Field monitoring and genetic diversity of the large copper butterfly *Lycaena dispar* (Lepidoptera: Lycaenidae)

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Abstract

The large copper butterfly *Lycaena dispar* (Haworth, 1803) (Lepidoptera: Lycaenidae), which is found across Europe and Asia, has been categorized as a near-threatened species (NT) in South Korea from 2012 mainly due to limited distribution. The species has been infrequently observed outside its traditional range in the northwestern region, but no nationwide survey has yet been conducted. In addition, the population genetic data of *L. dispar*, which is informative to evaluate its vulnerability, remains unknown. In this study, we visited 36 sites spread across all South Korean provinces to verify the distributional range of the species. Mitochondrial cytochrome oxidase subunit I (*COI*) was also partially sequenced for 53 individuals from nine sites. We observed *L. dispar* at 15 sites in six provinces, including the two previously known provinces, indicating a southward range expansion. The in-field monitoring and genetic data collectively suggested that *L. dispar* does not have a limited distribution nor is it isolated, indicating that it should be reclassified as less vulnerable. Our study demonstrates that the combination of field and genetic data can provide a more reliable assessment of the stability of a species.

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Introduction

The International Union for Conservation of Nature (IUCN) records the distribution and abundance of species worldwide and categorizes them based on their risk of extinction.[1] Similarly, the National Institute of Biological Resources (NIBR) in South Korea annually updates the list of species in Korea, evaluates their risk of extinction, and investigates potential threats (https://species.nibr.go.kr/index.do). It also publishes the Red Data Book (RDB), which currently lists approximately 5,200 insect species and categorizes them based on several factors, such as the change in their population size and their distributional range.[2]

In addition to species categorization, estimates of genetic diversity as an indicator of biodiversity that is indirectly associated with population stability have also been proven important for biological conservation.[3,4] For example, a genetic diversity study using the mitochondrial gene *CytB* for 1,036 bird species in Europe found that the non-threatened group had a ~4% lower genetic diversity than those categorized as threatened and that some species classified as non-threatened may be at risk of extinction, highlighting the need to reflect genetic diversity data during the evaluation process.[5] In South Korea, several insect species that are categorized as endangered have had their within-population genetic diversity and among-population genetic differentiation assessed, with strong consistency found between field observations and genetic data.[6,7] Genetic data are necessary for the evaluation of species stability because low genetic diversity and genetic isolation reduce the long-term viability of a population when faced with fluctuating environmental conditions, an increase in inbreeding, and genetic drift.[8,9]

The large copper butterfly (*Lycaena dispar* Haworth, 1803)

(Lepidoptera: Lycaenidae) is distributed widely across Europe and Asia.[10] In Asia the species is distributed from the temperate regions to as far as the Amur region, including China and Russia, to Korea.[11,12] In northwest Europe, the species is facing serious decline, mainly due to habitat loss,[13,14] and has been listed as an endangered species in the IUCN RDB since 1990, although the species has been expanding in central and northern Europe.[10,15] On the Korean peninsula, the species was initially recorded only in North Korea[16] but has since spread to South Korea, although the species has been reported to be restricted only to northern regions such as Gyeonggi and Gangwon Provinces.[2,17] In addition to its limited distribution, the risk of population loss due to habitat destruction via stream reconstruction and chemical spraying for agriculture has led the species to be categorized as near threatened (NT), which means that the species has a risk of local extinction, though it may be minimal.[2] However, an increase in the local population in specific areas due to the abundance of host plants, such as curly dock (*Rumex crispus* L., 1753) (Caryophyllales: Polygonaceae), has also been reported.[2,18]

In the present study, we visited 36 sites spread across all nine provinces in South Korea and three cities to verify the distributional range of *L. dispar*. We also sequenced the DNA barcoding region of mitochondrial cytochrome oxidase subunit I (*COI*) from 53 individuals collected from nine sites to investigate the population's genetic characteristics and their relationship with any recent changes in the species range. This DNA barcoding region has been widely used for various geographically-based genetic analyses and species identification,[19,20] including several insect species found on the Korean peninsula.[6,21,22]

Materials and Methods

Field observations

We visited a total of 36 sites across all nine South Korean provinces and three cities (Seoul, Daegu, and Ulsan) (Table 1). The sites were selected based on their potential as habitable areas such as wet grasslands near rivers, streams, or rice paddies, and personal observations made by two of authors and other naturalists with experience with butterflies. The field observations were conducted at least once at each site for 1 h from May to September 2023; for more than ten sites, more than one visit was made.

