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Review

Aquaculture-oriented genetic researches in abalone: Current status and future perspective

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Basic genetic and cytogenetic information including polymorphic DNA markers, chromosomes and genome size was summarized to get insights into phylogenetic or systematic relationship among abalone species belonging to the genus *Haliotis*. Hybridization, triploidization and genetic mapping were also briefly reviewed as aquaculture-oriented genetic techniques to improve growth and other commercially important traits. Cryopreservation and other biotechnologies potentially applicable on genetic improvement were also briefly mentioned as supporting tools for efficient breeding of abalone strains.

Key words: Chromosome, DNA markers, genetic map, genome size, *Haliotis*, hybrid, polyploidy, selection

INTRODUCTION

Abalone species in the genus *Haliotis* with relatively large body size are distributed in the temperate zone including Japan, Australia, New Zealand, USA, Mexico and South Africa (Elliott, 2000; Hara, 2008a, b). In tropical and subtropical regions, small or 'cocktail size' *Haliotis* species are also popular (Jarayabhand and Paphavasit, 1996). Abalone species are commercially important because they have large edible part (adductor muscle or foot), that is, highly appreciated and reaching good values on the international market (Oakes and Ponte, 1996; Eliott, 2000). Thus, abalone species have been exploited by capture fisheries and natural stocks were overexploited. Such exploitation led to many conservation strategies including re-stocking, hatchery production and release of artificial seedlings. However, such a kind of semi-natural production is not considered so successful. Pacific abalone *Haliotis discus hannai* is an economically important species in Japan and stable propagation techniques have been established and stocking of artificial abalone seedlings has begun since 1970's (Hara, 2008a, b). Recently, more than 20 million seedlings are released every year so as to rehabilitate natural resources in the shallow sub tidal zone, but the annual production still remains approximately 2000 metric tones in Japan (Hara, 2008a, b). Survival and fecundity of released abalone are closely related to successful rehabilitation of wild resources by re-stocking. The above-mentioned example is also in agreement with FAO (2010) regarding the global fisheries production, suggesting that other sustainable production systems are necessary. On the other hand, there are many environmental and socio-economical issues that hinder the sustainable production of coastal wild stock.

Aquaculture of abalone is considered to have big potential as a sea-food farming industry. The slow growth in abalone species is considered the most serious problems (Elliott, 2000). Thus, growth is the most important aquaculture trait in the case of abalone farming, because faster growth to the marketable size reduces the production time and cost as the methods to improve growth and other commercially important traits of aquaculture abalone populations, hybridization, triploidization and genetic mapping have been practiced. Here, we briefly reviewed the current status and future perspective of abalone genetics with special references to aquaculture practices with basic biological information of *Haliotis* species.

BASIC GENETICS AND CYTOGENETICS

About 70 *Haliotis* species are distributed from the sub frigid zone to the tropical zone and about 20 to 30 species

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species have been utilized as fresh, frozen, and processed foods as well as materials for industrial arts (Elliott, 2000; Hara, 2008a, b). Taxonomy and systematics based on morphology are generally difficult and inconclusive in abalone species. Recent molecular genetic approach using hemocyanin sequence suggests the presence of genetically two distinct groups (Streit et al., 2006). The first group includes European *Haliotis tuberculata,* South African *Haliotis midae*, Indo-Pacific *Haliotis asinina,* Japanese *Haliotis diversicolor diversicolor,* and Taiwanese *Haliotis d. supertexta.* The second group includes East Pacific *Haliotis fulgens*, *Haliotis corrugate*, *Haliotis wallanlensis*, *Haliotis rufescens, Haliotis cracherodii*, Japanese *H. discus hannai*, and *Haliotis gigantea*. The other genetic studies indicate that the following two abalone species are possibly the same single one: Northwest Pacific *Haliotis madaka* and *H. d. hannai*, Indo-Pacific *H. diversicolor aquatilis* and *H. d. supertexta*, and Mediterranean *H. tubercularta* and *H. lamellose*, based on molecular data from the sperm lysin gene (Lee and Vacquiere, 1995), 18S rDNA (Naganuma et al., 1998), and 16S rDNA (An et al., 2005). These results roughly provide us with a phylogenetic inter-specific relationship among abalone species in the genus *Haliotis*.

