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Geographic Genetic Contour of A Leaf Beetle, *Chrysolina aurichalcea* (Coleoptera: Chysomelidae), on the Basis of Mitochondrial COI Gene and Nuclear ITS2 Sequences

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The leaf beetle, *Chrysolina aurichalcea* (Coleoptera: Chysomelidae), is a pest damaging plants of Compositae. In order to understand the genetic diversity and geographic variation we sequenced a portion of mitochondrial COI gene (658 bp) and complete nuclear internal transcribed spacer 2 (ITS2) of the species collected from seven Korean localities. A total of 17 haplotypes (CACOI01~CACOI17), with the maximum sequence divergence of 3.04% (20 bp) were obtained from COI gene sequence, whereas 16 sequence types (ITS2CA01~ITS2CA16), with the maximum sequence divergence of 2.013% (9 bp) were obtained from ITS2, indicating substantially larger sequence divergence in COI gene sequence. Phylogenetically, the COI gene provided two haplotype groups with a high nodal support (≥87%), whereas ITS2 provided only one sequence type group with a high nodal support (≥92%). The result of COI gene sequence may suggest the presence of historical biogeographic barriers that bolstered genetic subdivision in the species. Different grouping pattern between COI gene and ITS2 sequences were interpreted in terms of recent dispersal, reflected in the ITS2 sequence. Finding of unique haplotypes and sequence types only from Beakryeng-Islet population was interpreted as an intact remnant of ancient polymorphism. As more samples are analyzed using further hyper-variable marker, further fruitful inference on the geographic contour of the species might be available.

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Introduction

The leaf beetle, *Chrysolina aurichalcea* (Coleoptera: Chysomelidae), damages young leaves of the species of Compositae and is a damaging pest along with the aphid, *Cryptosiphum artemisiae*, to the mugwort *Artemisia* species (Choo *et al*., 1992). The species damages most severely during May ~ June, tends to damage in night, and have death feigning behavior (http://100.naver.com/insect). The species is distributed in Korea, Japan, China, Taiwan, and eastern region of Russia including Sakhalin (Kimoto and Takizawa, 1994; Kim, 2001).

The species in Japan has been reported to have four different karyotypes, three of which are also present in Korea, although exact zoogeographic information for such diversification is unknown (Fujiyama, 1989). Subsequent study for Japanese populations has reported that 2n=31 (male) and 2n=41 (male) are two major karyotypes (Fujiyama and Okamoto, 1996), but have very few difference in external morphology, except for a slight difference in male reproductive organ (Fujiyama, 1989). Laboratory crossing experiment also supported reproductive isolation between the two types by both pre-mating and post-mating mechanisms (Fujiyama, 1989). In a subsequent study, the two types were found in the same microhabitats within sympatric zone, suggesting that post-mating mechanism is more strong (Kitamura *et al*., 2010).

In contrast to the recognition of reproductive isolation sequence and phylogenetic analyses using mitochondrial NADH dehydrogenase subunit 2 (ND2) gene from an

extensive sample of the two karyotypes have shown co-occurrence of identical haplotypes in different types in several localities and non-monophyly in either of the two karyotypes (Kitamura *et al*., 2008). These results collectively were used to suggest that chromosomal changes occurred rapidly and recently, possessing ancestral polymorphism in ND2 sequences (Kitamura *et al*., 2008).

Currently, studies on Korean populations of the *C. aurichalcea* is limited only to the status as pest and its host plant (Choo *et al*., 1992), whereas, no other genetic

