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COI-based genetic diversity and structure of Northeast Asian populations of *Assiminea hiradoensis* (Gastropoda, Assiminidae): conservation importance of brackish coastal habitats

Cho Rong Shin[1], Hyejeong Yu[2], Gyeongmin Kim[3], Bia Park[1], Eun Hwa Choi[1] and Ui Wook Hwang[1,2,4,5]

[1]*Department of Biomedical Convergence Science and Technology, Kyungpook National University, Daegu 41566, Korea*
[2]*Department of Biology Education, Teachers College and Institute for Phylogenomics and Evolution, Kyungpook National University, Daegu 41566, Korea*
[3]*School of Life Sciences, Graduate School, Kyungpook National University, Daegu 41566, Republic of Korea*
[4]*Institute for Korean Herb-Bio Convergence Promotion, Kyungpook National University, Daegu 41566, Korea*
[5]*Phylomics Inc., Daegu 41544, Korea*

ABSTRACT

Assiminea hiradoensis (Assiminidae, Gastropoda), a species native to Northeast Asia, is currently classified as Near Threatened (NT) on the Korean national Red List due to its restricted distribution and vulnerability to environmental changes. Given the threats posed by habitat degradation, including coastal development and climate change, understanding the genetic diversity and structure of *A. hiradoensis* populations is essential for its conservation. Between 2022 and 2023, we collected 113 *A. hiradoensis* individuals from 12 brackish coastal areas across Korea to examine the genetic diversity and population based on *COI*. Additionally, we incorporated two *COI* sequences from populations in Japan and China, retrieved from NCBI. A total of 34 unique *COI* haplotypes were identified from 115 *A. hiradoensis* individuals. Phylogenetic, TCS network, and PCoA analyses revealed weak spatial genetic structure among populations, suggesting that gene flow between populations is limited. However, relatively high genetic diversity was observed within local populations, indicating that these populations may be genetically distinct from each other. The localized genetic diversity appears to be influenced by specialized environmental factors specific to each site, as reflected in the analysis of molecular variance (AMOVA), which indicated that the majority of genetic variation occurs within populations rather than between them. These findings underscore the importance of conserving localized brackish coastal habitats, as they play a pivotal role in maintaining the genetic diversity of *A. hiradoensis*. In conclusion, this study highlights the urgent need for targeted conservation strategies to protect the specialized brackish coastal habitats of Northeast Asia.

Keywords: *Assiminea hiradoensis*, *COI*, brackish gastropods, molecular phylogeny, population genetics, Near Threatened

INTRODUCTION

The International Union for Conservation of Nature (IUCN) Red List of Threatened Species is widely regarded as the most authoritative and comprehensive tool for assessing the extinction risk of species and serves as a cornerstone for biodiversity conservation and sustainable development efforts (Betts *et al.*, 2020). Although the IUCN Red List is not mandatory, it plays an important role in setting policies and strategies for biodiversity conservation internationally (e.g. Brooks *et al.*, 2016; Mace *et al.*, 2018, Bennun *et al.*, 2018). Many countries also develop national red

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Corresponding author: Ui Wook Hwang
Tel: +82 (53) 950-6344, e-mail: uwhwang@knu.ac.kr
Co-Corresponding author: Eun Hwa Choi
Tel: +82 (53) 950-6344, e-mail: choice@knu.ac.kr
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lists based on IUCN criteria (e.g. Raimondo *et al.*, 2023; Hochkirch, 2023). The IUCN Red List assessment process requires assessors to follow scientifically rigorous guidelines and assign any species (excluding microorganisms) to 1 of 8 categories of extinction risk according to an objective set of criteria, based on data linked to population trend, size and structure and geographic range and their trends over time (IUCN, 2012).

