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Short Communication

Molecular comparison of *Lycorma delicatula* (Hemiptera: Fulgoridae) isolates in Korea, China, and Japan

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ABSTRACT

Lycorma delicatula (White) was recently introduced in Korea, acting as a grape insect pest. Since the introduction of this invasive insect, it initially spread rapidly throughout central and southern Korea, and is now distributed throughout the mainland. Here we developed new mitochondrial markers from NADH dehydrogenase subunit 2 and NADH dehydrogenase subunit 6 regions, and analyzed the regional isolates of *L. delicatula* collected from original locations in China, as well as invasive locations in Korea and Japan. All Korean and Japanese isolates were found to be genetically identical to those from Beijing, Tianjin, Qingdao, and Shanghai, China. Further isolates, from Zhejiang province, China, had two additional haplotypes.

Introduction

The lantern fly, *Lycorma delicatula*, suddenly arrived in the Korean Peninsula, and is now distributed throughout the mainland (Han et al., 2008). This species appears to have become fully established in Korea due to the increase of winter temperature, spurred by the global warming effect (Lee et al., 2011). Egg masses are often found in host plants, and huge numbers of first instar nymphs can be observed every spring. Because of the importance of the species as an invasive insect pest, many researchers have studied *L. delicatula* in terms of its taxonomy, biology, ecology, ethology, and control, within Korea (Han et al., 2008; Park et al., 2009; Shin et al., 2010; Choi et al., 2011; Kim et al., 2011c).

In Korea, this species has become a serious pest of grapes, and now brings substantial economic losses by causing serious damages to grapevines (Shin et al., 2010; Lee et al., 2011). *Lycorma delicatula* primarily damages its host plants through the sucking of plant sap (Kim et al., 2011b), and secondarily, through spreading sooty mold disease (Lee et al., 2009). Because sooty mold disease disrupts photosynthesis, leading to a decline in the quality and yield of grapes, this species is frequently controlled in vineyards through the use of chemical pesticides (Shin et al., 2010). Aside from grapes, this species can utilize 38 woody and four herbaceous species (Park et al., 2009). In fact, the main host of *L. delicatula* is the native Chinese tree called the tree of heaven, *Ailanthus altissima* Swingle, in which the lantern flies feed, mate, and overwinter as an egg form. This Chinese tree may contribute to the settlement and proliferation of *L. delicatula*, which has been already become widely distributed in Korea along roadsides (Lee et al., 2011). Based on the observed distribution patterns to date, it is unlikely that it will be possible to eradicate this species in order to protect the grapevines. Instead, it is necessary to understand the biology of *L. delicatula* to control it (Kim et al., 2011b).

The native range of *L. delicatula* was once contained within China, from the previous record (Liu, 1939). Until the 1930's, this species was distributed in only Shanxi, Shandong, and Hebei provinces in China (Liu, 1939). Recent studies of Chinese fauna have confirmed that this species has nation-wide coverage, being distributed in Anhui, Beijing, Guangdong, Henan, Hubei, Jiangsu, Shaanxi, Sichuan, Yunnan, and Zhejiang in China, and is also distributed in Taiwan, Vietnam, and India, where temperatures are relatively higher than those in northern China (Xiao, 1992; Hua, 2000). In Japan, this species was recorded in Okinawa, Honshu, and Kyushu since the 1930's. However, it was reportedly sporadically observed, and thereby, it's distribution throughout Japan was doubtful. More recently, mass occurrences of *L. delicatula* have been identified in Hakusan, Ishikawa Prefecture in 2008 where some

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Table 1
Collection list of *Lycorma delicatula*. Abbreviation of each location in parenthesis. KOR; Korea, CHN; China, and JPN; Japan.