Table 1. Inventory of samples and summary of sequencing result

Locality (no. of individuals)	Collection date	Sample number	COI				ITS2		
			Haplotype	GenBank acc. no.	Sequence type I *	G + C contents (%)	Size (bp)	GenBank acc. no.	Sequence type II*
1. Yeongwol, Gangwon-province	2009.07.10	CA3281	CACOI01	JN601816	ITS2CA01	43.48	483	JN601850	ITS2CA01
	2009.07.10	CA3283	CACOI02	JN601817	ITS2CA02	43.39	484	JN601851	ITS2CA02
	2009.07.10	CA3284	CACOI03	JN601818	ITS2CA03	43.69	483	JN601852	ITS2CA03
	2009.07.10	CA3285	CACOI04	JN601819	ITS2CA04	43.48	483	JN601853	ITS2CA04
	2009.07.10	CA3286	CACOI01	JN601820	ITS2CA05	44.30	474	JN601854	ITS2CA05
	2009.07.10	CA3287	CACOI05	JN601821	ITS2CA01	43.48	483	JN601855	ITS2CA01
2. Kwanak-gu, Seoul	2010.05.27	CA3288	CACOI06	JN601822	ITS2CA06	43.12	480	JN601856	ITS2CA06
	2010.05.27	CA3289	CACOI01	JN601823	ITS2CA07	44.30	474	JN601857	ITS2CA05
	2010.05.27	CA3293	CACOI01	JN601824	ITS2CA08	43.71	485	JN601858	ITS2CA07
	2010.05.27	CA3294	CACOI01	JN601825	ITS2CA09	43.51	485	JN601859	ITS2CA01
3. Andong, Gyeongsangbuk-province	2009.10.04	CA3298	CACOI01	JN601826	ITS2CA07	44.30	474	JN601860	ITS2CA05
	2009.10.04	CA3299	CACOI01	JN601827	ITS2CA07	44.30	474	JN601861	ITS2CA05
	2009.10.04	CA3300	CACOI07	JN601828	ITS2CA01	43.48	483	JN601862	ITS2CA01
	2009.10.04	CA3301	CACOI08	JN601829	ITS2CA01	43.48	483	JN601863	ITS2CA01
	2009.10.04	CA3302	CACOI01	JN601830	ITS2CA11	43.48	483	JN601864	ITS2CA08
	2009.10.04	CA3303	CACOI01	JN601831	ITS2CA12	43.27	483	JN601865	ITS2CA09
4. Kangseo-gu, Busan	2009.10.06	CA3306	CACOI09	JN601832	ITS2CA10	43.54	480	JN601866	ITS2CA08
	2009.10.06	CA3308	CACOI10	JN601833	ITS2CA07	44.30	474	JN601867	ITS2CA05
	2009.10.06	CA3309	CACOI01	JN601834	ITS2CA13	43.27	483	JN601868	ITS2CA10
	2009.10.06	CA3310	CACOI09	JN601835	ITS2CA14	43.39	484	JN601869	ITS2CA08
5. Eocheong-Islet, Gunsan, Jeollabuk-province	2009.06.29	CA1971	CACOI11	JN601836	ITS2CA15	43.75	480	JN601870	ITS2CA11
	2009.06.29	CA1972	CACOI02	JN601837	ITS2CA16	43.36	482	JN601871	ITS2CA12
	2009.06.29	CA1973	CACOI12	JN601838	ITS2CA01	43.48	483	JN601872	ITS2CA01
	2009.06.29	CA1974	CACOI02	JN601839	ITS2CA10	43.54	480	JN601873	ITS2CA08
	2009.06.29	CA1975	CACOI13	JN601840	ITS2CA17	43.36	482	JN601874	ITS2CA13
	2009.06.29	CA3312	CACOI01	JN601841	ITS2CA18	43.54	480	JN601875	ITS2CA01
6. Seogwipo, Jeju-province	2009.04.21	CA1965	CACOI02	JN601842	ITS2CA10	43.54	480	JN601876	ITS2CA08
	2009.04.21	CA1966	CACOI14	JN601843	ITS2CA19	43.30	485	JN601877	ITS2CA01
	2009.04.21	CA1968	CACOI02	JN601844	ITS2CA20	43.48	483	JN601878	ITS2CA01
	2009.04.21	CA1969	CACOI02	JN601845	ITS2CA02	43.39	484	JN601879	ITS2CA02
	2009.04.21	CA1970	CACOI01	JN601846	ITS2CA20	43.48	483	JN601880	ITS2CA01
7. Beakryeng-Islet, Incheon	2010.06.13	CA3317	CACOI15	JN601847	ITS2CA21	43.75	480	JN601881	ITS2CA14
	2010.06.13	CA3318	CACOI16	JN601848	ITS2CA22	43.57	482	JN601882	ITS2CA15
	2010.06.13	CA3320	CACOI17	JN601849	ITS2CA23	43.57	482	JN601883	ITS2CA16

I, Sequence type obtained before Gblocks analysis (Castresana, 2000) by which non-conserved blocks are removed in the alignment; and II, sequence type obtained after Gblocks analysis (Castresana, 2000) by which non-conserved blocks are removed in the alignment.

and molecular perspective study has yet been performed. In order to expand our understanding about the species for a diverse purpose including pest control strategy an inference on genetic population structure may offer us important biological information by enabling us to understand about magnitude of genetic diversity and genetic relationships among localities (Roderick, 1996). In particular, it would be important to investigate whether or not populations of the species occurring in Korean peninsula also hold ancestral polymorphisms in mitochondrial DNA (mtDNA) and distribution of the polymorphism.

In the present study, therefore, we investigated the geographic genetic contour of *C. aurichalcea* using partial sequence of mitochondrial cytochrome *b* oxidase subunit I (COI) gene and completely sequenced nuclear ribosomal internal transcribed spacer 2 (ITS2) in order to grab hold of population genetic information of the species. Previously, mitochondrial COI gene sequence has been utilized to illustrate several geographic genetic perspectives of insects occurring in the Korean peninsula (Jeong *et al*., 2009; Kim *et al*., 2009). Further, this region that is widely used as "DNA barcode region" (Hebert *et al*., 2003) has been suggested to provide early insight into the patterning of genomic diversity within species (Hajibabaei *et al*., 2007). The ITS2 that is located between 5.8S and 28S rRNA genes is one of the fast evolving non-coding regions of the nuclear ribosomal DNA (Arnheim, 1983; Miller *et al*., 1996). Thus, this region has been investigated in a broad array of studies including population genetic studies (Fritz *et al*., 1994; Mukabayire *et al*., 1999; Marcilla *et al*., 2001; Wörheide *et al*., 2002) including insects occurring in Korean peninsula (Oh *et al*., 2009; Kang *et al*., 2011).

