**A THESIS**
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# Overwintering ecology and population genetics of *Lycorma delicatula* (Hemiptera: Fulgoridae) in Korea

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**By**
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# Overwintering ecology and population genetics of *Lycorma delicatula* (Hemiptera: Fulgoridae) in Korea

Marana Park

## ABSTRACT

*Lycorma delicatula* (White) (Hemiptera: Fulgoridae) was recently introduced to an invasive alien species, and was also agricultural pest in particular grape vine, in Korea.

Overwintering is a key element of survival for *L. delicatula*. Ability for cold tolerance showed the temporal changes during the overwintering season of *L. delicatula*, in Korea. In the field, hatching rate (%) of egg masses showed 72.1, 33.1, 84.3 and 52.3 % in 2010, 2011, 2012, and 2013, respectively. January was well explained time by following population size of 1st nymph in Korea. Therefore, I suggested the relationship between hatchability and temperature in January. The

hatching rate of 1st nymph *L. delicatula* in field was well predicted in these models.

Diapause development was applied for the hatching model of overwintering *L. delicatula* eggs. The relationship between egg developmental rate and temperature was described in a linear model and a non-linear model. The lower developmental threshold temperature was 11.13 °C, and the thermal constant was at 293.26 degree days. Hatching model was well validated in 1st nymph of *L. delicatula* collected in field.

Seasonal occurrence of *L. delicatula* was investigated among three host plants (*Vitis vinifera, Ailanthus altissima* and *Morus alba*). Female ratio of *L. delicatula* sampled from *A. altissima* was ranged from 35 to 45 %. The occurrence data were fitted to the logistic model based on the degree days (base 11.13 °C). Therefore, accumulated degree days were calculated as 271, 492, 620 and 908 DD, at 1st, 2nd, 3rd and 4th instar nymph in the peak time of *A. altissima*, respectively. This model was not incorporated into adult stage of *L. delicatula*, because of its dispersal behavior on *A. altissima*.

We isolated and characterized eight microsatellite loci for *L. delicatula* by using a hybridization-biotin enrichment method. Population genetic structure was conducted among nine locations in Korea. Isolation

by distance (IBD) suggested that populations in South Korea have not yet reached genetic equilibrium, also genetic differentiation (global $F_{ST}$=0.0474) indicated in range expansion recently. Bayesian-based clustering analysis indicated the presence of at least three genetically unique populations in Korea, which showed a distinct genetic background in Cheonan and Samcheok. The assignment test suggested that long-distance dispersal of *L. delicatula* may have occurred over large areas of South Korea. More complex dispersal patterns may have occurred during *L. delicatula* invasion of heterogeneous landscapes in South Korea.

Also, we evaluated the sink-source metapopulations at four locations (Cheonan, Daegu, Suwon and Gwangju) in Korea. Cheonan showed relatively larger effective population size *($N_e$)* and lower immigration rate (*m*) than other regions. A comparison among life stages, AMOVA showed different genetic variation between nymph and post-oviposition populations. Also, subgraph was constructed by post-oviposition in full graph with 29 edges. Therefore, genetic parameters were partially implied by the oviposition and were triggered for active dispersal of *L. delicatula*.

Integrated genetics and population ecology may provide the improved management plans and insight for hidden invasion phenomena of *L. delicatula* in Korea.

Key words: *Lycorma delicatula*, overwintering, egg hatching, population genetics, biological invasion

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## PART I

**PARTⅡ**

# GENERAL INTRODUCTION

*Lycorma delicatula* (White) (Hemiptera: Fulgoridae) was recently introduced accidently in Korea. After colonization, it was rapidly spreading and damaged to the agricultural habitats, in mainly grape vine yard, including forestry and urban environment. Korea administration designated the ecosystem disturbing wild species which was expected the significant reduction or replacement of biodiversity in Korea (Ministry of environment, 2012).