No.	Date	Location	GPS-N	GPS-E
1	2010-06-28	Seoul, KOR (SL)	37.33.42.6	126.56.39.3
2	2010-07-03	Incheon, KOR (IC)	37.47.29.9	126.16.14.4
3	2010-08-17	Suwon, KOR (SW)	37.16.07.7	126.59.06.7
4	2010-08-19	Cheonan, KOR (CA)	36.44.01.6	127.15.08.1
5	2010-09-14	Cheongyang, KOR (CY)	36.26.15.8	126.46.05.0
6	2010-08-20	Gochang, KOR (GC)	35.25.50.8	126.43.09.7
7	2010-08-20	Buan, KOR (BA)	35.40.36.9	126.44.24.8
8	2010-07-31	Andong, KOR (AD)	36.32.31.1	128.47.49.4
9	2010-08-06	Yechoen, KOR (YC)	36.39.56.0	128.31.12.0
10	2010-09-29	Nonsan, KOR (NS)	36.13.20.6	127.0.55.1
11	2009-07-22	Beijing, CHN (BJ)	39.54.16.8	116.24.29.5
12	2010-07-05	Tianjin, CHN (TJ)	39.07.15.1	117.12.54.1
13	2011-08-15	Qingdao, CHN (QD)	36.19.21.6	120.23.36.9
14	2010-09-04	Shanghai, CHN (SH)	31.37.23.4	121.23.50.2
15	2010-09-05	Ningbo, CHN (NB)	29.47.42.4	121.47.37.4
16	2010-09-06	Tiantai, CHN (TT)	29.03.45.7	121.02.45.1
17	2010-09-07	Linan, CHN (LA)	30.14.01.9	119.43.29.0
18	2010-09-15	Hakusan, JPN (HS)	36.35.40.8	136.37.32.1

individuals were collected in this study (see Materials and methods). The occurrence of sudden outbreaks of *L. delicatula* in Japan is very similar to that of Korea, which has sparked our interest in an epidemiological investigation between the three adjacent countries, in order to determine whether this species has been naturally or artificially transferred.

Despite its relevance as an insect pest of grape, the sources of sudden emergences of *L. delicatula*, as well as the origin of the insects occurring in Korea, are, as yet, unknown. Like other invasive species, *L. delicatula* has rapidly spread throughout central and southern Korea over the past five years (Park et al., 2009). Recently, it is highly suggestive that *L. delicatula* was introduced from China, but this has not been scientifically proven. In addition, there is a question as to whether it could arrive from overseas by flight, because its morphology and wing structure are not appropriate for long distance migration, such as that exhibited by many other fulgorid species. An alternative theory regarding its invasion, and one which is, in many respects, more believable, is that they were artificially transferred through the import of products, via transportation as adults or egg masses. However, it is still not known where the invasive population originated, and genetic comparisons between source and invasive populations have not previously been performed. A better understanding of its genetic structures is also important for the effective control in grape vineyards, as well as for the establishment of quarantine policy for *L. delicatula*.

In this study, we aim to confirm the genetic relationships between different *Lycorma* isolates from Korea, China, and Japan. Here, we developed new mitochondrial markers from NADH dehydrogenase subunit 2 and NADH dehydrogenase subunit 6 regions, and then compared the regional isolates of *L. delicatula* collected from some original and invasive locations in Korea, China, and Japan.

Materials and methods

Taxon sampling

We collected 18 *Lycorma delicatula* isolates from various locations in Korea, China, and Japan (Table 1). These individual samples were collected from 10 central and southern locations of Korea, seven eastern and eastern costal locations of China, and from one emerging location in Japan (Fig. 1). Except for two samples from Shanghai, samples from China were located more than 50 km apart, so as to take a more representative sample, and increase the possibility of confirming the origin of invasive populations in Korea and Japan. All individuals were collected as female adults from the main host, *Ailanthus altissima*. All samples were stored in 80% ethanol as voucher specimens, and their genomic DNA was preserved at −20 °C. All samples and voucher specimens were preserved in Kunsan National University, Republic of Korea.

