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学位論文題目	Comparative study of the rare species barfin flounder (*Verasper moseri*) with its closely related species spotted halibut (*Verasper variegatus*), on the genetic monitoring of the stock enhancement program (希少種マツカワ (*Verasper moseri*) およびその近縁種ホシガレイ (*Verasper variegatus*) の種苗放流事業をめぐる遺伝的モニタリングに関する比較研究)
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論 文 内 容 要 旨

Chapter 1. General introduction. Significance of the genetic monitoring

In the present thesis, the comparative genetic monitoring of the stock enhancement program of two closely related flatfish species of the family Pleuronectidae, the rare barfin flounder, *Verasper moseri*, "matsukawa" in Japanese, and spotted halibut, *V. variegatus*, "hoshigarei" in Japanese, is introduced. Barfin flounder has been reported to be distributed through the northern part of Japan, Hokkaido and Northern Honshu, both Pacific and Japan Sea sides, as well as along the Tatar strait, the Southern Okhotsk Sea and the Kuril Islands; however, in the recent days it has been mainly caught along the east coast of Hokkaido. Barfin flounder fisheries situation has been recently declared to be highly critical, as the amount of natural resources has been drastically reduced since 1975. On the other hand, spotted halibut is distributed along Japan, from north Honshu southward, Peter the Great Bay, the Korean Peninsula, the Yellow Sea, the Gulf of Po-Hai and the East China Sea. However, at the present time spotted halibut fisheries situation is rather unstable, being its catches considerably decreased since 1980 to less than seven tons by year. Both species have special characteristics, as for example fast growth in cold water and high meat quality, which together with its recent scarceness and its consequent high market price made them ideal target species for sea farming in Japan. In 1987, JASFA- Akkeshi station, located in the east coast of Hokkaido, started the stock enhancement program of barfin flounder, since then about one million and a half hatchery-reared juveniles have been released into the natural water at several locations through Hokkaido. Besides, the stock enhancement program of spotted halibut started on 1993, in Fukushima prefecture, since then about 400.000 juveniles and adults have been released into the sea.

However, even though the aim of supportive breeding is to increase the amount of resources in the wild, meaning the total population size, through breeding part of the native population in a captive environment, this process also implies the reduction of the genetic variability even in first generation offspring, due to the small genetic contribution of just a few breeders. Therefore, if the level of genetic variation of the released stock is reduced, the success of the stock enhancement program could be failed, and even more the integrity and future of the wild stock could be threaten through introgressive hybridization of hatchery releases with indigenous populations.

Consequently, in the present study, genetic monitoring of barfin flounder and spotted halibut stock enhancement programs is proposed and developed as a essential measure to carry out a responsible supportive breeding, which includes:

- Genetic evaluation of the wild populations
- Genetic evaluation of the hatchery-offspring to be released and the recaptured stocks
- Assessment of the genetic divergence between wild and hatchery
- Estimation of the factors causing the genetic divergence between wild and hatchery through pedigree tracing analysis

Chapter 2. Development of microsatellite DNA markers as a tool for the monitoring study

In order to carry out the genetic evaluation of both species, a total of seventeen microsatellite DNA markers, eight from barfin flounder and nine from spotted halibut, were developed (Table 1). The number of alleles per locus ranged from 2 to 33 in barfin flounder, and from 2 to 21 in spotted halibut. Expected heterozygosities ranged from 0.57 to 0.98 in barfin flounder, and from 0.42 to 0.91 in spotted halibut. No deviations from Hardy-Weinberg equilibrium were observed in any of the primer sets, or when cross-amplifying between these two species. Therefore, the loci covered a wide range of genetic polymorphism, demonstrating their usefulness for population analysis, kinship evaluation, and stock enhancement monitoring. However, the loci *Vmo17* and *Vva8* exhibited deviations from the Mendelian mode of inheritance and the loci pair *Vmo2-Vmo6* showed significant linkage, therefore these loci should be used carefully.

Chapter 3. Evaluation of the genetic variability in the wild population of barfin flounder

In the recent years, barfin flounder's captures have been very scarce, as well, its distribution has been restricted to the east coast of Hokkaido, therefore the availability of only one population collected from Akkeshi permitted just to examine the genetic

condition of this species in the wild. Microsatellite DNA markers (msDNA) and the control region of the mitochondrial DNA (mtDNA) were used for the genetic evaluation.

