymerase Chain Reaction) amplify such regions, we have to find those DNA regions in each organism. We prepared the genomic library of Hanasaki crab, and then eight microsatellite regions (loci) considered useful markers, were identified. The primers for PCR amplification of these loci were successfully designed as shown in **Table 1.**

Table 1

Characterization of microsatellite DNA loci in Hanasaki crab

Locus	Repeat motif	Primer seaquences (5'-3')	Allele size (bp)	Anealing temp. (°C)
Pabre-3	$(AAT)_{18}$	F: CCTTATTGGGCTATCCTTAG R: TTCTGTTGCTGGCTGAT	202	53
Pabre-7	$(AAT)_{18}$	F: CAGCCAAATACACCAAAC R: AAATAAAAGAAAAGGGACAA	243	52
Pabre-13	$(CA)_{42}$	F: GGACACCAGGGAATAAA R: AAGTGAAGCGTTGTAATCTGA	147	54
Pabre-17	$(GT)_{26}$	F: CCTCCACAACCCTCCAAAAC R: AACTCAGGAAGCGGGACAATAC	327	48
Pabre-18	$(GA)_{35}+(GATA)_6GATG(GATA)_3$ $GATG(GATA)_3$	F: CCTCGTCCCTCTAGTAGGTAA R: AGGTCCCATACATACATTTTG	354	48
Pabre-22	$(ATT)_4+(ATT)_{18}$	F: TACACCAGCGAGTCAAATAAAT R: TCCCTCCCGATAGCATAA	301	48
Pabre-23	$(GT)_{23}$	F: TAACACCCTCCATACAGTCCAA R: CCAACGTGCGCTTTCTAA	216	48
Pabre-24	$(AG)_7(TG)_9(AG)_{14}$	F: AGAAAGAATAACAAGGCAAATC R: GCTCGGGTGAAGGTATC	144	48

F: Forward primer; R: Reverse primer.

For the validation of microsatellite DNA as a genetic marker, the mode of inheritance of microsatellite DNA alleles was confirmed using dams and its offspring. At first, we estimated the paternity for the three families (dam and the offspring) by comparing their allelic composition at all eight loci. The estimated paternity was always single for each pair. This result suggests that the larvae of Hanasaki crab hatched from a dam are produced by pair mating with single sire. The observed ratios of genotype segregation in each family were coincident with the ratios under Mendelian's low, except for *Pabre-13* locus showing null alleles. Thus the seven loci (*Pabre-3*, *-7*, *-17*, *-18*, *-22*, *-23*, and *-24*) would be useful to evaluate genetic diversity of Hanasaki crab populations.

(2) Mitochondrial DNA

In order to verify the relevance of the population structure identified by microsatellite DNA analysis, it is necessary to use not only the nuclear microsatellite DNA as the nuclear marker but also extra nuclear mitochondrial (mt) DNA. The sequence of mitochondrial cytochrome *c* oxidase subunit I gene (CO I) is frequently used for population genetic analyses in marine invertebrate organisms, but the results of examination on this sequence showed low variability in the case of Hanasaki

crab. The control region (called the AT-rich region, especially in Arthropod) is non-coding region and the mutation is seem to be neutral for natural selection, so we can predict that the region contain many polymorphic site being informative to resolve the population structure. However, the sequences of the region in Lithodidae including Hanasaki crab have not been reported. Hence we first aimed to identify the control region in mitochondrial genome of Hanasaki crab using bio-informatics method based on the sequences hermit crab, *Pagurus longicarpus* deposited in the DNA data bank, and then evaluated the property as genetic marker.

