Genetic and morphological variations in the Korean invasive barnacle *Amphibalanus eburneus* (Gould, 1841) (#67328)

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Genetic and morphological variations in the Korean invasive barnacle *Amphibalanus eburneus* (Gould, 1841)

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Invasive organisms often exhibit morphological variations across geographical populations as a phenotypic response to heterogeneous environments. This phenomenon may offer competitive advantage, allowing organisms to overcome instability due to rapid changes in habitat. The ivory barnacle *Amphibalanus eburneus* of the family Balanidae is a marine crustacean, which presents near-cosmopolitan distribution due to its extensive invasion potential and exhibits a wide spectrum of phenotypic variations. Here, we investigated the morphological and genetic variations in the Korean invasive *A. eburneus*, which has successfully settled in this region since the late 1980s, to elucidate the processes of adaptive evolution through invasion. We selected four populations representing all surrounding Korean waters and applied two-dimensional landmark-based geometric morphometrics to the scutum and tergum. Further, we amplified the mitochondrial genetic marker mtCOI and generated a haplotype network to visualize the population structure. We detected interpopulation variations in the shape and morphospace structures, and one population could be separated from the rest based on the distinct morphotype of the tergum. We also detected 14 haplotypes in four populations belonging to two distinct lineages based on the moderate intraspecific pairwise distance (3%). Since the observed phenotypic variations did not mirror the intraspecific genetic variations, we concluded that the natural variations in tergum morphology among the populations is primarily the outcome of local adaptation rather than selection driven by speciation.

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Abstract

Invasive organisms often exhibit morphological variations across geographical populations as a phenotypic response to heterogeneous environments. This phenomenon may offer a competitive advantage, allowing organisms to overcome instability due to rapid changes in habitat. The ivory barnacle *Amphibalanus eburneus* of the family Balanidae is a marine crustacean, which presents near-cosmopolitan distribution due to its extensive invasion potential and exhibits a wide spectrum of phenotypic variations. Here, we investigated the morphological and genetic variations in the Korean invasive *A. eburneus*, which has successfully settled in this region since the late 1980s, to elucidate the processes of adaptive evolution through invasion. We selected four populations representing all surrounding Korean waters and applied two-dimensional landmark-based geometric morphometrics to the scutum and tergum. Further, we amplified the mitochondrial genetic marker mtCOI and generated a haplotype network to visualize the population structure. We detected interpopulation variations in the shape and morphospace structures, and one population could be separated from the rest based on the distinct morphotype of the tergum. We also detected 14 haplotypes in four populations belonging to two distinct lineages based on the moderate intraspecific pairwise distance (3%). Since the observed phenotypic variations did not mirror the intraspecific genetic variations, we concluded that the natural variations in tergum morphology among the populations are primarily the outcome of local adaptation rather than selection driven by speciation.

Introduction

Successful biological invasion depends on the invader's potential to survive under changing environments at all stages of life leading to and following its introduction (Smith, 2009). Invasive species often undergo inevitable changes upon exposure to diverse biotic or abiotic components along the process of speciation. Meanwhile, they may evolve specific novelties as a result of a plastic or an epigenetic response to the external stimuli or inputs, without obvious intra-genetic diversification (Lee, 2002; Richards et al., 2006; Ghalambor et al., 2007; Prentis et al., 2008; Kistner & Dybdahl, 2014). Such biological responses to exotic conditions lead to changes in the morphology, physiology, development, behavior, or combinations of these attributes at any level of organization (Davidson, Jennions & Nicotra, 2011). Notably, invasive species can benefit from such adaptive evolution by rapidly producing optimal phenotypes that enable successful settlement (Bonduriansky, Crean & Day, 2011). In individuals exhibiting potent plasticity in expressing distinct phenotypes, these adaptive traits are advantageous and may be subject to selection (de Jong, 2005). Once these individuals become dominant in a population, the genotype conferring the optimal phenotype can be inherited through ontogeny, leading to the transmission of environmentally induced phenotypes to the subsequent generations (Bonduriansky, Crean & Day, 2011) and, ultimately, allowing the spread of the invasive species over broad geographic ranges (Hahn, Van Kleunen & Müller-Schärer, 2012; Geng et al., 2016; Zaccara et al., 2021).

Barnacles are dominant inhabitants of the coastal ecosystems. Many barnacle species are invasive and have spread across the globe (Gilg et al., 2010). Their cosmopolitan distribution may attribute to human-mediated translocation over the past centuries, primarily via the ballast water and skin fouling, serving as the vectors for planktonic larvae and sessile adults, respectively (Zardus & Hadfield, 2005; Davidson et al., 2009; Carlton, Newman & Pitombo, 2011). The invasive barnacle species with long-range dispersal and massive biomass often exhibit phenotypic variations across geographic populations. Specifically, morphological variations in certain fitness-related body parts in response to heterogeneous environments have been commonly documented, which allow for the comprehensive studies of adaptive evolution. For instance, wave exposure levels drive morphological variations in the calcareous structures and chitinous exoskeleton of individuals to protect the soft body inside (Pentcheff, 1991; Arsenault, Marchinko & Palmer, 2001; Miller, 2007; Neufeld & Palmer, 2008). Similarly, the cirrus morphology varies with frequent wave exposure to enhance particle capture efficiency (Arsenault, Marchinko & Palmer, 2001), altering between shorter and thicker forms than those noted in wave-protected zones (Marchinko & Palmer, 2003; Li & Denny, 2004; Miller, 2007; López et al., 2010). Furthermore, barnacles with bathymetric distribution often exhibit morphological variations in the cirri, being longer in individuals inhabiting the upper intertidal zone than in those inhabiting the middle-lower intertidal areas (Chan & Hung, 2005). Finally, the density at which the individuals grow is another significant factor, resulting in competition for space and mating, which further induces shell elongation (Barnes & Powell, 1950; Bertness, Gaines & Yeh, 1998) and variations in penis length (Hoch, 2008).

The ivory barnacle *Amphibalanus eburneus* (Gould, 1841) belonging to the family Balanidae is native to the east coast of the USA, distributed from Nova Scotia to Florida, including the Caribbean and Gulf of Mexico (Kaplan, 1988). However, the species has now become nearly cosmopolitan due to extensive ship fouling (Henry & McLaughlin, 1975; Larsen, 1985). Until recently, the range of *A. eburneus* distribution was limited to the European seas (Relini & Matricardi, 1979; Molnar et al., 2008; Jaberimanesh et al., 2019; Osca & Crocetta, 2020), Pacific Ocean (Henry & McLaughlin, 1975; Iwasaki, 2018), Indian Ocean (Biccard & Griffiths, 2016), and Canadian Arctic (Chan et al., 2015). Now, however, *A. eburneus* has become a harmful invader in many countries, inflicting incessant ecological and industrial damage due to its following distinct traits. First, *A. eburneus* is euryhaline, exhibiting a preference for waters with salinity ranging between 15 and 20 ppt, which allows them to endure drastic changes across diverse geographic habitats (Dineen & Hines, 1994). Second, this species follows variable temperature-dependent larval development; as such, at the optimum temperature of 26°C, the duration from the nauplius to the cyprid stage may vary from 7 to 13 days (Costlow & Bookhout, 1957). Finally, their special life-history traits at the sessile stages can enable pelagic larval dispersal, direct development, and high reproduction, which together facilitate survival (Streftaris, Zenetos & Papathanassiou, 2005). Given these optimized dispersal abilities, *A. eburneus* is an excellent model to study the morphological variations across geographic populations accompanying biological invasion, although a few aspects remain unexplored.

