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Original Research Articles

mtCO1-based population structure and genetic diversity of Pacific oyster *Crassostrea gigas* populations acquired from two farms in South Korea

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Since the early 1990s in South Korea, climatic and anthropogenic factors have incurred the reduction of the wild seeds of the Pacific oyster, *Crassostrea gigas*, which raised concerns about losing genetic diversity and accelerating genetic deterioration. We assessed the genetic diversity of *C. gigas* populations from two farms (Tongyeong and Gadeokdo) on the southern coast, where about 80% of the cultivated oysters in Korea are produced. Tongyeong showed slightly higher diversity than Gadeokdo, but both populations had a similar genetic structure characterized by low nucleotide diversity. Comparative haplotype analyses provided data supporting genetic features of the populations that include (1) weak genotype-locality relationship, (2) low levels of gene flow between populations, and (3) possible seasonal fluctuation of genetic variation within a population. Furthermore, the highly alike haplotype network patterns were observed between the wild and farm populations as well as among the populations in neighboring countries, which suggests that the genetic structure is conserved between wild and hatchery populations, and geographic proximity has minimal influence on the genetic composition.

INTRODUCTION

The Pacific oyster, *Crassostrea gigas* (*Magallana gigas*), is a seasonal delicacy beloved all over the world and has become one of the globally important aquaculture species.[1] It is also reputable for its high nutritional quality and health benefits; therefore, it has been dubbed "Milk of Sea" (USDA 2018). Since the new suspended culture techniques with longlines and rafts had been employed in Korea in the 1960s, the nationwide yearly production of *C. Gigas* dramatically increased and came to the point where it exceeded 300,000 metric tons, making the country the second-largest producer in the world as of 2019.[2,3] During which Korea's oyster industry also faced a significant reduction in natural seed production occurred by the climatic change and the country's rapid industrialization throughout the time.[4] Subsequently, many seeds got imported from other countries, such as Japan and the United States, to replenish the drastic shortage of seeds, and afterwards they blended into the wild populations.[4,5] In 2007, the 10,900 tons of crude oil released from the tanker MV Hebei Spirit, covered 375km of the shoreline of the western coast of Korea.[6] The toxicity of the oil wiped out most oysters at more than 90% of the oyster farms in that region, triggering the displacement of the natural spats by the ones from southern areas.[7] In consequence, this accident and the climatic and anthropogenic interference further depleted the wild stock on the West Coast and predictably impacted the diversity.

Mitochondrial DNA (mtDNA) analysis is one of the commonly adopted approaches to examine divergences in animals by its small size, maternal and non-recombining mode of inheritance, rapid evolution, extensive intraspecific polymorphisms, etc.[8] Among mtDNA markers, cytochrome oxidase *c* subunit I (mtCOI) is an eminently conserved protein-encoding gene in the mitochondrial genome.[9] The effectiveness of mtCOI analysis in studying genetic diversity and genetic structure of marine mollusk populations has been charted in blood cockle, estuarine clam, and hard clam.[10-12] In Korea, although a few studies have examined the genetic diversity of *C. gigas* using molecular-based

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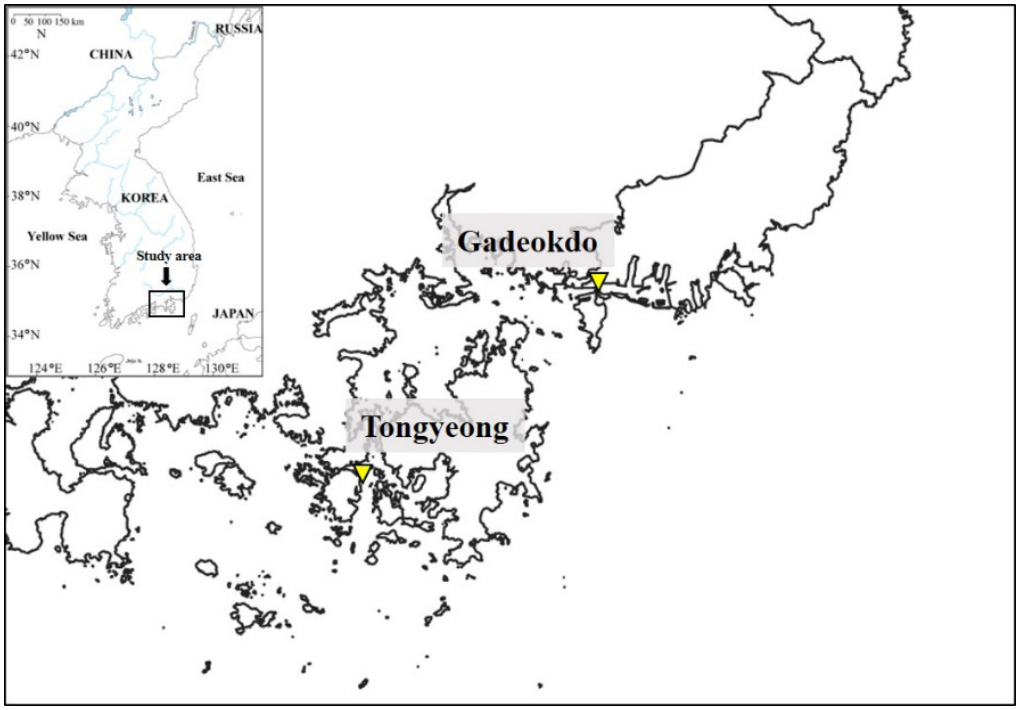