The primers were designed based on the sequences of Cytochrome *b* oxidase (Cyt *b*) and NADH dehydrogenase subunit I (ND I) genes presumably located on either side of the AT-rich region, and we could obtain the approximately 2.5kbp fragment by PCR method. The all sequences and genes arrangement of this fragment were determined by primer walking, homology search, and prediction for the secondary structure of tRNA. The size of presumed control region was 1249bp. Four ORF (Open Reading Frame) were predicted within the region. But any genes deposited in DNA data bank did not show homology to these ORFs and the ratio of adenine (A) and thymine (T) in the region was 73% being average value in other arthropod animals, indicating that it is reasonable to regard as the AT-rich region.

To assess polymorphism of the AT-rich region, 359bp of 5'-end of the region in 34 individual from Goyo-mai were sequenced and aligned. Totally 30 haplotypes were detected. Substitutions and insertion/deletion (indel) occurred at 25 and six sites, respectively, within 30 polymorphic sites (**Table 2**). The measures of genetic variability were approximately three times in number of haplotypes, 1.5 times in haplotype diversity (*h*), and five times in nucleotide diversity (π) higher than those of CO I. This result indicates that the sequences of the control region are quite useful to elucidate genetic diversity and population structure of Hanasaki crab.

Table 2

Comparison of genetic variability between two mtDNA regions in Goyomai population

Region	No. of sites	Sample size	No. variable sites	No. of haplotypes	Haplotype diversity (*h*)	Nucleotide diversity (π)
CO I	332	50	9	11	0.684	0.003
AT-rich	358	34	30	30	0.993	0.017

2) Stock structure

(1) Genetic variability and differentiation in Nemuro, South Kurilsky, Sakhalin and Kamchatka populations

Figure 1 shows a map of the sample collection sites. The each 48–50 adult samples were caught in Ochi-ishi, Goyo-mai, South-Kurilsky, Sakhalin and Kamchatka during June 2003 and November 2005. The DNA was extracted from muscle of pleopods of each crab. PCR products at seven microsatellite loci, *Pabre-3, -7, -17, -18, -22, -23*, and *-24*, were size fractionated on denaturing polyacrylamide gels, and allele size were determined with the ABI 377 automated DNA sequencer. Mitochondrial DNA AT-rich regions were also amplified and sequenced.

