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Intron sequence diversity of the asian cavity-nesting honey bee, *Apis cerana* (Hymenoptera: Apidae)

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Abstract

The Asian cavity-nesting honeybee, *Apis cerana* (Hymenoptera: Apidae), has been extensively studied for its biogeography and genetic diversity, but the molecules utilized in past studies were mainly ~90 bp long mitochondrial non-coding sequences, located between $tRNA^{Leu}$ and COII. Thus, additional molecular markers may enrich our understanding of the biogeography and genetic diversity of this valuable bee species. In this study, we reviewed the public genome database to find introns of cDNA sequences, with the assumption that these introns may have less evolutionary constraints. The six introns selected were subjected to preliminary tests. Thereafter, two introns, titled White gene and MRJP9 gene, were selected. Sequencing of 552 clones from 184 individual bees showed a total of 222 and 141 sequence types in the White gene and MRJP9 gene introns, respectively. The sequence divergence ranged from 0.6% to 7.9% and from 0.26% to 17.6% in the White gene and the MRJP9 introns, respectively, indicating higher sequence divergence in both introns. Analysis of population genetic diversity for 16 populations originating from Korea, China, Vietnam, and Thailand shows that nucleotide diversity (π) ranges from 0.003117 to 0.025837 and from 0.016541 to 0.052468 in the White gene and MRJP9 introns, respectively. The highest π was found in a Vietnamese population for both intron sequences, whereas the nine Korean populations showed moderate to low sequence divergence. Considering the variability and diversity, these intron sequences can be useful as non-mitochondrial DNA-based molecular markers for future studies of population genetics.

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Introduction

The biogeography and genetic diversity of *A. cerana* has extensively been studied for its distributional range in the temperate and tropical regions of Asia (De la Rúa *et al*., 2000; Deowanish *et al*., 1996; Sihanuntavong *et al*., 1999; Smith and Hagen, 1996; Smith *et al*., 2000; Smith *et al*., 2004; Takahashi *et al*., 2007; Tan *et al*., 2007). These studies exclusively used an internal spacer region located between mitochondrial $tRNA^{Leu}$ and COII to analyze this species' genetic diversity (Crozier and Crozier, 1993; Cornuet *et al*., 1991).

Nuclear loci require four times more the effective population size than mitochondrial loci (Brown, 1983). Thus, several more folds of nucleotides are required from nuclear DNA to obtain equivalent

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numbers of variable sites to mitochondrial loci in general (Zink and Barrowclough, 2008). Because of this condition, population genetics studies of *A. cerana* have focused on mitochondrial DNA. In fact, only a limited number of studies have utilized nuclear loci-originating markers, such as the ribosomal internal transcribed spacer 2 (ITS2) (Kim *et al*., 2010). Nevertheless, development of nuclear loci might be required to achieve a balance of information to expand our understanding of the population genetics aspects of *A. cerana*. In particular, recent studies have shown that intron sequences have a distinct rate of evolution, making them potentially useful in resolving relationships over a wide range of taxonomic levels (Yu *et al*., 2011). However, this method of study requires additional experimental efforts, such as isolation of alleles, PCR optimization, and alignment expertise due to insertion/deletion (indel) (Sang, 2002).

In this study, we reviewed the genome database to find introns of cDNA sequences for *Apis* species, including *A. cerana*. Initially, six introns were selected and primers were designed, and subsequently two introns, each having proper levels of diversity, were selected. These two introns were used to assess the potential of population genetic markers for *A. cerana* using 552 clones from 184 individuals of *A. cerana* collected from 16 localities in four countries including Korea.

Materials and Methods

Sampling

A total of 184 *A. cerana* workers collected from 16 localities in four countries (South Korea, China, Vietnam, and Thailand) were used in this study (Table 1). The field-collected bees were preserved in 95% ethanol for molecular experiments.