As a result, surprisingly, even though barfin flounder is considered to be a rare species, its level of genetic variation inferred from msDNA and mtDNA showed comparable levels to species with high abundance (He=0.86, A=16.7, h=0.922) (Table 2). Consequently, barfin flounder's east Hokkaido population seems to be of remnant nature still showing a large pool of alleles, thus its conservation management is of special need in order to maintain this reservoir of genetic diversity in the wild. However, the occurrence of rare alleles observed at the msDNA and the absence of low frequency haplotypes at the mtDNA could reflect the recent census population size decline.

Chapter 4. Evaluation of the genetic variability and structure in the wild population of spotted halibut

In the case of spotted halibut, the population census size is also low since its captures have been also greatly diminished, however, due to its wide distribution along the Japanese Pacific coast, and around Kyushu, its genetic population structure could be evaluated. For that, samples from Iwate, Fukushima, Ehime and Nagasaki were analysed. MsDNA and mtDNA markers were used for the genetic evaluation.

The level of genetic variability was similar in all four locations, although always lower in Nagasaki. Average allele richness ranged from 7.7 to 10.2, average effective numbers of alleles ranged from 4.5 to 5.4, and expected average heterozygosities ranged from 0.71 to 0.77. No deviations from Hardy-Weinberg expectations were observed over all loci (Table 3). The effective number of alleles was about 50% of the allele richness in all the cases, indicating a high occurrence of rare alleles. On the other hand, low levels of haplotype and nucleotide diversities were observed at Iwate and Fukushima, h=0.60 and 0.62 and π=0.001 and 0.002, respectively, and very low levels of haplotype and nucleotide diversities were observed at Ehime and Nagasaki, h=0.19 and 0.19 and π=0.0003 and 0.0003, respectively (Table 4). Concluding, on average, high genetic variability (He=0.75, A=10) was inferred from msDNA, although not as high as in barfin flounder, but comparable to other species with large population census size, like the Japanese flounder, and the plaice (Table 2). In contrast, low level of genetic variation was

inferred from the mtDNA (h= 0.421, π=0.001), comparable to the endangered ayu (Table 2). This fact, together with the occurrence of high number of rare alleles at the msDNA, reflected the recent population census size decline. Hence, spotted halibut's classification as rare species is recommended.

The genetic population structure analysis revealed the existence of two groups, North and South locations, differentiation which was slightly inferred from the msDNA F_{ST} test (Table 5), and highly supported by the mtDNA F_{ST} test, together with Neighbour-Joining dendrogram based on net nucleotide diversity, and the statistical parsimony haplotype network (Table 5 and Figure 1). These facts suggested that the designation of two specific Management Units (North and South) is needed in the conservation program and future stock enhancement activities of spotted halibut.

Chapter 5. Estimation of *Ne* (effective population size) in barfin flounder recaptured stock by pedigree analysis

The genetic divergence of the "tentative recaptured" stock (collected from Akkeshi and Kushiro fish markets), from the corresponding hatchery broodstock (used to produce the hatchery-juveniles released in Akkeshi bay), and the original wild stock, was evaluated in order to estimate the effectiveness of the stock enhancement program. The "tentative recaptured" stock was the fish captured from the sea, but whose origin, whether wild or recaptured, was unknown till physical and genetic tags were examined. "Tentative recaptured" stocks (Akkeshi and Kushiro), hatchery broodstock, and wild stock, significantly diverged among them based on the F_{ST} test together with Neighbour-Joining dendrogram based on Reynolds's genetic distance (Table 6 and Figure 2). Next, "real recaptured" individuals were identified through pedigree tracing. No wild individuals were found. The *Ne* of the "real recaptured" stock was estimated as 16.6. The factors affecting to the low *Ne* and high divergence between the recaptured stock and its corresponding broodstock were identified to be of hatchery origin, like the large variance in family size of the released hatchery-juveniles and the low number of contributing parents, since the family survivability of the released stock in the wild was rather high.