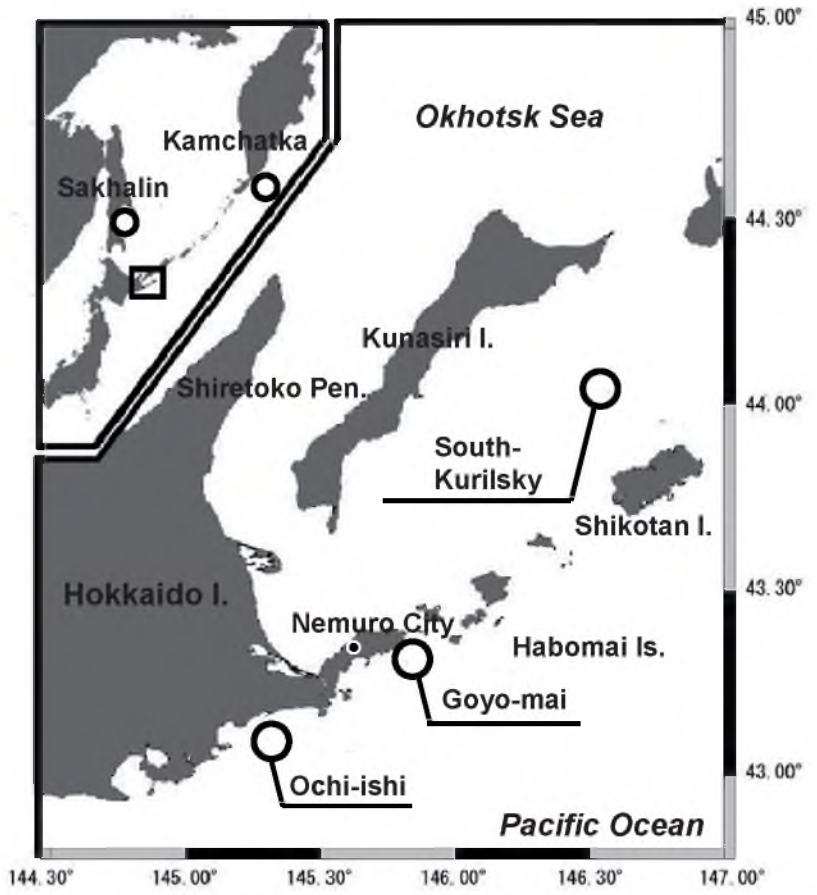

Fig. 1

The values of genetic variability (mean number of alleles, effective number of alleles, and expected heterozygosity) of seven microsatellite DNA loci in each population are shown in **Table 3.** The populations of coast of Nemuro Peninsula (Ochi-ishi and Goyo-mai) and South-Kurilsky showed high genetic variability similarly. However, the values of *Pabre-17* and *-23* in Kamchatka and Sakhalin showed remarkably lower than those of Nemuro Peninsula and South-Kurilsky populations. The mtDNA variability in each population is shown in **Table 4.** The populations in the coastal region of Nemuro Peninsula (Ochi-ishi and Goyo-mai) and South-Kurilsky showed relatively higher variability, except for nucleotide diversity (π), than those of Sakhalin and Kamchatka. These results suggest that genetic variability of the populations around Nemuro Peninsula is relatively higher than those of another populations and they are not fall into genetically threatened condition.

Table 3

Microsatellite DNA variability in four populations of Hanasaki crab

	Locus							Average
	Pabre-3	*-7*	*-17*	*-18*	*-22*	*-23*	*-24*	
Ochi-ishi								
No. of alleles	10	15	18	34	22	11	18	18.3
he	0.819	0.858	0.723	0.968	0.919	0.714	0.856	0.837
Goyo-mai								
No. of alleles	9	10	22	34	24	14	16	18.4
he	0.837	0.826	0.777	0.964	0.919	0.741	0.859	0.846
South-Kurilsky								
No. of alleles	13	16	18	34	21	11	19	18.9
he	0.871	0.857	0.779	0.971	0.907	0.725	0.862	0.853
Sakhalin								
No. of alleles	10	12	18	26	15	6	16	14.7
he	0.877	0.896	0.886	0.937	0.898	0.594	0.848	0.848

Table 4

MtDNA variability in five populations of Hanasaki crab

Population	Sample size	No. of haplotypes	Effective no. of haplotypes	Haplotype diversity (h)	Nucleotide diversity (π)
Ochi-ishi	38	26	17.6	0.969	0.017
Goyo-mai	34	30	27.5	0.993	0.017
South Kurilsky	34	29	22.4	0.986	0.016
Sakhalin	32	13	5.5	0.845	0.017
Kamchatka	42	21	8.5	0.904	0.017

The results of AMOVA (Analysis of Molecular Variance) to evaluate global genetic differentiation showed that 0.7% (microsatellite DNA) and 2.94% (mtDNA) of total genetic variability are explained by the difference among populations. Both values were significantly larger than 0. Pair-wise *F*ST analysis showed that global genetic differentiation among populations mainly caused by the differentiation between Sakhalin and Nemuro/South-Kurilsky or between Kamchatka and Nemuro/South-Kurilsky (**Table 5**). All values between Nemuro and South-Kurilsky populations were not significantly larger than 0, indicating that these populations are genetically homogeneous and can be regarded as single stock. Thus the genetically different stocks of Hanasaki crabs in these sea areas surveyed are the following three: Sakhalin, Kamchatka, and Nemuro/South-Kurilsky.

Table 5

Pair-wise FST values among five populations of Hanasaki crab using microsatellite and mitochondrial (mt) DNA markers

	Ochi-ishi	Goyo-mai	South Kurilsky	Kamchatka	Sakhalin
Ochi-ishi	–	–0,015	–0,009	0,015	0.096*
Goyo-mai	–0,009	–	–0,009	0,012	0.085*
South Kurilsky	–0,001	–0,001	–	0,016	0.100*
Kamchatka	0,004	0.008*	0.007*	–	0,017
Sakhalin	0.015*	0.015*	0.016*	0.014*	–

Above diagonal: mtDNA; Below diagonal: microsatellite DNA.