Molecular experiment

Total DNA was extracted from one or two legs using the Wizard Genomic DNA Purification Kit, in accordance with the manufacturer's instructions (Promega, Madison, WI, USA). While reviewing intron sequences of *Apis*, six cDNAs, which provide the exon–intron structure, were found in the GenBank: the White gene (Kawakita *et al*., 2008), MRJP9 gene (Peiren *et al*., 2008), OR2 gene (Unpublished, GenBank accession number FJ666105), 1 inosital 1,4,5-triphosphate receptor gene (Lo *et al*., 2010), long-wavelength rhodopsin (LWRh) gene (Kawakita *et al*., 2008), and mitotic checkpoint control protein (Bub3) gene (Kawakita *et al*., 2008). These cDNAs included one to three introns and two to four exons. Among each of these, one intron having a relatively longer size and/or a proper site for primer design was selected from each gene. Three or four primers from each intron were designed and tested for proper utility (e.g., amplification success). Detailed primer information and origin are provided in Table 2.

A 35-cycle amplification (94°C for 1 min, 50–52°C for 1 min, and 72°C for 1 min) process for PCR was conducted after initial denaturation at 94°C for 5 min and the final extension step continuing for 7 min at 72°C. To confirm successful DNA amplification, electrophoresis was carried out using 0.5× TAE buffer on 0.5% agarose gel. After purification with the PCR purification Kit (Qiagen, Germany), the amplicons were cloned into a pGEM-T Easy vector (Promega, USA). For the cloning process, XL1-Blue competent cells (Stratagene, USA) were transformed with the ligated DNA, and the resultant plasmid DNA was isolated using a Wizard Plus SV Minipreps DNA Purification System (Promega, USA). Two or three clones with limited sampling were sequenced for preliminary experiments and the obtained sequences were analyzed for their variability. Eventually, the White gene and the MRJP9 gene introns were selected and these introns were subjected to full individual screening, analyzing three clones per individual. DNA sequencing was conducted using the ABI PRISM® BigDye® Terminator ver. 3.1 Cycle Sequencing Kit with an ABI 3100 Genetic Analyzer (PE Applied Biosystems, USA). All products were sequenced from both strands.

Sequence analysis

Sequence delimitation was conducted by comparing newly acquired sequences to the GenBank-registered corresponding cDNA using MAFFT ver. 6 (Table 2; Katoh *et al*., 2002). When one or more nucleotide base or insertion/deletion (indel) position differed to the obtained sequences, the sequences were considered different sequence types.

Variability estimate

In order to estimate the variability of the intron sequences, population diversity estimates, such as sequence diversity and nucleotide diversity, both of which are reflective of genetic

Table 1. A list of trapping localities, sample numbers and GenBank accession numbers

