Variation in Genetics, ~~and morphological~~morphology, and recruitment ~~variation~~ in the ~~Korean~~ invasive barnacle *Amphibalanus eburneus* (Gould, 1841) in Korea

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Abstract

The ivory barnacle *Amphibalanus eburneus* ~~of the family Balanidae~~ is a marine crustacean, which presents near-cosmopolitan distribution due to extensive introduction and exhibits a wide spectrum of phenotypic variation. ~~Here~~To elucidate processes of adaptive evolution through invasion, we investigated variation in ~~the~~ genetic structure, ~~and~~ shell ~~morphological~~ morphology, and recruitment patterns ~~variation in~~ for *A. eburneus*, from the southern Korean Peninsula ~~which~~ where it ~~has successfully settled in the Korean Peninsula since~~ it has been established since the late 1980s~~, to elucidate the processes of adaptive evolution through invasion~~. ~~We amplified the mitochondrial genetic marker mtCOI and generated a haplotype network to visualize the population structure.~~ We selected samples from four ~~populations~~ ecologically relevant regions representing all surrounding South Korean waters. ~~and~~ From these we amplified the mitochondrial genetic marker cytochrome oxidase subunit I (COI) from 258 individuals and generated a haplotype network to visualize population structure. To examine morphological variation, we applied two-dimensional landmark-based geometric morphometrics to the scutum and tergum for a subset of individuals.~~-~~. Furthermore, we estimated the density and plate occupancy of ~~collected~~ year-old individuals in the field to ~~determine the~~compare ~~difference in their~~recruitment responses ~~habitat conditions~~ among localities. We detected 35 haplotypes ~~in~~ among the four ~~populations~~ locations belonging to three distinct clades based on ~~the~~ moderate intraspecific pairwise genetic distance (≥3.5%). The haplotypes in these clades ~~did~~ were not locality-specific ~~distribute~~in their distribution ~~locality-specific~~. ~~We~~ In constrast, we did detect~~ed~~ interpopulation variation in ~~the~~ opercular shape and morphospace structure~~s~~, and one population

could be separated from the rest based on its distinct tergum morphotype alone~~of the tergum~~. This morphologically distinct population was also differentiated by displaying the lowest mean recruitment density and ~~the~~ level of plate occupancy. Our results indicate that although there is no relationship between ~~the level of the~~molecular variation in the ~~at the mt~~COI gene and ~~the level of~~geographic regions in South Korea, association with locality for ~~variation in the~~ operculum morphology~~.~~ and recruitment response suggest ecological adaptation by this barnacle in a new habitat.

Introduction

Barnacles are often dominant inhabitants of ~~the~~ coastal ecosystems~~. Many barnacle~~ with some species that are ~~invasive~~ transportable and that have spread across the globe (Gilg et al., 2010). The~~ir~~ cosmopolitan distribution of a number of species may be attributed to human-mediated translocation recently and over the past centuries, primarily via ~~the~~ ballast water and ship-hulls~~,~~ serving as the vectors for planktonic larvae and sessile adults, respectively (Zardus & Hadfield, 2005; Davidson et al., 2009; Carlton, Newman & Pitombo, 2011). ~~Invasive~~ Widespread barnacle species with long-range dispersal and massive ~~biomass~~ population sizes often exhibit phenotypic variation~~s~~ across geographic populations. Specifically, morphological variation~~s~~ in fitness-related physical traits in response to heterogeneous environments ~~have~~ has been commonly documented, which allows for the comprehensive study 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). Similarly, cirrus morphology varies with wave exposure to enhance particle capture efficiency (Arsenault, Marchinko & Palmer, 2001), alternating between shorter and thicker in high exposure situations and longer and thinner forms 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 recruit and 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), the Indian Ocean (Biccard & Griffiths, 2016), and the Canadian Arctic (Chan et al., 2015). Now, however, *A. eburneus* has become a harmful invader in many countries, inflicting extensive ecological and industrial damage due to a suite of 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~~ differences in salinity across diverse geographic habitats (Bacon, 1971; Jaberimanesh et al., 2019~~Dineen & Hines, 1994~~). Second, this species ~~follows variable temperature-dependent~~undergoes larval development across a range of temperatures (Scheltema & Williams, 1982); 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). Given these high competitive abilities, *A. eburneus* is an excellent model to study ~~the~~ genetic, morphological, and ecological variation~~s~~ across geographic populations accompanying biological invasion.