Molecular experiments and DNA sequencing

A total of 53 *L. dispar* individuals were sequenced from nine sites (Table 1; Figure 1). The collected individuals were confirmed to be the target species by one of the authors (S.-S. Kim) via an examination of the external morphology. Total DNA was extracted from one leg using the Wizard Genomic DNA Purification Kit per the manufacturer's instructions (Promega, Madison, WI, USA). The DNA barcoding region of *COI* (658 bp) was amplified under the following conditions: an initial denaturation step at 94 °C for 2 min, 35 amplification cycles (94°C for 1 min, 50°C for 1 min, and 72°C for 1 min), and a final extension step of 10 min at 72°C. The following universal primers reported in Hebert *et al*.[23] were used:

LepF1, 5'-ATTCAACCAATCATAAAGATATTGG-3'
LepR1, 5'-TAAACTTCTGGATGTCCAAAAAATCA-3'

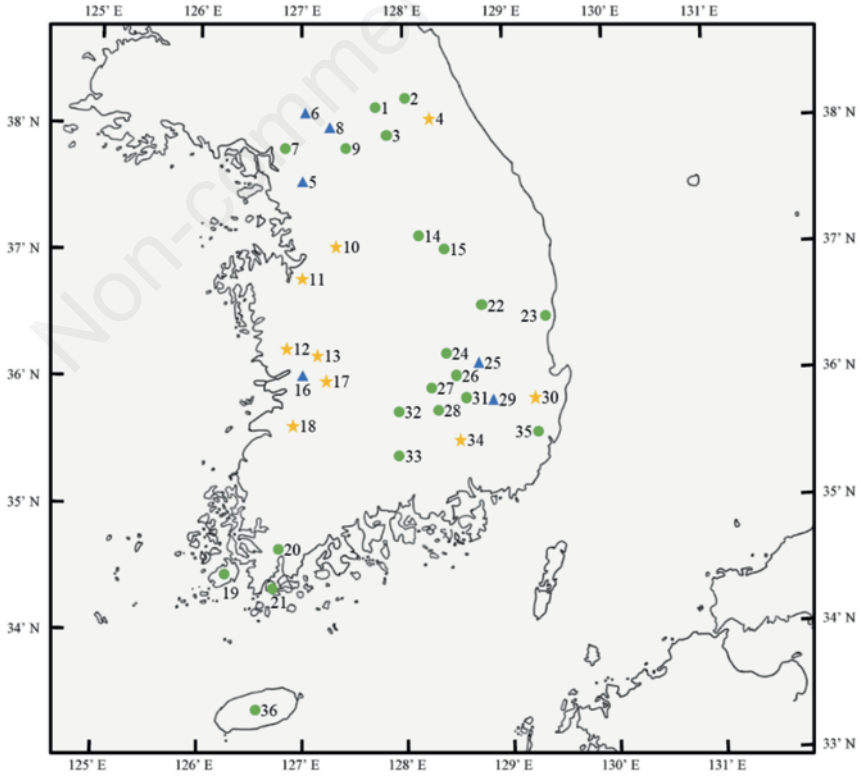

Figure 1. Sites for ecological investigation and DNA analysis. Green circles represent visited sites, blue triangles represent sites where *Lycaena dispar* was observed, and yellow stars represent sites where samples were collected for molecular sequencing. See Table 1 for site details.

Table 1. List of sites for the observation of *Lycaena dispar*, the observed number of individuals, and the number of individuals sequenced for mitochondrial *COI*.

No.	Site		Date	Number of individuals	
				Observation	Molecular sequencing
1	Gangwon-do	Hwacheon	2023-08-03	0	0
2		Yanggu	2023-06-01	0	0
		”	2023-08-02	0	0
3		Chuncheon	2023-04-22	0	0
4		Inje	2023-05-18	0	0
		”	2023-05-31	5	4
		”	2023-07-19	0	0
5	Seoul	Seoul	2023-05-04	6	0
6	Gyeonggi-do	Yeoncheon	2023-05-17	3	0
7		Paju	2023-05-17	0	0
8		Pocheon	2023-06-22	1	0
9		Gapyeong	2023-05-17	0	0
10		Anseong	2023-05-20	5	4
11	Chungcheongnam-do	Asan	2023-06-25	15	10
12		Buyeo	2023-05-10	9	5
13		Nonsan	2023-05-11	37	5
14	Chungcheongbuk-do	Jecheon	2023-04-13	0	0
		”	2023-04-27	0	0
		”	2023-04-28	0	0
15		Danyang	2023-05-08	0	0
		”	2023-05-20	0	0
16	Jeollabuk-do	Iksan	2023-05-10	27	0
17		Wanju	2023-06-23	15	10
18		Jeongeup	2023-07-20	6	5
19	Jeollanam-do	Jindo	2023-06-03	0	0
20		Gangjin	2023-06-11	0	0
21		Wando	2023-06-04	0	0
22	Gyeongsangbuk-do	Andong	2023-05-24	0	0
		”	2023-05-25	0	0
23		Yeongdeok	2023-06-25	0	0
		”	2023-08-23	0	0
24		Gumi	2023-06-28	0	0
25		Gunwi	2023-08-22	1	0
26		Chilgok	2023-05-01	0	0
27		Seongju	2023-04-30	0	0
		”	2023-06-29	0	0
		”	2023-09-17	0	0
28		Goryeong	2023-07-27	0	0
29		Gyeongsan	2023-09-06	8	0
30		Gyeongju	2023-05-13	5	4
31	Daegu	Daegu	2023-09-06	0	0
32	Gyeongsangnam-do	Geochang	2023-08-08	0	0
33		Sancheong	2023-08-09	0	0
34		Changnyeong	2023-07-02	19	6
35	Ulsan	Ulsan	2023-05-12	0	0
36	Jeju-do	Jeju	2023-06-07	0	0
Total (sites/individuals)				15/162	9/53

