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Geographic Variation and Genetic Diversity between Polluted and Unpolluted Sites of Korean *Littorina brevicula* (Gastropoda, Littorinidae) Based on the Mitochondrial Cytochrome *b* Gene Sequence

Jae-Hwa Suh[1,2,*], Sook-Jung Kim[1] and Jun-Im Song[1]
([1]Dept. Biological Science, Ewha Womans University, Seoul 120-750, Korea
[2]LMO Evaluation Team, Biodiversity Center,
National Institute of Environmental Research, Inchon 404-170, Korea)

ABSTRACT

MtDNA cyt *b* gene was used to investigate the geographic variation of 11 populations (106 individuals) of the planktonic developing, periwinkle *Littorina brevicula*, throughout Eastern, Western, and Southern coastal regions in Korea. The sequence of 500 base pairs and 13 different haplotypes were determined. Haplotype *Lb*A was predominated through the populations studied with frequence of 0.877. Haplotypes were shown different frequencies in each coastal region (0.82, 0.90, and 1.00, respectively). Genetic analysis of the 61 individuals of *L. brevicula* from the polluted and unpolluted sites yielded 8 distinct haplotypes. Haplotype *Lb*A also was most common, and it was shared by 0.872 of frequency among specimens.

Key words: geographic variation, mtDNA cyt *b* gene, *Littorina brevicula*, pollution, haplotype

INTRODUCTION

The degree of genetic differentiation among local populations provides important indirect evidence, reflecting pattern and scale of effective local dispersal (Reid, 1996; Heipel *et al.*, 1998;

* To whom correspondence should be addressed
Tel: 032-560-7264, Fax: 032-560-7253, E-mail: amphibia@me.go.kr

Bohonak, 1999). Most marine species with a high dispersal potential often show only limited population genetic differentiation, because gene flow is usually positively correlated with dispersal ability (Hansen, 1980; Kohn and Perron, 1994; Tatarenkov, 1995). In marine gastropods, for example, species with planktonic dispersing larvae display higher levels of gene flow and less population genetic differentiation (Benzie and Williams, 1992; Brown and Murray, 1995), than nonplankonic developing species with poor dispersal capacity (Johannesson, 1992; Johnson and Black, 1995). Nevertheless, even in species with high dispersal abilities there are several factors that may limit actual dispersal and/or gene flow, thus creating opportunities for genetic differentiation as well (Palumbi, 1994, 1996). These limitations include to invisible gene flow barrier, isolation by distance, behavioral limit to dispersal and selection (Palumbi, 1994, 1996).

The micro- or macro-geographic spatio-temporal genetic structures of planktonic *Littorina* species were previously investigated by allozyme variation (Johannesson *et al.*, 1995; Tatarencov, 1995; de Wolf *et al.*, 1998a, b, 2000). However, recent studies have been performed on DNA techniques (de Wolf *et al.*, 1998b; Reid, 1996; Kyle and Boulding, 2000; Song *et al.*, 2000). A disadvantage of allozyme studies is that variation at some allozyme loci in *Littorina* has been shown not to be neutral with respect to selection (Johannesson *et al.*, 1995; Johannesson and Tatarencov, 1997). Despite this problem, only a few population genetic studies of littorinid species have used DNA sequence information from the mitochondrial genome (e.g. Kyle and Boulding, 1998). Not only can mtDNA sequence polymorphisms in fast-evolving mitochondrial genes provide higher resolution of population genetic structure than allozymes, but, with very few exceptions (e.g. Malhotra and Thorpe, 1994), nearly all studies have found variation in mtDNA markers to be neutral with respect to selection (Avis, 1994). An additional advantage is that the mitochondrial genome is haploid and mater-lineal, nonrecombinant fashion (Moritz *et al.*, 1987).

Littorina brevicula (Pillippi) is homogeneous population with planktonic developing. The periwinkle is one of the most common snails found in the intertidal zone of Korea, and it has been widely used for the assesment of heavy metal pollution in the marine environment (Kang *et al.*, 1999, 2000). We found that heavy metal (especially Cd, Zn, Cu and Pb) is accumulated in the digestive gland, intestine and gill of *L. brevicula* (Lee *et al.*, 1996; Song *et al.*, 1997). We also investigated the status of marine pollution in Korea, using the combined k-dominance curve for species biomass and individual numbers. These results of Dokdong, Chundo and Daejungchun showed greater impact than the results of Isudo, Tangsa and Jinha (Rho *et al.*, 1997). Therefore, to clarify the genetic variation of *L. brevicula* between polluted and unpolluted sites, amylase polymorphism has been previously carried out by ourselves (Park *et al.*, 1999).