Recently, *A. eburneus* emergence was noted in an ecological monitoring project in the Korean harbors ("Improvement of management strategies on marine ecosystem disturbing and harmful organisms"). Being underway since 2013, the project is aimed at monitoring various alien marine organisms invading the Korean harbors through ship fouling or ocean currents. During a survey in April 2021, extensive *A. eburneus* spread was documented in four major harbors, namely Incheon, Tongyeong, Sokcho, and Hanlim, representing all surrounding Korean waters, including the Yellow Sea, Korean Strait, and East Sea (Sea of Japan). Considering the substantial geographic distances and heterogenous habitat conditions, we speculated whether *A. eburneus* presents genetic and morphological variations across geographies and what is the evolutionary history of their invasive ability.

To this end, in the present study, we first addressed these questions by analyzing the genetic structure of Korean *A. eburneus* populations using a mitochondrial genetic marker. Then, we examined variations in the shape and matching symmetry of the opercular and calcareous plates of *A. eburneus*, which are of evolutionary and taxonomic significance (Pitombo et al., 2017). For analysis, we employed two-dimensional landmark-based geometric morphometrics (LBGM)—a powerful statistical tool for quantifying morphological variations (Slice, 2007) to distinguish populations. Finally, we proposed the possible invasion pathway of *A. eburneus* in Korea based on the link between the evolutionary trends of operculum morphology and genetic traits.

Materials & Methods

Sample collection

We collected respectively 60, 55, 61, and 82 individuals of *A. eburneus* from the Incheon (37°27′41.4″N, 126°36′49.8″E), Tongyeong (34°49′38.1″N, 128°26′03.5″E), Sokcho (38°13′36.7″N, 128°35′19.6″E), and Hanlim (33°25′11.2″N, 126°15′40.1″E) harbors of Korea in April 2021 (Fig. 1A). We carried out the port access and the collection with the permission of the Korea Institute of Marine Science &Technology Promotion (KIMST, project number: 20190518). We collected the individuals using 10 pre-installed acrylic attachment plates (30×30 cm) (Fig. 1B) in April 2020, submerged at the depth of 1–3 m from the sea level and installed for 12 months, covering both warm and cold seasons. The warm season lasted from April to October 2020 (spring and summer), while the cold season lasted from November 2020 to March 2021 (fall and winter). Since the maximum age of the adult *A. eburneus* (Fig. 1C) remains unknown and their lifespan may vary with food availability and environmental factors, we collected individual with the basal diameter of 2–2.5 cm, according to the maximum reported diameter for the species (Gosner, 1978; Kaplan, 1988). We used a knife to remove the individuals from the plate and immediately preserved them in 95% ethanol. To examine the seasonal changes in water temperature and salinity, we collected the data of these parameters using a handheld YSI Pro30 temperature and conductivity meter (YSI, Yellow Springs, Ohio) at the four sampling sites during 2020–2021.

DNA amplification and genetic analyses

We randomly selected ten individuals from each locality sample and isolated genomic DNA with the aid of the LaboPassTM Kit (Cosmo, Seoul, Korea) following the manufacturer's protocols. We amplified the mitochondrial cytochrome c oxidase subunit I (mtCOI) partial sequences with polymerase chain reaction (PCR) using PCR premix (BIONEER. Co, Daejeon, Korea) in AllInOneCycler™ PCR system (BIONEER. Co, Daejeon, Korea). We used universal primer pair, jgLCO1490 (5`– TIT CIA CIA AYC AYA ARG AYA TTG G –3`) and jgHCO2198 (5`– TAI ACY TCI GGR TGI CCR AAR AAY CA –3`) (Geller et al. 2013). We used the amplification protocol for mtCOI consisted of initial denaturation at 94 °C for 2 min, 30 cycles of denaturation at 94 °C for 1 min, annealing at 48 °C for 1 min, extension at 72 °C for 1 min, final extension at 72 °C for 10 min, and storing at 4 °C. We purified the PCR products for sequencing reactions using the Labopass PCR Purification Kit (Cosmo, Seoul, Korea) following the instructions of the manufacturer. We sequenced DNA on an ABI automatic capillary sequencer (Macrogen, Seoul, Korea) using the same set of primers.

We performed a population genetic analysis at the continental scale, comparing the level of genetic differentiation between all obtained sequences. We confirmed the sequences with BLAST search (Altschul et al., 1990), and visualized using Finch TV, version 1.4.0 (http://www.geospiza.com/Products/finchtv.shtml) to check the quality of signal and sites with possible low resolution. We deposited all obtained sequences in GenBank (Article S1). We performed sequence alignment using the MAFFT v7.313 (Katoh & Standley, 2013) with those of *A. eburneus* already published and publicly available. We estimated uncorrected pairwise distances using Geneious prime (https://www.geneious.com/prime/). We used the TCS algorithm

implemented in PopART (Clement, Posada & Crandall, 2000; Leigh & Bryant, 2015) to evaluate genealogical relationships among mtCOI haplotypes by reconstructing a haplotype network. We used DnaSP 5.10 (Librado & Rozas, 2009) to estimate haplotype diversity (Hd) (Rozas & Rozas, 1999), nucleotide diversity (π) (defined as the average number of pairwise nucleotide differences, and their standard deviations) (Tajima, 1983; Nei, 1987).

Morphological data acquisition

We dissected the animals under a stereo dissecting microscope, Nikon SMZ 1000 (Nikon, Tokyo, Japan). For each individual, we first separated the left and right sides of the opercular plates from body and subsequently divided them into scutum and tergum. We used 5% sodium hypochlorite (NaClO) to clean the surface of dissected parts. We placed the dissected part on the petri dish covered by black paper with scale to take microscope images. To make a dorsal perspective angle perpendicular to the microscope objective, we manipulated the specimen using forceps. After each specimen was adequately positioned consistently, we took images of ventral view of scutum and tergum in multiple foci using a camera DP22 (Olympus, Tokyo, Japan) implemented in the dissecting microscope. To illustrate the three-dimensional inclusion better, we used stacking software Helicon Focus 7.7.5 (Kozub et al. 2008) to combine images.