** Significantly larger than F ST=0 (after Bonferroni correction).*

(2) DNA analysis of the populations on the coasts of northern Shiretoko Peninsula and the eastern Erimo Cape

Especially, remarkable genetic differentiation was detected between Nemuro/South-Kurilsky and Sakhalin in both DNA markers, and we can estimate no larval migration between these areas. However, the ocean current simulation using POM (Princeton Ocean Model) suggested that the larvae hatched on the south coast of Sakhalin are able to reach to the north coast of Shiretoko Peninsula in Hokkaido in 60 days after their hatching (**Fujita et al., 2007**). If this suggestion is propriety, the population on the north coast of Shiretoko Peninsula is genetically closed or homogeneous to the population on the Sakhalin, and the restriction of larval migration between the Sakhalin/Shiretoko and Nemuro/South-Krilsky would be occurred the Nemuro Channel. Thus analysis for the population of north coast of Shiretoko Peninsula would give us the insight for the boundary between different stocks and show the importance of joint work of the ocean current simulation and DNA analyses to reveal stock structure. Additionally, the simulation study indicated high probability that the larvae hatched on the coast of Nemuro Peninsula reach to the east coast of Erimo Cape being southern limit of the distribution Hanasaki crab. This result suggests that the crabs in the southern limit area belong to the stock of Nemuro/South-Krilsky. Hence the objective of this study was to assess the genetic variability of the populations on the north coast of Shiretoko Peninsula and the east coast of Erimo cape using microsatellite and mitochondrial DNA markers, and we examined genetic relationships among all populations including Nemuro/South-Krilsky, Sakhalin, and Kamchatka.

The 39 and 50 adult crabs were collected from the tidal zones on the north coast of Shiretoko Peninsula (Shari) and the east coast of Erimo Cape (Erimo), respectively, on October 2007 (**Fig. 2**). The DNA was extracted from muscle of pleopods of each crab. PCR products at six microsatellite loci, *Pabre-3*, *Pabre-7*, *Pabre-18*, *Pabre-22*, *Pabre-23*, and *Pabre-24*, were size fractionated on denaturing polyacrylamide gels, and allele size were determined with the ABI 377 automated DNA sequencer. Mitochondrial DNA AT-rich regions were also amplified and sequenced. The genotypic and haplotypic data of the Ochi-ishi, Goyomai, South-Kurilsky, Sakhalin, and Kamchatka obtained in above study were used for analyses.