Electrophoresis was used to confirm successful DNA amplification with 0.5× Tris-acetate-EDTA (TAE) buffer on 0.5% agarose gel. The Polymerase Chain Reaction (PCR) products were purified using a PCR Purification Kit (Qiagen, Germany). DNA sequencing was conducted for both directions using an ABI PRISM BigDye Terminator ver. 3.1 Cycle Sequencing Kit with an ABI 3100 Genetic Analyzer (PE Applied Biosystems, Foster City, CA, USA). To obtain the finalized individual sequences, the Clustal Omega program was used for sequence alignment (http://www.ebi.ac.uk/Tools/msa/clustalo/). Nucleotide sequences were translated based on the invertebrate mitochondrial DNA genetic code to check for the presence of pseudogene sequences. Individual sequences were assigned as haplotypes (e.g., LDBAR01, LDBAR02, and LDBAR03) when a new sequence differed by one nucleotide or more from other sequences based on alignment using Phylogenetic Analysis Using Parsimony (PAUP) ver. 4.0b.[24] With the same software, the unrooted pairwise genetic distance between haplotypes was calculated.

Haplotype relationships

Haplotype relationships were inferred using the maximum-likelihood (ML) method with the TPM2u+F model from IQ-TREE.[25] To root the tree, the homologous region of the violet copper, *Lycaena helle*, was downloaded from GenBank (acc. no. MW503602).[26] Branch support was assessed using 1,000 replicates of Ultrafast Bootstrap (UFBoot)[27] and the Shimodaira–Hasegawa approximate likelihood ratio test (SH-aLRT).[28] The generated tree was viewed using FigTree version 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree). The median-joining algorithm in POPART version 1.7 was also used to investigate the haplotype relationships.[29]

Genetic diversity indices

The haplotype diversity (h) and nucleotide diversity (π) for each population were estimated according to Nei[30] using Arlequin ver. 3.5.[31] Per-population values that deviated from the standard error (SE) were considered significant. Within-locality maximum sequence divergence was extracted from within-locality unrooted pairwise distance using PAUP.[24] Given that population-level analysis requires at least two haplotypes per unit, only four of the nine populations were analyzed in this manner (Table 1).

Genetic structure

The distribution, relationships, and abundance of the gene pools were also analyzed using Bayesian Analysis of Population Structure (BAPS) ver. 6.0.[32] This analysis was conducted using clustering, with a linked locus module and a codon model. In this process, mixture analysis was performed with K-values ranging from 1–10, and optimal clusters were identified based on the maximum log marginal likelihood values.

Results

Field monitoring

Field monitoring of 36 sites in nine provinces and three cities led to the observation of *L. dispar* at 15 sites in six provinces (Gangwon-do, GW; Gyeonggi-do, GG; Chungcheongnam-do, CN; Jeollabuk-do, JB; Gyeongsangbuk-do, GB; and Gyeongsangnam-do, GN) and Seoul, with no individuals observed in Chungcheongbuk-do (CB), Jeollanam-do (JN), Jeju-do (JJ), Daegu, and Ulsan (Figure 1; Table 1). Individuals were observed mostly in grassland close to or slightly distant from ponds or forests, and sometimes where only one host plant (*R. crispus*) was present (Figure 2). Except for GW, GG, and possibly Seoul, which neighbors GG, most of the sites in the other provinces and cities at which *L. dispar* was observed have never been officially reported before for sightings of this species,[2,17] although sightings have been infrequently reported at some of these sites by naturalists. Thus, our field monitoring indicates that *L. dispar* has extended its distribution to the southwest to JB and the southeast to GN (Changnyeong, site 34; Figure 1). In terms of the number of individuals observed, Nonsan in CN was ranked first (site 13; 37 individuals), followed by Iksan in JB (site 16; 27 individuals), Changnyeong in GN (locality 34; 19 individuals), Asan in CN (site 11; 15 individuals), and Wanju in JB (site 17; 15 individuals;