We generated two TPS files for the scutum and the tergum separately to evaluate the geometric variation in size and shape, including the asymmetry by the side (matching symmetry between left and right parts). We generated copy of all images and employed them with originals for producing TPS files using tpsUtil software (Rohlf, 2015). We digitized the chosen landmark (LM), all Type I (Bookstein, 1991), twice using tpsDig2 software (Rohlf, 2010) to estimate digitization-related errors (Klingenberg, Barluenga & Meyer, 2002). We selected four anatomical points of scutum for LM digitization (Fig. 2A): one located on apex (LM 1); one located on the posterolateral tip (LM 2); one located along the inflection point of basal margin (LM 3); one located along the inflection point of adductor margin (LM 4); one located on the lower point of basal ridge (LM 5). We selected ten anatomical points of tergum for LM digitization (Fig. 2B): one located on apex (LM 1); one located along the inflection point of lateral margin (LM 2); one located on the distolateral tip of scutal side (LM 3); one located on the basal and spur margin intersection on scutal side (LM 4); one located on the spur distolateral point (LM 5); one located on the spur distomedial point (LM 6); one located on the basal and spur margin intersection on tergal side (LM 7); one located along the inflection point of basal margin (LM 8); one located on the distomedial tip of tergal side (LM 9); one located on the proximomedial tip of tergal side (LM 10). The scutum and tergum datasets finally included 290, and 300 digitized images (from the original 145 and 150 images), respectively.

Geometric morphometric analyses

We employed algorithms implemented in Morpho J package software ver. 1.07d (Klingenberg, 2011) for all LBGM analyses. We aligned and superimposed all landmark configurations in six TPS files with Generalized Procrustes Analysis (GPA) to remove the effects of non-shape

variation (Rohlf & Slice, 1990). We converted the Procrustes shape coordinates into a covariance matrix (Brusatte et al., 2011). As a size proxy, we estimated the centroid size (CS) for each individual from the raw LM coordinates (Bookstein, 1989). We calculated the CS as the square root of the sum of squared distances for a set of centroid LMs (Mitteroecker et al., 2013). We performed Procrustes analysis of variance (ANOVA) test for group structuring evidence in the overall dataset using population and side as classifiers. We also used it to evaluate digitizing errors (Klingenberg & McIntyre, 1998). After the Procrustes ANOVA to test error terms, we employed the first digitization dataset and divided it into the left and right datasets for the subsequent LBGM analyses. We performed regressions of shape onto size to test allometry using regression scores and CS (Monteiro, 1999; Klingenberg, 2016). The null hypothesis states that shape develops isometrically; thus, a statistically significant result demonstrates that the shape changes with the increasing size according to a predictable model (Ponssa & Candioti, 2012). We applied a permutation test (Good, 2013) to assess the statistical significance against the null hypothesis. The number of shape variations determined by the regression were expressed as a percentage of the total variation percentage around the mean (Rodríguez-Mendoza, Munoz & Saborido-Rey, 2011). We estimated residual components to subtract the portion of shape variations predicted by the regression for further analyses. We used the residual shape component for principal component analysis (PCA), which is the most efficient method to examine the variation of multiple variables within a single sample. This analysis is frequently applied for the first exploratory analysis of a large dataset composed of several samples to provide a visual impression of overall shape variations (Mitteroecker et al., 2013). We performed PCA with the wire frame to visualize scutum and tergum's average shape variations along major PCA axes. We employed separated residual components for canonical variate analysis (CVA). The canonical variate analysis is a multivariate method, providing a criterion for reliably distinguishing among multiple groups preliminarily defined. The results of the method indicate the separation among populations by maximizing distances between group means relative to the variation within groups (Klingenberg & Monteiro, 2005). The analysis generated a multivariate statistical value as Mahalanobis Distances, (MD) (Timm, 2002). The permutation test assessed the statistical significance against the equal group means' null hypothesis.

Results

Population genetic diversity

Eighteen mtCOI patrial sequences were obtained (four each from Incheon, Tongyeong, and Sokcho and six from Hanlim). The final alignment was trimmed to the same length of 658 base pairs, of which 631 were constant and 27 were variable (6 singleton and 21 parsimony informative). No stop codons were detected, and the sequences encoded polypeptides of 219 amino acids. The average nucleotide frequency of the aligned sequences was 28.8, 38.1, 15.6, and 17.5% for each A, T, G, and C, respectively. In other words, the sequences were AT-rich (66.9%). The uncorrected pairwise distances between the four populations ranged between 0 and 3% (Table S1), with the highest value recorded between individuals H2 and S3.

The network (Fig. 3) detected 14 haplotypes from the obtained sequences, forming two distinct clades (A and B) separated by nine mutational substitutions. Clades A and B contained respectively six and eight haplotypes. However, the frequency of haplotypes in each clade was not locality-specific, and nearly all haplotypes detected presented widespread geographic distribution. The Incheon population included three haplotypes (Hd = 0.833, π = 0.012), belonging to both clades, and two of these three haplotypes were shared by the Tongyeong and Hanlim populations. The Tongyeong population included four haplotypes (Hd = 1, π = 0.045), all belonging to clade B. The Sokcho population included four haplotypes (Hd = 1, π = 0.019), two of which belonged to clade A. The Hanlim population included five haplotypes (Hd = 0.933, π = 0.018), two of which belonged to clade B.

Variations in size and shape

The ANOVA results (Table 1) yielded negligible digitizing errors for all datasets, with the individual variability mean square (MS) and F values far exceeding the error values. The individuals significantly varied in terms of the size of the scutum ($F = 7.92$, $p < 0.0001$) and tergum ($F = 7.92$, $p < 0.0001$). However, neither population nor asymmetry ~~by the side~~ contributed to the observed variations in the size of the scutum (population, $p = 0.0389$; asymmetry ~~by the side~~, $p = 0.6323$) and tergum (population, $p = 0.1104$; asymmetry ~~by the side~~, $p = 0.194$). The effect of asymmetry ~~by the side~~ was significant, contributing to ~~the~~ variations in the shape of the scutum ($F = 5.34$, $p < 0.0001$) and tergum ($F = 6.33$, $p < 0.0001$) and exceeding that of individual variability. The population most significantly contributed to ~~the~~ variations in the shape of the scutum ($F = 7.44$, $p < 0.0001$) and tergum ($F = 8.23$, $p < 0.0001$), and its contribution exceeded that of the asymmetry ~~by the side~~.