The univoltine life history of *L. delicatula,* they occurred from May to October in Korea. The first instar nymphs appear in May, and then these molt four times, becoming adults in late July. After turning adult, mating and then ovipositing the egg masses, which were found in late September (Park *et al*., 2009). Therefore, pre-oviposition of female *L. delicatula* showed long times, which estimated about two months in the Korea. Also, they laid more 500 individual eggs as far as known, egg mass was averagely included 30-40 individual eggs (Lee, 2009). Egg masses were discovered on the wide range of host plants including the non-host plants and inorganic object from late September. By closing the winter, adult dying off from late October and overwintering in egg stage for winter season. *L. delicatula* showed specific features of oviposition like a discrepancy between oviposition sites and host ranges of off spring in

Korea. But, its ovipositional characteristics (e.g. behavior, signal) were not revealed until now.

Rapid management decision was required against the sporadic pest (e.g. invasive pest), moreover, significant damage to crops in agriculture. Evaluation of key factors was needed response to biotic/abiotic factors inhabited environment of target species. Field monitoring may help catching the critical insight, like a vulnerable developmental stage and species specific behavior (e.g. feeding, mating system). Understanding species characteristic is fundamental process to acquire the ecological knowledge from on-the-ground experience. Also, species characteristic including physiological tolerance, diet breadth could measure in laboratory, if needed.

Overwintering ability was speculated the establishment as recently increased winter temperatures in Korea (Lee *et al*., 2011). Also, winter mortality of eggs was determinant for abundance and distribution of *L. delicatula* in Korea. The dynamics of egg diapause is key factors to understanding the cold tolerance and development of egg stage in Korea. Therefore, we were seasonally quantified the overwintering ability by measuring the cold tolerance in laboratory in winter season. And, relationship was evaluated between egg survival and winter temperature

in field condition, to predict the density following spring. Also, these parameters can provide information for assessing the risk of establishment and dispersal ranges, if further invasion occurred in other regions.

Although it was not effective for control, egg mass destruction was suggested and conducted by farmers so far. Therefore, forecasting the hatching time of eggs was critical, moreover, low mobility and gregarious behavior in this stage. Egg development was linked diapause phases, in particular temperate regions (Tauber *et al*., 1990). Environmental stimuli may comprehend the diapause phases by measuring hatching rate, duration as time interval sampling in winter season. Moreover, post-diapause development was critical for egg hatching of *L. delicatula*.

Listed on the 41 kinds of host plants and different degree of host damage was reported (Park *et al*., 2009). *Ailanthus altissima* (tree of heaven) were major host plants both China and Korea. *Vitis vinifera* L. seriously was damaged by *L. delicatula* in Korea; otherwise, this was not major host plants in China. In Korea, the problem of sooty mold disease was caused in grapevine yards, settled individuals of *L. delicatula* by sucking the trees and producing the honeydews. Its dispersal ability may be associated with different

host plant preferences among nymphs and adults. The host plant preferences of *L. delicatula* has changed during its life cycle, with a broad range of host plants in nymph stages, but only a few plant species, mainly *A. altissima and V. vinifera* in the adult stage (Kim *et al*., 2011). Feeding damage of *L. delicatula* was incurred throughout the growing season. But most critical time was when move to grapevine yard at adult stages, moreover, it oviposite in the vicinity of grapevine yard where causing a potential increases of population size. Seasonal occurrence of *L. delicatula* was required to know its short distance dispersal patterns across life stages and among host plants in fine scales.

The invasion of *L. delicatula* was presumably considered occurrence in the mid-2000s, because it was not discovered until 2004 (Han *et al*., 2008). Since then, it was recorded in Cheonan only a few adults in September for the first time (Kim and Kim, 2004). Significantly increased density was reported centrally in the mid-western region of South Korea, after 2006. And then, *L. delicatula* has spreading from this region following detected in some eastern areas, finally it become abundant across South Korea in present (Han *et al*., 2008; RDA reports).