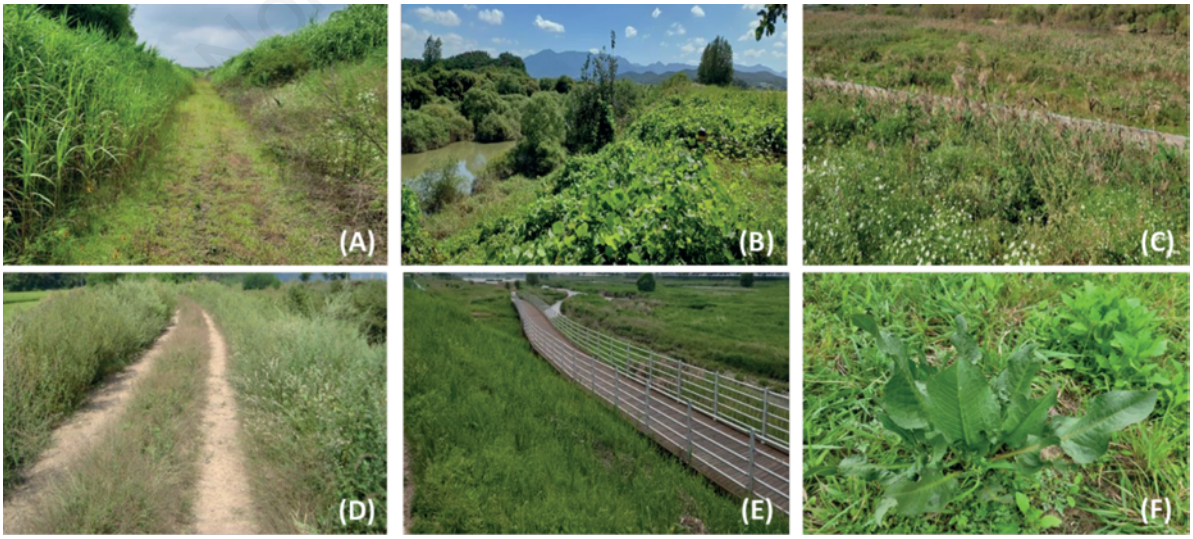

Figure 2. Photographs of the habitat and host plant of *Lycaena dispar*. A and B, Changnyeong, Gyeongsangnam-do (site 34); C and D, Jeongeup, Jeollabuk-do (site 18); E, Wanju, Jeollabuk-do (site 17); and F, the host plant, the curly dock (*Rumex crispus*).

Figure 3). Thus, the regions in which observations were made for the first time had a higher number of individuals than the previously known GG and GW, emphasizing the suitability of these new regions for *L. dispar*. Although we sighted *L. dispar* at only one of the three sites in GN, the number of *L. dispar* individuals observed at Changnyeong, which was the southernmost site with *L. dispar*, was the third highest (Figure 3). We were also able to observe all of the life stages of *L. dispar* at Changnyeong (Figure 4). These results collectively suggest that the species has permanently expanded its range to Changnyeong in GN.

Haplotype distribution

Four haplotypes were obtained by sequencing 658 bp of the *COI* gene from 53 *L. dispar* individuals collected from nine of the sites (Figure 1; Table 2). LBBAR01 was the most dominant haplotype across all sites, accounting for 47 individuals (88.7%). LBBAR02 was found in a single individual at Inje (GW; site 1) and two individuals at Jeongeup (JB; site 8). LBBAR03 was found in a single individual at Inje. LBBAR04 was found in one individual at Asan (site 3) and one at Nonsan (site 5) in CN (Table 2).

Table 2. List of *Lycaena dispar* individuals sequenced for the DNA barcoding region of *COI*.

No.	Collection site	Animal number	Haplotype (658 bp)	GenBank no.
1	Inje, Gangwon-do (4)	CNU15800	NDBAR01	PP267111
		CNU15802	NDBAR02	PP267112
		CNU15803	NDBAR03	PP267113
		CNU15804	NDBAR01	PP267114
2	Anseong, Gyeonggi-do (4)	CNU15806	NDBAR01	PP267115
		CNU15807	NDBAR01	PP267116
		CNU15808	NDBAR01	PP267117
		CNU15809	NDBAR01	PP267118
3	Asan, Chungcheongnam-do (10)	CNU15932	NDBAR01	PP267119
		CNU15933	NDBAR04	PP267120
		CNU15934	NDBAR01	PP267121
		CNU15935	NDBAR01	PP267122
		CNU15936	NDBAR01	PP267123
		CNU15937	NDBAR01	PP267124
		CNU15938	NDBAR01	PP267125
		CNU15939	NDBAR01	PP267126
		CNU15940	NDBAR01	PP267127
		CNU15941	NDBAR01	PP267128
4	Buyeo, Chungcheongnam-do (5)	CNU15811	NDBAR01	PP267129
		CNU15812	NDBAR01	PP267130
		CNU15814	NDBAR01	PP267131
		CNU15815	NDBAR01	PP267132
		CNU15816	NDBAR01	PP267133
5	Nonsan, Chungcheongnam-do (5)	CNU15817	NDBAR01	PP267134
		CNU15818	NDBAR04	PP267135
		CNU15819	NDBAR01	PP267136
		CNU15820	NDBAR01	PP267137
		CNU15821	NDBAR01	PP267138
6	Wanju, Jeollabuk-do (10)	CNU15920	NDBAR01	PP267143
		CNU15921	NDBAR01	PP267144
		CNU15922	NDBAR01	PP267145
		CNU15923	NDBAR01	PP267146
		CNU15924	NDBAR01	PP267147
		CNU15925	NDBAR01	PP267148
		CNU15926	NDBAR01	PP267149
		CNU15927	NDBAR01	PP267150
		CNU15928	NDBAR01	PP267151
		CNU15929	NDBAR01	PP267152
7	Jeongeup, Jeollabuk-do (5)	CNU16213	NDBAR02	PP267153
		CNU16214	NDBAR01	PP267154
		CNU16215	NDBAR02	PP267155
		CNU16216	NDBAR01	PP267156
		CNU16217	NDBAR01	PP267157
8	Gyeongju, Gyeongsangbuk-do (4)	CNU15823	NDBAR01	PP267139
		CNU15824	NDBAR01	PP267140
		CNU15825	NDBAR01	PP267141
		CNU15826	NDBAR01	PP267142
9	Changnyeong,Gyeongsangnam-do (6)	CNU16222	NDBAR01	PP267158
		CNU16223	NDBAR01	PP267159
		CNU16224	NDBAR01	PP267160
		CNU16225	NDBAR01	PP267161
		CNU16230	NDBAR01	PP267162
		CNU16231	NDBAR01	PP267163

