



Rapid Origin Determination of the Northern *Mauxia* Shrimp (*Acetes chinensis*) Based on Allele Specific Polymerase Chain Reaction of Partial Mitochondrial 16S rRNA Gene

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ABSTRACT: *Acetes chinensis* is an economically important shrimp that belongs to the Sergestidae family; following fermentation, *A. chinensis*' economic value, however, is low in China, and much of the catch in China is exported to Korea at a low price, thus leading to potential false labeling. For this reason, we developed a simple method to identify *A. chinensis*' origin using allele-specific polymerase chain reaction (PCR). Ten single nucleotide polymorphisms (SNPs) were identified from partial (i.e., 570 bp) DNA sequence analysis of the mitochondrial 16S rRNA gene in 96 Korean and 96 Chinese individual shrimp. Among 10 SNP sites, four sites were observed in populations from both countries, and two sites located in the middle with SNP sites at their 3'-ends were used to design allele-specific primers. Among the eight internal primers, the C220F primer specific to the Chinese *A. chinensis* population amplified a DNA fragment of 364 bp only from that population. We were able to identify the *A. chinensis* population origin with 100% accuracy using multiplex PCR performed with two external primers and C220F primers. These results show that the 16S rRNA gene that is generally used for the identification of species can be used for the identification of the origin within species of *A. chinensis*, which is an important finding for the fair trade of the species between Korea and China. (**Key Words:** *Acetes chinensis*, Single Nucleotide Polymorphism, Allele-specific Polymerase Chain Reaction, Origin Identification)

INTRODUCTION

Acetes is a genus of shrimp that belongs to the Sergestidae family. This family includes species of great abundance and therefore plays an important role in the ocean food chain (Omori, 1974). The genus *Acetes* comprises 14 species but only two species, *A. chinensis* and *A. japonicus*, have been reported in Korea (Kim, 1977). *A. chinensis* inhabits the Indo-West Pacific coast of India to Korea, Japan, China, and Indonesia. Traditionally, this species was used after drying or were fermented to make shrimp paste mixed with fermented anchovy and oyster to

make the distinctive seasoning kimchi.

In contrast to the great economic value of this species in Korea, it is economically less important in China; in 2011, 19,613.74 tons of *A. chinensis* were imported into Korea from China (<http://www.mof.go.kr>), and the difference in market price can lead to false labeling of the origin. Decreased natural resources, habitat sharing in the Yellow Sea, and false market identification necessitate the development of molecular markers that can be used for stock assessment and fishery management, population dynamics and phylogenetic relationship analyses, and origin identification.

Microsatellites are widely used as molecular markers in population genetics. However, with the development of high-throughput screening strategies, single nucleotide polymorphisms (SNPs) are increasingly being used as molecular markers due to several advantages, including processing efficiency, ease in both scoring and standardizing genotypes across laboratories, and the high

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density in which they are observed across most genomes (Vignal et al., 2002; Anderson and Garza, 2006). In this study, we analyzed SNPs in the 16S rRNA gene of *A. chinensis* originating from both Korea and China, and developed primers that could be used to differentiate the origin.

MATERIALS AND METHODS

Samples and DNA preparation

Over a thousand individual samples of *A. chinensis* were obtained from local markets in Ganghwado, an island located in the Yellow Sea, and Jindo, another island located in the South Sea. Two groups of samples caught in the coastal area of Shenzhen, Guangdong (E114, N22), China were obtained from importers in October 2013 and April 2014. From each sampling site, 48 individuals that were used for the 16S rRNA gene analysis were separately preserved in 100% ethanol at the sampling site and then transported to the laboratory for DNA extraction. The remainders of the samples from each site were pooled and preserved 100% ethanol. Total DNA was isolated from each of 48 individuals that were separately preserved using a MagExtractor MFX-6100 automated DNA extraction system (Toyobo, Osaka, Japan). The extracted genomic DNA was quantified using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and stored at -20°C until used for analysis.