Understanding the invasion process was essential for better actions for management in early invasion stage. *L. delicate* has dispersed throughout the Korea by overcoming the geographical barriers with time lag since first report. Quantifying dispersal, especially rare long distance, was difficult and time consuming when measured directly (e.g. mark-release-recapture method) (Roux and Wieczorek, 2009). Approaching the population genetics can easily measure the local movement in level of population and individuals in wild population. Microsatellite markers were used tracking the dispersal patterns and identifying the source population based on the population genetic models in fine scale (Kim and Sappington, 2006). Also, advanced genetic stochastic methods can evaluate the contemporary gene flow, drift and selection for ecological significance, so called by molecular ecology. Therefore, we developed the species specific microsatellite markers of *L. delicatula.* And, genetic diversity and structure was evaluated for invasion process and dispersal ability in spatiotemporal patterns in Korea.

Generally, *L. delicatula* was recently introduced invasive alien species, also agricultural pest in particular grape vine, in Korea. Therefore, we approached two points of view for management (1)

developed the forecasting system based on the overwintering ecology and phenology (2) evaluated the invasion dynamic and dispersal pathway perspective on the population genetics in this study. Integrated population ecology and genetics may provide the improved management strategy of *L. delicatula* in Korea.

# PART Ⅰ

## Chapter 1. Impact of winter temperatures on egg survival

## Abstract

Overwintering is a key element of survival for *L. delicatula* as it spends most of its life cycle during winter time in Korea. In this study, we investigated the cold tolerance and developed the forecasting model from overwintering eggs in Korea. Egg sampling was made five times, in monthly intervals from 22 November in 2011 to 1 April in 2012, in Cheonan. To examine the effect of cold acclimation, eggs were separated two groups; (1) acclimation for 7 days at 1 °C (AC) (2) no acclimation (NAC). And then, eggs were treated by the combinations of different temperatures (-5, -10, -15 and -20 °C) and exposure time (12hr, 24hr, 3days, 5days, 7days, 10 days and 15 days) at each sampling date, for both groups. Cold tolerance ability showed the temporal changes during the overwintering season of *L. delicatula*, in Korea. Also, $LLTemp_{100}$ (Low lethal temperature of 100% mortality) appears around -20 °C. For development of forecasting model, we evaluated the relationship (1) temporal changes for 4 years in Cheonan (2) survival and winter temperature from different six locations in 2011 from field collected eggs. Therefore, I suggested the relationship between hatchability and temperature; mean temperature ($y$=10.807$x$+106.24, $r^2$=0.92) and

minimum temperature ($y$=7.97$x$+131.61, $r^2$=0.97) in January. These models well predicted the hatching rate of 1st nymph *L. delicatula*, by showing the narrow deviation ranges; -1.97 - 0.97 °C (monthly minimum temperature in Jan) and -0.75 - 1.28 °C (monthly mean temperature in Jan) in field.

Key words: cold tolerance, egg mortality, winter temperature, forecasting model

## 1-1. Introduction

Overwintering ability was important traits for establishment and survival possibility, in particular, of invaders in temperate zone. Successfully established invaders negatively impacted on the ecosystem, moreover, if damaged to crop it become a serious pest in the recipient. Risk assessment and control agent has trouble with the unknown information of target invaders. Temperature is powerful effects on survival, distribution and abundance of insect (Stange and Ayres, 2010). Moreover, lethal temperature limits was essential to assess the potential of the establishment of invasive insect (Bale, 2002). Also, make it possible to prospect the range expansion boundary in introduced environment (Peterson *et al*., 2001).