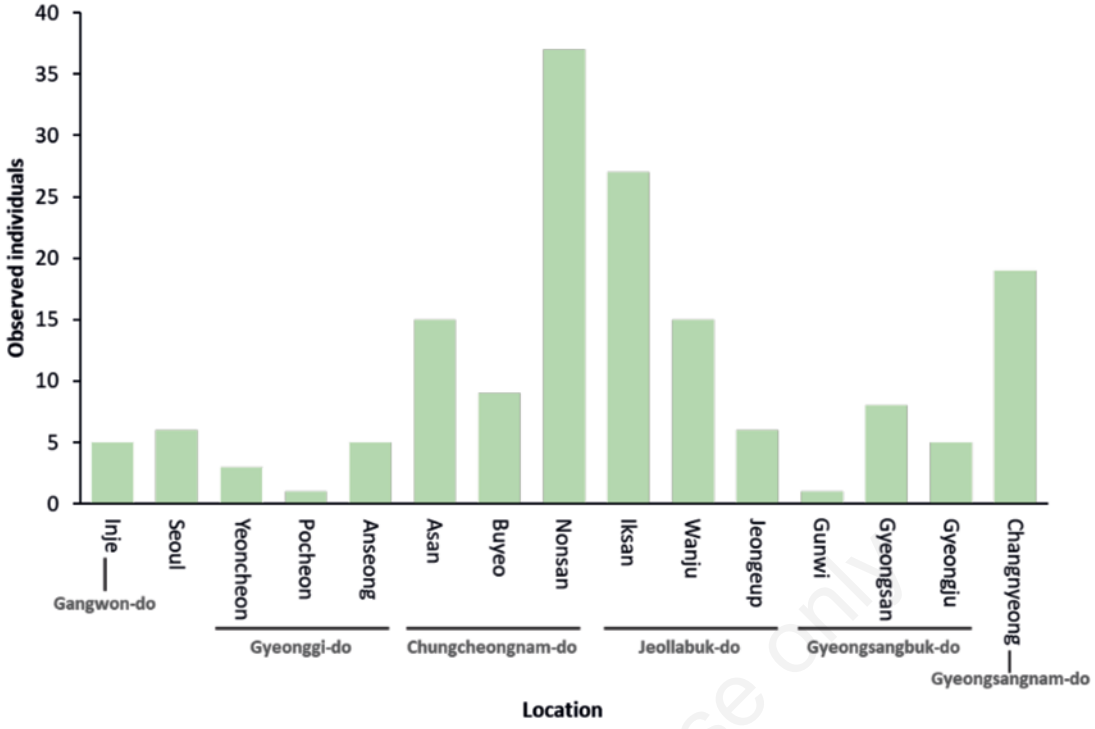

Figure 3. Number of observed *Lycaena dispar* individuals at 15 sites.