Figure 1. Pacific oyster, *C. gigas*, sampling sites in Tongyeong (TY) and Gadeokdo (GD), South Korea.

methods,[13,14] our understanding of the current genetic structure and diversity is still insufficient, given the above chronologically deteriorating circumstances and the economic value of this species.

As a preliminary work for full-on research on the populations throughout the Korean peninsula, the present study investigated the mtCOI gene to understand better the genetic variation and population structure of *C. gigas* from Tongyeong and Gadeokdo in the southern coast, where about 80% of the cultivated oysters in Korea are produced.[15,16]

MATERIALS AND METHODS

SAMPLE COLLECTION

Forty oysters each were obtained from aquaculture farms in Tongyeong (TY, 34°52'38.4"N 128°14'40.8"E) and Gadeokdo (GD, 35°04'19.0"N 128°50'28.6"E) with ten collected at each site in October 2020 and January, April, and July 2021 (Figure 1). They were immediately transferred on ice to the Laboratory of Physiology, Pukyong National University, Busan, Korea. Their fresh mantle edges were dissected and stored in 100% ethanol at room temperature until DNA isolation.

DNA EXTRACTION

The excised samples were incubated with 600 µL of TNES-urea buffer (10 mM Tris- HCl, pH 8.0; 125 mM NaCl; 10 mM EDTA, pH 8.0; 0.5% SDS; 6 M urea) and 10 µL proteinase K (20 µg/mL) at 60°C for at least 12 h, and total genomic DNA (gDNA) was extracted using the phenol/chloroform method.[17] The extracted gDNA was dried at room temperature, resuspended in TE buffer (10mM Tris-HCl; 1 mM EDTA, pH 8.0), and stored at −20°C for later use. Its quantity and quality were checked using a spectrophotometer (DS-11; DeNovix, DE, Wilmington, USA).

PRIMER DESIGN

To design a pair of primers for polymerase chain reaction (PCR), we retrieved mtCOI sequences from GenBank (https://www.ncbi.nlm.nih.gov/)) and aligned them using ClustalW in BioEdit 7.2.[18] After comparing the aligned nucleotide matrix, conserved regions of the *Magallana* sequences were selected as forward and reverse primers. Their melting temperatures and secondary structures were checked with the web service Sequence Manipulation Suite[19] before oligonucleotide synthesis.

PCR AMPLIFICATION AND SEQUENCING

PCR was conducted in a 20-µL reaction using *AccuPower*® PCR PreMix (Bioneer, Daejeon, South Korea) with 0.2 µM of the forward [CRA- MT-18171f (5'-TTTCG-CACTGGGCAGGTAT-3')] and reverse [CRA-MT-1865r (5'-ATTTCGCACTGGGCAGGTAT-3')] primers and 1 µL of gDNA template (20 ng/µL). The DNA was amplified using the ProFlex PCR System (Thermo Fisher Scientific, Waltham, MA, USA) at 95°C for 5 min, followed by 35 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 2 min, with a 5-min final extension at 72°C. The PCR product was electrophoresed in 1.5% agarose gel stained with ChamelGreen™ I (SFC Probes, South Korea) followed by gel purification with an *AccuPrep*® PCR Purification Kit (Bioneer, Daejeon, South Korea). The purified DNA was subsequently sequenced at Macrogen (Seoul, South Korea).