Locality (no. samples)	Sample number	GenBank accession number	
		White	MRJP9
1. Cheongsong-gun, Gyeongsangbuk-do, Korea (22)	AC5168-AC5189	KT267574-KT267588	KT267715-KT267736
2.Chungju-si, Chungcheongbuk-do, Korea (16)	AC5190-AC5205	KT267574, KT267589-KT267599	KT267715, KT267717, KT267727-KT267728, KT267737-KT267751
3. Hongcheon-gun, Gangwon-do, Korea (7)	AC5206-AC5212	KT267574, KT267600-KT267602	KT267715-KT267717, KT267752-KT267758, KT267930-KT267931
4. Namwon-si, Jeollabuk-do, Korea (5)	AC5213-AC5217	KT267574, KT267576, KT267603-KT267606	KT267715, KT267717, KT267728, KT267759-KT267761
5. Icheon-si, Gyeonggi-do, Korea (12)	AC5218-AC5229	KT267574-KT267575, KT267601, KT267607-KT267616	KT267715, KT267717, KT267727-KT267728, KT267762-KT267776
6. Yanggu-gun, Gangwon-do, Korea (9)	AC5230-AC5238	KT267574, KT267617-KT267623	KT267715, KT267717, KT267727-KT267728, KT267771, KT267777-KT267789
7. Cheongyang-gun, Chungcheongnam-do, Korea (6)	AC5239-AC5244	KT267574, KT267576, KT267588, KT267624-KT267629	KT267715, KT267727-KT267728, KT267753, KT267784, KT267790-KT267798
8. Pyeongchang-gun Gangwon-do, Korea (17)	AC5245-AC5261	KT267574, KT267576, KT267593, KT267601, KT267603-KT267604, KT267630-KT267640	KT267715-KT267717, KT267728, KT267772, KT267790, KT267799-KT267816, KT267932
9. Cheongwon-gun, Chungcheongbuk-do, Korea (24)	AC5262-AC5285	KT267574, KT267576, KT267601, KT267641-KT267655	KT267715-KT267717, KT267728, KT267784, KT267817-KT267836, KT267933
10. Kunming, Yunnamprvince, China (8)	AC5286-AC5293	KT267574, KT267618, KT267629, KT267656-KT267668	KT267715, KT267717, KT267734, KT267833-KT267844, KT267934
11. Yellow Mountain Anhui province, China (11)	AC5294-AC5304	KT267574, KT267576, KT267667, KT267669-KT267677	KT267715, KT267727-KT267728, , KT267834, KT267838 KT267845-KT267860, KT267935
12. Changbai Mountain, Jiling province, China (9)	AC5305-AC5313	KT267574, KT267601, KT267624, KT267671, KT267678-KT267682	KT267715, KT267717, KT267727, KT267861-KT267873

Table 1. A list of trapping localities, sample numbers and GenBank accession numbers

Locality (no. samples)	Sample number	GenBank accession number	
		White	MRJP9
13. Tianshui, Gansu province, China (8)	AC5314-AC5321	KT267574, KT267683-KT267687	KT267715, KT267727, KT267734, KT267874-KT267884, KT267936
14. Beijing, China (7)	AC5322-AC5328	KT267574, KT267576, KT267580, KT267601, KT267664, KT267601, KT267688-KT267690	KT267715-KT267717, KT267727, KT267885-KT267895
15. Vin Phuc, Vietnam (13)	AC5329-AC5341	KT267574, KT267576, KT267582, KT267588, KT267590, KT267618, KT267629, KT267691-KT267705	KT267715, KT267727-KT267728, KT267790, KT267826, KT267874, KT267879, KT267896-KT267911
16. Chanthaburi, Thailand (10)	AC5342-AC5351	KT267574, KT267618, KT267707-KT267714	KT267715, KT267727-KT267728, KT267880, KT267888, KT267912-KT267929

Table 2. List of primer sequences used to amplify and sequence the introns of *Apis cerana*