Materials and Methods

Insects

Adult *Chrysolina aurichalcea* were caught from seven Korean localities from June 2009 to May 2010. The samples were frozen at −70°C until being used in molecular analysis. Sampling information on collection locality, number, and date are provided in Table 1, and the locality map is shown in Fig. 1. The different sampling sizes may reflect the difference in population size at each locality, considering that a similar effort for sample collection was made.

Primer design

In order to amplify and sequence the COI gene we used the primer set COLCOIF1 (5'- AAACTAWTARCCTTCAAAG-3') and HCO2198 (5'- TAAACTTCAGGGTGACCAAAAAATCA-3'). HCO2198 was adopted from Folmer *et al*. (1994), but COLCOIF1 was designed in this study from the alignment of $tRNA^{Trp}$ and neighboring genes of mtDNA from several full-length mitochondrial genomes of Coleoptera. For the amplification of ITS2, primers NG02955 (5'-ATGAACATCGACATTTCGAACGCACAT-3') and AB052895 (5'-TTCTTTTCCTCCGCTTAGTAATATGCTTAA-3') located on the 5.8S and 28S rDNAs, respectively, were successfully used (Ji *et al*., 2003; Table 2).

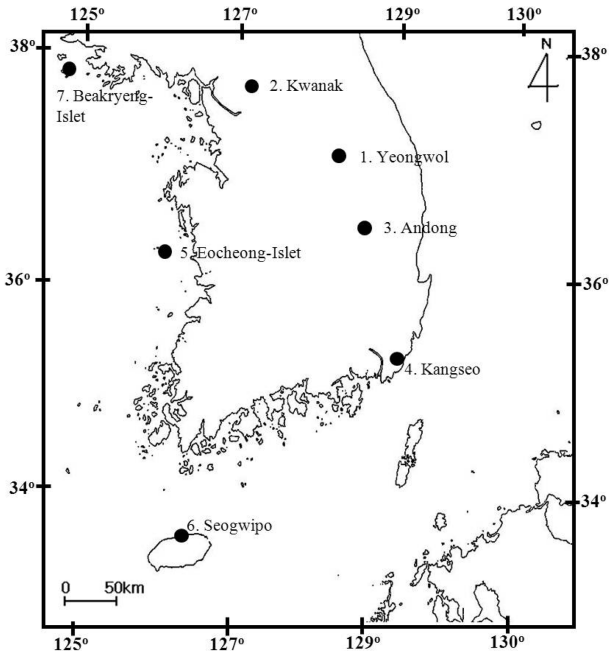

Fig. 1. Sampling location of *chrysolina aurichalcea* in Korea.

DNA extraction, PCR, cloning, and sequencing

From the frozen hind leg total DNA was extracted with a Wizard Genomic DNA Purification Kit using the manufacturer's instructions (Promega, USA). For the amplification of the 658-bp region of COI gene, PCR was conducted under the following conditions: an initial denaturation step at 94°C for 7 min, a 35-cycle amplification (94°C for 1 min, 47.8~56°C for 1 min, and 72°C for 1 min), and the final extension step for 7 min at 72°C. For PCR of ITS2, an initial denaturation step at 94°C for 7 min, a 35-cycle amplification (94°C for 40 sec, 55~60°C for 30 sec, and 72°C for 40 sec), and the final extension step for 2 min at 72°C were conducted. Successfully amplified PCR products were subjected to DNA purification using PCR purification Kit (Bioneer, Korea). The COI gene amplicons were directly sequenced, whereas those from ITS2 were cloned into a pGEM-T Easy vector (Promega). For the cloning process, XL1-Blue competent

Table 2. Type of substitution in the COI and ITS2 sequences of *Chrysolina aurichalcea*