Recent progress in molecular biology makes it possible to develop a large number of polymorphic DNA markers from nuclear genome of marine invertebrates. Microsatellite (MS) markers, as the most convenient co-dominant markers, were isolated and characterized by Sekino and Hara (2001), Sekino et al. (2005) and Li et al. (2002). Hara and Sekino (2005) genetically distinguished two resemble abalones, *H. d. discus* and *H. d. hannai* using MS markers. They also succeeded in showing clear inter-specific relationship among *H. gigantea, H. madaka, H. d. discus* and *H. d. hannai* by MS analyses (Sekino and Hara, 2007a). Luo et al. (2010) identified inter-specific hybrids between *H. d. hannai* and *H. gigantea* using amplified fragment-length polymorphism (AFLP) and MS. Hybrid identification by MS was also reported by Ibarra et al. (2005).

Cytogenetic results categorize abalone species into three groups based on chromosome numbers and karyotypes (Table 1). European species, *H. tuberculata* has 2n=28, while Indo-Pacific (or Tropical and Subtropical) species, *H. varia*, *H. planate*, *H.diversicolor diversicolor*, *H. d. aquatilis*, *H. asinina* and *H. ovina*, show 2n=32. In contrast, North East and North West Pacific species, *H. madaka*, *H. discus hannai*, *H. d. discus*, *H. gigantea*, *H. chracherodii*, *H. rufescens*, *H. fulgens*, and *H. corrugate* show 2n=36. Very recently, it was reported that African Indo-Pacific species, *H. midae*, has 36 chromosomes (Van der Merwe and Roodt-Wilding, 2008; Franchini et al., 2010).

Using the difference in karyotype between the parental species, identification of hybrid abalone was also cytogenetically conducted (Amar-Basulto et al., 2011). At present, however, molecular cytogenetic studies including chromosome banding and fluorescence *in situ* hybridization (FISH) have been poorly developed (Okumura et al., 1999; Gallardo-Escarate et al., 2005) and further detailed investigation is prerequisite to bridge near future linkage map and physical map.

There is a little knowledge about the genome size of abalone. C-value (genome size or cellular DNA content per haploid genome) was reported as approximately 2.14 pg in *H. corrugata*, 1.82 pg in *H. rufescens*, and 1.71 pg in *H. fulgens* (Gallardo-Escarate and Del Rio-Portilla, 2007), 1.43 pg in *H. midae* (Franchini et al., 2010), 1.84 pg in *H. d. hannai* and 1.45 pg in *H. d. aquatilis* (Adachi and Okumura, 2012).

HYBRIDIZATION

In hybrids, heterosis or hybrid vigor can be expected to improve traits such as growth. In Japan, artificial cross-breeding was performed in all the possible combination among *H. d. hannai*, *H. gigantea* and *H. madaka* (Ahmed et al., 2008). All hybridizing combinations gave viable progeny with true hybrid characteristics which were verified by allozyme genotypes and mtDNA restriction fragment length polymorphism (RFLPs); however lower fertilization rates than pure homospecific crosses were recorded. These hybrids exhibited normal or quasinormal gonad development similar to the parental species and they spawned fertile gametes. These hybrid abalones were reported to produce viable progeny by hybrid-hybrid F2 crosses and back crosses. In Mexican abalones, genetic certification was made on putative *H. fulgens* x *H. rufescens* hybrid abalone individuals by allozyme and microsatellite DNA analyses and then gonad development was examined in ‘true’ hybrid individuals (Ibarra et al., 2005). These hybrid males exhibited partial or total sterility because they showed development of spermatocytes and very few spermatozoa, but hybrid females showed vitellogenic or previtellogenic oocytes. These results suggest that fertile inter-specific hybrid abalone should arise from the cross-breeding between closely related species such as *H. discus discus - H. d. hannai - H. madaka - H. gigantea* combinations, and semi-fertile (male-sterile) hybrid abalones from *H. fulgens - H. rufescens* combination. However, reproductive capacity is not fully understood for hybrids between remotely related species due to the shortage of hybrid studies.

On the other hand, Koike et al. (1988) reported that growth rates of F1 hybrids (*H. madaka* female x *H. gigantea* male, *H. gigantea* female x *H. madaka* male, *H. gigantea* female x *H. discus* male, and *H. madaka* female x *H. discus* male) were better than their parental species in 6-month-old juvenile stages. They also reported that daily feeding rates and monthly growth rates were superior in the hybrid *H. madaka* female x *H. gigantean* male. The outperformance in growth was also reported in *H. gigantea* female x *H. discus discus* male hybrid group

Table 1. Diploid chromosome numbers and karyotypes reported in abalone species.