Allometry and size-corrected shape variations among populations

Regression analysis revealed an allometric effect in the left (4.33%, $p = 0.011$) and the right (3.6%, $p = 0.0459$) scutum datasets, thus the null hypothesis regarding isometric shape development was rejected. PCA based on residuals revealed major shape variations of the left (Fig. 4A) and right scutum (Fig. 4B), with the first two axes explaining respectively 69.6% (PC1 = 37.5%; PC2 = 32.1%) and 66.5% (PC1 = 41.3%; PC2 = 25.2%) of the total variance. Although PCA on both datasets did not reveal apparently distinguishable clustering among the populations, the Incheon population was recognizable in the PC1 morphospace of the left scutum based on a slightly different trend. Specifically, the Incheon individuals occupied the space between −0.15 and 0.05 for the left scutum, with a negative center of gravity. The wireframe demonstrated the shape variations in Incheon individuals corresponding to PC1. As such, the left (Fig. 4C) and right scuta (Fig. 4D) were horizontally narrower than the average due to medially shifted LMs 1–4 and LMs 1–3, respectively.

Regression analyses rejected the allometric association in the left (1.983%, $p = 0.127$) and right (1.597%, $p = 0.3037$) tergum datasets. The first two PC axes of the left (Fig. 5A) and right tergum (Fig. 5B) datasets explained respectively 38.9% (PC1 = 23.7%; PC2 = 15.2%) and 39.3% (PC1 = 24.6%; PC2 = 14.7%) of the total variance. The PC1 of both datasets emphasized the

variations in the shape of the tergum among the populations, with the morphospace clustering of the Tongyeong population on one side (with the center of gravity in the negative part of the PC1). The Sokcho population was clustered on the negative side in the left tergum dataset but not in the right one. Meanwhile, the Incheon and Hanlim populations displayed clustering on the positive side of PC1 morphospace. The wireframe corresponding to PC1 (Fig. 5C, D) showed a narrow and elongated shape for the Tongyeong and Sokcho populations due to proximally shifted LMs 1, 2, and 8; medially shifted LMs 3, 4, 9, and 10; and distally shifted LMs 5, 6, and 7.

Population differentiation based on morphological distance

Based on the Mahalanobis distances (Table 2), the Incheon population was distinguishable from the rest, with the highest values of the left and right scutum morphology. The Tongyeong population was ~~the~~ most distantly related to the rest, with ~~the~~ highest values of the left and right tergum morphology. The permutation test of CVA rejected the PE null hypothesis for equal group means between populations ($p < 0.0001$). CVA of the left and right scutum datasets showed that the first two axes explained respectively 86.5% (CV1 = 54.5%; CV2 = 32%) and 92.1% (CV1 = 62.7%; CV2 = 29.4%) of the total variance. In the CV1 morphospace, the Incheon population was the most distinct, occupying the space between −4 and 1 for the left scutum and between −4 and 0 for the right one. The wireframe demonstrated variations in the shape of the left and right scutum in the Incheon population. As such, the left and right scuta were horizontally narrower than the average due to medially shifted LMs 2–4. The Tongyeong population occupied the CV1 morphospace between −6 and 0 for the left scutum and between 0 and 8 for the right one. The wireframe corresponding to CV1 described a narrow and elongated tergum due to proximally shifted LMs 1, 2, and 8; medially shifted LMs 3, 9, and 10; and distally shifted LMs 5, 6, and 7.

Discussion

The present study investigated the adaptive phenotypic responses of ~~the~~ genetic and morphological traits in ~~the~~ Korean *A. eburneus* populations. Based on the genetic distances of ~~the~~ mtCOI sequences, *A. eburneus* populations showed low genetic diversity. The diversification rate ranged between 0% and 3%, falling within the intra-specific values, compared to the much higher inter-specific values of mtCOI sequences in other balanomorph barnacles (Tsang et al., 2008; Chen et al., 2014). ~~Our~~ genetic analysis confirmed ~~the~~ differentiation between ~~the~~ clades at the population level. Haplotype network analysis revealed detailed genetic characteristics of the populations, establishing two separate clades, A and B, with 14 haplotypes. Interestingly, the clade separation in the network was not locality-specific, and nearly all haplotypes in the populations were ~~sporadically~~ placed in the two clades. Furthermore, the haplotype and nucleotide diversities of all ~~population~~ were comparable to or higher than ~~the~~ previously reported values in various acorn barnacles (Geller et al., 2008; Torres-Pratts, Schärer & Schizas, 2009;

Chen et al., 2014; Wu et al., 2015; Wrange et al., 2016). This trend may be attributed to the extensive and long-term gene flow between populations, occurring multiple times since *A. eburneus* overcame the geographical barrier after its first invasion near the Korean Strait in the late 1980s (Kim, 1988). Given that the spread of *A. eburneus* was initiated from the southern coast of Korea, the haplotypes in the Tongyeong and Hanlim populations may be more closely related due to geographic connectivity, and the gene flow to the adjacent areas, including Incheon and Sokcho, may have begun gradually. Our results support this scenario, as the Tongyeong population showed evidently higher nucleotide diversity, indicating that the individuals in this population exhibit the potential to adapt to heterogeneous environments due to high degree of DNA polymorphism. Tongyeong is the geographical intersection between the Yellow Sea and the East Sea, serving as the bridge mediating the dispersal of larvae and adults in both directions and multiple times and ultimately leading to the accumulation of genetic diversity. However, to verify this speculation, further analyses using additional sequences are warranted for investigating the frequency and abundance of haplotypes, which would offer insights into the dispersal mechanisms of this species.

Our LBGM analysis revealed substantial geometric variations in the opercular plates of *A. eburneus*, which are reported for the first time in a study on barnacles. We identified two different aspects of morphological variations in the datasets: allometry and shape variations between sides and among populations. Allometry is a known morphological factor contributing to integration (Klingenberg, 2016), and group discrimination can often be improved following size correction (Sidlauskas, Mol & Vari, 2011). The examined individuals significantly varied in terms of the size of the scutum and tergum, indicating moderate allometric effects on the scutum morphology. Although we could not markedly improve population discrimination following size correction, the discrepancy in the presence of allometry between the scutum and tergum is noteworthy. In recent years, with increase in the number of studies analyzing the invertebrate morphology using LBGM, cases of the independent evolution of specific body parts regardless of being physically connected to one another have been noted (Karanovic & Bláha, 2019; Karanovic, Huyen & Brandão, 2019; Budečević et al., 2021). In this context, our findings suggest that the scutum and tergum of *A. eburneus* have evolved in size independently, despite being connected to each other to form the opercular plate.