Region	Nucleotide position	Type of substitution	Category	Amino acid substitution
COI	15	T↔C	Ts	ATC (Ile)↔ ACC (Thr)
	28	A↔G	Ts	
	46	A↔C	Tv	
	49	T↔C	Ts	
	50	T↔C	Ts	
	61	A↔G	Ts	
	82	C↔T	Ts	
	127	A↔G	Ts	
	133	C↔T	Ts	
	169	A↔G	Ts	
	181	T↔A	Tv	
	196	A↔G	Tv	
	217	T↔C↔A	Ps	
	220	C↔T	Ts	
	262	G↔A	Ts	
	271	A↔C	Tv	
	286	C↔T	Ts	
	289	G↔A	Ts	
	318	C↔T	Ts	GCA (Ala)↔ GTA (Val)
	346	C↔A	Tv	
	373	C↔T	Ts	
	433	A↔G	Ts	
	466	A↔G	Ts	
	473	G↔A	Ts	GTT (Val)↔ ATT (Ile)
	477	G↔A	Ts	GGA (Gly)↔ GAA (Glu)
	502	G↔A	Ts	
	506	T↔C	Ts	TCA (Ser)↔ CCA(Pro)
	535	A↔G	Ts	
	544	G↔A	Ts	
	550	A↔G	Ts	
	559	A↔G	Ts	
	571	C↔T	Ts	
	577	A↔G	Ts	
	578	C↔T	Ts	
	586	T↔C	Ts	
	54	G↔A	Ts	
	76	A↔G	Ts	-
	125	T↔A	Tv	-
	140	G↔A	Ts	-
	141	C↔T	Tv	-
	202	A↔G	Ts	-
	268	A↔G	Ts	-
	279	G↔A	Ts	-
	309	G↔A	Ts	-
	354	T↔C	Ts	-
	358	T↔C	Ts	-
	369	C↔T	Ts	-
	399	C↔T	Ts	-
ITS2	403	G↔A	Ts	-
	416	T↔A	Tv	-
	417	A↔G	Ts	-
	418	A↔T	Tv	-
	423	G↔A	Ts	-
	426	C↔T	Ts	-
	430	T↔deletion	Indel	-
	431	T↔deletion	Indel	-
	440	T↔deletion	Indel	-
	441	T↔deletion	Indel	-
	442	T↔deletion	Indel	-
	443	T↔deletion	Indel	-
	446	T↔G	Tv	-

Ts, transition; Tv, transversion; Ps, polymorphic site; Indel, insertion/deletion; and -, not applicable.

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). 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 and phylogenetic relationships

Bi-directionally sequenced individual sequences were aligned using MAFFT ver. 6 (Katoh *et al*., 2002). COI gene sequences were delimitated to the site just after each

primer site, resulting in 658 bp. Comparison of several *C. aurichalcea* sequences registered in Bold System (http://www.barcodinglife.com/) turned out to be 98% sequence homology to one intraspecific sample (AY_796210). On the other hand, the boundary of the ITS2 sequence was delimited using the Hidden Markov Model-based ITS2 annotation software, with the model selection set to either Metazoa or Diptera and the maximum E-value set as the default value, E < 0.001 (Keller *et al*., 2009). When the homologous sequences from two individuals differed by one or more nucleotide base (for both COI and ITS2) or one insertion/deletion (indel) position (for ITS2), the sequences were considered either different haplotypes (for COI) or sequence types (for ITS2). Haplotype or sequence type designations were applied to new sequences as they were discovered (i.e., CACOI01 and CACOI01 and so forth for COI, and ITS2CA01, ITS2CA02, ITS2CA03 and so on for ITS2).

Unlikely COI haplotypes ITS2 sequences contain indel positions, which make alignment inaccurate. Thus, the aligned sequences were subjected to GBlocks analysis (ver. 0.91b) in order to select conserved regions with the maximum number of contiguous non-conserved positions set to four (Castresana, 2000). Resultantly, 14 indel positions were reduced to seven positions and this equivalent to the sequence length from 515 positions reduced to 481 positions (93% of original positions). Further, subsequence sequence analysis was performed using "after-GBlocks sequence" in order to avoid uncertainty.

Phylogenetic analysis among the COI haplotypes and ITS2 sequence types was respectively conducted via the maximum-parsimony (MP) method (Fitch 1971) using Phylogenetic Analysis Using Parsimony and Other Method (PAUP) ver. 4.0b10 (Swofford, 2002). The reliability of the trees was assessed by 1,000 iterations of bootstrapping (Felsenstein, 1985). To root trees, the homologous region of a within-familial species, *Crioceris duodecimpunctata* for COI gene (Stewart and Beckenbach, 2003) (GenBank accession number AF467886) or within-generic species *C. timarchoides* for ITS2 (Gomez-Zurita *et al*., 2000) was used as outgroup, respectively.

Gene flow

The genetic distance was estimated from ITS2 data using Arlequin ver. 3.0 software (Excoffier *et al*., 2005). Population genetic distance between pairs of locality (F_{ST}) and its permutation test to obtain the statistical significance of the value (1,000 bootstraps) were determined in accordance with the approach described by Excoffier *et al*. (1992), with the selection of algorithm, Kimura' two-parameters method, to calculate the distance between DNA sequences (Kimura, 1980). Pairwise N_m (the product of the effective population size, N_e, and migration rate, m) values were used to estimate the pairwise F_{ST} based on the equilibrium relationship: $F_{ST} = 1 / (4N_m + 1)$.

Results and Discussion

Sequence analysis

A total of 17 COI haplotypes and 16 ITS2 sequence types (23 types before GBlocks analysis to select conserved blocks for ITS2) were obtained by sequencing 34 individuals of *Chrysolina aurichalcea* collected from seven localities in Korean peninsula (Table 1). Individual sequence information was deposited in the GenBank database (Table 1). Although COI gene revealed no indels, presenting all identical 658 bp, ITS2 was variable in length, ranging from 474 bp to 485 bp. Sequence alignment of COI gene revealed 35 variable nucleotides, 30 of which are transitions (13 TC and 17 GA), 4 of which are transversions (3 AC and 1 TA), and one of which is polymorphic site (TCA) (Table 2). Among them, five of nucleotide substitutions caused amino acid change. In the case of ITS2 a total of 26 variable sites were detected. This consisted of 16 sites of transitions (5 TC and 11 GA), 4 sites of transversions (1 TG and 3 TA), and 6 indels (Table 2).