The Korean Peninsula is surrounded by four marginal seas: the Yellow Sea, East China Sea, Korea Strait, and East Sea (Sea of Japan). These regions provide ~~a~~ distinct habitat conditions for marine life due to their unique ocean dynamics and physical ~~factors~~environments, leading to biogeographic division of three ecoregions characterized by different biota (Rebstock & Kang, 2003; Han & Lee, 2020; Spalding et al., 2007). The western coast of Korea is strongly affected by the Yellow Sea. It generally shows low salinity in summer and high salinity in winter (Spalding et al., 2007). The general characteristic of the southern coast of Korea is a relatively warm current with ~~a~~ high salinity under the influence of the Tsushima Current of the Korea Strait which branches out of the East China Sea (Lim et al., 2019). The East Sea is characterized by low temperature and high salinity, which is known to have an ~~ocean~~ oceanogeographic intersection ~~in which~~where warm and cold currents meet. Naturally, barnacles inhabiting Korea show species-specific geographic distribution based on their preference for such ocean characteristics (Kim et al., 2020). Kim et al. (2020) ~~have~~ collected 20 species of native and ~~invasive~~ introduced barnacles in 44 localities of Korea from 2016 to 2018 and tracked changes in their distribution along with water temperature and salinity changes. They found that *A. eburneus* populations had ~~settled~~established in the southwestern coast of Korea representing the East China Sea ecoregion. They also found that *A. eburneus* preferred relatively warm water ~~rather than inhabiting in the cold one~~over the cooler water in both northeastern and northwestern parts of Korea.

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"). 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, East China Sea, Korea Strait, and East Sea (Sea of Japan). Considering the substantial geographic distances and heterogeneous habitat conditions among these regions, we speculated that *A. eburneus* might exhibit adaptive genetic and morphological variation across geographies ~~and morphological variations across geographies~~ and ~~what is a~~ possible routes of ~~the~~ invasion ~~pathway~~.

To this end, in the present study, we first addressed the question of genetic variability by analyzing the genetic structure of Korean *A. eburneus* populations using a mitochondrial genetic marker. Then, we examined variations in the shape and left-right symmetry of the opercular ~~and calcareous~~ plates of *A. eburneus*, which are of evolutionary and taxonomic significance (Pitombo et al., 2017), to evaluate morphology relative to habitat. 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 estimated the density and plate occupancy of ~~collected~~ *A. eburneus* individuals to ~~determine~~ assess ~~the~~ potential differences in recruitment ~~their habitat condition~~ among localities ~~that might explain the evolutionary trend of operculum morphology evaluated.~~

Materials & Methods

Sample collection

We accessed ports and made collections with the permission of the Korea Institute of Marine Science &Technology Promotion (KIMST, project number: 20190518). Individual barnacles were randomly collected from 10 pre-installed acrylic attachment plates (3×3 dm) (Fig. 1B) per location in April 2020, submerged at a depth of 1–3 m from sea level. Plates were deployed for a duration of 12 months to include exposure across both warm and cold seasons. ~~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 accessed ports and made collections with the permission of the Korea Institute of Marine Science &Technology Promotion (KIMST, project number: 20190518). We randomly collected the individuals using 10 pre-installed acrylic attachment plates (3×3 dm) (Fig. 1B) in April 2020, submerged at the depth of 1–3 m from the sea level and lasted 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 only individuals with ~~the~~ basal diameters of 2–2.5 cm, ~~according to~~ the maximum reported diameter for the species (Kaplan, 1988; Gosner, 1999). 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 photographed ~~the~~ each settlement plate before ~~the~~ barnacle collection using a digital camera (Olympus TG-5) and used a knife to remove the individuals from the plate and immediately preserved them in 95% ethanol~~. We returned the plates at the same place after the collection.~~ To examine the seasonal changes in water temperature and salinity, we collected these 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. (How often??)