Currently, molecular research is being conducted actively in the study of biological species, and despite the development of genetic research of the species, genetic factors have not yet been incorporated into the IUCN Red List's evaluation criteria. Previous studies have emphasized the need to include genetic diversity in conservation strategies, suggesting that population genetics and distribution modeling should be part of the Red List evaluation process (Santamaria and Mendez, 2012; Daniels *et al.*, 2020; Garner *et al.*, 2020). Additionally, the current IUCN criteria are largely tailored to vertebrates, creating challenges in assessing invertebrate species due to difficulties in obtaining ecological information, population size fluctuations, and distribution patterns (Cardoso *et al.*, 2011). In response to these limitations, Petit-Marty *et al.* (2021) proposed utilizing molecular markers to measure genetic diversity, particularly in situations where population size trends are difficult to determine.

The species *Assiminea hiradoensis*, found exclusively in Northeast Asia, inhabits estuarine and brackish environments, typically on sandy substrates (Lee and Min, 2019). Classified as Near Threatened (NT) on the Korean national red list, its distribution is limited to specific areas in South Korea, namely Jangheung-gun, Goheung-gun in Jeollanam-do, and Seogwipo-si in Jeju-do (Lee and Min, 2002; Min *et al.*, 2004; Lee and Gil, 2018). While some studies document its habitat and distribution, further ecological data remains scarce.

Salt marshes, critical vegetated zones within estuaries that undergo tidal flooding, support a diverse array of species adapted to these dynamic conditions (Boorman, 1995; Boorman, 2003). These habitats offer essential ecosystem services, such as providing nurseries for fish, purifying water, mitigating climate change, buffering waves, and offering cultural value (Lee *et al.*, 2014; Kelleway *et al.*, 2017). As brackish environments often host species with narrow distributions, they are particularly vulnerable to environmental changes (Shin *et al.*, 2021). On the Korean Peninsula, human activities such as coastal reclamation and the destruction of salt marshes have led to severe habitat degradation. On a global scale, the loss of salt marshes has been significant, with an estimated 719 km^2lost between 2000 and 2019 (Campbell *et al.*, 2022), highlighting the urgent need for conservation and restoration efforts (Foster *et al.*, 2013; Adam, 2019; Mori, 2024).

Mitochondrial *COI* maker is the most frequently used in examining population genetic diversity and structures in metazoan species (Baek *et al.*, 2013; Kim *et al.*, 2014; Baek *et al.*, 2020; Choi *et al.*, 2020, 2021; Shin *et al.*, 2021; Hong *et al.*, 2023; Kim and Hwang, 2023; Kim *et al.*, 2023). In this study, we aim to underscore the importance of conserving brackish coastal habitats in Korea by investigating *COI*-based genetic diversity and structure of *Assiminea hiradoensis* populations. Additionally, it seeks to demonstrate the utility of genetic data in reassessing Red List categories for threatened brackish gastropod species. The findings from this research could play a crucial role in guiding revisions to IUCN Red List evaluation criteria, supporting updates to national red lists, and informing future research directions. By examining the genetic health of *A. hiradoensis*, this study highlights the critical role of localized coastal habitats in maintaining species diversity and stability, thereby enhancing conservation strategies for brackish ecosystems and the species that depend on them.

MATERIALS AND METHODS

To identify the habitat of *Assiminea hiradoensis*, a field survey was conducted along selected coastal regions of Korea. A total of 113 *A. hiradoensis*

Table 1. Sampling localities of 115 individuals of *Assiminea hiradoensis* including Korea, Japan, and China

Population	Locality	N
WE	Nudong-ri, Gonam-myeon, Taean-gun, Chungcheongnam-do	11
EA	Opo-ri, Ganggu-myeon, Yeongdeok-gun, Gyeongsangbuk-do	10
SE	Dadae-dong, Saha-gu, Busan	14
	Naegan-ri, Geoje-myeon, Geoje-si, Gyeongsangnam-do	10
	Gohyeon-dong, Geoje-si, Gyeongsangnam-do	6
SM	Daejin-ri, Gonyang-myeon, Sacheon-si, Gyeongsangnam-do	10
	Gasan-ri, Chukdong-myeon, Sacheon-si, Gyeongsangnam-do	17
	Mangdeok-ri, Jinwol-myeon, Gwangyang-si, Jeollanam-do	4
SW	Gunnong-ri, Hoecheon-myeon, Boseong-gun, Jeollanam-do	4
	Sadang-ri, Daegu-myeon, Gangjin-gun, Jeollanam-do	8
	Samdu-ri, Gunoe-myeon, Wando-gun, Jeollanam-do	7
JJ	Hamori, Daejeong-eup, Seogwipo-si, Jeju-do	12
JP	Urakami, Nagasaki, Nagasaki, Japan	1
CN	China	1
	Total	115