Uncorrected pairwise comparison between pairs of haplotypes and sequence types is displayed in Tables 3 and 4, respectively. The sequence divergence among 17 haplotypes ranged from 0.152% to 3.04% (1~20 bp) and the highest estimate was found in a comparison between CACOI13 and CACOI15. On the other hand, uncorrected pairwise distance among 16 sequence types showed the maximum divergence of 1.699% (8 sites) and the highest estimate was found in a comparison between ITS2CA05 and ITS2CA06 (Table 4). This estimate for COI is quite high considering other relevant studies, which utilized homologous COI gene. For example, the estimate was 0.2% for the domestic silkworm (Kim *et al*., 2000), 0.2% and 1.2% for two species of mushroom flies (Bae *et al*., 2001), 0.3% for a bumblebee, *Bombus ardens* (Kim *et al*., 2009), 0.45% for the tiny dragonfly (Kim *et al*., 2007b), 0.76% for the mason bee (Kim *et al*., 2008), 0.9% for the diamondback moth (Li *et al*., 2006), 0.91% for the swallowtail butterfly (Jeong *et al*., 2009), 0.91% for a ground beetle, *Scarites aterrimus* (Wang *et al*., 2011), 1.67% for the cabbage butterfly (Jeong *et al*., 2009), 4.0% for the firefly, *Luciola lateralis* (Kim *et al*., 2001), 5.0% for another firefly, *Pyrocoelia rufa* (Lee *et al*., 2003), and 11% for the oriental mole cricket (Kim *et al*., 2007a). The two firefly species and the mole cricket have been reported to have a taxonomic implication (Kim *et al*., 2001; Lee *et al*., 2003; Kim *et al*., 2007). Thus, excluding

Table 3. Pairwise comparisons among 17 haplotypes obtained from mitochondrial COI gene sequences of *Chrysolina aurichalcea*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1. CACOI01	-	0.01368	0.00152	0.00152	0.00456	0.00152	0.00152	0.00152	0.0152	0.00152	0.01064	0.00152	0.00912	0.00608	0.02584	0.02432	0.02584
2. CACOI02	9	-	0.0152	0.0152	0.01824	0.0152	0.0152	0.0152	0.00152	0.0152	0.0152	0.0152	0.01672	0.01368	0.02584	0.02432	0.02584
3. CACOI03	1	10	-	0.00304	0.00608	0.00304	0.00304	0.00304	0.01672	0.00304	0.01216	0.00304	0.01064	0.0076	0.02736	0.02584	0.02736
4. CACOI04	1	10	2	-	0.00608	0.00304	0.00304	0.00304	0.01672	0.00304	0.01216	0.00304	0.01064	0.0076	0.02736	0.02584	0.02736
5. CACOI05	3	12	4	4	-	0.00608	0.00608	0.00608	0.01976	0.00608	0.0152	0.00608	0.01368	0.01064	0.0304	0.02888	0.0304
6. CACOI06	1	10	2	2	4	-	0.00304	0.00304	0.01672	0.00304	0.01216	0.00304	0.01064	0.0076	0.02736	0.02584	0.02736
7. CACOI07	1	10	2	2	4	2	-	0.00304	0.01672	0.00304	0.01216	0.00304	0.01064	0.0076	0.02736	0.02584	0.02736
8. CACOI08	1	10	2	2	4	2	2	-	0.01672	0.00304	0.01216	0.00304	0.01064	0.0076	0.02736	0.02584	0.02736
9. CACOI09	10	1	11	11	13	11	11	11	-	0.01672	0.01672	0.01672	0.01824	0.0152	0.02432	0.02584	0.02736
10. CACOI10	1	10	2	2	4	2	2	2	11	-	0.01216	0.00304	0.01064	0.00456	0.02736	0.02584	0.02736
11. CACOI11	7	10	8	8	10	8	8	8	11	8	-	0.01216	0.00152	0.01064	0.02888	0.02736	0.02584
12. CACOI12	1	10	2	2	4	2	2	2	11	2	8	-	0.01064	0.0076	0.02736	0.02584	0.02736
13. CACOI13	6	11	7	7	9	7	7	7	12	7	1	7	-	0.00912	0.0304	0.02888	0.02736
14 CACOI14	4	9	5	5	7	5	5	5	10	3	7	5	6	-	0.02432	0.0228	0.02432
15. CACOI15	17	17	18	18	20	18	18	18	16	18	19	18	20	16	-	0.00152	0.00304
16. CACOI16	16	16	17	17	19	17	17	17	17	17	18	17	19	15	1	-	0.00152
17. CACOI17	17	17	18	18	20	18	18	18	18	18	17	18	18	16	2	1	-

Numbers above the diagonal are mean distance values and numbers below the diagonal are absolute distance values.