In climatology, winter temperature showed seasonality in early-, mid- and late winter season with stepwise variation of winter temperature in Korea (Fig. 1). Cold tolerance capacity of insect was changed successive as the season progressed in temperate zone (Jang and Kang, 2004; Watanabe, 2002). Therefore, observation of seasonal changes was required to evaluate the organism's native characteristics linked its physiological process (e.g. diapause). Only evaluating super cooling temperature (SCP) was not good indicators, especially chill tolerant insect,

by over/under estimated the real tolerance range at low temperature (Bale, 1989; Ma *et al*., 2006; Hiiesaar *et al*., 2009). Survival at low temperature which exposed to various time and temperature was one alternative way as determined LTime (Low lethal time) and LTemp (Low lethal temperature), statistically (Bale, 1991). Also, cold tolerance capacity was affected by acclimation levels before exposure to low temperature, moisture, physiological status and so on (Danks, 1978).

Winter mortality was critical for population dynamics, especially overwintering insect, by adjusting initial population size following generation in the field. Therefore, tracking the survival by winter temperature can make the forecasting system perspective on the pest management. Experienced temperature in overwintering habitats may indicate the different survival ability. Namely, field validation may compensate for limited conditions of laboratory experiment by naturally prolonged exposure to low temperature. Also, only laboratory experiment has been limited to figure out the values for ecological significance (Renault, 2002).

*L. delicatula* eggs overwinter 6~7 months/year from early winter to mid spring in Korea. Eggs, laid on the surface of oviposition substrate, are directly exposed to low temperature during the winter. Previously,

relationship between hatching rate and winter temperature was explained from different three locals in Korea (Lee *et al*., 2011). But, they did not fully provide cold tolerance of *L. delicatula* eggs in Korea.

Therefore, cold tolerance of *L. delicatula* eggs was assessed seasonally in full overwintering season of *L. delicatula* eggs in this study. Relationship between hatching rate and winter temperature was investigated from field observation in spatiotemporal scales in Korea. Also, we aimed the development of forecasting system for overwintering *L. delicatula* eggs based on the response to low temperature in laboratory and field experiments in Korea.

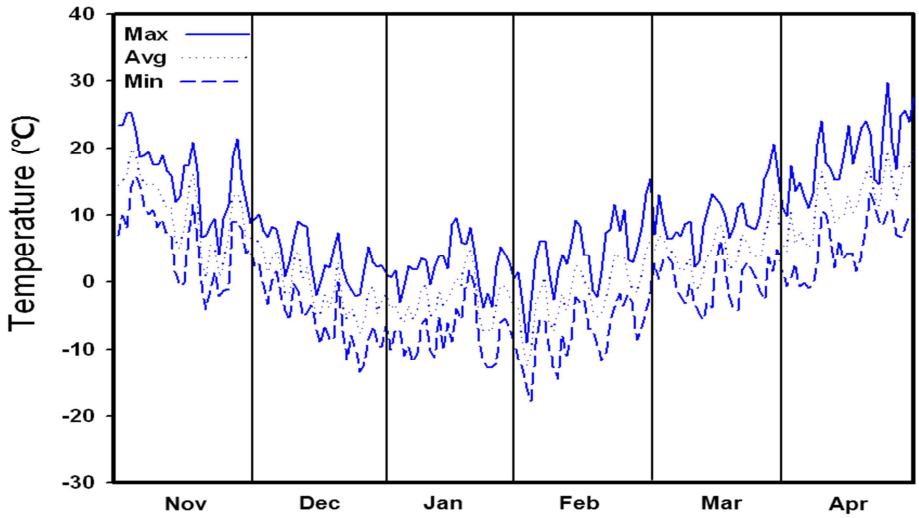



Fig. 1. Change of winter temperature (°C) on *L. delicatula* eggs from November in 2011 to April 2012 in Cheonan. Minimum temperature was -17.8 °C.