Distribution	Chromosome number	Karyotype						NOR or rDNA	Reference
***Haliotis* Species**	**2n**	**M**	**M/SM**	**SM**	**SM/ST**	**ST**	**T**		
European									
H. tuberculata	28	16		12					Arai and Wilkins (1986)
	28a								Colombera and Tagliaferri (1983)
H. lamellose	28a								Colombera and Tagliaferri (1983)
Indo-Pacific									
H. varia	32	26		6					Nakamura (1985)
	32	18		12	2				Arai et al. (1988)
	32	16		16					Jarayabhand et al. (1998)
H. planata	32	18		12	2				Arai et al. (1988)
H. diversicolor	32	16		14		2			Arai et al. (1988)
H. d. aquatilis	32	16		10	4	2			Nakamura (1985)
H. asinina	32	20		12					Jarayabhand et al. (1998)
H. ovina	32	18		12			2		Jarayabhand et al. (1998)
H. midae	36								Van der Merwe and Roodt-Wilding (2008)
	36	6	1	9		2			Franchini et al. (2010)
Pacific Northwest									
H. discus discus	36	20		16					Arai et al. (1982)
	36	20		16					Miyaki et al. (1997)
H. d. hannai	36	20		16					Arai et al. (1982)
	36	22		14					Wang et al. (1988)
	36	22		14				*	Okumura et al. (1999)
H. madaka	36	20		16					Miyaki et al. (1999)
H. gigantea	36a								Nakamura (1986)
	36	20		16					Miyaki et al. (1997)
Pacific Northeast									
H. cracherodii	36	16		16		4			Minkler (1977)
	36	16		16		4			Gallardo-Escarate and Del Rio-Portilla (2007)
H. fulgens	36	20	6	8	2				Hernandez-Ibarra et al. (2004)
	36	16		16		4		**	Gallardo-Escarate et al. (2005)
H. corrugata	36	20		14		2		***	Gallardo-Escarate et al. (2005)
	36	20		14		2			Gallardo-Escarate and Del Rio-Portilla (2007)
H. rufescens	36	16	12	6	2				Gallardo-Escarate and Del Rio-Portilla (2007)
	36	16	2	16	2				Gallardo-Escarate et al. (2004)
	36	16		18		2			Gallardo-Escarate and Del Rio-Portilla (2007)

[a]Diploid chromosome number was estimated from haploid number in meiotic cells; *Ag-NOR number 5 and 6 pairs; **18S-5.8S-28S rDNA located at the terminal region of the long arms of two pairs of SM chromosomes (number 4 and 11 pairs); ***18S-5.8S-28S rDNA located at the terminal region of the long arms of two pairs of SM chromosomes (number 2 and 4 pairs).

Table 2. Summary of triploidization treatments and viability of resultant larvae in abalone based on Okumura (2006).

Haliotis species	Treatment*	Viability of larvae	Reference
H. discus hannai	CS, HS, HPS	Survival 60-90%	Arai et al. (1986)
	CS, CB	Hatch 33-89%	Sun et al. (1993)
	CS	Survival 3-52%	Hasekura (1993)
	HS	Survival 1-64%	Hasekura (1993)
	CB	Survival 12-100%	Hasekura (1993)
	HS+Caffeine	Survival 1-74%	Hasekura (1993)
	Caffeine	Normal 18-75%	Okumura et al. (1996a)
	Caffeine	Normal 31-94%	Okumura et al. (2007)
H. diversicolor diversicolor	CS	Normal 62-74%	Kudo et al. (1991)
H. d. supertexta	CS	Survival 12-16%	Yang et al. (1997)
	6-DMAP	Normal 50%	Yan et al. (2001)
H. d. aquatilis	6-DMAP	Survival 50-55%	Yan and Chen (2002)
H. midae	CB	Survival 12-16%	Stepto and Cook(1998)
H. asinina	6-DMAP	Normal 78-83%	Norris and Preston (2003)
H. rufescens	CB	Survival 1-55%	Maldonado et al. (2001)
H. rubra	6-DMAP, CB	Survival 30-55%	Liu et al. (2004)
H. laevigata	6-DMAP	Survival 17%	Dunstan et al. (2007)
	CB	Hatch 18-57%	Li et al. (2007)