Table 4. Pairwise comparisons among 16 sequence types obtained from nuclear ITS2 sequences of *Chrysolina aurichalcea*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. ITS2CA01	-	0.00208	0.00208	0.00208	0.01057	0.00626	0.00208	0.00208	0.00208	0.00624	0.00418	0.00208	0.00208	0.00418	0.00417	0.00208
2. ITS2CA02	1	-	0.00417	0.00417	0.01271	0.00835	0.00417	0.00417	0.00417	0.00833	0.00626	0.00417	0.00417	0.00626	0.00625	0.00417
3. ITS2CA03	1	2	-	0.00416	0.01268	0.00835	0.00416	0.00416	0.00416	0.00832	0.00626	0.00417	0.00417	0.00626	0.00625	0.00417
4. ITS2CA04	1	2	2	-	0.01268	0.00835	0.00416	0.00416	0.00416	0.00832	0.00626	0.00417	0.00417	0.00626	0.00625	0.00417
5. ITS2CA05	5	6	6	6	-	0.01699	0.01268	0.01268	0.01268	0.00423	0.01486	0.01271	0.01271	0.01486	0.01483	0.01271
6. ITS2CA06	3	4	4	4	8	-	0.00835	0.00418	0.00835	0.01253	0.00626	0.00835	0.00835	0.00626	0.01044	0.00835
7. ITS2CA07	1	2	2	2	6	4	-	0.00416	0.00416	0.00832	0.00626	0.00417	0.00417	0.00626	0.00625	0.00417
8. ITS2CA08	1	2	2	2	6	2	2	-	0.00416	0.00832	0.00209	0.00417	0.00417	0.00209	0.00625	0.00417
9. ITS2CA09	1	2	2	2	6	4	2	2	-	0.00832	0.00626	0.00417	0.00417	0.00626	0.00625	0.00417
10. ITS2CA10	3	4	4	4	2	6	4	4	4	-	0.01044	0.00833	0.00833	0.01044	0.01042	0.00833
11. ITS2CA11	2	3	3	3	7	3	3	1	3	5	-	0.00626	0.00626	0.00418	0.00835	0.00626
12. ITS2CA12	1	2	2	2	6	4	2	2	2	4	3	-	0.00417	0.00626	0.00625	0.00417
13. ITS2CA13	1	2	2	2	6	4	2	2	2	4	3	2	-	0.00626	0.00625	0.00417
14. ITS2CA14	2	3	3	3	7	3	3	1	3	5	2	3	3	-	0.00835	0.00626
15. ITS2CA15	2	3	3	3	7	5	3	3	3	5	4	3	3	4	-	0.00625
16. ITS2CA16	1	2	2	2	6	4	2	2	2	4	3	2	2	3	3	-

Numbers above the diagonal are mean distance values numbers below the diagonal are absolute distance values

these species, the maximum sequence divergence was roughly ≤ 1.0% within species.

On the other hand, the divergence of ITS2 is substantially smaller than that of COI. Generally, it is known that ITS2 is fast evolving regions, relatively free from structural and functional constrains (Tang *et al*., 1996; Navajas *et al*., 1998).

Geographic genetic contour

Phylogenetic analysis was investigated to know evolutionary relationships among haplotypes and sequence types and to find out any discernable groups in connection with geographic affinity (Fig. 2 and 3). Seventeen COI haplotypes were largely unresolved, presenting multibranches with low bootstrap values, but two groups were recognizable (groups A and B). Group A that has 87% of nodal support is composed of two haplotypes (CACOI11 and CACOI13), which are two among five haplotypes found in locality 5 (Eocheong-Islet). Group B that has 94% nodal support is composed of three haplotypes (CACOI15, CACOI16, and CACOI17), which are all the haplotype members found in locality 7 (Beakryeng-Islet) (Fig. 2). In the case of ITS2 only one group (group A) composed of two sequence types (ITSCA05 and ITSCA10) was strongly supported (92% of nodal support; Fig. 3). Geographically, ITSCA05 was found in four localities such as locality 1 (Yeongwol), locality 2 (Kwanak-gu), locality 3 (Andong), and locality 4 (Kangseo-gu), whereas ITSCA10 was found only in locality 4 (Table 5).