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 partial sequences of the mitochondrial gene cytochrome c oxidase subunit I (~~mt~~COI) ~~partial sequences~~ with polymerase chain reaction (PCR) using PCR premix (BIONEER. Co, Daejeon, Korea) in an 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~~ with the following amplification protocol: ~~for mtCOI consisted of~~ initial denaturation at 94 °C for 2 min~~,~~ followed by 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~~ sequence identities 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) and performed a population genetic analysis at the continental scale, comparing the level of pairwise genetic differentiation between all obtained sequences and~~We deposited all obtained sequences in GenBank (Article S1)~~. We performed sequence alignment using the MAFFT v7.313 (Katoh & Standley, 2013) ~~with~~ and included 13 additional sequences ~~those~~ of *A. eburneus* already published and publicly available in GenBank: one from Los Angeles (North Pacific Ocean, KU204257), one from Ketchikan (North Pacific Ocean, KU204298), four from Tangier Sound (North Atlantic Ocean, MK308058, MK308095, MK308188, MK308249), four from Mastic beach (North Atlantic Ocean, MZ595234, MZ595235, MZ595236, MT192780), and three from Gomishan Wetland (Caspian Sea, MK240317, MK240318, MK240319).. We estimated uncorrected pairwise distances using Geneious prime (https://www.geneious.com/prime/)~~. We~~ and used the TCS algorithm implemented in PopART (Clement, Posada & Crandall, 2000; Leigh & Bryant, 2015) to evaluate genealogical relationships among ~~mt~~COI 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). We examined the degree of gene flow among populations based on parameters F_{ST} (Hudson, Slatkin & Maddison, 1992) to check ~~whether a~~ phylogeographic structure ~~existed for all~~among populations.

Morphological data acquisition

How many barnacles were measured? The same individuals used for sequencing above? 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 the 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 parts on a petri dish covered by black paper and included a scale to take calibrated 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 on 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 depth of field more fully, 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 in side-by-side pairs (matching symmetry between left and right parts) (Ho et al., 2009). We generated copies~~y~~ of all images and employed them along 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 five anatomical reference points of the 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 medial margin (LM 4); one located on the lower point of basal ridge (LM 5). We selected ten anatomical references of the 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 carinal side (LM 7); one located along the inflection point of basal margin (LM 8); one located on the distomedial tip of cranial side (LM 9); one located on the proximomedial tip of cranial 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 implementing the Procrustes ANOVA to test error terms, we employed the first digitization dataset and divided it into the left and right datasets. We carried out typical LBGM analyses on the left and right datasets, including regression, principal component analysis (PCA), and canonical variate analysis (CVA), following the procedure previously described in Kim et al. (2021). We performed regressions of shape onto size to test allometry based on regression scores and CS (Monteiro, 1999; Klingenberg, 2016). We applied a permutation test (Good, 2013) to assess the statistical significance against the null hypothesis. 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 PCA, which 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 used the wire frame to visualize scutum and tergum's average shape variations along major PCA axes. We employed separated residual components for CVA, which is a multivariate method, producing a criterion for reliably distinguishing among multiple groups preliminarily defined. 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.

Measurement of density and plate occupancy of recruited barnacle

We calculated the density of *A. eburneus* ~~recruited~~ per attachment plate across sampling areas as the number of barnacles found in ~~the~~ each plate photograph by its area (9 dm^2). We measured the ~~occupied~~ area occupied ~~of~~ by *A. eburneus* ~~with other macroinvertebrates~~ based on pixel analysis of photographs using ImageJ software (Schneider, Rasband & Eliceiri, 2012). We used the plates ~~where~~ from which *A. eburneus* was collected to calculate the occupancy of animals as the ratio of the covered area (%) to the total area of the ~~attachment~~ plate. We estimated the mean, standard deviation, maximum, and minimum occupancy~~. This method has been used for a long time~~ as has been done ~~to measure~~for measuring the recruitment potential and abundance of various sessile marine organisms (Harriott & Fisk, 1987; Fisk & Harriott, 1990; Guy-Haim, Rilov & Achituv, 2015).