Table 1. mtCOI sequences of various *C. gigas* populations

No.	Haplotype locality	mtCOI NCBI accession number	References
	China	KP099007, KP099008, KP099009, KP099010, KP099011, KP099012, KP099013, KP099017, KP099024, KP099029, KP099030, KP099031, KP099032, KP099033, KP099034, KP099044, KP099050, KP099051, KP099052, KP099038, KP099039, KP099040, KP099041, KP099043, KP099019, KP099021, KP099022, KP099023, KP099015, KP099016, KP099018, KP099045, KP099046, KP099047, KP099048, KP099014, KP099049	Li et al.[20]
2	Incheon, Korea	KP099020, KP099025, KP099026, KP099027, KP099028	Li et al.[20]
3	Suncheon Bay, Korea	KP099042	Li et al.[20]
		AB641329, AB641330, AB641331, AB641332, AB641333, AB641334	Hong et al., 2011[21]
4	Saiki, Japan	KP099035, KP099036, KP099037	Li et al.[20]
	Matsushima Bay, Japan	AB736493, AB736494, AB736495, AB736496, AB736497, AB736498, AB736499	Sekino et al., 2012[22]
5	Canada	KF643857, KF643604, KF643519	Layton et al., 2014[23]
6	Taiwan	KX345121, KX345122, KX345123, KX345124, KX345125, KX345126, KX345127, KX345128	Hsiao, 2016[24]
7	Brazil	KX436124, KX436125, KX436126, KX436127, KX436128, KX436129, KX436130, KX436131	Moreira et al., 2016[25]
8	Mexico	KT317429, KT317430, KT317431, KT317432, KT317433, KT317434, KT317435, KT317436	Raith et al., 2016[26]
9	Netherlands	DQ659367, DQ659368, DQ659369, DQ659371	Cardoso et al. (unpublished)
10	United States	MT219482, MT219483, MT219484, MT219485, MT219486, MT219487, MT219488	Sutton et al., 2020[27]

DATA ANALYSIS

The raw sequence data were imported to Sequencher v. 5.4.5 (Gene Codes, Ann Arbor, MI, USA) to check the base manually calls on the chromatograms. Using the Clustal W function in BioEdit 7.2.5, we aligned all sequences with the default parameters. After the manual correction of obvious misalignments, a nucleotide sequence matrix of 863-bp mtCOI fragments from the 80 *C. gigas* specimens from TY and GD was generated. DnaSP v. 6[28] was employed to estimate the total number of haplotypes (H) and haplotype distribution among populations. The haplotype diversity (h), nucleotide diversity (π), and mean number of pairwise differences (k) were calculated using ARLEQUIN v. 3.5.[29]

A parsimony network was generated and plotted with TCS analysis[30] using PopART v. 1.7[31] to evaluate the genealogical relationship of all *C. gigas* haplotypes. The network was constructed from 80 sequences from the TY and GD populations, alongside 117 sequences from various countries, which had been examined in the previously published studies (**Table 1**).

The genetic structure of the *C. gigas* populations from the TY and GD was assessed by analysis of molecular variance (AMOVA) on ARLEQUIN v. 3.5. The fixation index (FST) value ranges from 0 (no differentiation between subpopulations) to 1 (complete differentiation of populations).[32] The significance of each pairwise comparison was tested with 10,000 random replicates.

RESULTS

AMPLIFICATION OF MTCOI GENE FROM A CHRONOLOGICAL SET OF C. GIGAS

Eighty *C. gigas* samples collected from aquaculture farms in Tongyeong (TY) and Gadeokdo (GD) over the 4 consecutive seasons (i.e., 10 TY and 10 GD samples per season) were used for this study. Initially, the mtCOI gene of each individual was amplified by PCR. All samples generated a single 2000-bp amplicon, indicating the presence of an intact mtCOI gene in each individual (Figure 2). Subsequent sequencing confirmed that all of the amplicons are mtCOI-specific (data not shown).