Analysis of the 16S rRNA gene

Partial sequences (570 bp) of the 16S rRNA gene were analyzed from *A. chinensis* individuals from Korea ($n = 96$) and China ($n = 96$). The target DNA was amplified using the 16S rRNA 16Sbr universal primer (5'-CCGGTCTGAACTCAGATCACGT-3') and 16Sar universal primer (5'-CGCCTGTTTATCAAAAACAT-3') set whose melting temperatures (T_m) are 55.7°C and 53.9°C , respectively. Polymerase chain reaction (PCR) was performed using a thermocycler (PTC-2040; Bio-Rad, Hercules, CA, USA) in 20- μL volumes with 10 to 20 ng of DNA, 0.5 units of DNA polymerase (Anti-HS Taq, TNT

Research, Seoul, Korea), 250 μM of each dNTP, and 1 \times PCR buffer containing 2 mM MgCl_2 and 10 pmol of each primer. The PCR amplification consisted of an initial denaturation at 95°C for 10 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 7 min. The PCR fragments were purified using Expin PCR SV (GeneAll Biotechnology, Seoul, Korea) according to the manufacturer's instructions. Approximately 8 to 20 ng of purified product was used as a template for sequencing using the ABI BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster, CA, USA).

Allele-specific polymerase chain reaction reaction

After SNP identification in the partially amplified 16S rRNA gene, two of four SNPs that differed between Korean and Chinese *A. chinensis* samples were selected as allele-specific PCR primers (Table 1). Two SNPs located at positions 53 and 502 were excluded because they produced PCR products of the same size as that of the 16Sbr and 16Sar primers. Each primer listed in Table 1 was tested along with 16Sbr or 16Sar primers using gradient annealing temperatures. After internal primer selection, a multiplex PCR reaction was conducted with the 16Sbr, 16Sar, and the selected C220F primer, and the PCR products were analyzed on a 2% agarose gel.

RESULTS AND DISCUSSION

Mitochondrial DNA is a popular molecular marker because it is uniparental and has a high evolutionary rate, lack of introns, large copy number in every cell, and limited recombination (Galter et al., 2009). Although the cytochrome *c* oxidase subunit I (COI) gene is the most well-known molecular marker for the analysis of intraspecific and interspecific relationships in many marine fish and shellfish, the 16S rRNA mitochondrial gene has also proven to be a good marker to differentiate marine species (Craig et al., 2001). In our study, we first attempted to amplify the *A. chinensis* COI gene using universal primers, but did not observe any amplification and,

Table 1. Primers used to develop allele-specific PCR

Primers ¹	Sequences	Length	T_m
K207F	5'-CACATAATATTTGTAGTAAGTAAAG-3'	26	52
C207F	5'-CACATAATATTTGTAGTAAGTAAAA-3'	26	52
K207R	5'-TATAATAAATTAAGTGTTTTAAAC-3'	24	44
C207R	5'-TATAATAAATTAAGTGTTTTAAAT-3'	24	44
K370F	5'-GCCTTTTCACTTAAAAAGTTAAG-3'	22	56
C370F	5'-GCCTTTTCACTTAAAAAGTTAAA-3'	22	56
K370R	5'-GCTGTCTCAGTTTAAAAATTAAC-3'	25	54
C370R	5'-GCTGTCTCAGTTTAAAAATTAAT-3'	25	54

PCR, polymerase chain reaction; T_m , melting temperature; F, forward; R, reverse.

¹ First letter of the primer (i.e., K or C) designates the *A. chinensis* country of origin: Korea or China.

CCGGTCTGAACTCAGATCACGTA AAAATTTTAAAGGTCGAACAGACCTCCTT
ACTTTTAACGGCTACATTAAAAAAGGAATTTTAAATTCAACATCGAGGTGCGAA
 CCCTTCCTGTGATTTGGACTCTCAAGGTAGATTACGCTGTTATCCCTAAA
 GTAACTTAAATTTTAAATCTTTATTAAAGGATCATTTAATA CACAATAATA
TTTGTAGTAACTAAAATTTAAACAGTTAATTTATTATATTTTCATCGCCC
 CAATGAAACTGATCAAAACTCAGATAAGTTTATACTTAATAACTAATATCA
 AAAAATTTAAACAGTAAAGCTTTATAGGGTCTTATCGTCCCTCTAATTTAT
 TTAAGCCTTTTCACTTAAAAGTTAAATTTAATTTTAAAACTGAGACAGCT
 TTCTTTTTGTACAACCATTTCATACAAGCCTTCAATTTAAAAGGCTAATTATT
 ATGCTACCTTCGCACGGTCAGAATACCGCGGCTCTTTAACTAGATAAGTCA
 GTGAGCAGGCTATACTTTATATAACTATAATCATAAAGAGATGTTTTTGGAT
 AACAGGCG

Figure 1. Nucleotide sequence of a partial DNA fragment (570 bp) of the *A. chinensis* 16S rRNA gene. The forward 16Sbr and reverse 16Sar primers at each end are underlined and the internal allele-specific C220F primer is shaded. Single nucleotide polymorphism locations are designated in bold and underlined.

therefore, used the 16S rRNA gene as an alternative.