In this paper we explore geographic variation in mitochondrial haplotype frequencies of *L. brevicula* in Korea by surveying mitochondrial cytochrome *b* gene, and compare to genetic diversity between polluted and unpolluted sites, by effect of heavy metals.

MATERIALS AND METHODS

In this study, 11 populations of *L. brevicula* (n = 106) were collected at Eastern, Southern and Western coastal regions in Korea from 1998 to 2001 (Table 1). Of these regions, polluted sites

Table 1. Collecting localities and number of sample of *Littorina brevicula*

Sampling site (abbreviation)	Localities	Dates	No. of Sample
Eastern coastal populations			
1. Sokcho (SC)	MoolChi port, Sokcho-shi, Kangwon-do	May 1, 1999	8
2. Uljin (UJ)	Uljin-gun, Kyongsangbuk-do	May 2, 1999	10
3. Tangsa (TS)	Tangsa-ri, Ulsan-shi, Kyongsangnam-do	Dec. 18, 1998	10
4. Chundo (CD)	Bangdo-ri, Onsan-up, Ulsan-shi, Kyongsangnam-do	Dec. 18, 1998	9
5. Daejungchun (DJC)	Ijin-ri, Onsan-up, Ulsan-shi, Kyongsangnam-do	Dec. 18, 1998	10
6. Jinha (JH)	Onsan-up, Ulsan-shi, Kyongsangnam-do	Dec. 18, 1998	10
Southern coastal populations			
7. Dukdong (DD)	Ukdong-ri, Masan-shi, Kyongsangnam-do	Dec. 19, 1998	11
8. Isudo (ISD)	Wepo-ri, Kojedo, Kyongsangnam-do	Dec. 19, 1998	11
9. Chejudo (CD)	Taejong, Pukcheju-gun, chejudo	Jun 15, 2001	9
Western coastal populations			
10. Haenam (HN)	Haenam-gun, Chollanam-do	Dec. 18, 1999	9
11. Shinjindo (SJD)	Taean-gun, Chungchongnam-do	Aug. 21, 2000	9

were Dokdong, Chundo, and Daejungchun and unpolluted sites were Isudo, Tangsa and Jinha. The height of the collected *L. brevicula* extends from 5 mm to 15 mm.

Samples preserved in pure ethanol were repeatedly rinsed with sterile distilled water before procedure. DNA was extracted from the foot of each individual. The tissue was homogenized in extraction buffer of the same volume, and then the genomic DNA was extracted using modified protocols of Micheli and Bova (1997). The fragment of the mt cyt *b* gene was amplified using polymerase chain reaction (PCR) with the following primer sets : LB-F (5′-TTTTGGTTCTTTAC-TAGGCC-3′) and LB-R (5′-AATCCTAAGGGATTATTCGA-3′) which yield a 609 bp fragment. PCR condition were: 5 min at 94°C followed by 30 cycles, each of 1 min at 94°C, 1 min at 42°C and 1 min at 72°C was added to allow complete extension of all amplified fragment.

For analysis of sequence variation, PCR product was sequenced using an ABI PRISM 310 DNA sequencer. A sum of 106 individuals of 11 populations was sequenced. The sequencing reaction was performed using the ABI PRISM BigDye Terminator Cycle Sequencing Kit (with AmpliTaq DNA polymerase, FS) following the manufacturer's instruction. Alignment of nucleotide sequences was performed by the multisequencing editing program CLUSTAL W (Thompson *et al.*, 1994). *L. brevicula* of Japan, available in the Genbank (accession number: U46794), was aligned as a reference species. The DNA sequences were analyzed using the MEGA program (Kumar *et al.*, 1993). The different haplotypes determined from our data were compared with the sequences of Japanese *L. brevicula* as a standard.

RESULTS AND DISCUSSION

Genetic variation among the 11 populations of *L. brevicula* was estimated and then compared with Genbank data of the same species in Japan (assession number U46794; Fig. 1). Neither deletion nor insertion was found within any populations. Transitions were more frequent than transversions at synonymous sites and were dominated at the third position of the codon. The analysis of 106 individuals from 11 populations for planktonic developing *L. brevicula* revealed 13 haplotypes. Unique haplotypes (i.e., sequences represented by only one individual) were found in every collection site. Of 13 haplotypes, haplotype *Lb*A was predominanted in all the populations studied with frequencies from 0.7 to 1.0 (Table 2), however, the others were rare (0.00-0.13). Haplotype distribution of *L. brevicula* in each three coastal region is shown in Table 3. The frequencies of haplotype *Lb*A in eastern, southern, and western coastal regions were 0.820, 0.901 and 1.000, respectively. The haplotypes differed from one another by one or two substitutions (Fig. 1). This result is similar to that founded by Kyle and Boulding (2000), whereby common haplotype (mtDNA cyt *b*) was at a relatively constant frequency (approximately 87%) in *L. scutulata* (gastropods) of all population. Among 13 different haplotypes, numbers of haplotype detected in eastern coast were 10 (77%) which were higher than those of southern and western coast (4 and 1, respectively). On the other hand, all individuals of western coast was monomorphic to the haplotype *Lb*A. This result led us to consider that Korean current affected the spatial distribution of haplotype variation. Korea is bounded on the east, south, and west sides by the East sea, Korea straits, and Yellow sea. The coastal waters of Korea are usually divided into four regions for geographical analysis: East Sea, Korea Straits, Jeju island waters, and Yellow sea (Seo, 1996).