Finding of phylogenetically subdivided COI haplotypes suggest the presence of polymorphism and this, in turn, suggests that the species occurring in Korean peninsula experienced historical biogeographic barriers that bolstered genetic subdivision. This result may be consistent with the ND2-based phylogeny with an extensive Japanese sample of the two karyotypes: co-occurrence of identical haplotypes in different types in several localities and non-monophyly in either of the two karyotypes (Kitamura *et al*., 2008). Their reasoning for such polymorphism

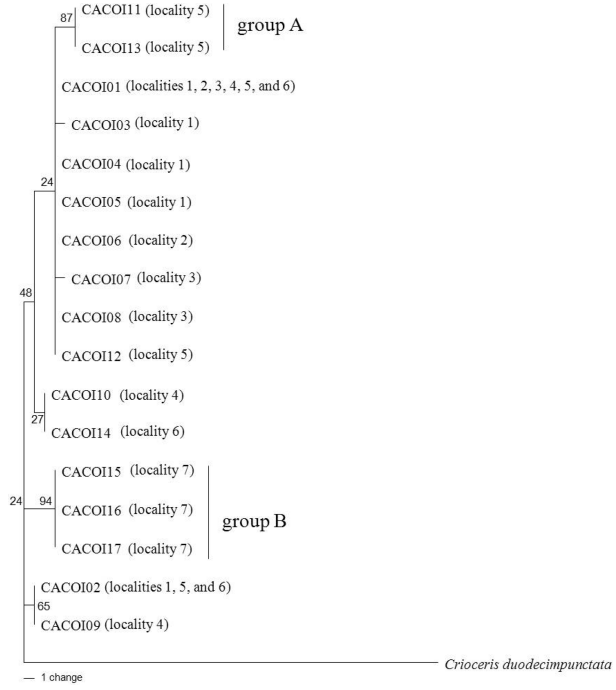

Fig. 2. Phylogenetic analysis among 17 COI haplotypes of *Chrysolina aurchalcea.* The tree was acquired via the MP method incorporated in the PAUP software (Swofford, 2002). *Crioceris duodecimpunctata* (Genbank accession number AF467886) was used as an outgroup in order to root tree. The numbers on (under) the branches represent bootstrap values of 1,000 replications. Parentheses indicate the locality name, from which the particular haplotype was obtained. Two groups with relatively high node supporting value (> 87%) were recognized.

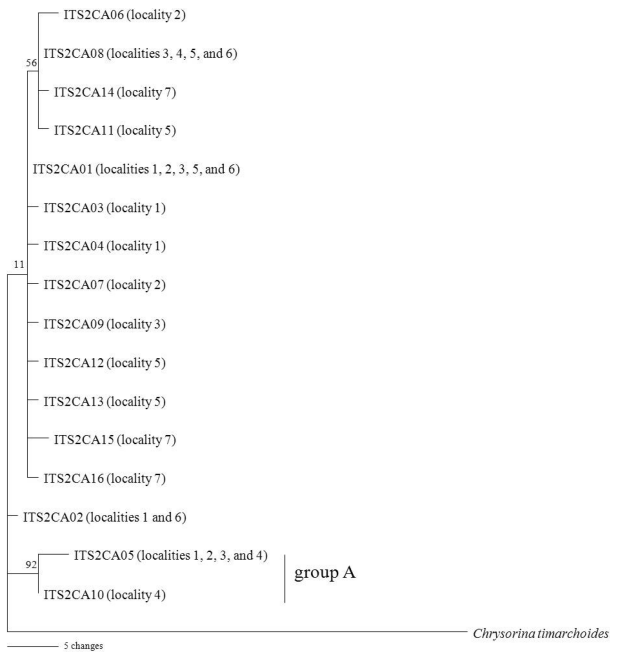

Fig. 3. Phylogenetic analysis of 16 ITS2 sequence types of the *Chrysolina aurichalcea*. The tree was acquired via the MP method incorporated in the PAUP (Phylogenetic Analysis Using Parsimony and Other) ver. 4.0b10 software (Swofford, 2002). *Chrysorina timarchoides* (Genbank accession number AJ279788.1) was used as an outgroup in order to root tree. The numbers on the branches represent bootstrap values of 1,000 replications. Parentheses indicate the locality name, from which the particular sequence type was obtained. One group with relatively high node supporting value (> 92%) was recognized.

Table 5. Relative frequencies of COI haplotypes and ITS2 sequence types through the populations

Region	Types	Locality						
		1 (6)	2 (4)	3 (6)	4 (4)	5 (6)	6 (5)	7 (3)
COI	CACOI01	0.33 (2)	0.75 (3)	0.67 (4)	0.25 (1)	0.17 (1)	0.20 (1)	
	CACOI02	0.17 (1)				0.34 (2)	0.60 (3)	
	CACOI03	0.17 (1)						
	CACOI04	0.17 (1)						
	CACOI05	0.17 (1)						
	CACOI06		0.25 (1)					
	CACOI07			0.17 (1)				
	CACOI08			0.17 (1)				
	CACOI09				0.50 (2)			
	CACOI10				0.25 (1)			
	CACOI11					0.17 (1)		
	CACOI12					0.17 (1)		
	CACOI13					0.17 (1)		
	CACOI14						0.20 (1)	
	CACOI15							0.33 (1)
	CACOI16							0.33 (1)
	CACOI17							0.33 (1)
ITS2	ITS2CA01	0.33 (2)	0.17 (1)	0.34 (2)		0.34 (2)	0.60 (3)	
	ITS2CA02	0.17 (1)					0.20 (1)	
	ITS2CA03	0.17 (1)						
	ITS2CA04	0.17 (1)						
	ITS2CA05	0.17 (1)	0.17 (1)	0.34 (2)	0.25 (1)			
	ITS2CA06		0.17 (1)					
	ITS2CA07		0.17 (1)					
	ITS2CA08			0.17 (1)	0.50 (2)	0.17 (1)	0.20 (1)	
	ITS2CA09			0.17 (1)				
	ITS2CA10				0.25 (1)			
	ITS2CA11					0.17 (1)		
	ITS2CA12					0.17 (1)		
	ITS2CA13					0.17 (1)		
	ITS2CA14							0.33 (1)
	ITS2CA15							0.33 (1)
	ITS2CA16							0.33 (1)