Development of DNA markers

We developed two new markers based on the complete mitochondrial genome sequences of *L. delicatula* (Song et al., 2012), which was retrieved from GenBank (accession number EU909203). Among all

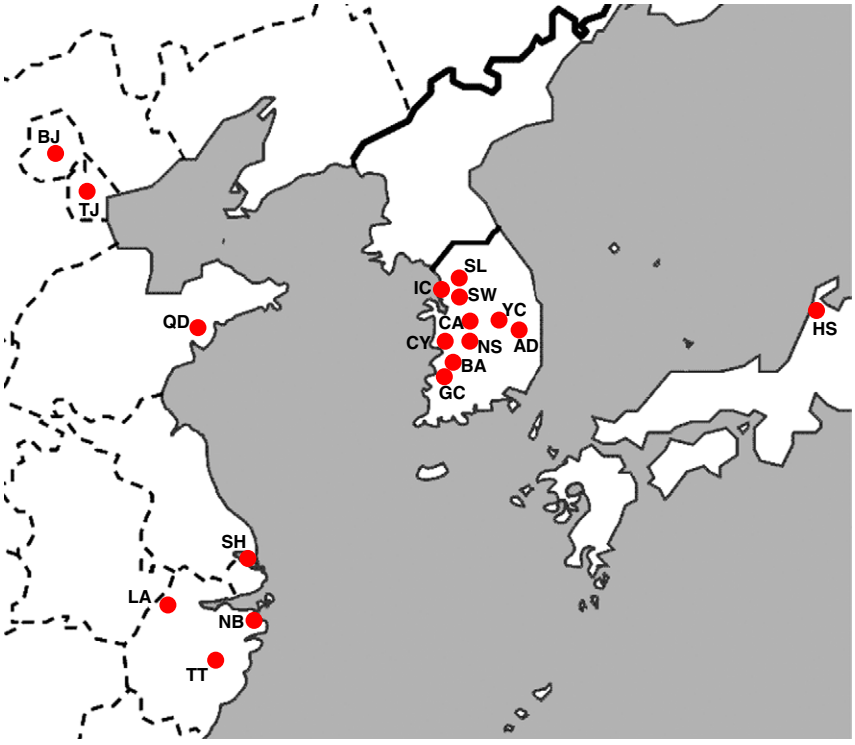

Fig. 1. Approximate locations of collection sites listed in Table 1.

Table 2
Sequence information of *ND2* and *ND6* primer sets used in this study.

Target region	Primer name	Sequence
ND2	ND2-238F	5′-AATTGCCCCATTAATGAAAGA-3′
	ND2-866R	5′-TTTGATTTGGTTATTGTAGGGATT-3′
ND6	ND6-87F	5′-TCAAACAGCCTTAATGTGCAG-3′
	ND6-480R	5′-TGGTCCTTCAAATGTTCTTACG-3′

mitochondrial regions, 966 bp of NADH dehydrogenase subunit 2 (*ND2*) and 501 bp of NADH dehydrogenase subunit 6 (*ND6*) were selected to design new primer sets (Table 2). The *ND2* and *ND6* regions are known for having highly variable nucleotide sites among mitochondrial genes in Insect and Crustacea (Cameron et al., 2004). Pairs of primers on the sequences applicable for *ND2* and *ND6* regions were designed using PRIMER 3 (Rozen and Skaletsky, 2000).

DNA extraction, amplification, sequencing, and alignment

Total genomic DNA extraction was performed using a LaboPass® tissue miniprep kit (COSMOGENETECH, Corp., Daejeon), according to the manufacturer's protocol. All tissue samples for extraction were obtained from prothoracic muscles to avoid the amplification of parasites or symbiotic bacteria. The target fragments of *ND2* and *ND6* were amplified by polymerase chain reaction (PCR) using AccuPower® PCR preMix (BIONEER, Corp., Daejeon). We used the following thermal cycle parameters for 20 μL amplification reactions: initial denaturation for 5 min at 95 °C, followed by 34 cycles for 30 s at 95 °C, 40 s at 52.5 °C, and 50 s at 72 °C and a subsequent final extension at 72 °C for 10 min. PCR products were tested by electrophoresis on an agar gel to observe a conspicuous band, and were then purified using a LaboPass® PCR purification kit (COSMOGENETECH, Corp.). PCR products were directly sequenced in both directions using an automated sequencer (ABI Prism 3730XL DNA Analyzer). The resulting chromatograms were evaluated for miscalls and ambiguities, and were assembled into contigs in SeqMan™ Pro (version 7.1.0, 2006; DNAstar™ Inc., Madison, WI). The sequences were individually checked for protein coding frame-shifts to avoid mitochondrial pseudogenes (Bensasson et al., 2001). Consensus files were aligned by a Clustal X (Thompson et al., 1997) method using MEGA 5 (Tamura et al., 2011). The sequences generated in this study were all deposited to the National Center for Biotechnology Information (NCBI) GenBank as the following accessions: KC422353–KC422370 for *ND2* and KC422371–KC422388 for *ND6*.