Results

Population genetic diversity

~~Seventy~~ Fifty seven partial ~~mt~~COI ~~patrial~~ sequences were obtained: ten from Incheon, 12 from Tongyeong, 13 from Sokcho, 22 from Hanlim, ~~one from Los Angeles (North Pacific Ocean, KU204257), one from Ketchikan (North Pacific Ocean, KU204298), four from Tangier Sound (North Atlantic Ocean, MK308058, MK308095, MK308188, MK308249), four from Mastic beach (North Atlantic Ocean, MZ595234, MZ595235, MZ595236, MT192780), and three from Gomishan Wetland (Caspian Sea, MK240317, MK240318, MK240319).~~ The final alignment was trimmed to a length of 627 base pairs, of which 578 were constant and 49 were variable (17 singleton and 32 parsimony informative). No stop codons were detected, and the sequences encoded polypeptides of 227 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 3.5% (Table S1), with the highest value recorded between the individuals of Sokcho and Hanlim.

The parsimony network generated with TCS (Fig. 3) detected 35 haplotypes (Hd = 0.961, π = 0.016) from ~~the~~ all obtained sequences, forming three distinct clades (A, B, and C). Clade A was separated from Clade B by 12 mutational substitutions. Clade C was distinguished from Clades A and B by 12 and 6 substitutions, respectively. Overall, the frequency of haplotypes in each clade was not locality-specific but Clade A contained only individuals matching closely with those from the Atlantic Ocean and Caspian Sea, Clade B contained only indivduals matching closely with those from the Pacific Ocean and Caspian Sea, whereas Clade C individuals were intermediate with no identifiable match with any sea or ocean. Nearly all haplotypes detected presented widespread geographic distribution except for those of Tangier Sound, which were only found in clade A. The Incheon population included seven haplotypes (Hd = 0.933, π = 0.01304) belonging to clades A and B. Four of these seven haplotypes were shared by Tongyeong, Hanlim, Los Angeles, Tangier Sound, and Mastic beach populations. The Tongyeong population comprised eight haplotypes (Hd = 0.924, π = 0.01352) belonging to clades A and B. Two of these eight haplotypes were shared by Sokcho, Hanlim at the former clade. The Sokcho population included nine haplotypes (Hd = 0.897, π = 0.01795), two of which exclusively belonged to clade A. The Hanlim population included 14 haplotypes (Hd = 0.957, π = 0.01606), two of which belonged to clades A and B. ~~Throughout~~ Encompassing all ~~populations~~ haplotypes of *A. eburneus*, except for those of Los Angeles and Ketchikan represented by only single sequences, the evidence ~~of~~ for population genetic structuring in Korea was low as revealed by Fst analysis (Table 1). ~~All~~ But, all Korean populations were significantly differentiated from North Atlantic populations, Tangier sound, and Mastic beach, with Fst values ranging from 0.3634 (Hanlim) to 0.63869 (Tongyeong). While the Caspian population, Gomishan wetland, was more closely related to Korean ones, with Fst values ranging from -0.01160 (Sokcho) to 0.09686 (Tongyeong), than North Atlantic ones, with Fst values of 0.2037 for Tangier Sound and 0.20952 for Mastic Beach. Within Korean populations, Tonyeong was most distantly related to populatios of Sokcho and Hanlim, with Fst values of 0.04338 and 0.07844, respectively.

Variations in size and shape

The ANOVA results (Table 2) yielded negligible digitizing errors for all datasets, with the individual variability mean square (MS) and F values far ~~exceeding~~ exceeded 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 side-by-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 side-by-side was significant, contributing to ~~the~~ variation~~s~~ 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 3), 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.

Density and plate occupancy of *Amphibalanus eburneus* with other macroinvertebrates

Table 4 summarizes plate density of *A. eburneus* ~~recruited~~ recruitment in the surveyed areas. The Hanlim population showed the most significant mean density at 65.27 indi/dm^2, followed by Incheon, Sokcho, and Tongyeong populations (density at 54.89, 32.04, 8.67 indi/dm^2, respectively).