GENETIC DIVERSITY

To characterize the genetic diversity of *C. gigas* populations from TY and GD, multiple sequence analysis was performed using an 863-bp nucleotide sequence of the mtCOI from the 80 samples. This analysis identified 27 different single nu-

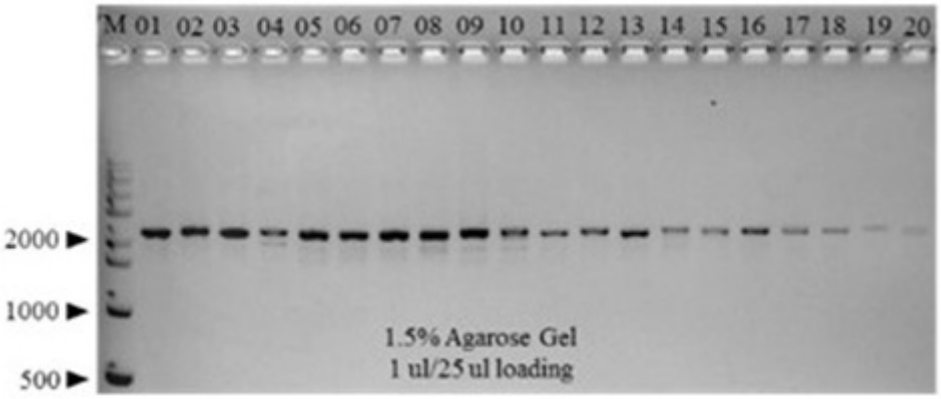

Figure 2. PCR amplification of mitochondrial *cytochrome c oxidase subunit 1* from *C. gigas* samples collected from TY (01 to 10) and GD (11 to 20) in Fall. The other seasons' amplification had the same band pattern. A pair of primers, CRA-MT-18171f and CRA-MT-1865r, were used to amplify a 2000-bp fragment that includes the gene. M, 1 kb size marker.

Table 2. Diversity indices of *C. gigas* from TY and GD.

Sampling sites	n	s	H	h ± SD	π	k
TY	40	16	13	0.5154 ± 0.0982	0.000927	0.80
GD	40	10	9	0.4397 ± 0.0983	0.000689	0.59
Mean				0.4760 ± 0.0710	0.00081	0.70

n, number of individuals sampled per site; s, number of polymorphic sites; H, number of haplotypes; h, haplotype diversity; π, nucleotide diversity; and k, mean number of pairwise differences.

cleotide polymorphism (SNP) sites in total, consisting of 21 transitions (a/g, c/t) and 4 transversions (t/g), with no indels being detected. The translated amino acid sequences of the mtCOI fragments from the 80 *C. gigas* were 100% identical, indicating that the sequence variation of *C. gigas* mtCOI is predominantly due to synonymous substitutions (data not shown).

Based on the SNP profiles, a total of 22 haplotypes were determined: 13 in TY and 9 in GD (Table 2), which indicates that the genetic diversity is slightly greater in TY (h=0.5154) than GD (h=0.4397) during the study period (**Table 2**)". Haplotype 1 (HAP_1) was the most abundant haplotype shared by 58 samples (28 in TY and 30 in GD), accounting for 72.5% of the total sample size. Other 20 haplotypes differed from HAP_1 by 1-3 nucleotides. As expected, TY and GD populations displayed low nucleotide diversity (0.000927 and 0.000689, respectively), with TY slightly more diverse (**Table 2**). Consistently, the haplotype diversity of TY was higher than that of GD. The mean number of pairwise differences (k) measuring the genetic diversity within a population was higher in TY than in GD (Table 2). Overall, TY and GD populations shared a similar genetic profile characterized by low genetic diversity with the predominant HAP_1 and diverse minor haplotypes.

POPULATION GENETIC STRUCTURE

AMOVA analysis was conducted to characterize the genetic structure of the 2 *C. gigas* populations. As shown in **Table 3**, most of the total molecular variance was present within populations (99.81%) rather than between the two populations. Accordingly, the fixation index (FST), a measure of population differentiation due to genetic structure, was 0.00188 but was not statistically significant (*p*-value = 0.49853).

CHARACTERIZATION OF HAPLOTYPES

To further delineate the genetic relationship of the two *C. gigas* populations, a haplotype network was constructed using the 863-bp mtCOI alignment matrix. As shown in Figure 3, HAP_1 formed a core of the network with other 20 satellite haplotypes. Except for HAP_19 and HAP_20, all satellite haplotypes were shown to be a singleton with a distinct SNP profile. All satellite haplotypes differ from HAP_1 by 1-3 nucleotides, resulting in the characteristic 'star-like' network pattern.