individuals were collected from 12 coastal sites across the Korean Peninsula between 2022 and 2023 (Table 1). The specimens were preserved in 95% ethanol and stored at -20℃ in the laboratory until DNA extraction. Genomic DNA was extracted from the foot muscle tissue using the DNeasy Blood and Tissue Kit (QIAGEN, Valencia, California, USA). DNA concentration was measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA).

To amplify partial mitochondrial DNA fragments of the cytochrome c oxidase subunit I gene (*COI*), PCR was performed using the universal primers LCO1490 and HCO2198 (Folmer *et al.*, 1994). The PCR reaction mixture consisted of 10-20 ng of genomic DNA, 10 mM dNTPs, 10 pM of each primer, and 0.15 units of Taq DNA polymerase (Solgent Inc., Daejeon, South Korea), in a total volume of 30 μL. Thermal cycling conditions included an initial denaturation at 95℃ for 3 minutes, followed by 35 cycles of denaturation at 95℃ for 1 minute, annealing at 40℃ for 1 minute, and extension at 72℃ for 1 minute, with a final extension at 72℃ for 10 minutes. After amplification, 1 μL of each PCR product was analyzed via electrophoresis on a 1% agarose gel and visualized under UV light. The PCR products were then sequenced using an ABI Prism 3730 DNA sequencer (PerkinElmer Inc., USA) and the Big Dye Terminator Sequencing Kit (PerkinElmer Inc., USA). All *COI* sequences obtained in this study were submitted to GenBank under accession numbers PQ721342-PQ721374.

A total of 115 *COI* sequences, consisting of the present results obtained from 113 individuals and two additional sequences from the NCBI GenBank, were used for the present phylogenetic and population genetic analyses. Sampling sites were grouped into eight populations based on ocean currents and geographic distance (Table 1). The nucleotide sequences of *A. hiradoensis*were aligned with BioEdit 7.2.5 (Hall, 1999). Genetic diversity analysis was conducted using DnaSP v6.0 (Rozas *et al.*, 2017), including Tajima's D and Fu's Fs neutrality tests. Parameters such as the number of haplotypes (*h*), haplotype diversity (Hd), variable sites, segregation sites (S), nucleotide diversity (π), average nucleotide

Table 2. Genetic diversity and neutrality indices of *Assiminea hiradoensis* populations inferred from 34 *COI* haplotype sequences

Pop.	N	Nh	*h*	π	S	k	Tajima's *D*	Fu's *Fs*
WE	11	3	0.345	60	2	0.36364	−1.42961	−1.246
EA	10	6	0.889	0.00530	9	3.20000	0.02564	−0.661
SE	30	18	0.920	0.00568	23	3.42989	1.44583	−10.133
SM	31	9	0.544	0.00115	8	0.69247	−1.97233*	−7.327
SW	19	4	0.614	0.00523	8	3.15789	1.29853	3.264
JJ	12	2	0.409	0.00271	4	1.63636	0.82793	3.699
Total	113	34	0.845	0.00532	34	3.21556	−1.50764	−22.117

Diversity parameters are given for each locality: N = the number of *COI* sequences (individuals), Nh = the number of haplotypes, *h* = haplotype diversity, π = Jukes-Cantor corrected estimates of nucleotide diversity, S = the number of segregation sites and k = the average number of pairwise nucleotide differences. Statistically significant values are written in bold: *P < 0.05.