Chapter 6. Estimation of *Ne* (effective population size) in spotted halibut recaptured stock by pedigree analysis

Similarly, the same procedure performed in barfin flounder was carried out in the evaluation of spotted halibut's "tentative recaptured" stock. The "tentative recaptured" stock, captured at two different times (2002 and 2003-04, from Miyako bay and Miyako fish market, respectively) diverged significantly from the wild captive broodstock used to produce the hatchery-juveniles released in Miyako bay (Table 7 and Figure 3). "Real recaptured" stock was also identified using pedigree tracing. *Ne* of the "real recaptured" stock was estimated as 7.7. The factors affecting to the low *Ne* and high divergence between the recaptured stock and its corresponding broodstock were also suggested to be of hatchery origin, like the large variance in family size of the released hatchery-juveniles and the low number of contributing parents, since the family survivability between both recaptured groups was fairly high.

Chapter 7. Conclusions and recommendations about barfin flounder and spotted halibut stock enhancement programs suggested from the hatchery and wild stock genetic assessment

From the present study it could be concluded that:

i) Barfin flounder's and spotted halibut's wild stocks showed high genetic variability at the msDNA markers comparable to other abundant species, however, the recent population decline could be detected on the high number of rare alleles observed. Moreover, spotted halibut depicted lower level of genetic variability compared to the rare barfin flounder. Thus, taking into consideration its small population census size, its designation as rare species would be recommended.

Spotted halibut population structure could be particularly drawn from the mitochondrial DNA control region analysis. Two groups or probably two different spawning grounds, North and South, were differentiated and present restricted gene flow was detected. The designation of two specific Management Units, North and South, was suggested for the stock enhancement activities and conservation management of this species.

ii) "Genetic tagging" successfully identified the recaptured stock in the wild. Barfin flounder's and spotted halibut's recaptured stocks, which could be called future "pseudo-wild" due to the low population census size, both showed *Ne* far smaller than 50, which is the Minimum Viable Population size recommended for short term fitness preservation of the species. The reason for it was recognized to be the lack of policy for broodstock management, which includes lack of effective breeding designs to enhance *Ne* and lack of offspring equalization.

A proposal for the future broodstock management to minimize loss of genetic variation in the stock enhancement program of spotted halibut and barfin flounder is described in Figure 4. Depending on the candidate broodstock, whether its pedigree is known or unknown, two different procedures should be carried out:

- If the pedigree of the candidate broodstock is unknown, an inter-individual coefficient of relationship should be calculated using molecular markers in order to estimate the degree of relationship among the available broodstock. Afterwards, minimal kinship selection procedures should be carried out in order to select those individuals which are less related with the rest.
- On the other hand, if the pedigree of the candidate broodstock is known, direct cross-breeding between individuals from unrelated families is believed to give better performance than applying minimal kinship selection procedures.

Afterwards, factorial breeding designs should be applied to mate the less related individuals. Factorial breeding designs, although time- and cost- consuming, have demonstrated to maintain genetic diversity in supplemental breeding programs, aspect that is extremely necessary in the case of stock enhancement programs intended for endangered species.

Table 1: Designed microsatellite DNA markers, observed number of alleles per locus, observed (*Ho*) and expected (*He*) heterozygosities, in barfin flounder (BF) and spotted halibut (SH), and DDJB accession numbers

Locus	No. of observed alleles BF	SH	*Ho* BF	*He* BF	DDJB Accession no.
Vmo1	*	8	*	*	AB110616
Vmo2	33	10	0.97	0.98	AB110617
Vmo4	3	3	0.50	0.57	AB110618
Vmo6	9	2	0.84	0.81	AB110619
Vmo7	*	2	*	*	AB110620
Vmo8	9	6	0.81	0.75	AB110621
Vmo13	26	12	0.94	0.95	AB110622
Vmo17	27	17	0.84	0.97	AB110623

Locus	No. of observed alleles SH	BF	*Ho* SH	*He* SH	DDJB Accession no.
Vva6	8	6	0.84	0.76	AB110624
Vva7	6	24	0.75	0.71	AB110625
Vva8	9	5	0.81	0.79	AB110626
Vva9	6	1	0.75	0.67	AB110627
Vva11	11	3	0.72	0.73	AB110628
Vva13	21	21	0.79	0.91	AB110629
Vva14	11	2	0.81	0.83	AB110630
Vva16	5	7	0.41	0.42	AB110631
Vva18	8	2	0.59	0.66	AB110632