Gene name	Primer name	Direction	Sequence (5'- 3')	Intron size (bp)	GenBank Number	Reference
white gene	**AC-White-F1**	F	CAAGCTTGCACTTTTTTTGAAAC	158	EU184853	Kawakita *et al.* (2008)
	AC-White-R1	R	ACGTTTCTTGTCCAGGTAC			
	AC-White-R2	R	TCGCATACAGTGTTTATCGCAT			
MRJP9 gene	**AC-MRJP9-F1**	F	CTGGTATTACAAGTGTTTACAGA	310	FJ666103	Cristino *et al.* (Unpublished)
	AC-MRJP9-R1	R	AATTTTGAAGTTTTTAGATC			
	AC-MRJP9-R2	R	ATTTATGTTTCCAGTAGTAGA			
OR2 gene	AC-OR2-F1	F	ATGGTGTAGCTTTGCTGCTACA	124	FJ666105	Cristino *et al.* (Unpublished)
	AC-OR2-F2	F	TGTTGACTACTACTATTACG			
	AC-OR2-R1	R	TGCTGACAAACAATCTGTAC			
	AC-OR2-R2	R	GAAAAACTTTGCCCCTGAAATCG			
1 inositol 1,4,5-triphosphate receptor gene	**AC-TRG-F1**	F	TCTTATAAACAAATAAAGTCAG	94	FJ932636	Lo *et al.*(2010)
	AC-TRG-F2	F	TTTATAAATCTAAAGCATCAG			
	AC-TRG-R1	R	TTTTATCTGACCACCTATCG			
	AC-TRG-F1	R	TGTGTACTCCAACATTACG			
long-wavelength rhodopsin (LWRh) gene	AC-LWRH-F1	F	ATTTGGACAATGACGATGATCGC	94	EU184839	Kawakita *et al.* (2007)
	AC-LWRH-F2	F	AGCTCTCATTCGTATAATAGC			
	AC-LWRH-R1	R	TAAACCCAAATGCCGTAGC			
	AC-LWRH-R2	R	ACTAGTATTTTGATTTTCAG			
mitotic checkpoint control protein (Bub3) gene	**AC-MITOTIC-F1**	F	GTAAAACTTTGGGATCCTAG	98	EU184823	Kawakita *et al.* (2007)
	AC-MITOTIC-F2	F	ATAAGTTTGTAGTTGGTAC			
	AC-MITOTIC-R1	R	ATTTTCTTTTATCCTATGAC			
	AC-MITOTIC-R2	R	TAAGTTGAATGAAAACTAATAGC			

Mainly used primers are bold-faced.

Table 3. Preliminary information of intron sequenced

Intron	Number of intron sequenced	Size (bp)	Number of sequence type	Divergence (%)
White gene	53	158~160	20	0.63~3.16
MRJP9 gene	18	310~313	12	0.31~5.11
OR2 gene	24	118~126	11	0.79~7.14
1 inosital 1,4,5-triphosphate receptor gene	31	92~94	8	1.06~3.19
Lomg-wavelength rhodopsin (LWRh) gene	27	94	2	0.00~1.06
Mitotic checkpoint control protein (Bub3) gene	29	98	1	0

Table 4. Characteristic of intron sequences

Taxon	Size (bp)	A/T content (%)	G/C content (%)	Divergence (%)	Sequence type*
White gene	157~163	72.2	27.8	0.6~7.9	552/222
MRJP9 gene	307~357	80.5	19.5	0.26~17.6	552/141

*First values are total number of clones sequenced and second values are number of sequence type.

diversity within a population, were determined using Arlequin ver. 3.5 (Excoffier and Lischer, 2010). The maximum sequence divergence within each locality was estimated via extraction of the within-locality estimates of unrooted pairwise distances from PAUP ver. 4.0b (Swofford, 2002).

Results and Discussion

Selection of two introns

As a preliminary experiment, sequencing was performed only for limited individuals: 53 clones from the intron of the White gene, 18 from MRJP9 gene, 24 from OR2 gene, 31 from 1 inosital 1,4,5-triphosphate receptor gene, 27 from LWRh gene, and 29 from Bub3 gene. Six introns provided multiple numbers of sequence types (8–20), but the introns of the Bub3 gene and the LWRh gene provided one and two sequence types, respectively (Table 3). When diversity of sequence types was considered, the intron of one inosital 1,4,5-triphosphate receptor gene showed the lowest as eight (25.8%), excluding the introns of Bub3 gene and LWRh gene. Furthermore, this intron was shorter in original length at 92–94 bp and this may be problematic, considering the potential of variable sites in a given length. The intron of OR2 gene provided the second highest diversity of sequence type (45.83%) and the highest sequence divergence (0.79–7.14%), but the original sequence length of the intron is only the third (118–126 bp), after the introns of the White gene and the MRJP9 gene (158–160 bp and 310–313 bp, respectively). Thus, these were excluded from subsequent full individual screening, and finally, the introns of the White gene and the MRJP9 gene were selected for full investigation to evaluate their potential as nuclear population genetic markers.