The PCR products of approximately 570 bp were obtained from both China and Korea samples by amplification with the 16S rRNA universal primers. Sequence analysis of the PCR products showed over 99% sequence identity to the 16S rRNA gene of *A. chinensis* (GenBank accession No. JN689221.1).

In total, 10 polymorphic sites were identified in the sequence analysis of the 570-bp fragment among a total of 192 *A. chinensis* individuals from China and Korea (Figure 1). Based on these polymorphic sites, six haplotypes were identified (Table 2). Only one haplotype (haplotype 1) was observed in all 96 individuals from China. The same haplotype 1 was not detected in any individual from Korea. Although five haplotypes were observed in the Korean *A. chinensis* population, haplotype 2 was most frequent (78%) and the frequency of the remaining haplotypes was very low and resulted in a low nucleotide diversity of 0.057 (Table 2). Among the nine polymorphic sites, four of them (i.e., locations 53, 220, 383, and 502) were unique to individuals from either Korea or China. As shown in Table 1, all individuals from China had C, A, A, and G at these

locations, respectively, in contrast to individuals from Korea which possessed T, G, G, and T, respectively.

Considering the number of individuals ($n = 96$) analyzed in this study, the presence of only one haplotype in the Chinese *A. chinensis* population is very interesting. In the year 2013, we analyzed a total of 48 individuals originating from China and found only one haplotype. To confirm this result, we obtained samples from China in 2014. Therefore, acquiring samples from the same stock was not possible. No current reports exist regarding *A. chinensis* population genetics in China, most probably due to the species' low economic value; thus, further work is necessary to determine the reason behind the presence of a single haplotype in the Chinese *A. chinensis* population. Although five other haplotypes were present in the Korean *A. chinensis* population, haplotype 2 was the major haplotype (78%). Sample haplotypes did not differ between Kanghwa-do and Jin-do, which are separated by 350 km. Therefore, depletion of genetic diversity was observed in samples from both Korea and China, which could result in a rapid crash of this economically valuable species following abrupt environmental changes, such as an increase in water

Table 2. Locations and sequences of 10 single nucleotide polymorphisms identified from the analysis of 192 individuals

	Polymorphic site										Frequency	
	53	158	195	220	301	349	383	398	441	502	Korea	China
Haplo_1	C	C	C	A	A	C	A	C	A	G		1
Haplo_2	T	A	C	G	A	A	G	C	T	T	0.78	
Haplo_3	T	A	C	G	G	A	G	C	T	T	0.06	
Haplo_4	T	A	C	G	A	A	G	A	T	T	0.06	
Haplo_5	T	C	C	G	A	C	G	C	A	T	0.06	
Haplo_6	T	C	T	G	A	C	G	C	A	T	0.06	
Nucleotide diversity (%)											0.0507	0
											±0.0702	
No. of haplotypes											5	0
Haplotype diversity											1	1
No. of polymorphic sites											6	0

temperature.

Although sequence analysis of the 570 bp fragment of the 16S rRNA gene can differentiate the origin with 100% accuracy, the procedure is a tedious and time consuming processes which includes PCR, purification of the PCR products and sequencing. Therefore, a more simple and rapid allele-specific PCR-based method similar to sequence characterized amplified region marker (Wang et al., 2011) was attempted.

To identify the *A. chinensis* country of origin based on allele specific PCR, we designed eight internal primers, which included a nucleotide corresponding to the SNP at their 3'-ends. The two SNPs close to each end (locations 53 and 502) were excluded because they produced PCR products of similar size as those of the 16Sbr and 16Sar pair. When each of the primers was paired with the external primers (16Sbr or 16Sar), some were not origin-specific; some primers specifically designed for the Chinese population amplified DNA fragments from the Korean *A. chinensis* population and vice versa (data not shown). It has been reported that mismatch at the 3' end might not enough for the detection of single base changes and additional mismatches are necessary (Little, 1997). The other factor causing the non-specific amplification could be the differences in the melting or annealing temperature of the primers. The melting points of 16Sbr forward and 16Sar reverse primer are 55.7°C and 53.9°C, respectively and those for the internal primers range from 44°C to 56.4°C (Table 1). Despite PCR using gradient annealing temperatures, most of the primers did not show origin specific amplifications (Data not shown).