Furthermore, similar trends were noted in the size-corrected shape variations between the sides. As such, the left and right sides of the plates were not described by the identical direction of LM shift (see Figs. 4C, D and 5C, D). Indeed, the asymmetry by the side of the opercular plates is not a new finding in barnacles. Specifically, members of the order Verrucomorpha, which inhabit deep-sea hydrothermal vents, exhibit an asymmetric form of the scutum, with one movable and the other fixed side (Newman, 2000). However, apart from that in such taxa presenting apparent disparity, the geometric asymmetry between the sides of the opercular plates has never been documented in acorn barnacles, with the exception of a previous study in which linear measurements of asymmetry were reported (Barnes & Healy, 1971). In our dataset, individuals from the Sokcho population showed obvious asymmetry in the tergum, forming a distinct cluster

in the left and right morphospace of the PCA biplot (see Fig 5A, B). Therefore, the two sides of the opercular plate have likely traced independent evolutionary paths in terms of shape, which may lead to incorrect population or species ~~differ~~ ~~ation~~ depending on which of the two sides is selected for examination.

Regarding population differentiation, the Tongyeong population could be clearly differentiated from the rest based on ~~the~~ variations in the shape of the tergum (narrow and elongated) on both sides of the opercular plate. According to Barnes and Healy (1971), water temperature is one of the major factors ~~sha~~ ~~g~~ ~~the~~ variations in the opercular plate morphology of *A. eburneus*. Based on linear measurement results, the authors reported that the growth rate of *A. eburneus* decreased in the cold season, resulting in the development of a smaller tergum, but increased in the warm season, resulting in the development of a larger tergum. Lively (1986) reported shape variations in the barnacle *Chthamalus anisopoma* Pilsbry, 1916 exposed to the predatory snail *Mexacanthina lugubris angelica* Oldroyd, 1918; as such, under predation pressure, some juveniles developed a bent morphology. Jarrett (2008) reported that *Chthamalus fissus* Darwin, 1854, which possesses an oval operculum, manages predation risk by changing the shape of the plate to become narrower, which is advantageous in escaping predators. Unfortunately, in the present study, we failed to determine the possible correlation of the observed shape variations in Tongyeong populations with water temperature and salinity due to the lack of differences in these environmental parameters among the localities throughout the two seasons (Table S2). In addition, we did not observe any evidence of ~~the~~ predation-related characteristics of the *A. eburneus* communities in the monitoring plates. Compared with direct observations in natural habitats, surveys using monitoring plates offer limited opportunities to witness natural phenomena representing the relationships among organisms due to limited space and resource accessibility. Nonetheless, based on our genetic analyses, evidently higher nucleotide diversity in the Tongyeong population implies greater plasticity for adaptation, which can drive the variations in ~~the~~ shape of the tergum in response to the external stimuli. Therefore, further studies are warranted on *A. eburneus* in ~~the~~ adjacent natural habitats using additional information on their community structure and trophic relationships.

Conclusion

Using mitochondrial gene sequence and LBGM analyses, the present study successfully unveiled the genetic and morphological diversity of ~~the~~ invasive *A. eburneus* in Korea. The genetic comparisons among the four populations confirmed two clades based on 3% pairwise genetic distances and 14 haplotypes, albeit without regional specificity. Furthermore, the quantitatively expressed size and shape variations resulted from phenotypic responses to heterogeneous environments. Nonetheless, the present study has limitations in that we could not accurately predict the major factors driving the dispersal of and morphological variations in *A. eburneus* due to the small number of sequences and insufficient information on environmental variables, which we intend to incorporate in future studies.

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Figure 1

Study plan of the study

(A) Map of ~~Korea~~ displaying the collection sites of *Amphibalanus eburneus* for the study. (B) Photograph of 10 acrylic attachment plates for monitoring. (C) Photograph of *A. eburneus* in ~~the~~ dorsal view.

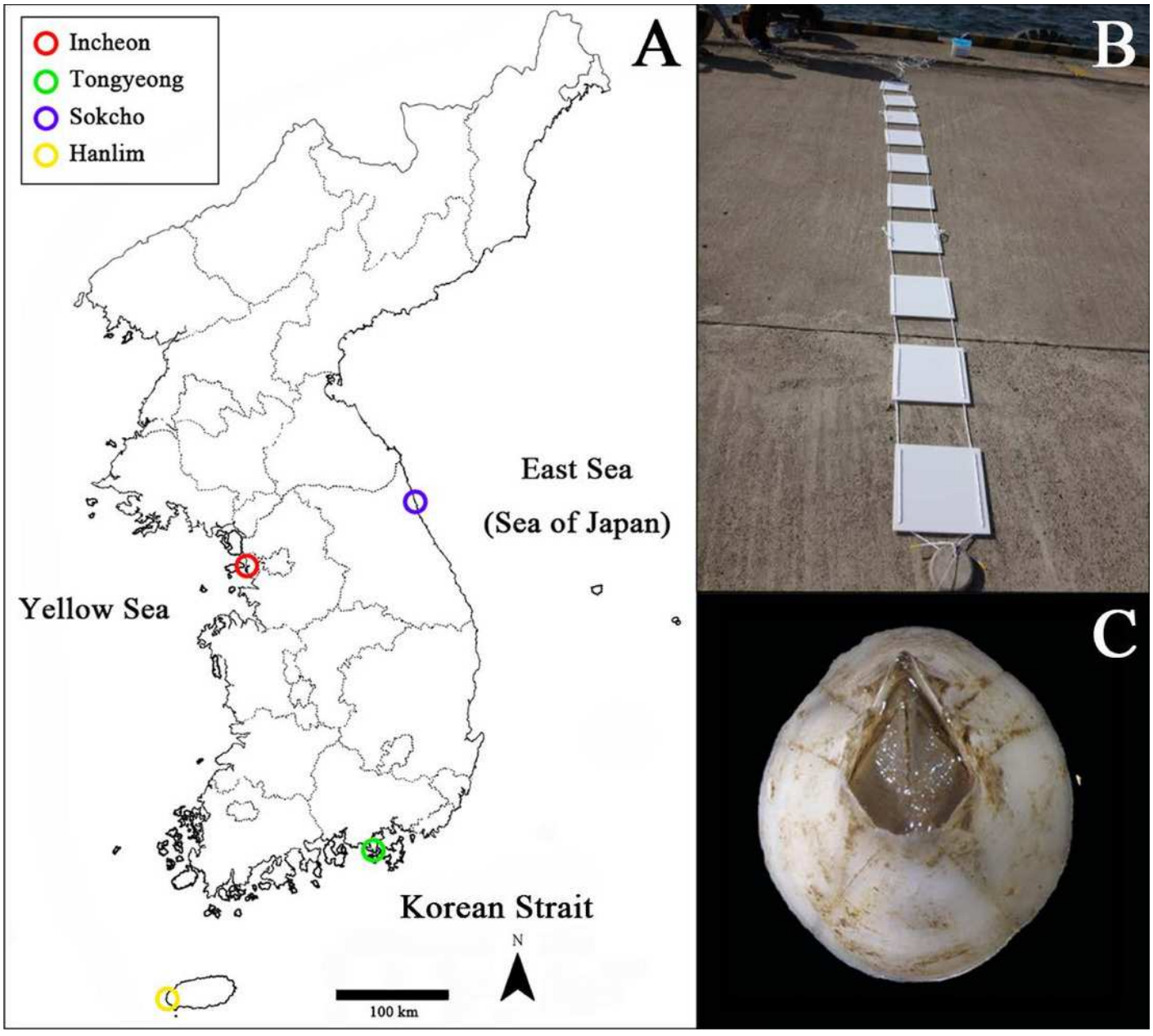

Figure 2

Anatomical points of the opercular plates.