Next, 117 mtCOI sequences of 8 different country origins and two Korean origins (Incheon and Sucheon) were retrieved from the NCBI GenBank and included in the haplotype network analysis (Table 1). Li et al.[20] previously characterized 46 HAPs in the *C. gigas* population with 12 geographical origins in the northwestern Pacific. Selected mtCOI sequences representing 37 haplotypes, including HAP_1 (KP099007), out of the 46 HAPs were included in this analysis (see Table 1). Similar to Figure 3, the haploid network consisted of the dominant HAP_1 and the satellite HAPs. While a majority of satellite haplotypes was a singleton, 11 satellite haplotypes shared by different geographical origins were identified (Figure 4). Despite the geograph-

Table 3. Analysis of molecular variance (AMOVA) among *C. gigas* mtCOI sequences

Source of variation	Degrees of freedom	Variance components	Percentage of variation
Among population	1	0.0006 Va	0.19
Within population	78	0.34872 Vb	99.81
Fixation index	F_{ST} = 0.00188	*p*-value = 0.49853	

ical proximity, only 3 haplotypes (i.e., HAP_3, HAP_11, and HAP_14) were shared by Chinese and Korean *C. gigas* populations. No significant relationship was observed between the Korean origins (TY, GD, Incheon, and Sucheon).

DISCUSSION

A total of 21 haplotypes were identified in the two populations (TY and GD, hereafter) based on nucleotide sites within the mtCOI gene. The subsequent genealogical analysis showed that these haplotypes form a characteristic shallow network consisting of the major haplotype (i.e., Hap_1) at the center and 20 minor satellite haplotypes diverging from HAP_1 (Figure 3).

Genetic differences between the two populations were shown to lie in their distinctive satellite haplotypes; TY had 12 satellite haplotypes while GD had 8, but none of the satellite haplotypes were shared between the two. Each satellite haplotype differed from HAP_1 by 1-3 nucleotides and mostly formed a singleton, suggesting these satellite haplotypes have emerged recently. Previously, Li et al. reported a similar "star-like" network based on 228 mtCOI sequences of wild *C. gigas* collected from the coasts of China, Korea, and Japan.[20] Furthermore, HAP_1 accounted for more than 70% of both wild and cultured populations, suggesting the population genetic structure of *C. gigas* is conserved between wild and hatchery populations. The sharing of HAP_1 at equally high frequencies among the geographically diverse populations indicates that HAP_1 likely represents the ancestral spat oyster from Japan that has become prevalent worldwide since the early 20th century.[1] Weak genetic differentiation (FST = 0.00188) between TY and GD is, thus, more likely due to the history of monophyly and large-scale distribution associated with the current *C. gigas* industry, rather than distance-dependent genetic homogenization between the two populations.

C. gigas populations were further characterized by analyzing a haplotype network of 117 mtCO1 sequences from 12 different geographic origins (**Figure 4**). This extended network consisted of the predominant HAP_1 and 56 satellite haplotypes, most of which were singleton. Only 3 satellite haplotypes (i.e., HAP_3, HAP_11, and HAP_14) were shared between analyzed Chinese and Korean *C. gigas* populations. This data could imply that geographic proximity might not exert much influence on the genetic makeup of *C. gigas* populations, which needs further investigations on a larger scale.

The loss of genetic diversity reduces the fitness and adaptability of a species and the size of its population, possibly increasing the risk of species extinction. The rapid decline of *C. gigas* populations in recent years necessitates systemic management to improve the sustainability of *C. gigas* populations. In the present study, both *C. gigas* populations (Tongyeong and Gadeokdo), located on the southern coast of Korea, were shown to possess low levels of genetic diversity, which is in parallel with the extant Pacific oyster *C. gigas* populations.[14,20]

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AUTHORS' CONTRIBUTION PER CREDIT

Methodology: Thanh T. Biet (Equal), Hong-Keun Park (Equal), Dongjin Park (Equal). Formal Analysis: Thanh T. Biet (Equal), Su-Jin Park (Equal). Writing – original draft: Thanh T. Biet (Equal), Hong-Keun Park (Equal). Writing – review & editing: Thanh T. Biet (Equal), Hong-Keun Park (Equal), Dongjin Park (Equal), Youn-Hee Choi (Equal). Investigation: Su-Jin Park (Equal). Resources: Su-Jin Park (Lead). Conceptualization: Youn-Hee Choi (Lead). Funding acquisition: Youn-Hee Choi (Lead). Supervision: Youn-Hee Choi (Equal).