Plate occupancies of *A. eburneus* with other sessile macroinvertebrates in the surveyed areas are summarized in Table 5. In Incheon, *A. eburneus* was recruited across plates with five macroinvertebrate species. *Amphibalanus eburneus* showed much higher mean occupancy of 43.89% than other animals, with the maximum occupancy of 59.23%. Of the 11 species recruited in Tongyeong plates, *Didemnum vexillum* Kott, 2002, called " sea vormit" belonging to Chordata, was the most dominant group with a mean occupancy of 19.64%. *Balanus trigonus* Darwin, 1854 was the second most dominant barnacle species with a mean occupancy of 17.04%. While *A. eburneus* was the fourth most dominant one (6.21%) after the third most dominant one, *Halichondria (Halichondria) bowerbanki* Burton, 1930, the sea sponge (12.99%). Five macroinvertebrate species appeared in the Sokcho plates. *Amphibalanus eburneus* was the second most dominant taxon in Sokcho with an occupancy of 13.31% following the most dominant one of *Ascidiella aspersa* (Müller, 1776), a species of solitary sea squirts. In the Hanlim plates, seven species were observed, of which *A. eburneus* was the most dominant taxon with a mean occupancy of 29.27%. *Amphibalanus amphitrite* (Darwin, 1854) showed the second most mean occupancy at 4.86%.

Discussion

The present study investigated ~~the~~ adaptive phenotypic responses ~~of~~ in the genetics, morphology, and ~~morphological~~ recruitment ~~traits in~~ of ~~the~~ Korean populations of the alien barnacle *A. eburneus* ~~populations~~. Based on the pairwise distance of ~~the~~ mtCOI sequences, *A. eburneus* populations showed a low diversification rate. The values ranged between 0% and 3%, falling within the level of intra-specific ~~level~~ variability for this species, compared to the much higher inter-specific values of mtCOI sequences in other balanomorph barnacles (Tsang et al., 2008; Chen et al., 2014). Haplotype network analysis revealed detailed genetic characteristics of the populations, establishing three separate clades. Interestingly, the clade separation was not locality-specific, and nearly all haplotypes of the populations were randomly placed in the three clades. Fst value comparison between Korean populations confirmed that *A. eburneus* ~~had~~ has extensively ~~settled down~~established in all directions of the Korean Peninsula without ~~a~~

significant genetic differentiation suggesting that dispersal and/or delivery is intermixed. As s~~S~~uch *A. eburneus*'s invasion success could be primarily attributed to its wide range of adaptability in various salinity and water temperatures (Costlow & Bookhout, 1957;~~-~~3 new refs ~~Dineen & Hines, 1994~~), comparable to other ~~invasive~~ introduced balanomorph species. *Amphibalanus improvisus* (Darwin, 1854) is known to exhibit a complex haplotype admixture with global distribution (Chen et., 2014; Wrange et al., 2016) based on a high degree of euryhalinity and eurythermy (Pansch et al., 2013). It showed a similar distribution in the Korean Peninsula (Kim et al., 2020). Contrarily, another balanomorph invasive species, *Perforatus perforates* (Bruguière, 1789), showed a distribution confined to eastern and southern parts of the Korean Peninsula owing to its weak capability to ~~physical variation~~vary physically (Kim et al. 2020). Indeed, the capability to endure environmental fluctuation is known as a significant biological factor determining various marine invertebrates' geographical distributions (Chang et al. 2017; Seo et al 2021).

~~The~~ Parsimony network analysis revealed that Korean populations shared haplotypes with North Atlantic, North Pacific, and the Caspian Sea populations. However, the genetic differentiation occurred more significantly between Korean and ~~the~~ North Atlantic populations than between Korean and Caspian Sea populations. Lacking genetic differentiation is noteworthy given that the Caspian Sea is a geographically isolated region with a large distance from the Far East. Wrange et al. (2016) have reported a similar phenomenon based on their global scale population genetics study of *A. improvises*. They showed that the Caspian population shared haplotypes with Japan's, with nearly no genetic differentiation. However, they did not provide any specific hypothesis on the dispersal process between both regions. Unfortunately, it is also challenging to determine the actual invasion pathway into the Korean Peninsula due to insufficient alien genetic information. We can hypothesize that the first introduction of *A. eburneus* into the Korean Peninsula might have occurred from the Caspian Sea, and the later genetic exchange has taken place with new populations from North Atlantic and North Pacific Oceans, significantly contributing to the genetic structure with a high haplotype diversity in these populations. Shipping and other anthropogenic activities might have played an important role in shaping the current population genetic structure of *A. eburneus*. Fouling organisms including barnacles often form dense populations on ship hulls as large founding populations and/or potential admixture are essential for the invaders to overcome the founder effect and demographic bottlenecks (Roman & Darling, 2007; Dlugosch & Parker, 2008). Previous monitoring surveys support our speculation (Park et al., 2017; Kim et al., 2020)~~, reporting~~ that Korean *A. eburneus* populations are more frequently found in ~~the~~ ports than in ~~natural~~ exposed habitats. It is also likely that ~~the~~ shipping along the coastline is primarily responsible for the gene flow ~~within the~~among Korean populations because ~~the~~ internal shipping ~~of~~ across the Korean Peninsula is generally ~~inactive~~ impossible due to the mountain range developed along the eastern part precluding direct linkage between the east and west coast.