differences (K), and other genetic diversity metrics were calculated (Table 2). Mismatch distributions of *A. hiradoensis* populations were performed using DnaSP 6 v6.0 (Rozas *et al.*, 2017) to test whether demographic processes were consistent with the mismatch distribution test statistics (Fig. 1). A population typically displays a unimodal mismatch distribution when it has passed a recent demographic expansion, while a multimodal mismatch distribution suggests that the population is relatively stable

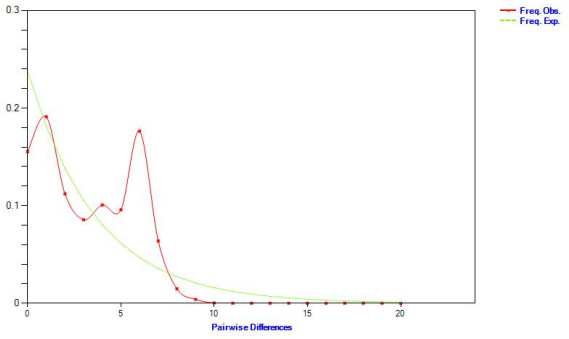

Fig. 1. Mismatch distribution analysis for total populations of *Assiminea hiradoensis* using DnaSP 6. The X axis shows the observed distribution of pairwise nucleotide differences, and the Y axis shows the frequencies. The red line with dots represents the observed frequency of pairwise differences, and the green lines show the expected values under the sudden population expansion model.

(Larery, 1996).

A phylogenetic tree based on 34 *COI* haplotypes was constructed using the maximum likelihood (ML) algorithm in the IQ-tree web server (http://iqtree.cibiv.univie.ac.at), with 1000 ultrafast bootstrap replicates (Fig. 2). The most appropriate nucleotide substitution models were selected using the Bayesian Information Criterion (BIC) implemented in ModelFinder (Kalyaanamoorthy *et al.*, 2017) in the IQ-tree web server. The resulting ML tree was visualized using FigTree 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/). To visualize gene genealogies, PopArt v1.7 software (Leigh andBryant, 2015) was used to generate a haplotype TCS network, which was further refined using Adobe Illustrator 2024 (Fig. 3). Principal Coordinate Analysis (PCoA) was applied to represent the genetic structure and differentiation of tested populations performed by DARwin software v6 (Perrier, 2003) and edited using Adobe Illustrator 2024 (Fig. 4).

Analysis of molecular variance (AMOVA) was conducted using Arlequin 3.5 (Excoffier and Lischer, 2010) (Table 3), and interpopulation genetic differentiation (Fst) and gene flow (Nm) were calculated. Gene flow was estimated based on the equation Nm = (1 − Fst) / 4Fst (Table 4).