## 1-2. Material and Methods

### Laboratory experiment

To know the seasonal cold tolerance, eggs were collected five times in monthly interval during overwintering season. Egg masses were collected in Cheonan (CA) (N36° 52′, E127° 10′) from November in 2011 to April in 2012. Detached egg masses were separated from oviposition sites (e.g. barks of the *Vitis vinifera* and *Prunus serrulata*) carefully. Egg masses were placed in the laboratory condition (RH 60~70 %, 25±1 °C) and, then individual eggs were separated by forceps gently. Ten eggs were placed on the petri dish (Ø 50 mm, height 15mm) randomly. Dry cotton was spread in the bottom for avoiding the adverse effects as direct contact the plastic surface. Sample size was 30 individual eggs at each treatment, respectively. Therefore, we separated the two groups, AC (provided acclimation for 7 days at 1 °C before temperature treatment) and NAC (without acclimation).

For mortality assay, eggs of *L. delicatula* were kept in different combinations of temperatures (-5, -10, -15 and -20 °C) and time (12, 24 hr, 3, 5, 7, 10 and 15 days) for both AC and NAC groups. Experiment was conducted five times. During the experiment, internal temperature of the freezer was checked using the HOBO ware at each treatment. After

treatment, it was taken out the freezer and transferred to the insect rearing laboratory (25±1 ℃, 60~70 % RH) for recording egg hatching.

## Field experiment

For field validation, we selected the sampling sites by considering the winter temperature zone in the Korea. Therefore, overwintering eggs were collected from different six locations Chuncheon (CC; N37° 52′, E127° 44′), Suwon (SW; N37° 15′, E126° 59′), Cheonan (CA) (N36° 52′, E127° 10′), Yeongdong (YD; N36° 08′, E127° 48′), Daegu (DG; N35° 09′, E126° 55′), Gunsan (GS; N35° 57,′ E128° 30′) on 22~25 February in 2011 (Fig. 2). Fifty egg masses were examined at each sampling site. Hatching condition was RH 60~70 % and 25±1 ℃ in the insect rearing room.

To identify yearly variation, hatching rate was investigated in successive four years from 2010 to 2013 in CA. We observed the hatching rate in the field. Therefore, we brushed out the dust, covered with egg masses, using the soft brush. Hatching was easily distinguished by naked eyes, because there is opened after emergence. This study was conducted in June, because that time was completely finished hatching the eggs in the field. Egg masses were selected randomly nearby the vine

yard, in same location. In each egg mass, total number of the eggs and opened exit-lid (=operculum) were recorded. Sample size was indicated in Table 4.