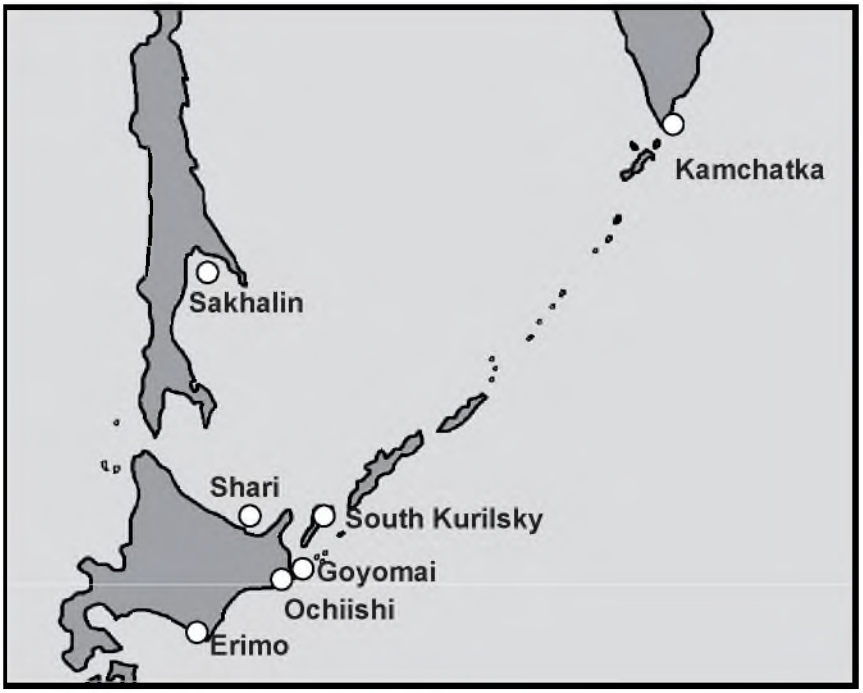

Fig. 2

Genetic variability (number of alleles and heterozygosity) of each population measured by microsatellite DNA is shown in **Table 6.** The level of values at all loci of Eriomo were almost same the populations of Nemuro/South-Kurilsky. But the values at *Pabre-23* of Shari were remarkably lower than those populations and same level to Sakhalin.

The mitochondrial DNA (AT-rich region) variability (No. of haplotypes, effective no of haplotypes, haplotype diversity, and nucleotide diversity) of each putative population are shown in **Table 7.** As well as the results of microsatellites, the values of Erimo were similar to that of Nemuro/South-Kurilsky, and the values of Shari were remarkably lower than Nemuro/South-Kurilsky.

To measure amount of genetic differentiation among/between populations, AMOVA and pair-wise *F*ST analyses were examined in both DNA markers. The result of AMOVA showed that significant genetic differentiation occurs among all putative populations (0.66% and 5.01% in microsatellite and mitochondrial DNA, respectively). Pair-wise *F*ST analysis indicated no significant differentiation between Erimo and Nemuro/South-Kurilsky in both DNA markers, but all values between those populations and Shari were significantly larger than 0. On the other hand, the values between Shari and Kamchatka were not significant (**Table 8**).

Table 6

Microsatellite DNA variability in seven populations of Hanasaki crab

	Locus						Average
	Pabre-3	*-7*	*-18*	*-22*	*-23*	*-24*	
Sakhalin							
No. of alleles	10	12	26	15	6	16	15.3
he	0.877	0.896	0.937	0.898	0.594	0.848	0.845
Kamchatka							
No. of alleles	12	13	37	22	11	16	18.5
he	0.871	0.856	0.971	0.896	0.655	0.851	0.850
South-Kurilsky							
No. of alleles	13	16	34	21	11	19	19.0
he	0.871	0.857	0.971	0.907	0.725	0.862	0.866
Goyo-mai							
No. of alleles	9	10	34	24	14	16	17.8
he	0.837	0.826	0.964	0.919	0.741	0.859	0.858
Ochi-ishi							
No. of alleles	10	15	34	22	11	18	18.3
he	0.819	0.858	0.968	0.919	0.714	0.856	0.856
Erimo							
No. of alleles	14	13	31	20	12	20	18.3
he	0.837	0.821	0.963	0.899	0.717	0.904	0.857
Shari							
No. of alleles	10	12	23	16	6	12	13.2
he	0.851	0.877	0.954	0.900	0.580	0.874	0.839

Table 7

Mitochondrial DNA variability in seven populations of Hanasaki crab

Population	Sample size	No. of haplotypes	Effective no. of haplotypes	Haplotype diversity (h)	Nucleotide diversity (π)
Sakhalin	32	13	5.5	0.845	0.017
Kamchatka	42	21	8.5	0.904	0.017
South Kurilsky	34	29	22.4	0.986	0.016
Goyomai	34	30	27.5	0.993	0.017
Ochiishi	38	26	17.6	0.969	0.017
Erimo	44	35	24.8	0.982	0.019
Shari	39	18	8.0	0.899	0.013