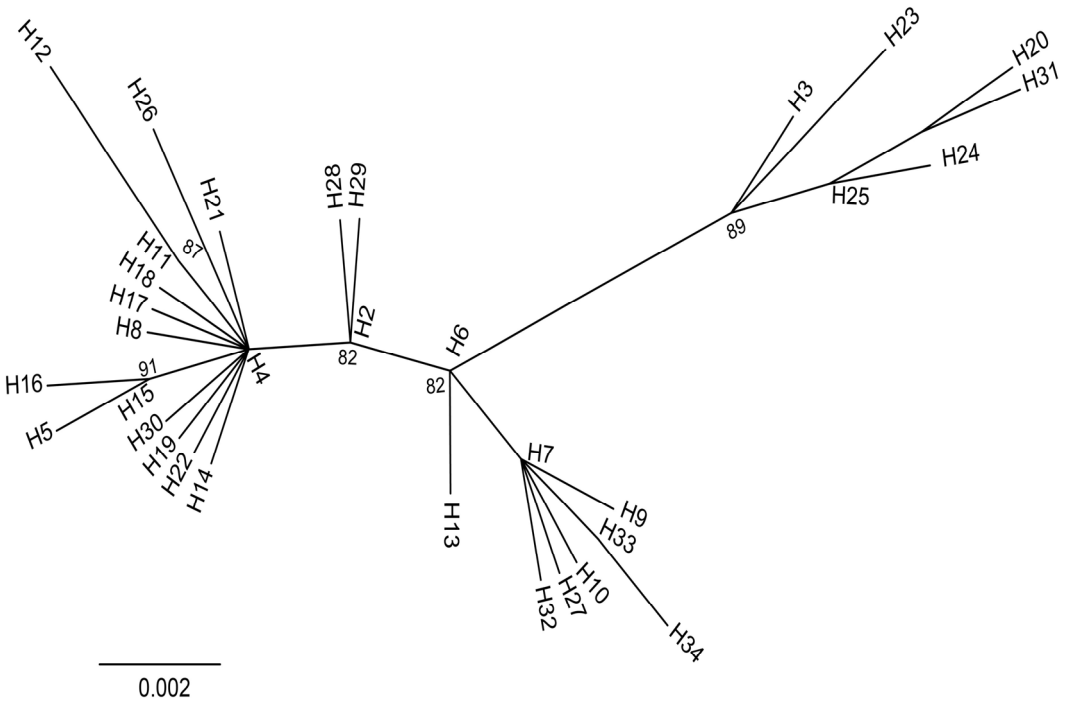

Fig. 2. Unrooted maximum likelihood tree showing phylogenetic relationships among 34 *COI* haplotypes of *Assiminea hiradoensis*. The bootstrapping value under 80 was not shown.

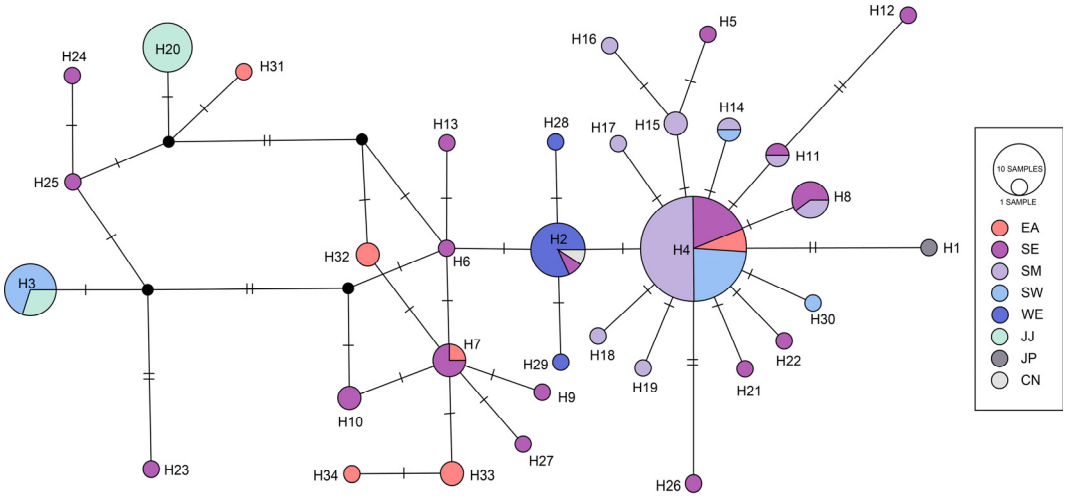

Fig. 3. TCS genetic network based on 34 *COI* haplotypes of *Assiminea hiradoensis*. The abbreviations of eight populations and further details of the haplotypes refer to Table 1 and Supplementary Table 1.