*CS, Cold shock; HS, heat shock; HPS, hydropressure shock; CB, cytochlasin B; 6-DMAP, 6-dimethylaminopurine.

by Miyaki et al. (1995) and they found that survival rate was lower than parental *H. gigantea* throughout the experimental period (341 days), but the growth of hybrid was better than the parental species in the period from the end of October to March. Viable hybrids were also produced by cross-breeding between *H. d. hannai* female and *Haliotis kamtschatkana* male, despite the very low fertilization success (Hoshikawa et al., 1998). The hybrid abalone outperformed in growth than parental species at 18°C, while at low temperature condition, about 8°C hybrids outperformed than *H. d. hannai*, but not *H. kamtschatkana.* The above mentioned studies concluded that some inter-specific hybrid abalone show increased growth than parental species and also suggest that inter-specific hybridization is a potential means to improve growth traits of aquaculture strains.

TRIPLOIDIZATION

There are relatively large numbers of triploid studies in aquatic invertebrates (Elliott 2000; Okumura 2006; Pifferrer et al., 2009) since the first pioneer trial that was done by Arai et al. (1986). Table 2 summarizes triploidization methods and approximate survival of larvae after treatments in abalone examined species. To induce triploidy, the release of the first polar body or the second polar body was inhibited by optimum dose (intensity and duration of treatment) of physical (cold-, heat- or hydrostatic pressure shock) or chemical (cytocharasin B (CB), 6-dimethylaminopurine (6-DMAP), or caffeine) treatments at the optimum starting timing. Precise temperature regulation apparatus such as “water-bath” is required for temperature treatments (cold or heat shock) of the large quantity of fertilized eggs and very expensive “French Press” is required for hydrostatic pressure treatment. Mass treatment is easier when the fertilized eggs are soaked into the chemical solution. But, CB and 6-DMAP are required to consider the risk-control for environment and workers in hatchery and are very expensive when cost-performance is estimated. For chemical treatment, the caffeine is considered the best because it is a natural product and recognized as safe food-additive and frequently the caffeine treatment achieves 100% triploidization (Okumura et al., 2007). Moreover, it is inexpensive if compared with CB and 6-DMAP as emphasized by Okumura (2006) who calculated that price required for the effective triploidization by caffeine is approximately 1/130 to CB and 1/50 to 6-DMAP treatment.

Generally, due to the side effect of the treatment, high mortalities were recorded in the stages just after the treatment and during larval stages, but later, survival rates become more stable (Okumura 2006). For determination of ploidy status of treated animal, chromosome observation was conducted in the early studies (Arai et al., 1986; Kudo et al., 1991; Okumura, 1991; Okumura et al., 1996a and b), but flow-cytometrical analysis is now a major tool for rapid ploidy determination by automated measurements of nuclear DNA contents of samples (Norris and Preston, 2003; Liu et al., 2004; Li et al., 2007; Okumura et al., 2007). Flow cytometry makes it possible to determine ploidy status of a small piece of tissue sample and individual veliger larva or early juvenile within a relatively short time (Eguchi et al., 2003). Diverse

results have been obtained about gonad development and reproductive capacity of triploid abalone. Hasekura (1993) found the spawning in 4-year-old triploid *H. d. hannai* females and obtained fertilized eggs at high percentages, but they developed abnormally. On the contrary, he did not detect any sperm from triploid males. In small size abalone *H. diversicolor diversicolor,* 2-year-old triploid females had mature oocytes, but the males exhibited no spermatozoa (Kudo et al., 1994). In triploid *H. rubra,* more abnormal gonadal development and gametogenesis were reported (Liu et al., 2009). Triploid females externally lacked an ovary, but they had only a thin layer of oogonia in microscopic level. On the contrary, triploid males showed gonadal development, but the spermatogenesis was arrested at spermatocytes.