Table 8

Pair-wise FST values among seven populations of Hanasaki crab using microsatellite and mitochondrial (mt) DNA markers

Population	Sakhalin	Kamchatka	South Kurilsky	Goyomai	Ochiishi	Erimo	Shari
Sakhalin	–	0.015	0.112*	0.086*	0.100*	0.075*	–0.001
Kamchatka	0.004	–	0.037*	0.019	0.026*	0.029*	0.030*
South Kurilsky	0.015*	0.006*	–	–0.007	–0.012	–0.002	0.159*
Goyomai	0.014*	0.007*	0.000	–	–0.016	–0.001	0.126*
Ochiishi	0.010*	0.004	0.000	–0.001	–	0.003	0.142*
Erimo	0.014*	0.006*	0.000	0.000	–0.001	–	0.125*
Shari	0.004	0.007*	0.017*	0.016*	0.013*	0.013*	–

Above diagonal: mtDNA; Below diagonal: microsatellite DNA.
** Significantly larger than FST=0 (after Bonferroni collection).*

To examine genetic relationship among populations, Nei's genetic distance in microsatellite DNA and nucleotide divergence in mitochondrial DNA among populations were computed and the dendrograms were constructed by Neighbor-joining method. In both dendrograms, Erimo joined to the populations of Nemuro/South-Krilsky, and Shari joined to Sakhalin (**Fig. 3**).

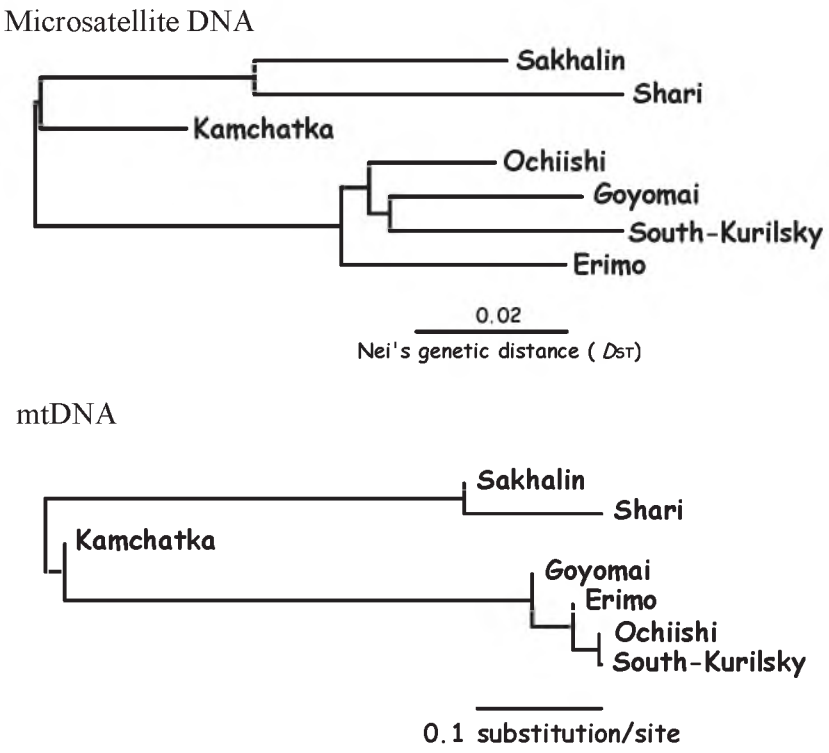

Fig. 3

These results suggest that the populations of Hanasaki crab inhabiting are divided into genetically three stocks, i. e., Sakhalin/Shiretoko Nemuro/South-Krilsky/Erimo, and Kamchatka. The migration of Hanasaki crab including their larvae between Sakhalin/Shiretoko and Nemuro/South-Kurilsky/Erimo stocks would be restricted by Nemuro Channel as predicted by the POM simulation (**Fujita et al., 2007**).