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 factor contributing to morphological integration (Klingenberg, 2013; 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, asymmetry side-by-side of the opercular plates is not unknown 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 plate 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; Chen et al., 2014). 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 identification depending on which of the two sides is selected for taxonomic examination.

Regarding population differentiation, the Tongyeong population could be clearly differentiated from the rest based on variations in the shape of the tergum (narrow and elongated) on both sides of the opercular plate. In general, a high recruitment density can determine the barnacle shape, often resulting in the columnar shell in response to intensive competition for the space and foraging (Barnes & Powell, 1950; Bertness, Gaines & Yeh, 1998; Hills & Thomason 2003). Our analysis suggested a less possibility of the relationship between shape variation and intra-specific competition as the Tongyeong population showed the lowest value among populations. On the other hand, it was found that *A. eburneus* in Tongyeong ~~had~~ did not compete~~d~~ as favorably with ~~the most~~other diverse groups of macroinvertebrates~~, which was opposite to~~as it did in other localities. ~~in which~~ *A. eburneus* was one of the most dominant groups among collected animals in all other locations. A threat by the complex relationship among collected animals in Tongyeong might have exceeded that of intra-specific space competition and enforced *A. eburneus*'s phenotypic change. For example, *Didemnum vexillum* (Stefaniak, 2017), known as a worldwide invasive colonial tunicate, covered the plate over two times *A. eburneus*’s mean occupancy in Tongyeong. At some plates, this tunicate had dominantly occupied the plate by covering not only the empty space, but also the top of the *A. eburneus* assemblage (see Tongyeong in Figure S1), disturbing significantly the movement of the opercular plates and the cirri, which are essential behaviors of feeding, respiration, and copulation. This hypothesis needs further survey given that there has been no experimental record so far on the shape variation of barnacle’s opercular plate caused by such relationship with other sessile neighbors.

According to Barnes and Healy (1971), water temperature is also another major factor affecting variations in the opercular plate morphology of *A. eburneus*. Based on linear measurement results, they reported that the population of *A. eburneus* in the cold region differed markedly from those in the warm region by showing the basal margin of the carinal side of the tergum deeply hollowed out. 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 predatory gastropod, *Mexacanthina lugubris lugubris* Sowerby, 1821. Unfortunately, in the present study, we failed to determine possible correlations of the observed shape variations in Tongyeong populations with a year record of water temperature and salinity due to the lack of a significant differences with other localities (Table S2). In addition, we did not observe any evidence of 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, the genetic diversity in the Korean *A. eburneus* populations imply a great plasticity for adaptation, which can drive variations in shape of the tergum in response to certain 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 ~~in the ~~invasive ~~alien barnacle *A. eburneus* in Korea. ~~The ~~G~~g~~enetic comparisons among ~~the ~~four ~~populations ~~regions confirmed three clades based on 3.5% pairwise genetic distances and 35 haplotypes, albeit without regional specificity. ~~Furthermore~~In contrast, ~~the ~~quantitatively ~~expressed ~~described variations in opercular plate size and shape ~~variations appeared in one ~~did identify a unique convergence in narrow elongated tergums in the population from Tongyeong. This population was also differentiated from all others ~~in which the ~~by reduced recruitment density and plate occupancy ~~are differentiated ~~it~~from others~~. ~~Nonetheless, the present study has limitations in that we could not accurately~~Despite the inability to predict ~~the ~~major factors driving ~~the ~~dispersal ~~of ~~and morphological variation~~s~~ in *A. eburneus* in South Korea, ~~due to insufficient number of sequences and information on environmental variables, which we intend to incorporate in future studies.~~we demonstrated a similar phenotypic response in *A. eburneus* among diverse genotypes from a single location.

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