Table 2. Genetic variability at 6 species: barfin flounder, spotted halibut, Japanese flounder, plaice, red sea bream, and endangered Ayu. Number of alleles (*A*), expected heterozygosity (*He*), haplotype and nucleotide diversities are indicated for each species

	A	*He*	*Haplotype diversity*	*Nucleotide diversity*	*Remarks*
Barfin flounder	16.7	0.86	0.922	0.002	Present study
Spotted halibut	10.0	0.75	0.421	0.001	Present study
Japanese flounder	15.3	0.75	0.998	0.027	*1,2
Plaice	15.0	0.73	0.970	0.028	*3
Red sea bream	23.7	0.86	0.913	0.028	*4,5
Ayu (endangered)	2.4	0.20	0.356	0.001	*6,7

*1: Sekino and Hara (2001) *4: Pérez-Enríquez et al. (1999) *7: Ikeda et al. unpublished
*2: Sekino et al. (2002) *5: Tabata and Taniguchi (2000)
*3: Hoarau et al. 2004 *6: Takagi et al. (1999)

Table 3: Spotted halibut genetic variability depicted by the number of alleles (*A*), allele richness (*An*), effective number of alleles (*Ae*), observed and expected heterozygosities (*Ho* / *He*), and significant deviation from Hardy-Weinberg Exact Test, averaged over all nine msDNA loci

	Iwate	Fukushima	Ehime	Nagasaki
A	11.8	9.9	9.9	8.3
An	10.2	9.9	9.0	7.7
Ae	5.1	5.4	5.3	4.5
Ho / He	0.76 / 0.76	0.72 / 0.77	0.80 / 0.76	0.68 / 0.71

Table 4. Genetic variability of the mtDNA control region of spotted halibut

	Iwate	Fukushima	Ehime	Nagasaki
Haplotypes / n° samples	4 / 29	6 / 25	4 / 30	4 / 30
Polymorphic sites (ts / tv)	4 (4 / 0)	6 (6 / 0)	3 (2 / 1)	3 (3 / 0)
Haplotype diversity	0.603	0.620	0.193	0.193
Nucleotide diversity	0.001	0.002	0.0003	0.0003

Table 5. Multilocus pairwise F_{ST} estimator and associated *P*-value between spotted halibut sampling locations, calculated by msDNA and mtDNA control region

	msDNA	*mtDNA*
Iwate – Fukushima	0.002	-0.020
Iwate – Ehime	0.011*	0.250*
Iwate – Nagasaki	0.027*	0.250*
Fukushima – Ehime	0.012	0.178*
Fukushima - Nagasaki	0.023*	0.178*
Ehime - Nagasaki	0.045*	-0.023

*: *P*-value <0.008, after Bonferroni correction *k*=6

Table 6. Multilocus pairwise F_{ST} estimator and associated *P*-value in brackets, between barfin flounder wild, broodstock, and tentative recaptured Akkeshi and Kushiro

	Wild	Broodstock	Tentative Rec. Akkeshi
Broodstock	0.022 (0.001*)		
Tentative Rec. Akkeshi	0.039 (0.000*)	0.031 (0.000*)	
Tentative Rec. Kushiro	0.043 (0.000*)	0.026 (0.001*)	0.003 (0.202)

*: *P*-value<0.008 (after Bonferroni correction, *k*=6)

Table 7. Multilocus pairwise F_{ST} estimator and associated P-value in brackets, between spotted halibut wild captive broodstock, and tentative recaptured 2002 and 2003-04

	Captive Broodstock	Tentative Rec. 2002
Tentative Rec. 2002	0.019 (0.000*)	
Tentative Rec. 2003-04	0.019 (0.001*)	0.002 (0.217)

*: P-value<0.008 (after Bonferroni correction, k=6)