3) Development of new DNA extraction method without damage to the individual's fitness and commercial value

The DNA of large crustacean species, including Hanasaki crab, has been extracted from the muscle of amputated pleopods. Amputated leg would be regenerated after several molting, but individual's fitness might be depressed, which is fear the abundance decline. Additionally, the commercial value of legless individual is low.

Kudo (2007) reported that cutting the side of carapace would be useful for new tag system to evaluate seeds releasing effectiveness to the wild environment. If a piece of carapace is available for population genetic analysis, we will construct new DNA analysis system without damage to the individual's fitness and to commercial value of Hanasaki crab. The genetic data obtained from a piece of carapace would be direct evidence whether the captured individual is artificial seed or not. The purpose of this study is development of new DNA extraction system using hard tissues including a piece of carapace of the crab for examining individual genotype.

Six fresh adult crabs kept at –20°C were used for this examination. The side of carapace of each individual was removed as described by **Kudo (2007)**. A piece of carapace (about 5 mm square) was separated into three parts, shell, inner membrane, and soft rim. Besides three parts of tissues mentioned above, muscles (10mg) and the tip (5mm in length) of pleopod (toe) were used for DNA extraction. Method of TENES-Urea/Phenol/Chlorofolm was used for DNA extraction. The DNA extracted was dissolved by 50μl of TE buffer and measured absorbance for 260nm and 280nm. Five microsatellite DNA loci (*Pabre-3*, *Pabre-18*, *Pabre-22*, *Pabre-23* and *Pabre-24*) and two mitochondrial DNA regions (2.0kbp of AT-rich region and 0.5kbp of CO I gene) were amplified.

The amount of DNA of all samples from inner membrane showed above 200ng/μl and the values were greater than those of muscle. Although small amount (about 10ng/μl), DNA extraction was succeed in the case of shell and tip of pleopods. The ratio of 260nm to 280nm indicating degree of purity of all samples from membrane showed 1.8–2.0 same as the case of muscle, meaning high purity. However in the case of other tissue the values were greatly fluctuated.

The results of PCR amplification to the AT-rich region of mitochondrial DNA are shown in **Figure 4.** The amount of PCR products from inner membrane and rim were enough for sequencing analysis as well as muscle. But in the case of shell and toe, the amount of PCR products was slight, while several samples were failed to amplify. The results of CO I gene were same to AT-rich region. The DNA from inner membrane was applicable to the all microsatellite loci examined and amplified allele bands were easy to genotype for each individual (**Table 9**).