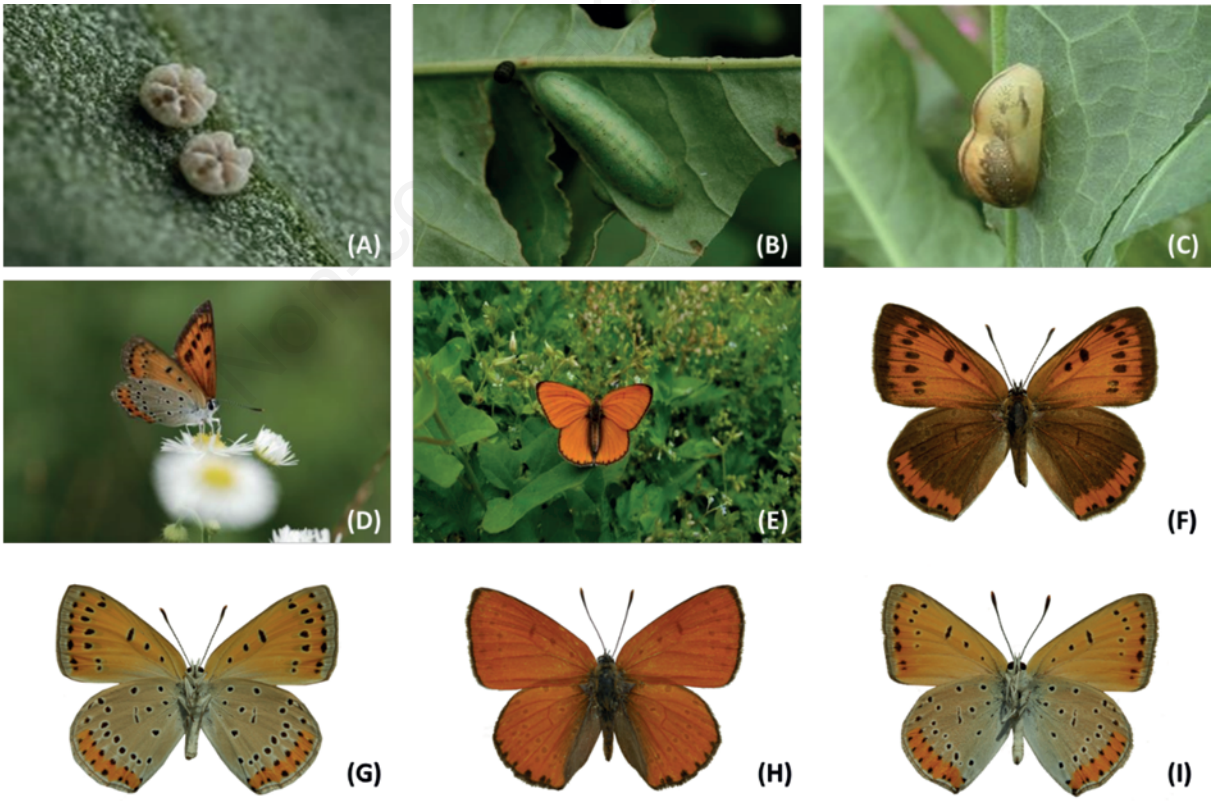

Figure 4. Life stages and specimen of *Lycaena dispar*. A, egg; B, larva; C, pupa; D, female adult; E, male adult; F, upperside of female adult; G, underside of female adult; H, upperside of male adult; and I, underside of male adult. All stages were observed in Changnyeong, Gyeongsangnam-do (site 34).

Haplotype relationships

The pairwise comparison revealed a sequence divergence ranging from 0.152% (1 bp) to 0.456% (4 bp), which is relatively low (Table 3). The maximum sequence divergence occurred between NDBAR02 (one individual from Inje and two from Jeongeup) and NDBAR03 (one from Inje). Phylogenetic analysis of the haplotypes revealed a large single group, although LBBAR03 and LBBAR04 formed a slightly discernable sub-group with higher nodal support (SH-aLRT=81.5, UFboot=71; Figure 5). Network analysis was also conducted (Figure 6), with LBBAR01, which was the most frequently detected and most widespread haplotype, located at the center of the network. From this central haplotype, NDBAR02 and NDBAR04 were derived first, with NDBAR03 then derived from NDBAR04, producing a simple star-like diversification pattern.

Genetic diversity

Genetic diversity was estimated only for the four populations with more than two haplotypes (Inje, Asan, Nonsan, and Jeongeup; Table 4). The haplotype diversity (h) ranged from 0.2 (Asan) to 0.8333 (Inje), while the nucleotide diversity (π) ranged from 0.000304 (Asan) to 0.002280 (Inje). Thus, Inje had the highest estimates, but these values did not have any statistical significance when standard error was considered.

Genetic structure

BAPS analysis divided the *L. dispar* individuals into four optimal gene pools (K=4; Figure 7). All populations commonly had one dominant gene pool (red), while Inje had three gene pools (red, green, and blue), with the green gene pool specific to Inje and the blue gene pool shared with Jeongeup. Nonsan and Asan had two gene pools, which were shared (red and yellow).

Discussion

Field monitoring

Our field monitoring has important implications for the species distribution of *L. dispar*. Except for GW, GG, and Seoul, our study officially reported for the first time new sightings of *L. dispar* in other provinces.[2,17] *L. dispar* has extended its distribution to JB and GN at a latitude of ~35.5′ N (Figure 1). These newly reported regions should not be considered ephemeral because the number of individuals at these sites was mostly higher than those observed in the previously known range (GG, GW, and Seoul; Figure 3). The observation of all *L. dispar* life stages at Changnyeong also indicat-

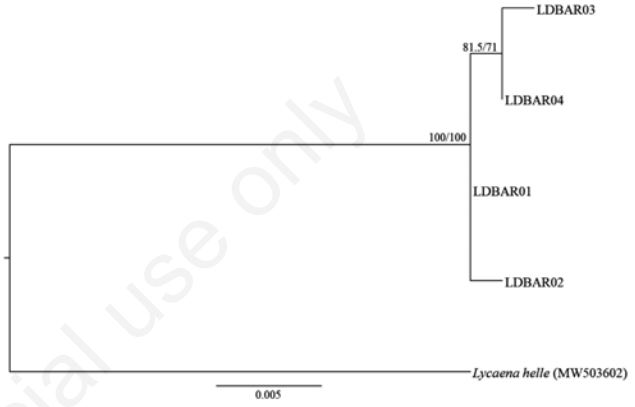

Figure 5. Phylogeny of the *COI* haplotypes of *Lycaena dispar* using the maximum-likelihood method. The numbers at each node indicate SH-aLRT (the first number) and UFBoot (the second number). *Lycaena helle* was used as an outgroup.

Table 3. Pairwise comparisons of the four *COI* haplotypes of *Lycaena dispar*.

Haplotype	1	2	3	4
1. NDBAR01	-	0.152	0.304	0.152
2. NDBAR02	1	-	0.456	0.304
3. NDBAR03	2	3	-	0.152
4. NDBAR04	1	2	1	-

Numbers above the diagonal, marked with '-', are the percent distance; numbers below the diagonal are the absolute distance.

Table 4. Within-population genetic diversity estimates for *Lycaena dispar* based on *COI* sequences.