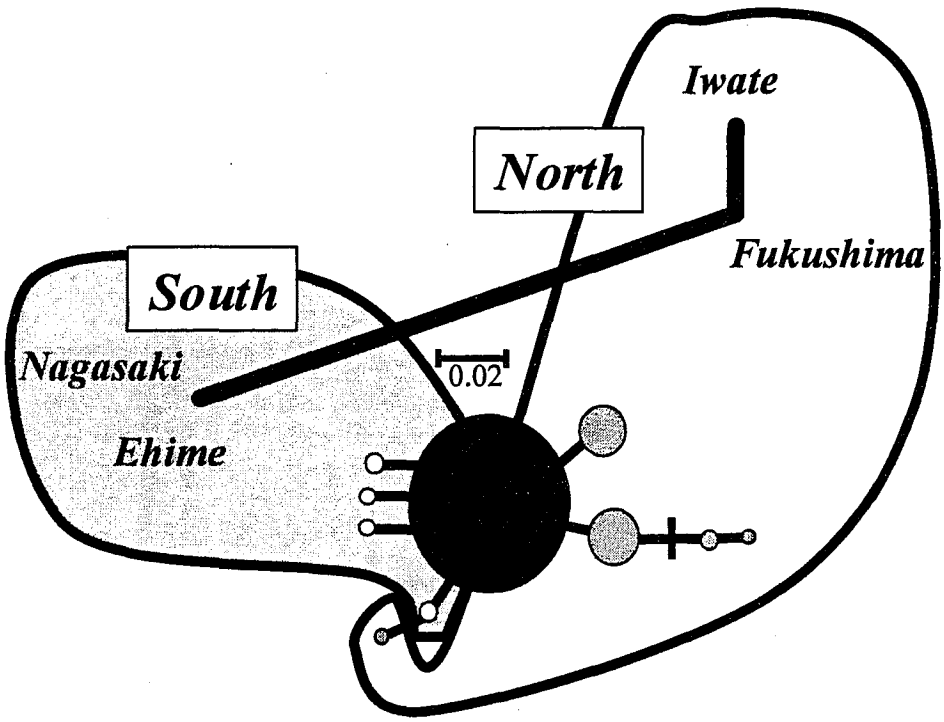

Figure 1. Spotted halibut population structure. Unrooted Neighbor-Joining dendrogram based on Net Nucleotide Diversity as genetic distance. Simultaneously, statistical parsimony network of spotted halibut mtDNA control region haplotypes.

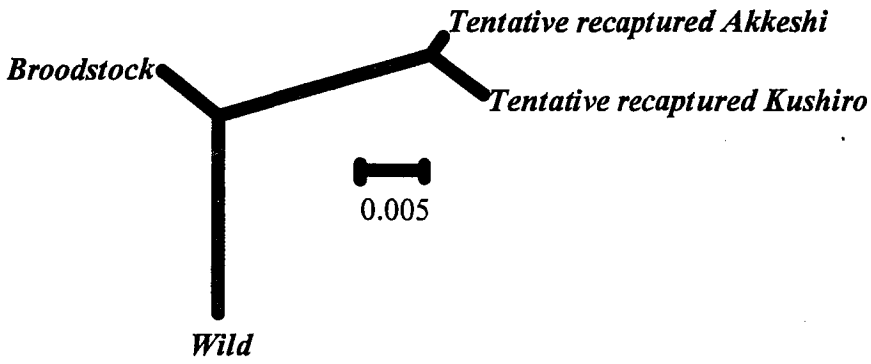

Figure 2. Genetic divergence of barfin flounder wild, broodstock and tentative recaptured stocks. Unrooted Neighbour-Joining tree constructed based on Reynolds's genetic distance.