RESULTS AND DISCUSSION

The population of *A. hiradoensis*was observed in halophyte-dominated communities, including reeds and tidal grasses, along brackish sandy flats. High population densities were recorded under favorable habitat conditions, whereas low densities or absence were observed in unsuitable environments. In most areas where *A. hiradoensis* was confirmed, other species from the same family, such as *A. japonica*, *A. grayana*, and occasionally *Pseudomphala miyazakii*, were also present. Population densities tended to

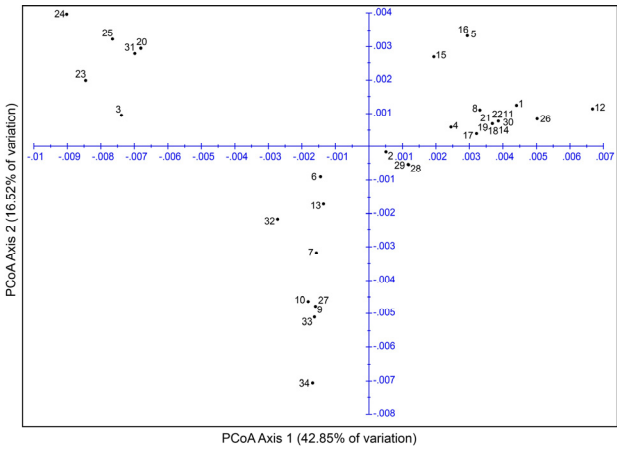

Fig. 4. A principal coordinate analysis (PCoA) of 34 *COI* haplotypes based on dissimilarity matrix according to Kimura (1980).

decrease in river estuaries with stronger seawater influence. This study identifies a previously unreported habitat for *A. hiradoensis*on the Korean Peninsula, expanding its known range beyond the southwestern sea and Jeju region to Taean on the west coast and Yeongdeok on the east coast (Table 1). These findings expand the known distribution of the species and suggest that *A. hiradoensis* may occupy otherestuarine habitats across South Korea.

A 604 bp-long *COI* sequence obtained from 115 *A. hiradoensis* individuals revealed 34 polymorphic sites (22 singleton variable sites and 12 parsimony-informative sites) with no insertions or deletions between them. From these data, a total of 34 *COI* haplotypes were identified (Supplementary Table 1),

Table 3. Analysis of molecular variance (AMOVA) test for estimating genetic variation among or within populations of *Assiminea hiradoensis*.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among groups	3	21.032	0.07212 Va	4.09
Among populations within groups	4	48.495	0.62777 Vb	35.61*
Within populations	107	113.760	1.06317 Vc	60.30*
Total	114	183.287	1.76306	

Statistically significant values are written in bold: * P < 0.05

Table 4. Pairwise genetic differentiation (Fst; below diagnoal) and gene flow (Nm; abovediagnoal) between eight populations of *Assiminea hiradoensis* based on 34 *COI* haplotype sequences

	JP	CN	SW	WE	JJ	SE	EA	SM
JP		0.00000	0.68183	0.03226	0.06429	1.34653	0.44444	0.10414
CN	1.00000		−1.36364	−0.50000	0.12162	−0.73716	−1.33333	0.26136
SW	0.26829	−0.22449		0.57164	0.25020	3.09851	0.82694	0.54340
WE	0.88571	−1.00000	**0.30427*****		0.06167	1.43305	0.42109	0.15276
JJ	0.79545	0.67273	**0.49980*****	**0.80213*****		0.23650	0.19861	0.04770
SE	0.15659	−0.51318	**0.07466***	**0.14854*****	**0.51387*****		3.19828	1.31436
EA	0.36000	−0.23077	**0.23214*****	**0.37253*****	**0.55728*****	**0.07250*****		0.22104
SM	0.70594	0.48889	**0.31510*****	**0.62071*****	**0.83978*****	**0.15981*****	**0.53074*****	

Statistically significant values are written in bold: * P < 0.05, ** P < 0.01, *** P < 0.001

Supplementary Table 1. List of 34 *COI* haplotypes along with eight populations of *Assiminea hiradoensis*