(A) Ventral view of the left scutum with five anatomical points for landmark digitization (marked by red circles). (B) Ventral view of the left tergum with ten anatomical points for landmark digitization. Scale bar: 1 mm.

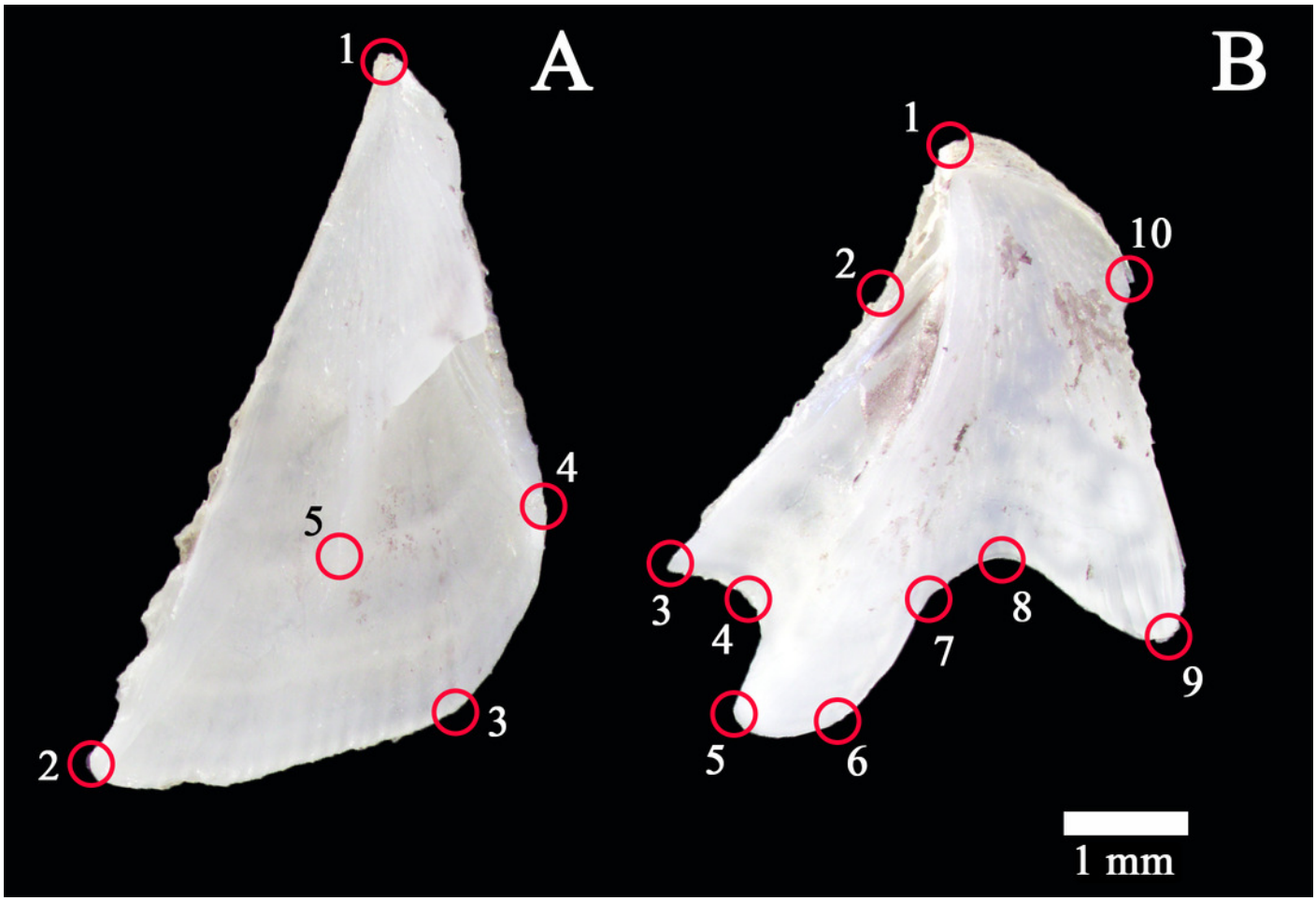

Figure 3

TCS haplotype network generated using 18 mtCOI sequences of *Amphibalanus eburneus* collected from four sites in Korea.

Different colors represent different collecting sites. Sizes of the nodes and pie segments are proportional to the haplotype frequency. Length of the branches is proportional to the number of mutational changes in haplotypes. Abbreviations: (I) Incheon, (T) Tongyeong, (S) Sokcho, (H) Hanlim.