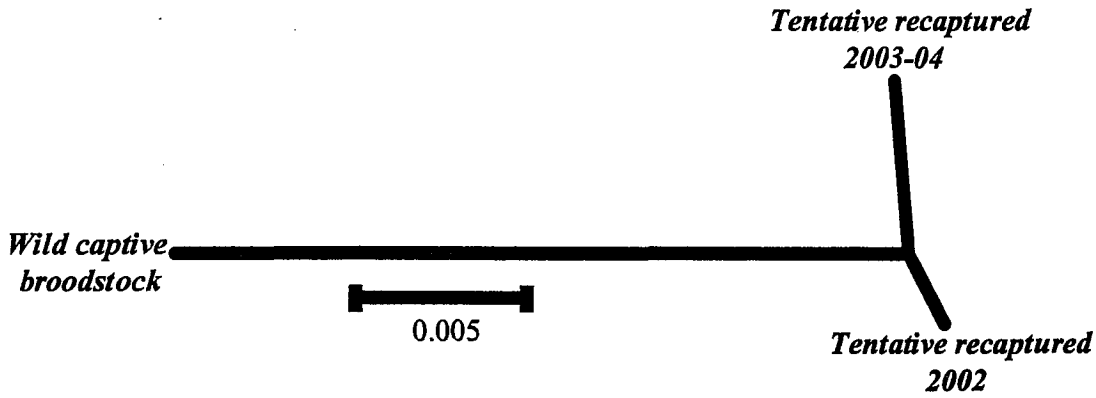

Figure 3. Genetic divergence of spotted halibut wild captive broodstock and tentative recaptured 2002 and 2003-04 stocks. Unrooted Neighbour-Joining tree constructed based on Reynolds's genetic distance.

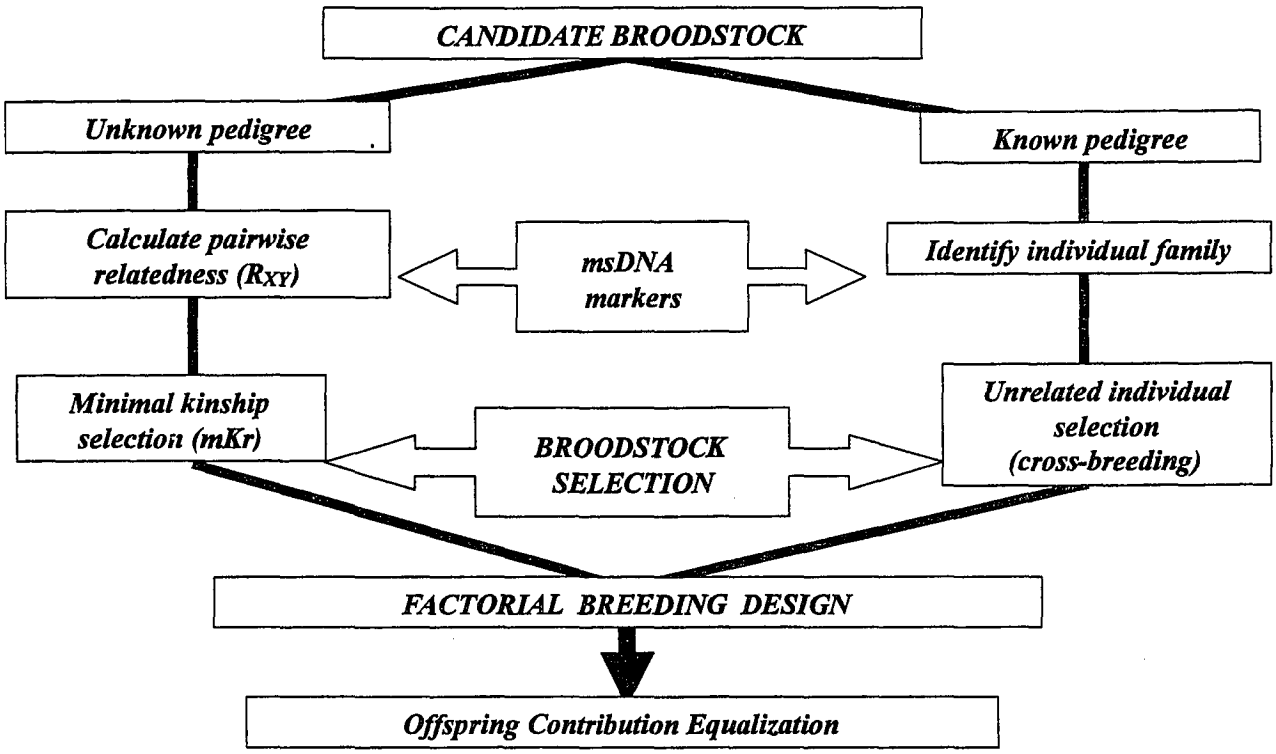

Figure 4. Diagram of the proposal for broodstock management to minimize loss of genetic variation in the stock enhancement program