Numbers in parentheses indicate sample size at each population. Locality names are as follows: 1, Yeongwol; 2, Kwanak-gu; 3, Andong; 4, Kangseo-gu; 5, Eocheong-Islet; 6, Seogwipo; and 7, Beakryeng-Islet.

without consistency to karyotype data was a rapid and recent chromosomal change, possessing ancestral polymorphism in ND2 sequences (Kitamura *et al*., 2008). In order to further test such possibility, nevertheless, more sensitive molecular markers revealing co-dominant nature might be required. Collectively, if individuals or populations carrying different karyotypes are truly present in Korean peninsula, one possible scenario that fulfills the present data would be re-hybridization of once isolated populations, which carries different karyotypes. Nevertheless, this interpretation may not fully explain the mechanism by which individuals with different karyotypes can reproduce.

In contrast to COI gene sequences, the ITS2 data do not concordant with COI result in that the individuals involved in the clustering in COI sequences (sample num-

Table 6. Fixation indices (F_{ST}) and migration rate (Nm) between pairs of populations of *Chrysolina aurichalcea* obtained from ITS2 sequence

	1	2	3	4	5	6	7
1. Yeongwol		F_{ST} = 0.15527	F_{ST} = -0.09038	F_{ST} = -0.22667	F_{ST} = -0.20229	F_{ST} = 0.12787	F_{ST} = 0.00141
		Nm = 2.72026	Nm = inf	Nm = inf	Nm = inf	Nm = 3.41014	Nm = 354.00000
2. Kwanak-gu			F_{ST} = -0.04833	F_{ST} = 0.06545	F_{ST} = 0.12601	F_{ST} = -0.06616	F_{ST} = -0.05830
			Nm = inf	Nm = 7.13918	Nm = 3.46800	Nm = inf	Nm = 8.07692
3. Andong				F_{ST} = -0.14176	F_{ST} = -0.09697	F_{ST} = 0.00323	F_{ST} = -0.04577
				Nm = inf	Nm = inf	Nm = 154.50000	Nm = inf
4. Kangseo-gu					F_{ST} = -0.19717	F_{ST} = 0.04414	F_{ST} = -0.09091
					Nm = inf	Nm = 10.82667	Nm = inf
5. Eocheong- Islet						F_{ST} = 0.12609	F_{ST} = 0.02743
						Nm = 3.46552	Nm = 17.72727
6. Seogwipo							F_{ST} = -0.02632
							Nm = inf
7. Beakryeng-Island							

*p < 0.05.
inf, infinite.

bers CA1971, CA1975, CA3317, CA3318, CA3320) are not involved in the clustering in ITS2 (sample numbers CA3286, CA3289, CA3298, CA3299, and CA3308) (Table 1) and their localities never overlap between the two data sets (Table 5). One of the possible interpretations for this inconsistency might be recent dispersal, considering that several sequence types (four among 16) are simultaneously found in more than one locality (Table 5). In geographic perspective, in fact, most localities have one or more sequence types that are also found in other localities, except for locality 7 (Beakryeng-Islet). Under the interpretation that ITS2 sequence is one of the fast evolving non-coding regions in insects (Navajas *et al*., 1998) sharing of identical sequence types among localities might be interpreted as the result of gene flow. In fact, genetic distance (F_{ST}) between pairs of populations suggest no statistically significant genetic separation (p < 0.05) and migration rates (Nm) estimates show at least more than two individuals per generation (between localities 1 and 2) by ITS2 sequences (Table 6). Thus, the result of our determinations of gene flow can be surmised that the *C. aurichalcea* populations on the Korean peninsula are quite well connected to each other.

Beakryeng-Islet population

Unlikely other populations Beakryeng-Islet population (locality 7) possesses unique haplotypes (CACOI15, CACOI16, and CACOI17) and sequence types (ITS2CA14, ITS2CA15, and ITS2CA16) that have never been found in other populations. Although small sample size and lack of clustering among the three sequence types in ITS2 phylogeny renders somewhat decisive conclusion impossible finding of unique haplotypes and sequence types from Beakryeng-Islet population imply that the population might be an intact remnant of ancient polymorphism. If this is the case, this location might be a plausible ground worth to search to find individuals with consistent haplotype and karyotype. This is particularly likely, because this locality is northwestern most one among our sampling localities. As more samples are analyzed using further hyper-variable marker, further detailed information on this largely unknown species will be possible.

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