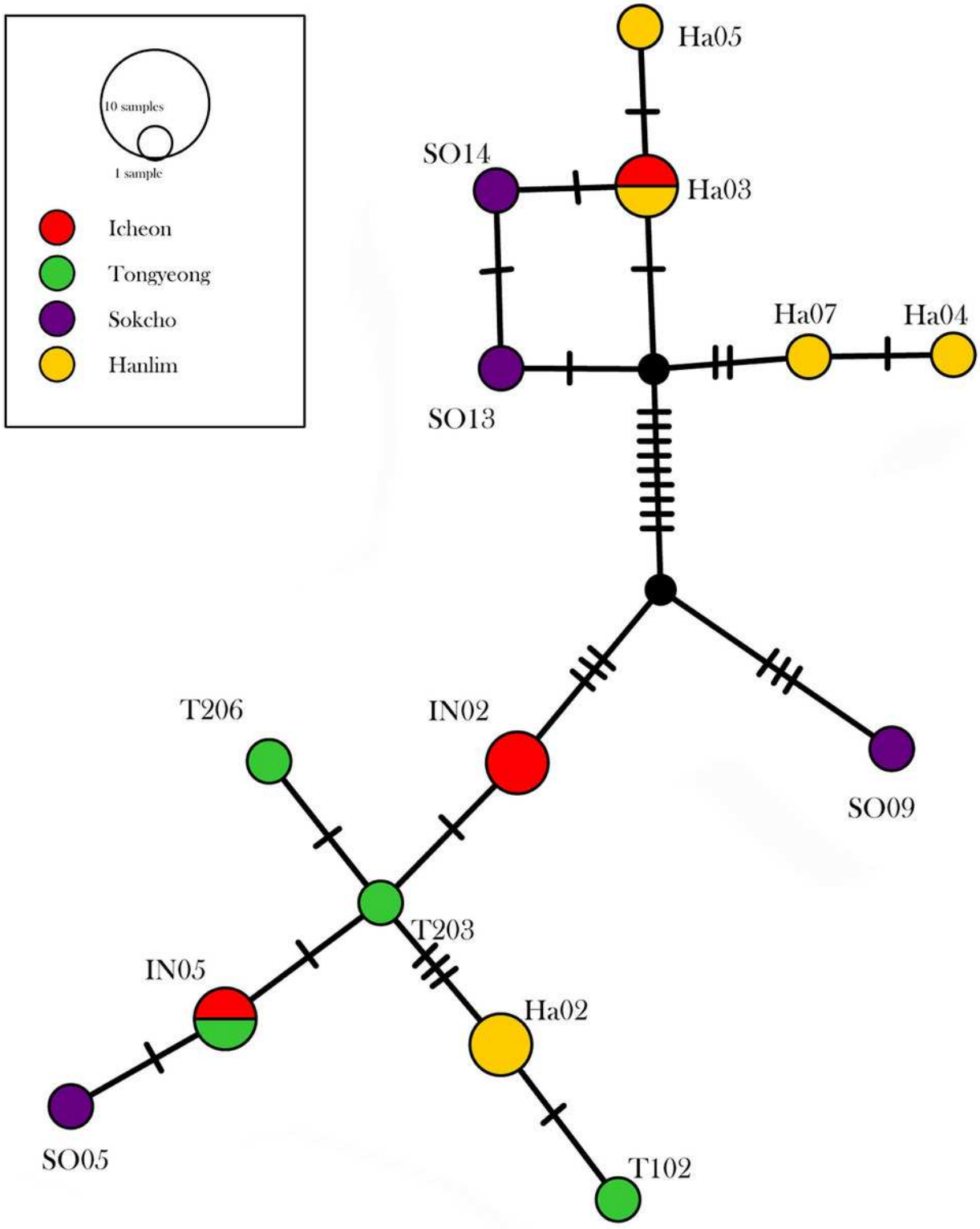
10 samples
1 sample
Icheon
Tongyeong
Sokcho
Hanlim
Ha05
SO14
Ha03
Ha07
Ha04
SO13
T206
IN02
SO09
T203
IN05
Ha02
SO05
T102

Figure 4

Principal component analysis of the residuals of the scutum shape coordinate.

(A) Scatter plot of the left scutum; principal components 1 and 2 are indicated on x- and y-axis, respectively. (B) Scatter plot of the right scutum. Red circles: Incheon individuals. Green circles: Tongyeong individuals. Purple circles: Sokcho individuals. Yellow circles: Hanlim individuals. (C) Wireframes of shape change in the left scutum (blue line) corresponding to the PC score against the mean shape (red line). (D) Wireframes of the shape change in the right scutum.

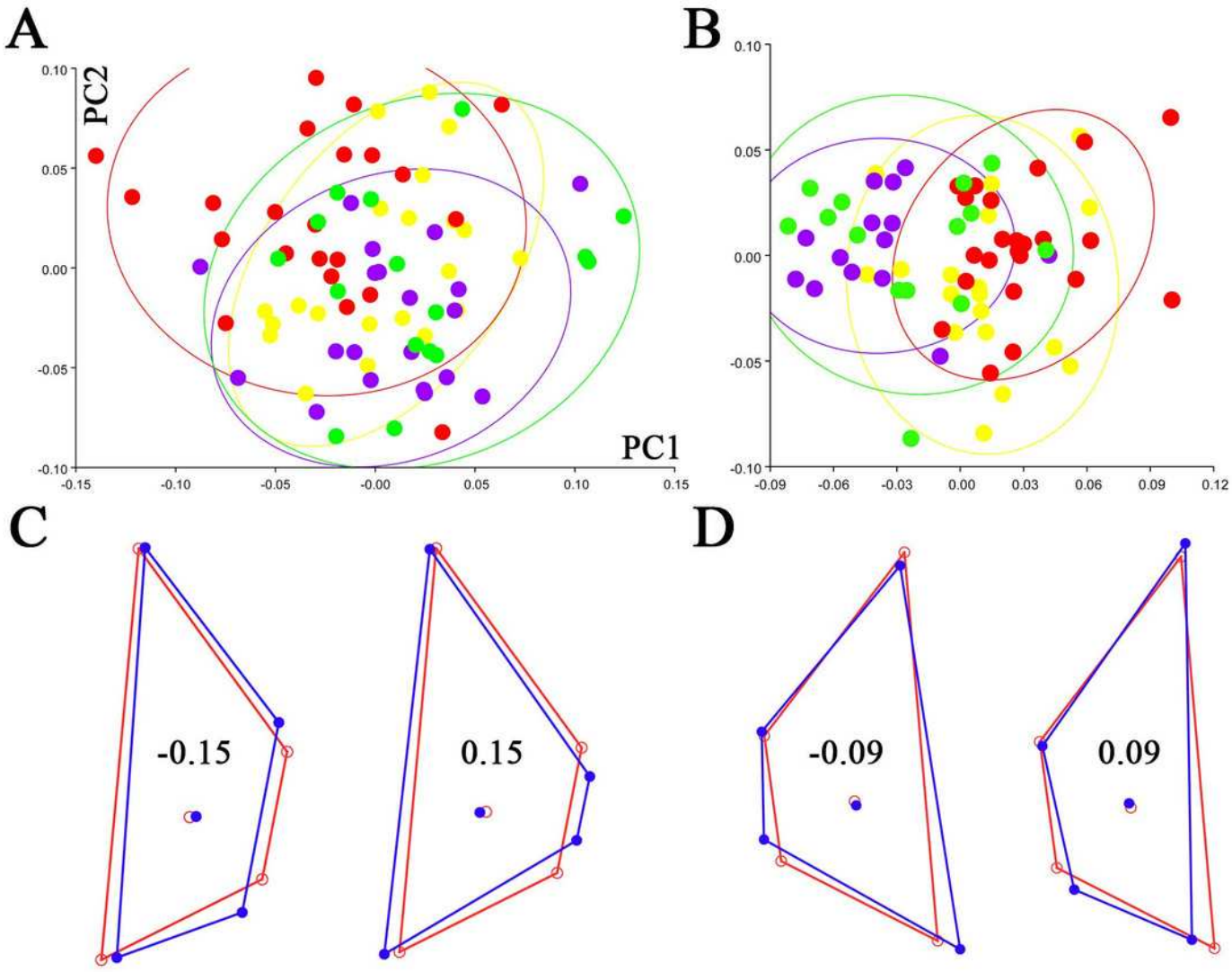

Figure 5

Principal component analysis of the residuals of the tergum shape coordinate.