	WE	EA	SE	SM	SW	JJ	JP	CN	
H1							1		1
H2	9		1					1	11
H3					7	3			10
H4		3	8	21	10				42
H5			1						1
H6			1						1
H7		1	3						4
H8			3	2					5
H9			1						1
H10			2						2
H11			1	1					2
H12			1						1
H13			1						1
H14				1	1				2
H15				2					2
H16				1					1
H17				1					1
H18				1					1
H19				1					1
H20						9			9
H21			1						1
H22			1						1
H23			1						1
H24			1						1
H25			1						1
H26			1						1
H27			1						1
H28	1								1
H29	1								1
H30					1				1
H31		1							1
H32		2							2
H33		2							2
H34		1							1
	11	10	30	31	19	12	1	1	115

with a haplotype H4 being the most prevalent across populations. Seven haplotypes including H2, H3, H4, H7, H8, H11, and H14 were shared by at least two populations, while 25 haplotypes were unique to specific populations (Supplementary Table 1). The average haplotype diversity (Hd) across populations was 0.843 (0.345-0.920), with an average nucleotide diversity (π) of 0.00532 (0.00060-0.00568) and a mean nucleotide difference (K) of 3.216 (Table 2). The haplotype diversity was high, but nucleotide diversity was moderate. Notable differences in genetic diversity were observed among populations. For instance, the WE population exhibited low levels of haplotype and nucleotide diversities, while the EA and SE populations showed significantly higher genetic diversity (Table 2).

Neutrality tests (Tajima's D and Fu's Fs) indicated negative values for most populations, suggesting a trend of population expansion. However, statistically significant results were observed only in the SM population for Tajima's D ($P < 0.05$), and Fu's Fs did not show statistical significance in any population (Table 2). In the mismatch distribution (Fig. 1), there were many parts where the frequency of expectation did not match the frequency of observation, indicating that *A. hiradoensis* didnot follow the theoretical expansion model. The mismatch distribution analysis revealed discrepancies between observed and expected frequencies, suggesting that *A. hiradoensis* populations may have experienced complex historical fluctuations or mixing of genetic lineages, rather than a single recent expansion event (Fig. 1).

Phylogenetic tree reconstruction, TCS network analysis, and Principal Coordinates Analysis (PCoA) revealed a relatively limited spatial structuring and complex dynamics of connectivity and differentiation of *A. hiradoensis* across Northeast Asia. A phylogenetic tree (Fig. 2) was constructed using the ML method with the HKY+F+I model selected by best-fit testing. The ML tree supported a monophyly of 34 *COI* haplotypes with high bootstrap value but did not form distinct subclades as haplotypes dispersed without geographical clustering. The result obtained by a TCS network analysis (Fig. 3) was consistent with that of the phylogenetic analysis (Fig. 2), revealing no geographic or regional separation. A star-like topology centered on the most common *COI* haplotype (H4), representing 36.5% (42/115) of the *A. hiradoensis* individuals. No notable genetic differences between *COI* haplotypes could be identified, but some haplotypes such as H3, H20, H23, H24, H25, and H31 differed from other haplotypes by 4-5 nucleotide steps. Haplotype H4 may represent the ancestral or population expansion center, but the clustering of unique *COI* haplotypes within specific populations and the lack of extensive haplotype sharing across regions may be interpreted as a result caused by limited gene flow. The high genetic diversity observed within populations of *A. hiradoensis* suggested that genetic mutations have accumulated over time rather than through recent expansions. Furthermore, it is thought that the presence of multiple sub-haplotypes radiating from central haplotypes reflected the species' microevolutionary processes. The PCoA plot (Fig. 4) provided additional evidence supporting the results of phylogenetic tree (Fig. 2) and TCS network (Fig. 3).

Pairwise genetic differentiation (*F*st) showed diverse values, ranging from –1.00000 to 1.00000, with significant differentiation observed in 15 of 28 comparisons (Table 4). In Korean populations, all Fst values were statistically significant, while comparisons involving Japanese (JP) and Chinese (CN) populations generally showed no significant differentiation. Among the significant *F*st values, the lowest was 0.07466 (SW-SE), and the highest was 0.83978 (SM-JJ). In general, *F*ST values of 0-0.05 represent little differentiation, values of 0.05-0.25 indicate moderate differentiation and values higher than 0.25 indicate great differentiation among populations (Wright, 1978). Gene flow (Nm) was classified as low for $Nm < 1$, high for $1 < Nm < 4$, and very high for $Nm > 4$ (Boivin *et al.*, 2004). Nm values also varied widely, with most values below 1, indicating restricted gene flow. However, SE exhibited higher connectivity ($Nm > 1$) with all populations except CN and JJ, suggesting a high degree of