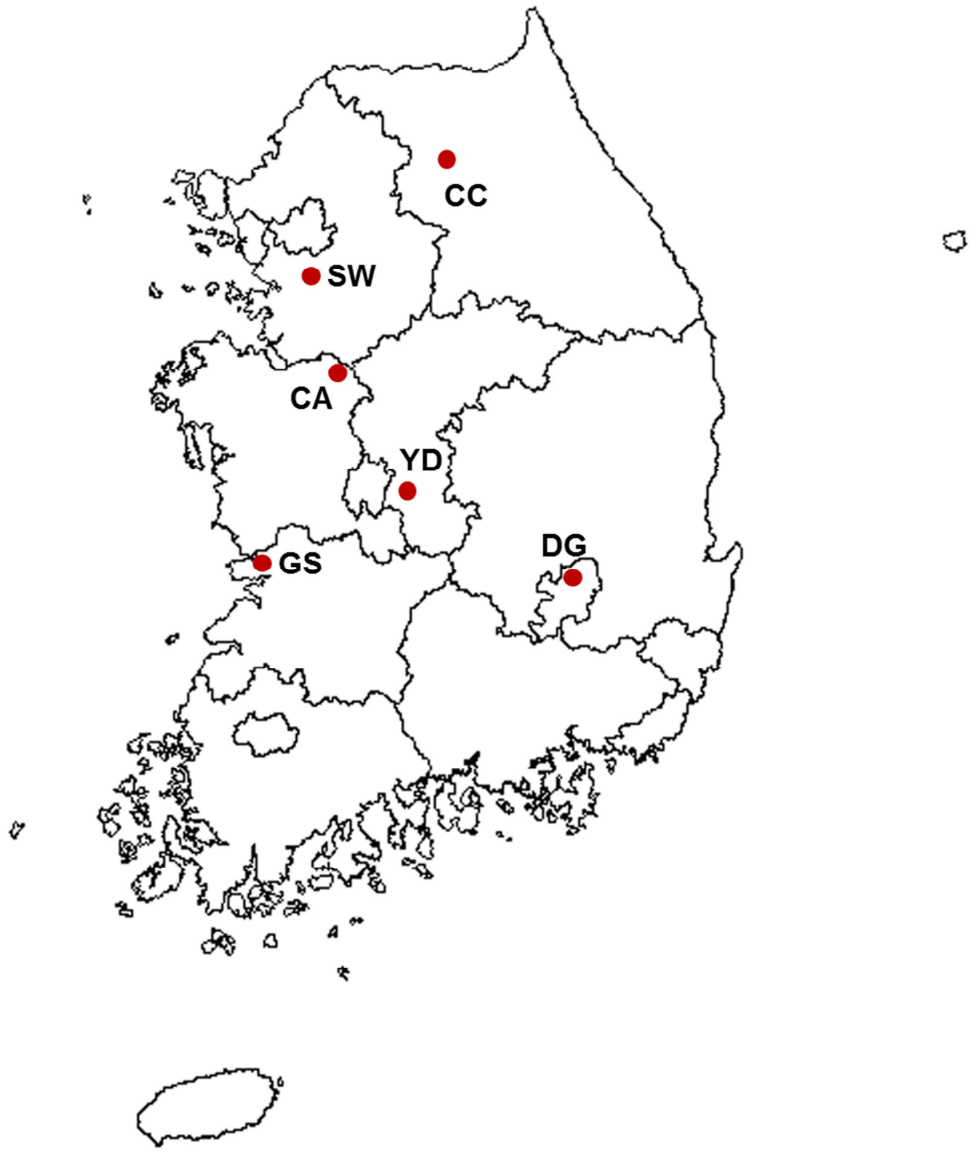



Fig. 2. Map indicated the sampling locations of *L. delicatula* eggs in Korea.

## Statistical analysis

Data for hatching rate was compared with acclimation, sampling date in the laboratory experiment using the one-way ANOVA. To know the well predicted calendar months, we analyzed the relationship between hatching rate and temperature data. We applied the combination of winter temperature as three ways (1) only January (2) only February (3) January and February at each collecting year. Temperature data was used daily mean and minimum temperatures along three categories. And annual variance of the temperature data was compared among four years, from 2010 to 2013. ANOVA test with post hoc using Tukey HSD was used.

After analyzing temporal variance, we analyzed the linear regression between egg hatching rate (%) and temperature data (the average of the daily mean and minimum air temperature in January) at six different sampling site in 2011.Therefore, we draw up the table on the estimated values of hatching rate (%) using the average of daily mean and minimum temperatures in January. To verify the estimated models, we compared the hatching rate (%) between observed and estimated. Temperature data for analyzing was obtained from Korea Meteorological Administration (KMA).

## 1-3. Results

### Laboratory experiment

After exposure to 24 hours, hatching rate of overwintering egg *L. delicatula* fluctuate and decreased sharply around February, in both AC and NAC conditions (Fig. 3). Hatching rate was significantly different between AC and NAC groups at all exposed temperatures (-5 °C: $f_{1,298}$=4.373, $p$=0.037; -10 °C: $f_{1,298}$=25.878, $p$=0.000; -15 °C: $f_{1,298}$=4.819, $p$=0.029). Also, hatching rate showed significant difference according to the sampling times, at -5 °C ($f_{4,10}$=12.25, $p$=0.001) and -15 °C ($f_{4,10}$=3.733, $p$=0.041) by NAC treatment. Otherwise, cold acclimation was only significantly different of hatching rate at -10 °C ($f_{4,10}$=7.694, $p$=0.004) among seasonal samples (Table 1).