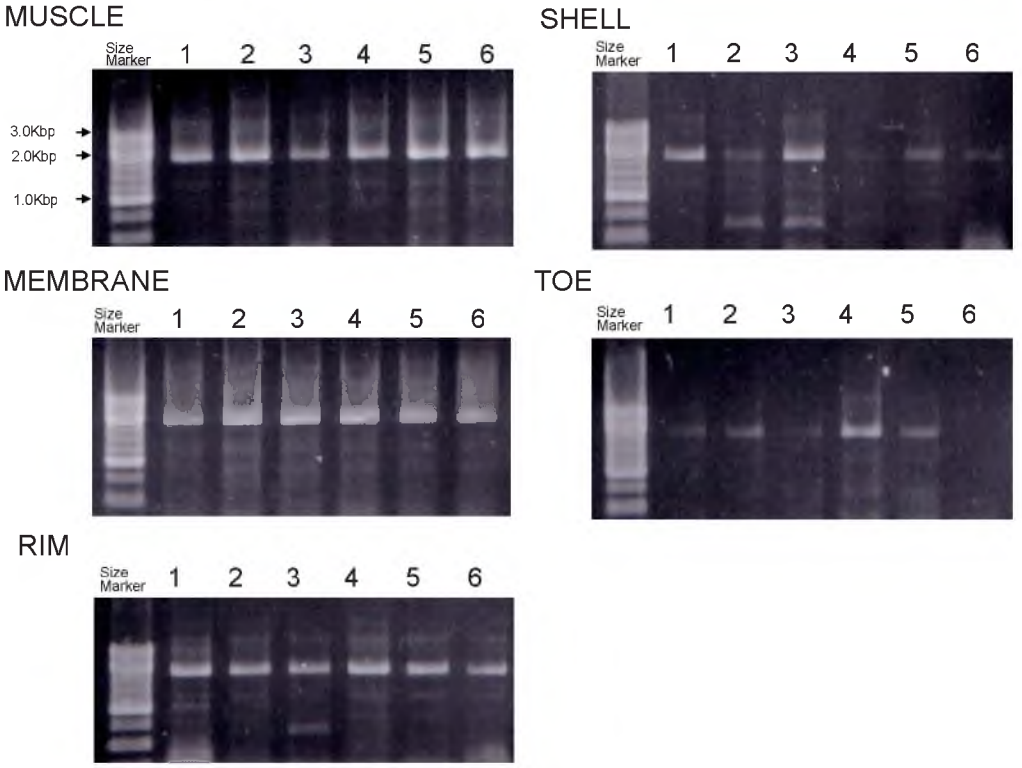

Fig. 4

Table 9

Microsatellite DNA genotypes amplified from DNA extracted from five exoskeleton parts of Hanasaki crab

Locus	Sample No.	Genotype				
		Muscle	Membrane	Rim	Shell	Toe
Pabre-3	1	191/191	191/191	191/191	191/191	FA
	2	206/200	206/200	206/200	SU	SU
	3	203/185	203/185	203/185	203/185	SU
	4	203/203	203/203	203/203	203/203	SU
	5	200/182	200/182	200/182	200/182	SU
	6	191/185	191/185	191/185	191/185	SU
Pabre-18	1	438/364	438/364	438/364	FA	FA
	2	392/386	392/386	SU	FA	FA
	3	400/380	400/380	400/380	400/380	FA
	4	392/386	392/386	392/386	392/386	FA
	5	344/344	344/344	344/344	SU	FA
	6	392/386	392/386	392/386	SU	FA
Pabre-22	1	306/295	306/295	306/295	306/295	306/295
	2	302/300	302/300	302/300	302/300	302/300
	3	306/296	306/296	306/296	306/296	306/296
	4	320/300	320/300	320/300	320/300	320/300
	5	302/302	302/302	302/302	302/302	302/302
	6	308/296	308/296	308/296	308/296	308/296

Locus	Sample No.	Genotype				
		Muscle	Membrane	Rim	Shell	Toe
Pabre-23	1	204/202	204/202	204/202	204/202	204/202
	2	204/185	204/185	204/185	204/185	204/185
	3	204/204	204/204	204/204	204/204	204/204
	4	204/202	204/202	204/202	204/202	204/202
	5	225/204	225/204	225/204	225/204	225/204
	6	225/202	225/202	225/202	225/202	FA
Pabre-24	1	159/143	159/143	159/143	159/143	159/143
	2	161/145	161/145	161/145	161/145	161/145
	3	151/145	151/145	151/145	151/145	151/145
	4	147/145	147/145	147/145	147/145	147/145
	5	154/151	154/151	154/151	154/151	154/151
	6	151/147	151/147	151/147	151/147	SU

FA: Fail to amplify; SU: Success to amplify but signals were unreliable.

Consequently, the most superior tissue for DNA extraction without damage to individual's fitness and commercial value is the inner membrane. We would be able to identify the stocked crabs by combined application of the two new tagging systems (carapace clipping and genotyping using the inner membrane), when we evaluate the effectiveness of artificial seed stocking.

ACKNOWLEDGEMENT

We appreciate Utoro, Shari-Daiichi, and Hiroo Fisherman's Associations for their help to collect samples. We thank Mr. M. Ueno and T. Matsumura (Hiroo FA) for giving us useful suggestion to collect samples.

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