Site	SS[a]	NH[b]	h[c]	NP[d]	MSD[e] (%)	MPD[f]	π[g]
1. Inje	4	3	0.8333±0.2224	3	0.456	1.500000±1.120934	0.002280±0.002034
2. Anseong	4	1	-	-	-	-	-
3. Asan	10	2	0.2000±0.1541	1	0.152	0.20000 0±0.269133	0.000304±0.000463
4. Buyeo	5	1	-	-	-	-	-
5. Nonsan	5	2	0.4000±0.2373	1	0.152	0.400000±0.435050	0.000608±0.000773
6. Wanju	10	1	-	-	-	-	-
7. Jeongeup	5	2	0.6000±0.1753	1	0.152	0.600000±0.562226	0.000912±0.000999
8. Gyeongju	4	1	-	-	-	-	-
9. Changnyeong	6	1	-	-	-	-	-

[a]Sample size; [b]number of haplotypes; [c]haplotype diversity with standard error; [d]number of polymorphic sites; [e]maximum sequence divergence; [f]mean number of pairwise differences; [g]nucleotide diversity with standard error; -, not available due to a single haplotype.

ed that these new regions were habitable (Figure 4). Nevertheless, the observation of *L. dispar* at only three of the nine sites in GB (Figure 1) suggests that further field monitoring is required to determine more precisely the distributional range of this species. This is particularly necessary because the six sites in GB at which *L. dispar* was not sighted are all located north of Changnyeong, the southernmost site for *L. dispar* observed in the present study.

Although the reasons for its range expansion were not investigated in the present study, we can speculate that it is associated with an increase in the abundance of its host plant, *R. crispus*. In Central and Western Europe, the decline and extinction of *L. dispar* was ascribed to the loss of wetland habitats, where host plants grow. Bink[33] showed that succession in the marsh leads to oligotrophic conditions, which reduce the growth and nutrient quality of the host plants, leading to a decrease in the survival and reproduction rates of *L. dispar*. Thus, the fluctuation of *L. dispar* seems to be closely associated with factors such as the size and quality of host plant clusters. *R. crispus* is distributed nationwide in South Korea and exhibits high environmental adaptability.[34] It is found on the ridges between rice fields, in orchards, along roadsides, in grassland, on the side of ditches and streams, and in wasteland.[2] The population size of *R. crispus* has greatly increased in Seoul due to the artificial reconstruction of the river system and the banks of Han River, which runs through Seoul, GG, and CB,[2] to increase the water storage volume and improve the ecosystem of the river system. Three other rivers, the Geum River in CN, the Yeongsan River in JN, and the Nakdong River in GB and GN, also underwent artificial reconstruction under the four-river refurbishment project from 2008 to 2012.[35,36] This artificial reconstruction of the four major river systems may have increased the abundance of *R. crispus* nationwide, which may have facilitated the range expansion of *L. dispar* in South Korea. However, this potential explanation requires further analysis.

Population genetic data

Genetic diversity and isolation, which are conceptually interconnected, are strongly associated with species stability. A larger population is more likely to maintain a higher genetic diversity, but isolated populations tend to have smaller population sizes, which increases the chance of sharing identical haplotypes, thus decreasing genetic diversity and increasing inbreeding, which is detrimental to long-term population survival.[8,9] Indeed, a positive relationship between species vulnerability and genetic estimates has been demonstrated for several insect species in South Korea. For example, mitochondrial *COI* analysis of the silver-stripped skipper *Leptalina unicolor* (Lepidoptera: Hesperiidae), which is listed as a critically endangered species in South Korea, has very low genetic diversity, with only one to two haplotypes per population and nearly complete genetic isolation of each population.[37] On the other hand, mitochondrial *COI* analysis of two well-observed butterfly species, the swallowtail butterfly *Papilio xuthus* (Lepidoptera: Papilionidae), and the cabbage butterfly *Pieris rapae* (Lepidoptera: Pieridae), has

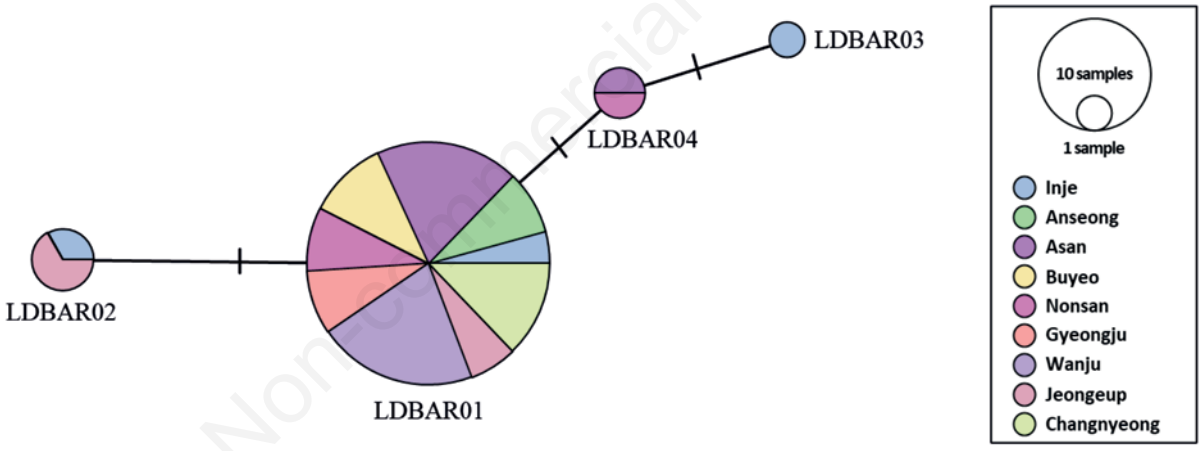

Figure 6. Median-joining network of the *COI* haplotypes of *Lycaena dispar*. Each circle represents a haplotype, and its colors represent a site. The circle size indicates the relative frequency of the sequences belonging to a particular haplotype. Hatch marks along the network branches indicate the number of substitutions.