Sequence analysis of the two introns

The sequence analyses of 552 clones from 184 individual bees provided a total of 222 sequence types, ranging in size from 157 to 163 bp for the White gene intron and 141 sequence types, ranging in size from 307 to 357 bp for the MRJP9 intron (Table 4). The G/C content was ~27.8% (72.2% in A/T content) in the White gene intron and 19.5% (80.5% in A/T content) in the MRJP9 intron, indicating that the two introns are highly biased for A/T nucleotides. The sequence divergence of the two introns was 0.6%–7.9% in the White gene intron and 0.26%–17.6% in the MRJP9 intron, indicating moderate to substantial divergence. Previously, Lee *et al*. (2015) reported the sequence divergence of two mitochondrial non-coding sequences (NC1 and NC2) from the same individual bees as 3.093% (three positions) to 1.031% (one position) and 2.597% to 0.433%, respectively. The NC2 has been proven to be useful for genetic diversity and biogeographic study for worldwide *A. cerana* populations (Smith and Hagen, 1996; Smith *et al*., 2000; Smith *et al*., 2004; Warrit *et al*., 2006; Takahashi *et al*., 2007). The NC1 was newly developed in Lee

Table 5. Within-locality diversity estimates of *Apis cerana* from White gene intron

Locality	SS[a]	NH[b]	*H*[c]	NP[d]	MPD[e]	π[f]
1. Cheongsong-gun, Gyeongsangbuk-do	66	15	0.6513	21	1.105828	0.006702
2. Chungju-si, Chungcheongbuk-do	48	12	0.4406	15	0.664894	0.004030
3. Hongcheon-gun, Gangwon-do	21	4	0.4714	3	0.514286	0.003117
4. Namwon-si, Jeollabuk-do	15	6	0.6571	6	0.800000	0.004848
5. Icheon-si, Gyeonggi-do	36	13	0.5619	16	0.941270	0.005705
6. Yanggu-gun, Gangwon-do	27	8	0.4587	8	0.592593	0.003591
7. Cheongyang-gun, Chungcheongnam-do	18	9	0.7059	9	1.183007	0.007170
8. Pyeongchang-gun, Gangwon-do	51	18	0.6322	19	0.923922	0.005600
9. Cheongwon-gun, Chungcheongbuk-do	72	18	0.4190	19	0.658451	0.003991
10. Kunming, Yunnam prvince, China	24	16	0.9384	17	2.206522	0.013373
11. Yellow Mountain, Anhui province, China	33	13	0.8068	12	1.212121	0.007346
12. Changbai Mountain, Jiling province, China	27	9	0.7322	10	1.202279	0.007287
13. Tianshui, Gansu province, China	24	6	0. 4420	7	0.659420	0.003996
14. Beijing, China	21	9	0.8143	10	1.409524	0.008543
15. Vin Phuc, Vietnam	39	23	0.9622	26	4.263158	0.025837
16. Chanthaburi, Thailand	30	10	0.6345	11	1.721839	0.010435

[a]Sample size
[b]Number of haplotypes
[c]Haplotype diversity
[d]Number of polymorphic sites
[e]Mean number of pairwise differences
[f]Nucleotide diversity

et al. (2015) and also showed near equal variability to NC2. Further, these non-coding sequences each provided ten in NC2 and nine haplotypes in NC1 from the 184 individual bees (Lee *et al*., 2015). Compared to the above result, in this study, the sequence divergence of the two introns are much larger in terms of number of sequence type and sequence divergence. Thus, these intron sequences might be useful as non-mitochondrial DNA-based molecular markers for future population studies.

Genetic diversity

In order to understand the population genetic diversity of *A. cerana* in populations, several diversity estimations were made (Tables 5 and 6). In the case of the White gene intron, the nucleotide diversity (π) ranged from 0.003117 (locality 3, Hongcheon-gun) to 0.025837 (locality 15, Vin Phuc, Vietnam), showing at least an eight-fold difference between them (Table 6). For the MRJP9 intron, diversity ranged from 0.016541 (locality, 4, Namwon-si) to 0.052468 (locality 15, Vin Phuc, Vietnam), showing at least a three-fold difference (Table 6). Thus, the MRJP9 intron showed two to five fold greater diversity than that of the White gene intron.