Eggs survived after exposure for 15 days at -5 °C (Fig. 4a). Hatching rate was significantly different at -10 °C ($f_{4,13}$=3.952, $p$=0.000) between AC and NAC treatments (Fig. 4b). Critical temperature appears around -20 °C because all eggs treated at -20 °C failed to hatch (Fig. 4d).

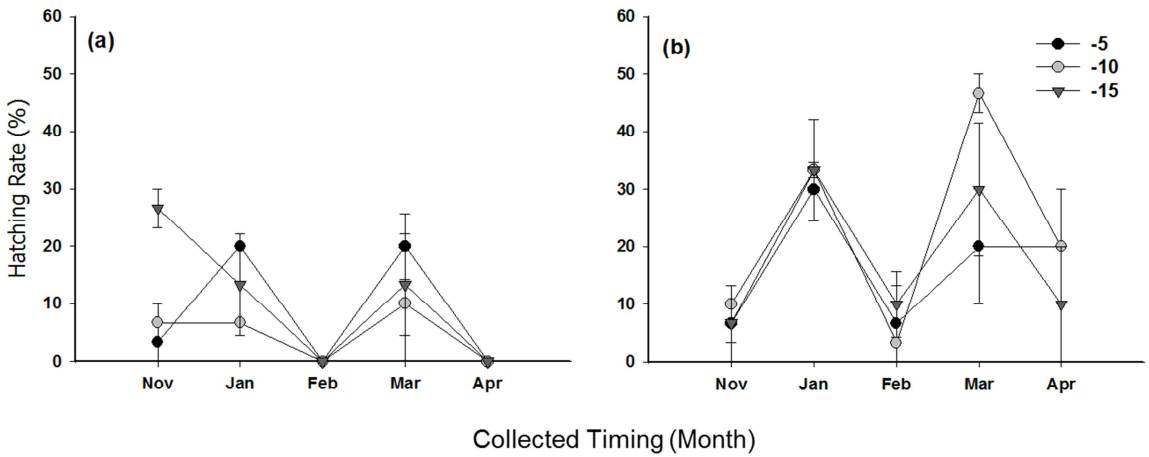



Fig. 3. Hatching rate (%) of *L. delicatula* eggs after exposed for 24 hr exposure to different temperatures in 2011-2012 (a) NAC : non-acclimation (b) AC : acclimation for 7 days at 1 °C.

Table 1. The variance of the hatching rate according to the five sampling time between NAC and AC (ANOVA, *P*>0.05).

| Temp (℃) | NAC | AC |
|---|---|---|
| -5 | $f_{4,10}$ = 12.25 $p$=0.001 | $f_{4,10}$ = 1.73 $p$=0.219 |
| -10 | $f_{4,10}$ = 0.818 $p$=0.542 | $f_{4,10}$ = 7.694 $p$=0.004 |
| -15 | $f_{4,10}$ = 3.733 $p$=0.041 | $f_{4,10}$ = 1.625 $p$=0.243 |