	23	62	86	101	107	110	116	160	176	191	302	308	383	411	469
Reference	T	G	T	C	T	G	T	T	A	A	A	C	T	A	C
*Lb*A	.	C	.	.	.	.	.	.	.	.	.	T	.	.	.
*Lb*B	.	C	.	.	.	.	.	.	.	.	G	T	.	.	.
*Lb*C	.	C	.	A	.	.	.	.	.	.	.	T	.	.	.
*Lb*D	.	C	C	.	.	.	.	.	.	.	.	T	.	.	T
*Lb*E	.	C	.	.	.	.	.	.	.	.	.	T	.	.	.
*Lb*F	.	C	.	.	.	.	.	C	.	.	.	T	.	.	.
*Lb*G	.	C	.	.	.	A	.	.	.	.	.	T	.	.	.
*Lb*H	.	C	.	.	.	.	.	.	.	G	.	T	.	.	.
*Lb*I	.	C	.	.	.	.	.	.	G	.	.	T	.	.	.
*Lb*J	C	C	.	.	.	.	C	.	.	.	.	T	.	.	.
*Lb*K	.	C	.	.	C	.	.	.	.	.	.	T	.	G	.
*Lb*L	.	C	.	.	.	.	.	.	.	.	.	T	.	.	.
*Lb*M	.	C	.	.	.	.	.	.	.	.	.	T	C	.	.

Fig. 1. Variable positions of mt cyt *b* gene sequences of *L. brevicula*. *Japanese L. brevicula* was used as a reference species (assession number U46794). Dots designate the same sequence as a reference species.

Table 2. Distribution of the 13 cytochrome *b* haplotypes identified in *L. brevicula*

Sites / Haplotype	Eastern Coast						Southern Coast			Western Coast		Average of
	SC	UJ	TS**	CD*	DJC*	JH**	DD*	ISD**	CJD	HN	SJD	Frequence
*Lb*A	6 (0.75)	7 (0.7)	9 (0.90)	8 (0.89)	9 (0.90)	8 (0.80)	10 (0.90)	9 (0.81)	9 (1.00)	9 (1.00)	9 (1.00)	93 (0.87)
*Lb*B	1 (0.13)	0	0	0	0	0	0	0	0	0	0	1 (0.01)
*Lb*C	1 (0.13)	0	0	0	0	0	0	0	0	0	0	1 (0.01)
*Lb*D	0	1 (0.10)	0	0	0	0	0	0	0	0	0	1 (0.01)
*Lb*E	0	1 (0.10)	0	0	0	0	0	0	0	0	0	1 (0.01)
*Lb*F	0	1 (0.10)	0	0	0	0	0	0	0	0	0	1 (0.01)
*Lb*G	0	0	1 (0.10)	0	0	1 (0.10)	0	0	0	0	0	2 (0.02)
*Lb*H	0	0	0	1 (0.11)	0	0	0	0	0	0	0	1 (0.01)
*Lb*I	0	0	0	0	1 (0.10)	0	0	0	0	0	0	1 (0.01)
*Lb*J	0	0	0	0	0	1 (0.10)	0	0	0	0	0	1 (0.01)
*Lb*K	0	0	0	0	0	0	1 (0.10)	0	0	0	0	1 (0.01)
*Lb*L	0	0	0	0	0	0	0	1 (0.09)	0	0	0	1 (0.01)
*Lb*M	0	0	0	0	0	0	0	1 (0.09)	0	0	0	1 (0.01)
Total	8	10	10	9	10	10	11	11	9	9	9	106 (1.00)

* : polluted site
** : unpolluted site

These regions exhibit substantial differences in ecological conditions such as current, salinity, transparency, topography, and temperature. Especially, East coastal regions have cold water and higher levels of current than those of others. For this reason, we consider that haplotype variation of this region has been shown higher than that of others. Song *et al.* (2000) had reported that populations from eastern coast of *Granulilittorina exigua* showed higher variability than those from southern and western coasts. There were minor nucleotide differences among individuals and populations. We presumed that the periwinkle had a high dispersal and gene flow potential because of its planktonic development. It is, therefore, expected to show little population genetic differentiation (de Wolf *et al*, 2000).