(A) Scatter plot of the left tergum; principal components 1 and 2 are indicated on the x- and y-axis, respectively. (B) Scatter plot of the right tergum. Red circles: Incheon individuals. Green circles: Tongyeong individuals. Purple circle: Sokcho individuals. Yellow circles: Hanlim individuals. (C) Wireframes of shape change in the left tergum (blue line) corresponding to the PC score against the mean shape (red line). (D) Wireframes of the shape change in the right tergum.

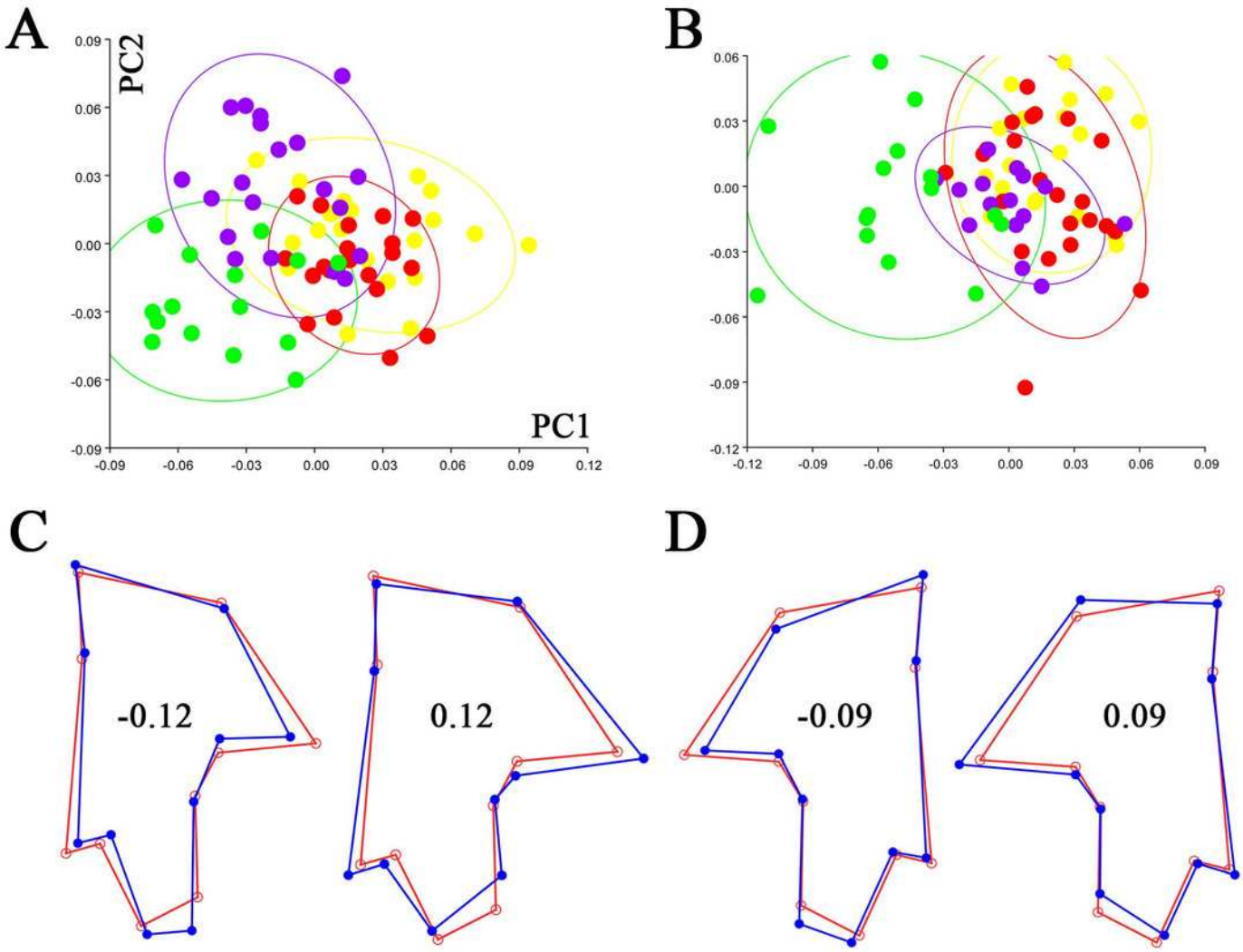

Table 1(on next page)

~~Variations~~ in the size and shape of the scutum and tergum inferred by Procrustes ANOVA using a randomized permutation procedure (10,000 iterations).

SS: sum of squares, MS: mean squares, df: degrees of freedom, F: Goodall's F critical value, P: probability of finding a random value larger than the observed value.

Part	Factor	SS	MS	df	F	P
			Size			
Scutum	Population	27.982413	9.327471	3	2.92	0.0414
	Individual	181.80021	3.134486	58	7.99	<0.0001
	Side	0.091607	0.091607	1	0.12	0.7304
	Digitizing	0.003736	0.000032	117	5.29	0.1721
			Shape			
	Population	0.25211324	0.0140062911	18	7.39	<0.0001
	Individual	0.65532580	0.0018831201	348	1.88	<0.0001
	Side	0.03222771	0.0053712850	6	5.49	<0.0001
	Digitizing	0.00123864	0.0000017644	702	0.78	0.7787
			Size			
Tergum	Population	14.863276	4.954425	3	2.09	0.1104
	Individual	146.846511	2.368492	62	3.58	<0.0001
	Side	1.139178	1.139178	1	1.72	0.1940
	Digitizing	0.10168	0.000776	131	7.79	0.2792

		Shape			
Population	0.31311889	0.0065233103	48	8.23	<0.0001
Individual	0.78594949	0.0007922878	992	3.53	<0.0001
Side	0.02271775	0.0014198593	16	6.33	<0.0001
Digitizing	0.00117952	0.0000005627	2096	0.78	0.7956

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Table 2(on next page)

Comparison of ~~variations~~ in the mean shape of the scutum and tergum among populations using canonical variate analysis.

Left score: Mahalanobis distance; right score: probability of finding a random value larger than the observed value.

Locality	Incheon	Tongyeong	Sokcho
Tongyeong	2.085/0.0001		
Sokcho	2.3851/0.0001	2.0506/0.0001	
Hanlim	1.7702/0.0001	1.3815/ 0.0173	1.7065/0.0001
Tongyeong	3.3156/0.0001		
Sokcho	2.9474/0.0001	1.9373/0.0005	
Hanlim	2.5981/0.0001	2.0773/0.0001	2.655/0.0001
Tongyeong	3.9417/0.0001		
Sokcho	3.7791/0.0001	4.3321/0.0001	
Hanlim	2.5423/0.0001	4.3213/0.0001	3.4708/0.0001
Tongyeong	4.2982/0.0001		
Sokcho	2.7491/0.0001	5.3365/0.0001	
Hanlim	3.2629/0.0001	5.7166/0.0001	2.6632/0.0001

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