Growth is the most attractive trait in abalone farming, because slow growth in abalone is always a serious problem that hinders the sound development of aquaculture. Thus, the estimation of performance has been concentrated to growth in most triploid abalone papers. In *H. d. hannai* and *H. d discus*, triploid significantly outperform in shell length and body weight than diploid control in the age from 1 to 5-year-old (Hasekura 1993; Sun et al., 1993; Chen et al., 2002). Similar outperformance in growth traits of adult abalones was also reported in *H. diversicolor aquatilis* (Yan and Chen 2002), *H. rubra* (Liu et al., 2004) and *H. laevigata* (Dunstan et al., 2007; Li et al., 2007), while in *H. diversicolor diversicolor*, there were no significant differences in shell length and body weight between triploid and control diploid, but edible part (muscle part) of triploid abalone was significantly larger than that of the control diploid (Kudo et al., 1994). Similar results were obtained in triploid *H. rubra*, in which no significant difference was seen between triploid and diploid, but triploid abalone had a more elongated shell and greater foot muscle (Liu et al., 2009).

Biochemical characteristics have been poorly studied in triploid abalone. Sun et al. (1993) reported higher glycolgen content in triploids than diploids. Almost the same amino acid composition between diploid and triploid abalone was reported by Chen et al. (2002). Recently, Dunstan et al. (2007) reported that fatty acid composition of the meat was not affected by ploidy statuses in *H. laevigata*.

There is an argument about the mechanism responsible for better growth in triploid abalone. Outperformance of triploid growth, especially in maturation and spawning season, has been generally explained in fish by the energy reallocation from sexual maturation to somatic growth due to sterility of triploid animals (Piferrer et al., 2009). However, gigantism, such as proportional correlation between ploidy status and body size, which has been found in polyploid plant (Blakeslee, 1941), is likely involved in abalone growth based on examinations on body size (Okumura and Yamamori, 2002), cell size (Harigaya et al., 2001), and cell numbers (Okumura et al., 2002). The involvement of gigantism in better growth is strongly suggested in bivalve triploids in the dwarf surfclam (*Mulinia lateralis*) (Guo and Allen, 1994) and the Pacific oyster (*Carassostrea gigas*) (Guo et al., 1996).

INDUCED GYNOGENESIS AND TETRAPLOIDIZATION

Gynogenesis is a development without any genetic contribution of paternal genome after triggering the initiation of development by fertilization of eggs with genetically inactivated sperm. Gynogenesis is considered an important manipulation technique to produce monosex populations as well as inbred lines. Arai et al. (1984) induced the gynogenesis with UV-irradiated sperm in *H. d. hannai* for the first time, but resultant progeny were inviable haploid larvae. Induction of gynogenetic development is generally difficult in shellfish due to the susceptibility of spermatozoa to UV irradiation (Li et al., 2000a, b), but a few studies reported the production of gynogenetic diploid abalone by inhibition of the polar body release after initiation of haploid development (Fujino et al., 1990b; Cai et al., 2004; Li et al., 1999; Li and Kijima, 2005; Nie et al., 2011).

Tetraploid abalone is important as the source of diploid gametes because mass production of triploid can be simply realized by cross breeding between diploid (haploid gametes) and induced tetraploid (diploid gametes). Tetraploid abalone can be theoretically induced by the possible occurrence of endomitosis after inhibition of the first cleavage, but no viable progeny has been produced by this method (Zhang et al., 2000). Alternatively, Okumura et al. (1998a, b) reported the production of tetraploid abalone larvae by inhibition of both the first and the second polar body release with CB and caffeine, respectively, after the initiation of gynogenetic haploid development by fertilization of eggs with UV-irradiated sperm. Inhibition of both meiotic divisions by cold shock was also examined (Okumura et al., 1996b). However, no viable tetraploid progeny has been produced.

GENETIC MAP AND RELATED QTL ANALYSES FOR MARKER-ASSISTED SELECTION

Genetic mapping is important not only for quantitative trait loci (QTL) analysis to elaborate aquaculture technology (Yu and Guo, 2006), but also for comparative synteny of genomes to get insights into evolutionary process of the target species (Barbazuk et al., 2000; Naruse et al., 2004). Since relatively large numbers of polymorphic microsatellite markers were successfully developed in *H. d. hannai* (Sekino and Hara, 2001, 2007; Sekino et al., 2005, 2006). The first linkage mapping for the abalone was conducted based on 180 microsatellite DNA markers (Sekino and Hara, 2007b). The numbers of linkage groups in the female and male map were 19 and 18, respectively, and almost or just same to the haploid chromosome number (n=18). As in linkage maps of other marine animals and fishes, female map (about 900 cM) is much

longer than male map (about 700 cM), suggesting higher recombination rates in females than males. The maps are useful to identify gene(s) related to or influenced on the commercially attractive traits such as growth, survival, disease resistance and environment adaptability. Recently, linkage map was also constructed by using 308 micro-satellite markers in *H. diversicolor* (Zhan et al., 2012). The number of linkage groups was consistent with the haploid chromosome number (n=16) and female map (about 760 cM) was longer than male map (about 680 cM) as observed in the map of *H. discus hannai* (Sekino and Hara, 2007b).