Genetic analysis of the 61 *L. brevicula* individuals from the polluted and unpolluted sites yielded 8 distinct haplotypes (Table 4). Haplotype *Lb*A was most common, and it was shared by 87.2% of

Table 3. Haplotype distribution of *L. brevicula* in each coastal region.

Haplotype \ Sites	Eastern Coast	Southern Coast	Western Coast
*Lb*A	47 (0.820)	28 (0.901)	18 (1.000)
*Lb*B	1 (0.018)	–	–
*Lb*C	1 (0.018)	–	–
*Lb*D	1 (0.018)	–	–
*Lb*E	1 (0.018)	–	–
*Lb*F	1 (0.018)	–	–
*Lb*G	2 (0.036)	–	–
*Lb*H	1 (0.018)	–	–
*Lb*I	1 (0.018)	–	–
*Lb*J	1 (0.018)	–	–
*Lb*K	–	1 (0.033)	–
*Lb*L	–	1 (0.033)	–
*Lb*M	–	1 (0.033)	–
Total	57	31	18

Table 4. Haplotypes distribution of *L. brevicula* in polluted and unpolluted sites.

Haplotype \ Sites	Polluted	Unpolluted	Total
*Lb*A	27 (0.901)	26 (0.839)	53 (0.872)
*Lb*B	–	–	–
*Lb*C	–	–	–
*Lb*D	–	–	–
*Lb*E	–	–	–
*Lb*F	–	–	–
*Lb*G	–	2 (0.065)	2 (0.032)
*Lb*H	1 (0.033)	–	1 (0.016)
*Lb*I	1 (0.033)	–	1 (0.016)
*Lb*J	–	1 (0.032)	1 (0.016)
*Lb*K	1 (0.033)	–	1 (0.016)
*Lb*L	–	1 (0.032)	1 (0.016)
*Lb*M	–	1 (0.032)	1 (0.016)
Total	30	31	61

specimens, whereas others were 1.6-3.2%. The frequence of haplotype *Lb*A in unpolluted site (0.839) was lower than that of pollutes sites (0.901). However, the number of haplotypes in unpolluted site (5) was slightly higher than that of polluted site (4). Similarly, Kovatch *et al.* (2000)

had reported that *Microarthridion littorale* (copepod) inhabited in unpolluted site showed high nucleotide (mtDNA cyt *b*) diversity and differentiated from polluted site. The presence of more diverse genotypes in a population increase the probability that certain genotypes will have higher fitness in a stressful environment and that certain stress linked with genotypes will be detectable in the environment (Nevo *et al.*, 1986). In general, however, even though this phenomenon occurs the only for nuclear genes, reduced mtDNA diversity also has been attributed indirectly to the presence of contaminants through stochastic genetic processes such as genetic drift (Street and Montagna, 1996). Future studies on *L. brevicula* will include comparison with different mtDNA genes to clarify evolutionary changes between polluted and unpolluted sites.

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미토콘드리아 Cytochrome *b* 유전자의 염기서열 분석을 이용한 한국산 총알고둥(복족강, 총알고둥과)의 지리적 변이 및 오염·비오염지역간의 유전적 다양성

서 재 화[1,2] · 김 숙 정[1] · 송 준 임[1]

([1]이화여자대학교 생물과학과; [2]국립환경연구원 생물다양성센터 LMO 심사단)

요 약

한국산 총알고둥(*Littorina brevicula*)의 지리적 변이를 조사하기 위하여 동해안, 남해안, 서해안에서 총 11개 집단 106개체를 대상으로 미토콘드리아 DNA cytochrome *b* 유전자의 염기서열을 분석하였으며, 분석 결과 총 500 bp의 염기서열을 검출하였다. 검출된 염기서열을 대상으로 염기치환 유무 및 치환 장소를 비교한 결과 13종류의 haplotype으로 구분되었으며, 그 중 *Lb*A가 주 haplotype으로 나타났다. *Lb*A의 평균 출현빈도는 0.877이었으며, 동해안은 0.82, 남해안 0.90, 서해안 1.00으로 각각 나타나 동해안 집단이 타 집단에 비해 haplotype의 다양성이 더 높았다. 특히 오염지역과 비오염지역간의 비교에서는 8종류의 haplotype이 구분되었으며, 역시 *Lb*A가 주 haplotype으로 나타났다.