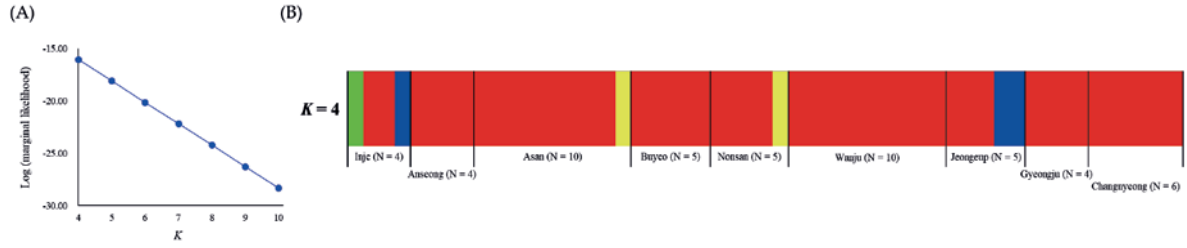

Figure 7. Bayesian clustering analysis of 53 *Lycaena dispar* individuals using *COI* haplotypes. (A) Log marginal likelihood values. The optimum number of clusters (*K*) was 4. (B) Bar plots of estimated membership of each individual in *K*=4 clusters. Black bars separate the nine populations. Different colors represent different haplogroups.

revealed that these species are genetically stable, with a moderate level of sequence divergence (maximum of 0.91%) among 15 haplotypes (2–8 haplotypes per population) and a moderate-to-high level of sequence divergence (maximum of 1.67%) among 30 haplotypes (4–12 haplotypes per population), respectively, with very few genetically isolated populations.[21]

The four haplotypes of *L. dispar* found in this study had a maximum divergence of only 0.456% (4 bp; Table 3) and formed one highly inclusive group (Figure 5 and 6), suggesting that *L. dispar* occurring in South Korea has relatively low genetic variation. Being confined for a long period to the northern region, such as GW and GG,[2,16,17] with its limited habitat and host plants may have restricted the population size in this region and the immigration of divergent haplotypes from North Korea, where larger populations are present.[16]

Nevertheless, BAPS analysis indicated that Inje, which is in the original distributional range for *L. dispar*, had the highest number of gene pools, with a unique green gene pool, a rare blue gene pool, which it shared with Jeongeup, and the common red gene pool (Figure 7). It has been suggested that the distribution of mitochondrial DNA lineages is proportional to their age under a simple isolation-by-distance model.[38] Thus, the oldest haplotypes will be most widespread with the highest frequencies, whereas their progeny are expected to be found close to the areas where they arose at lower frequencies.[39] Therefore, as range expansion continues, the green and blue gene pools present at Inje and possibly those in North Korea may expand to southern populations, increasing the genetic diversity. It is possible that the blue gene pool found at Inje and Jeongeup could be a consequence of this range expansion.

Collectively, the *L. dispar* populations in South Korea do not have an intrinsically high genetic diversity, but the presence of restricted gene pools, such as that found only at Inje and that found only at Inje and Jeongeup, indicates that there is potential for the South Korean populations to increase their genetic diversity. Moreover, the sharing of an identical haplotype and gene pool across all regions means that no single population in South Korea can be classified as isolated and thus of conservation concern.

Conclusions

Currently, *L. dispar* is classified as an NT species,[2] meaning that, while it is not classified as critically endangered, endangered, or vulnerable, it has the potential to be included in these categories shortly. This species was classified as NT mainly due to its limited distribution in South Korea and small population size. However, our field observations indicate that *L. dispar* is no longer confined to local areas. In particular, field observations lasting for only 1 h led to the sighting of a high number of individuals at some sites (Figure 3), indicating that the species has formed stable populations in some areas. Moreover, the population genetic analysis indicated that no population was isolated from the others, although more time is required for the genetic diversity to increase in the regions to which *L. dispar* has newly expanded. Considering this, we suggest that *L. dispar* should not be categorized as NT solely due to its limited distributional range and genetic status. A classification status of least concerned (LC) may be more suitable, which indicates a widespread species with a "good" number of individuals.

The present study has some limitations, such as the short field observational period and limited site visitation time. Moreover, the population genetic analysis was conducted using only mitochondrial DNA, which reflects the history of maternal inheritance. Thus, an expanded field monitoring program and co-dominant DNA data are essential to verify the results of this study and more confidently determine the range of *L. dispar*. Nevertheless, this study used field monitoring and molecular data to demonstrate that the combination of the two has the potential to allow a more reliable evaluation of the conservation status of wildlife in South Korea and other countries.

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