Gene (marker)-centromere map was reported for the allozyme loci by half-tetrad analysis in triploid *H. d. hannai* (Fujino et al., 1987; 1988a, b, 1989, 1990a) and in triploid *H. diversicolor diversicolor* (Fujino et al., 1991, 1992). By such approaches, allozyme loci were mapped in relation to the centromere of chromosomes. Half-tetrad analysis using polymorphic molecular markers and gyno-genetic diploid families successfully created much more precise gene(marker)-centromere map in *H. d. hannai* (Li and Kijima, 2005; Nie et al., 2011).

CRYOPRESERVATION

Cryopreservation of germ cells, gametes (egg and sperm) and zygotes (fertilized eggs, embryos, larvae) is very important from the view point of preservation of biodiversity as well as efficacy improvement in aqua-culture production. According to Tsai and Chao (1994), abalone sperm can be successfully cryopreserved for long time and they maintain high fertilizability about one year after storage and thawing. Such a successful result in cryopreservation of abalone sperm can make it possible to hybridize two different species that are distributed in geographically different places and that reproduce in different seasons. However, at present, no studies have been done on hybridization using cryopreserved sperm. Preservation of eggs and embryos has not been reported yet.

TRANSGENICS

Transgenic technology is necessary to study its future potential for genetic improvement as well as its application to expand our basic information on genes, genetics and genomics of aquatic organisms. At present, however, there are many arguments on public concerns about the use of genetically modified (GM) fish and shellfish from the viewpoint of food safety and security as well as environment safety including bio-diversity preservation. Gene transfer into abalone was tried by the electroporation of sperm in *Haliotis iris* by Sin et al. (1995) and then in *H. diversicolor supertexta* by Tsai et al. (1997). Gene transfer by the electroporation of embryos was also reported in *H. rufescens* by Powers et al. (1995). As mentioned above, transgenic in abalone was done by electroporation and no micro-injection was reported presumably due to difficulties of the very small size of eggs.

CONCLUSION

The present review paper concludes that both hybridization and triploidization are useful to improve growth traits in abalone. Thus, the combination of both methods, such as production of hybrid triploid strain (allo-triploidization) is potentially effective to improve growth. Although such approach is considered promising as a farm strain, their production has not been attempted yet. For further progress on chromosome manipulation studies in abalone, experimental hybridization between remotely related abalone species, for example between the abalone with 2n=32 chromosomes and those with 2n=36 chromosomes, are interesting. The successful sperm cryopreservation technique may enable us to cross-breed between the two species which are distributed in geographically different regions or presents reproductive asynchrony. Allo-triploidization between the remotely related abalone species is very interesting, because resultant allo-triploidy is likely to disrupt normal meiosis and gametogenesis and then increase the probability of sterilization than diploid hybrid and auto-triploid situations, due to the presence of one additional heterospecific genome (chromosome set).

Construction of much more precise genetic map will promote QTL analyses and then realize marker-assisted selection (MAS) to form aquaculture strains in abalone. However, the present genetic linkage map is still primitive because of the low coverage of whole genome. Further mapping using more co-dominant DNA markers is necessary to construct more detailed map for successful selection and gene identification. In addition, quantitative genetic studies are also required for abalone selection program. Although studies on quantitative traits have been done by many groups (Hara, 1990; Hara and Kikuchi, 1992; Kobayashi and Fujio, 1994, 1996; Kawahara et al. 1997), results are still fragmentary and inconclusive.

In conclusion, strategic studies on genetic improvement programs are necessary in aquaculture abalone strains by using classical genetic as well as modern biotechnological approaches. Further, genetic and genomic studies are now necessary to deepen our knowledge and then to expand it to application.

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