Data analysis

Pairwise sequence divergences in both *ND2* and *ND6* datasets were calculated using a *p*-distance model (Kimura, 1980), and a number of different nucleotides were also obtained using MEGA 5. Neighbor joining (NJ) analyses were conducted for each gene dataset with the *p*-distance model in MEGA 5. Because of the short sequence length and low taxa number, bootstrap values were not tested.

Results and discussion

The aligned lengths of sequences were 552 bp in *ND2* and 337 bp in *ND6*. Neighbor joining trees based on *ND2* and *ND6* were almost congruent (Fig. 2), which both showed that terminal units clustered into three different haplotypes. Among the 18 isolates from Korea, China, and Japan, 15 are identical in both *ND2* and *ND6* sequences (*ND2*Hap1 and *ND6*Hap2). Comparing the source and invasive isolates, ten Korean and one Japanese isolates were genetically matched to those from Beijing, Tianjin, Qingdao, and Shanghai. The remaining three, from Zhejiang province, China, had two different haplotypes. The two isolates from Ningbo and Tiantai were identical, but differed to that from Linan.

In the pairwise sequence divergences of *ND2* (Table 3), *ND2*Hap1 and *ND2*Hap2 differed by 1.3%, and *ND2*Hap1 and *ND2*Hap3 differed by 1.4%. However, sequence divergence between *ND2*Hap2 and *ND2*Hap3 was just 0.2%. Of the 552 bp in *ND2* sequences, eight site-specific polymorphisms were reported as a singleton. In the pairwise sequence divergences, *ND6*, *ND2*Hap1 and *ND2*Hap2 differed by 1.2%, and *ND2*Hap1 and *ND2*Hap3 differed by 0.9%. Whereas, sequence divergence between *ND2*Hap2 and *ND2*Hap3 was just 0.3%. Of the 337 bp in *ND2* sequences, four site-specific polymorphisms were reported as a singleton. These sequence divergences seem to be intraspecific difference levels between the local isolates. Formerly, when we compared two COI barcode sequences from Korean and Chinese *L. delicatula* samples generated in the two different studies (Han et al., 2008; Song et al., 2012), the sequences were 100% identical (data now shown). The *ND2* and *ND6* regions did not provide better resolution than the COI barcode