Among the 16 populations, the highest π was found in Vin Phuc, Vietnam (locality 15) in both intron sequences. The second highest estimate were found in Chanthaburi population, Thailand (locality 16) as 0.010435 in the White gene intron and Beijing, China (locality 14) as 0.027619 in the MRJP9 intron. On the other hand, the nine Korean populations ranged from 0.003117 to 0.007170 and 0.015984 to 0.022876 in the White gene intron and MRJP9 gene intron, respectively. This indicates that the Korean populations are relatively low in diversity of the two intron sequences, although their locality of seven (Cheongyang-gun) ranked the fifth in the MRJP9 intron and eighth in the White gene intron. In fact, Lee *et al*. (2015), based on NC2, has also shown that the India, Indonesia, Malaysia, Philippines, and Burma populations were several-fold higher in genetic diversity than the Korean populations, indicating the presence of small effective populations in Korea. Similar to π estimates, haplotype

Table 6. Within-locality diversity estimates of *Apis cerana* from MRJP9 gene intron

Locality	SS[a]	NH[b]	*H*[c]	NP[d]	MPD[e]	π[f]
1. Cheongsong-gun, Gyeongsangbuk-do	66	22	0.8084	45	6.481585	0.017057
2. Chungju-si, Chungcheongbuk-do	48	19	0.8741	38	7.047872	0.018547
3. Hongcheon-gun, Gangwon-do	21	12	0.8238	31	6.390476	0.016817
4. Namwon-si, Jeollabuk-do	15	6	0.7905	20	6.285714	0.016541
5. Icheon-si, Gyeonggi-do	36	19	0.8905	39	7.265079	0.019119
6. Yanggu-gun, Gangwon-do	27	18	0.9544	35	7.703704	0.020273
7. Cheongyang-gun, Chungcheongnam-do	18	14	0.9673	29	8.692810	0.022876
8. Pyeongchang-gun, Gangwon-do	51	25	0.8729	40	6.661176	0.017529
9. Cheongwon-gun, Chungcheongbuk-do	72	26	0.8310	44	6.073944	0.015984
10. Kunming, Yunnam prvince, China	24	14	0.9058	41	7.369565	0.019394
11. Yellow Mountain, Anhui province, China	33	22	0.9621	47	8.356061	0.021990
12. Changbai Mountain, Jiling province, China	27	16	0.8917	33	6.498575	0.017102
13. Tianshui, Gansu province, China	24	15	0.9529	52	9.326087	0.024542
14. Beijing, China	21	14	0.9524	51	10.495238	0.027619
15. Vin Phuc, Vietnam	39	22	0.9447	96	19.937922	0.052468
16. Chanthaburi, Thailand	30	23	0.9747	58	9.995402	0.026304

[a]Sample size
[b]Number of haplotypes
[c]Haplotype diversity
[d]Number of polymorphic sites
[e]Mean number of pairwise differences
[f]Nucleotide diversity

diversity (*H*) also was the highest in Vin Phuc, Vietnam (locality 15) for both intron sequences. The *H* of the nine Korean populations ranged from 0.4190 to 0.7059 in the White gene intron and 0.7905 to 0.9673 in the MRJP9 gene intron. These estimates are not high; however, they are better than those of π found in several Korean localities showing more improved ranks in the *H* than those of π. Nevertheless, it is obvious that the Korean populations have an overall low diversity.

In summary, we selected two intron sequences from the public genome database and sequenced 552 clones from 184 individuals of *A. cerana* collected from 16 localities in four countries. The high variability and diversity of the intron sequences show their high potential for use as non-mitochondrial DNA-based molecular markers for future population genetics studies.

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