connectivity in these regions. (Table 3). In marine invertebrates, gene flow and genetic connectivity were strongly influenced by the mode of larval dispersal (Selkoe and Toonen, 2011; Modica *et al.*, 2017; Shin *et al.*, 2021). Species with long planktonic larval durations (PLD) generally showedhigher connectivity due to extended dispersal potential, yet specific environmental factors and regional selection pressures can still lead to significant genetic structuring (Lester *et al.*, 2007; Pascual *et al.*, 2017, Esser *et al.*, 2023). Comparative studies have shown that species with shorter or no PLD often exhibit stronger genetic differentiation due to limited dispersal (Wilke and Davis, 2000; Guzmán *et al.*, 2011; Villamor *et al.*, 2014; Narváez-Barandica *et al.*, 2023). The observed genetic patterns in *A. hiradoensis*, characterized by both gene flow and genetic differentiation, indicated a complex interplay between larval dispersal and local environmental adaptation. The moderately differentiated Fst values between some population pairs suggested a degree of connectivity, but the high Fst values across other regions indicated more restricted gene flow. It might be due to environmental barriers like salinity gradients and estuarine landscape features that limit movement between habitats (López-Márquez *et al.*, 2021).

For AMOVA analysis, populations were divided into four groups: Japan (JP), China (CN), Western Korea (WE, SW, and JJ), and Eastern Korea (EA, SM, and SE). The variation among the groups was 4.09%, which was not statistically significant. Conversely, the variation among populations within groups (38.70%) and within populations (61.30%) was high and statistically significant ($P < 0.001$), and the proportion of variation within populations was higher than that between populations (Table 3). The AMOVA results indicated that genetic variation within populations, rather than among regional groups, accounts for most of the observed genetic diversity (Table 3). This pattern was commonly found in coastal species with limited connectivity, where local selection pressures, environmental heterogeneity, and habitat fragmentation influence population structure (e.g. Miller *et al*, 2019; Gates *et al.*, 2021; Choi *et al.*, 2024). Estuarine environments, such as the brackish habitats where *A. hiradoensis* reside, often exhibit unique salinity gradients and distinct ecological conditions that influence gene flow and shape population connectivity (Bilton *et al.*, 2002; Cloern *et al.*, 2017). In this species, restricted gene flow despite a planktonic larval stage may be due to specific ecological traits or environmental factors that inhibit dispersal (Bilton *et al.*, 2002; Watts and Johnson, 2004).

A. hiradoensis faced potential conservation concerns despite its signs of stable genetic structure. Habitat degradation, driven by river management and human activities in estuarine areas, poses a significant threat to the species (Kennish, 2002; Alda *et al.*, 2022). Although extinction risk may not be immediate, the long-term impact of habitat loss and environmental stressors could compromise population stability and genetic diversity (Mimura *et al.*, 2017; Pinto *et al.*, 2024). Effective conservation strategies should focus on protecting estuarine habitats, which serve as critical ecological transition zones for species like *A. hiradoensis*. Preserving these habitats will support genetic resilience and maintain the ecosystem functions critical for survival of this species.

Lastly, the results underscored the importance of incorporating genetic data into conservation decision-making. For brackish gastropod species like *A. hiradoensis*, which showed unique genetic structures and limited dispersal abilities in specialized habitats, genetic analyses can offer insights into population health and connectivity. These findings supported the idea that genetic considerations should be integral to evaluating species for protection under frameworks like the IUCN Red List. Safeguarding habitat of *A. hiradoensis* can contribute to preserving genetic diversity, enhancing the resilience of coastal ecosystems, and maintaining biodiversity.

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