Only one primer, C220F, specific to Chinese *A. chinensis* population with an A at the 3'-end that corresponded to SNP location 220 did not amplify any DNA fragments from the Korean *A. chinensis* population samples at a specific annealing temperature. As shown in Figure 1, C220F primer amplified the target from *A. chinensis* samples from China at all of the annealing temperatures ranging from 45°C to 60°C with the same efficiency. This primer also amplified the corresponding DNA from *A.*

chinensis samples from Korea at annealing temperatures from 45°C to 54°C with same efficiencies. However, amplification of target decreased at 57°C and there was no amplification at the annealing temperature of 60°C at which no difference was detected in the samples from China (Figure 2).

The other factor that needed to be optimized for the triplex reaction containing two external primers (16Sbr and 16Sar) and the internal C220F primers was the ratio of the three primers. Among several combinations, it was found that a ratio of 1 16Sbr:3 C220F:4 16Sar was optimum for PCR for origin identification (Data not shown). Although C220F primer was tested at 60°C for the differentiation with the 16Sar primer (Figure 2), the annealing temperature for the triplex reaction was further adjusted to 58°C for clear differentiation, and the result is shown in Figure 3. A common PCR product of 570 bp was produced from the external 16Sbr and 16Sar primer pair, and a PCR product of 364 bp was produced from the C220F and 16Sar primers. For further confirmation, we pooled the Korean samples and Chinese samples separately to make two large sample groups (Korea and China samples), and made groups of 100 individuals, extracted the DNA and conducted PCR as above, and obtained the same result (data not shown). This result demonstrated that the origin of *A. chinensis* could be determined by a simple PCR reaction with three primers. We developed similar strategies to identify green and red sea cucumbers that cannot be distinguished at young stages when they are released for resource enhancement. PCR with an internal primer designed to include an allele-specific SNP at the 3' end differentiated between the two variants, 100% and 4.2% amplification in green and red variants, respectively (Kang et al., 2011).

Although further analysis of samples from both Korea and China is necessary, the information described here could prevent false labeling of imported *A. chinensis* and contribute to the food safety. Also these results show that the 16S rRNA gene that is generally used for the identification of species (Ivanova et al., 2007) can be used for the identification of the origin within species, which is

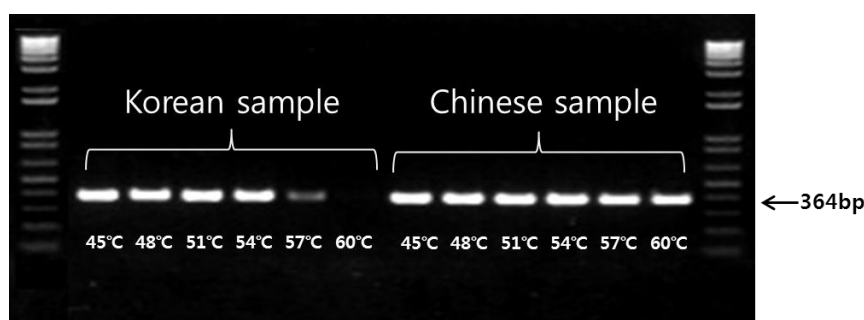


Figure 2. Amplification of target DNA from *A. chinensis* samples from Korea and China using Chinese origin specific C220F primer and common 16Sar reverse primer at different annealing temperature. The 3' end of the C220F primer has A residue and the corresponding nucleotides in *A. chinensis* samples from Korea and China are C and T, respectively.

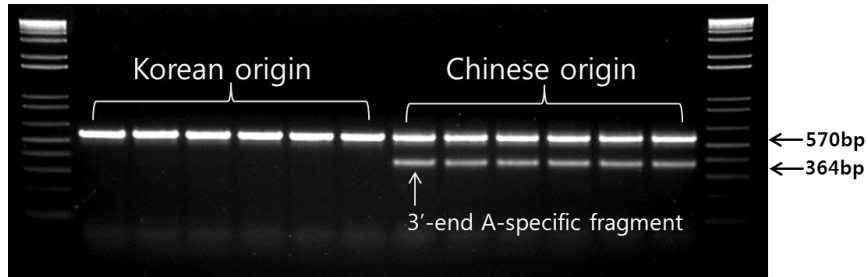


Figure 3. Identification of *A. chinensis* origin using polymerase chain reaction with a common primer pair and an internal primer. The 570-bp fragment is from the common forward and reverse primer. The 364-bp Chinese *A. chinensis*-specific fragment is from the 3'-end-specific internal forward primer and common reverse primer. Results from six representative samples among 96 individuals of *A. chinensis* from Korea and China are shown.

an interesting finding of this study.

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