論文審査結果要旨

マツカワ（*Verasper moseri*）およびその近縁種ホシガレイ（*Verasper variegatus*）は，水産資源として重要性が高い魚種であるが，両種は近年の濫獲により急激に資源が減少し，希少種または絶滅が危惧される状態に至っている。本研究は，資源水準の著しい低下が両種の集団にもたらす遺伝的影響を評価することを目的として，両種集団の遺伝的多様性に関する調査研究を行ったものである。また，両種の資源回復を目指して人工種苗の放流事業がすすめられるなかで，この事業が野生集団におよぼすインパクトを評価・予測し，インパクトの回避手法に関しても合わせて考案することを目指してしたもので，研究の結果は以下の通りである。

1）両種の遺伝的評価を行なうため，遺伝的モニタリング用マイクロサテライトDNAマーカーの開発を試み，マツカワについて8つ，ホシガレイについて9つの合計17マイクロサテライトDNAマーカーを検出した。これらのマーカーは広範囲にわたる遺伝的多型の検出，集団解析，血縁度の評価，放流に関するモニタリングについて利用可能であった。

2）マツカワの野生集団の遺伝的多様性調査をマイクロサテライトDNAマーカー（msDNA）およびミトコンドリアDNA（mtDNA）マーカーのコントロール領域を使用して実施した。その結果，マツカワは希少種であると考えられているが，msDNAとmtDNAから推測された遺伝的変異性は相対的に高く維持されていることが判明した（*He*=0.86，*A*=16.7，*h*=0.922）。

3)ホシガレイ野生集団における遺伝的変異性を評価するため,日本の各地で採集した標本を用いて，msDNAとmtDNAのマーカーを検出した。本種のmsDMAマーカーでは，遺伝的変異性が高かったが（*He*=0.75，*A*=10），マツカワに比べ低い値であった。msDNAおよびmtDNAマーカーによる遺伝的集団構造分析では，日本の北部および南部の明瞭な2群の存在が明らかとなった。ホシガレイの保全計画や将来の種苗放流活動を行なう上でこれら2群は管理ユニットとして設定する必要がある。

4）親子鑑定による集団の有効サイズ（*Ne*）の推定

種苗放流の遺伝的多様性を評価するため，野生集団（親魚候補），放流用人工種苗およびそれらの採捕個体群の遺伝的多様性を比較したところ，これら3標本群間には，統計的に有意の遺伝的分化が確認された。次に，放流種苗の再捕個体の家系判定の結果，検査個体のほぼすべてが再捕されたものと判定され，*Ne*は16.6と推定された。

ホシガレイについても，家系判別の結果，放流種苗の再捕個体の，*Ne*は7.7と推定された。有効集団サイズが小さい原因は種苗生産現場において使用される親魚数が小さいこと，および親魚の寄与率における大きな変動によることが示唆された。

本研究では，マツカワおよびホシガレイの種苗放流事業の問題点を解決するための方法に関して以下の提案を行っている。

1. 北海道東岸に生息する近年のマツカワ集団は遺伝的特性から残存集団であることが推定され，これらは絶滅危惧種として位置づけられるべきである。

2. ホシガレイは，マツカワより遺伝的多様性が低く，希少種あるいは絶滅危惧種としての認定が望まれる。また，日本列島の北部と南部の異なる2つの管理ユニット（Management Units）の設定が必要と考えられた。

3. マツカワとホシガレイの放流種苗の野生化集団（“pseudo-wild”）は，ともに集団の最小保全可能サイズ（Minimum Viable Population）（*Ne*=50）以下であった。このような状況を脱却するためには，有効な非近縁個体選択交配計画および次世代生産における親魚の寄与率の均等化などを計り*Ne*を高めるなど親魚の遺伝的管理を強化することが求められる。

本研究は，資源水準が顕著に低下した水産業上有用なマツカワおよびホシガレイの遺伝的多様性が現状において絶滅危惧種から希少種の水準にあることを解明し，さらに，遺伝的多様性の保全のための親魚管理方策に関して提言を行うと言った成果を上げた。よって,審査員一同は本研究者が博士（農学）の学位を授与するに値すると認定した。