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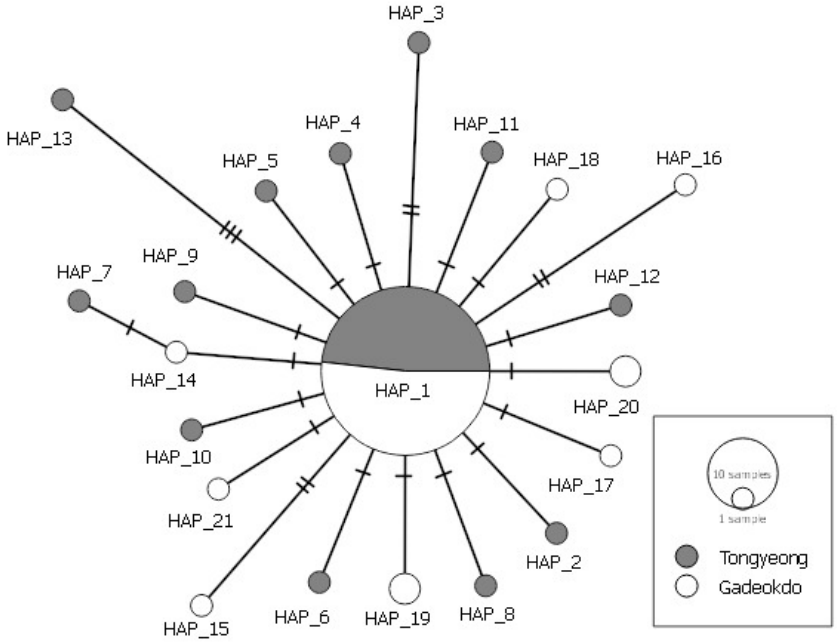

Figure 3. Haplotype network of mtCO1 of *C. gigas* populations from TY and GD. Each circle corresponds to a unique haplotype sized in proportion to its frequency. The hash marks on the lines indicate the number of substitutions that separate haplotypes.

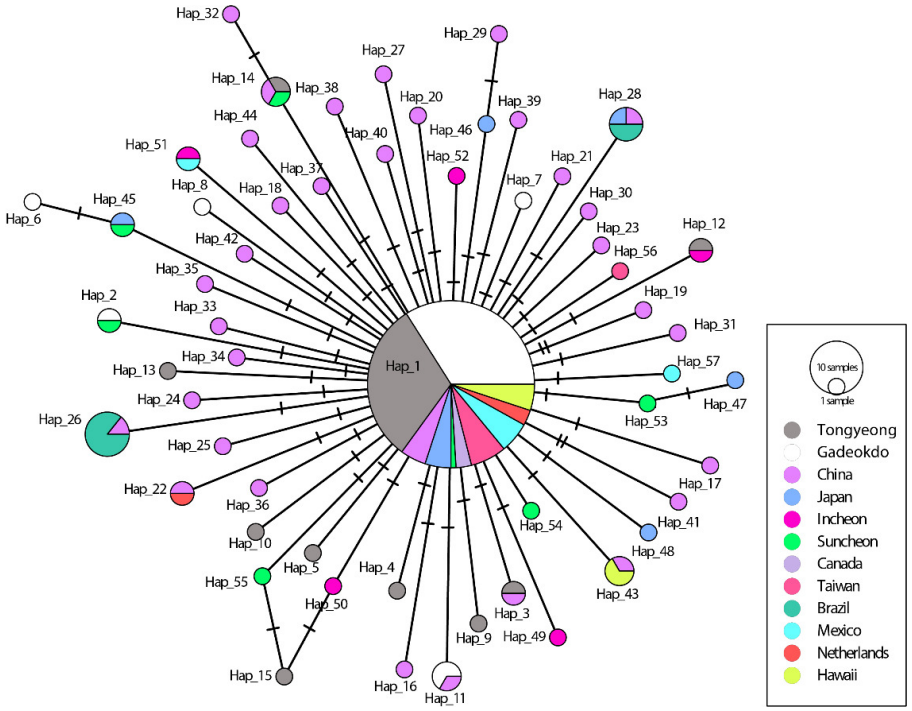

Figure 4. Haplotype network of mtCOI of 12 *C. gigas* populations, including TY and GD. Each of the different colors represents a geographic sampling site. Each circle corresponds to a unique haplotype sized in proportion to its frequency. The hash marks on the lines indicate the number of substitutions that separate haplotypes.

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