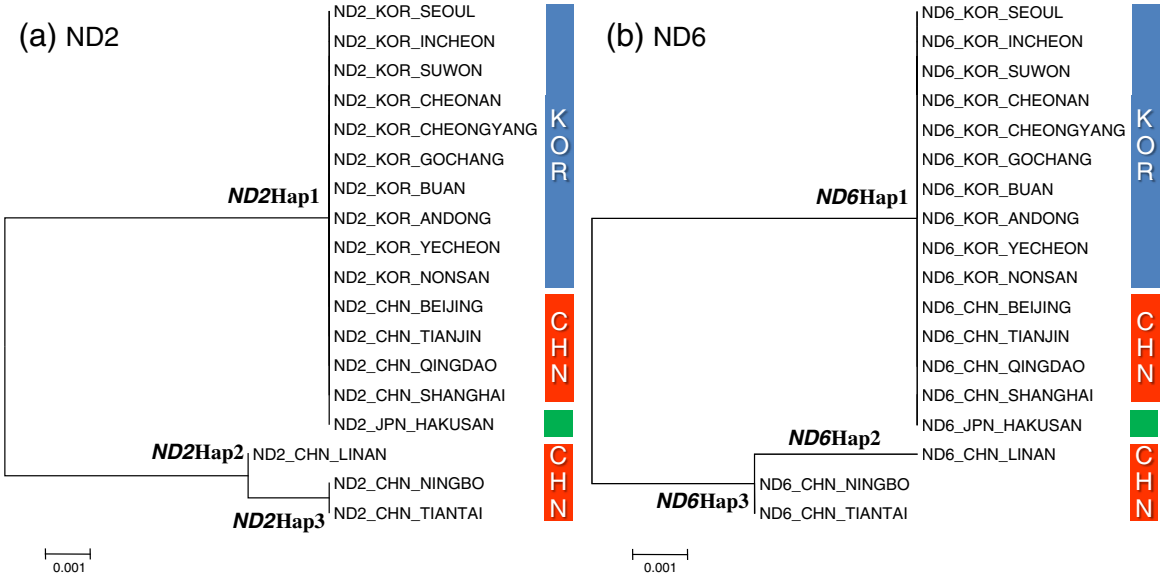

Fig. 2. Neighbor joining trees for 18 *L. delicatula* isolates based on *ND2* (a) and *ND6* (b). Different haplotypes (e.g., *ND2*Hap1) are established on nodes, which correspond to Table 3. Scale bar indicates the number of substitutions per site.

Table 3
Site-specific sequence polymorphisms and pairwise sequence divergence for *L. delicatula* *ND2* (a) and *ND6* (b). Positions of polymorphisms listed vertically above each site correspond to positions in the associated GenBank. In diagonal matrix of divergence, lower left indicates % divergence, and upper right indicates number of different nucleotides.

(a)	312	309	345	396	434	483	494	515	Divergence		
									*N2*H1	*N2*H2	*N2*H3
*ND2*Hap1	C	C	C	T	T	T	C	T	–	7	8
*ND2*Hap2	T	T	T	C	A	A	T	T	1.3	–	1
*ND2*Hap3	T	T	T	C	A	A	T	A	1.4	0.2	–

(b)	87	172	207	297	Divergence		
					*N6*H1	*N6*H2	*N6*H3
*ND6*Hap1	T	A	A	C	–	4	3
*ND6*Hap2	C	G	A	T	1.2	–	1
*ND6*Hap3	C	G	G	T	0.9	0.3	–

between local populations, but indicated that the invasive populations of *L. delicatula* came from China, based on confirmation of the additional identical sequences.

Our results, based on molecular comparison, suggested that the invasive isolates in both Korea and Japan more likely came from north of the Yangtze River, China, rather than the southern area. Although the collection sites at Shanghai, located in the Chongming island at the mouth of Yangtze River, were rather geographically close to other sampling sites in the southern area, such as Ningbo, the island was considerably isolated from the mainland. Additionally, all the host trees sampled in this area were artificially maintained for ornamental use after transferal from northern areas. Historically, this species was known to be mainly distributed in Shanxi, Shandong, and Hebei provinces in China, and was reported to be more common in the northern area than in the southern area (Liu, 1939). It is suggestive that *L. delicatula* has expanded into more southerly areas of the Yangtze River from the northern area of China. In addition, finding just two haplotypes (Fig. 2) relatively close to the southern locations suggests that some migratory populations might be isolated from the northern origins during the process of expansion.

In this study, newly designed *ND2* and *ND6* markers were successful used to identify the *L. delicatula* species and some local haplotypes from China, whereas, it seems to fail to reveal the geographic relationships between the 18 sample sites from Korea, China, and Japan, due to the low resolution of haplotype variation. In the previous study, two individual samples from Korea and China were found to be genetically identical in mitochondrial COI barcode region (Han et al., 2008). Our results, based on *ND2* and *ND6* regions, are in agreement with the previous results, and also indicate that the origin of *L. delicatula* is likely the north area of the Yangtze River, China.

Recently, molecular studies were performed to develop microsatellite (MS) markers, with the purpose of finding the introduction route and migration pattern of this insect pest (Kim et al., 2011a; Park et al., 2012). It is important to find the source of invasion in order to curtail additional introductions. Also, tracing the source region is important to support control strategies, such as the introduction of natural enemies (Torchin et al., 2003). We expect that the population-level studies based on MS loci may clarify the genetic relationships between different locations and the origin of *L. delicatula* in the near future.

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