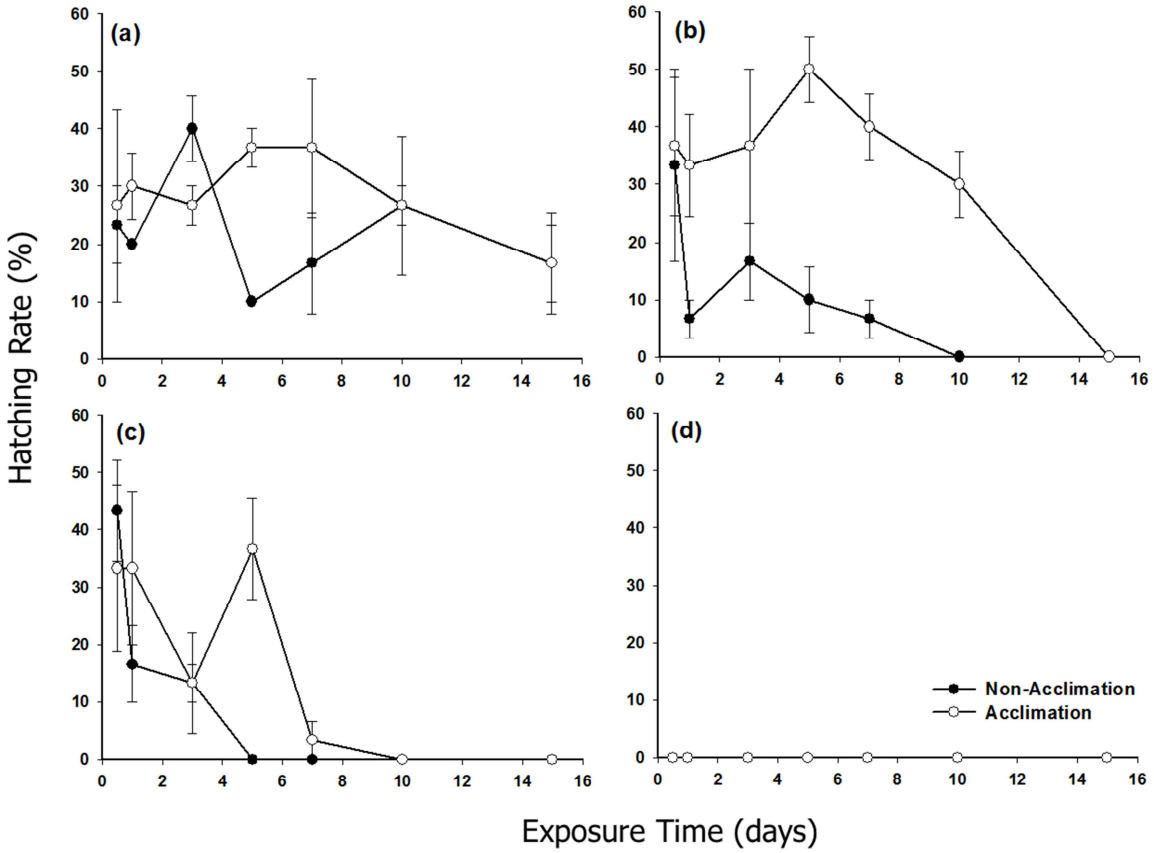



Fig. 4. Cold tolerance assay of *L. delicatula* eggs collected in January. (a) -5 °C (b) -10 °C (c) -15 °C, and (d) -20 °C .

## Field experiment

In the field, hatching rate (%) of egg masses showed 72.1, 33.12, 84.33, and 52.3 % in 2010, 2011, 2012, and 2013, respectively. Hatching rate was significantly different at each year in CA ($F_{3,239}$=29.744, $p$=0.00) (Table 4). Temperature was significantly varied in January (Mean: $F_{3,120}$=7.15, $p$<0.001; Min: $F_{3,120}$=6.504, $p$<0.001) and February (Mean: $F_{3,109}$=4.177, $p$=0.008; Min: $F_{3,109}$=6.147, $p$=0.001). Combined January and February temperature were not significantly different among sampling years (Mean: $F_{3,233}$=1.665, $p$=0.175; Min: $F_{3,233}$=2.365, $p$=0.072). Also, February temperature was not explained the relationship of the hatching rate (%) among sampling years (Fig. 5, 6). Therefore, January was considered well predicted time in calendar months, therefore, we used that time further analyzing.

Temperature was significantly different at six sampling sites in January (Mean: $F_{5,180}$=38.116, $p$<0.001; Min: $F_{5,180}$=50.457, $p$<0.001). Also, hatching rate (%) was varied from different six sampling sites in 2011 (Table 2). The linear regression was shown for the daily minimum ($y$=7.97$x$+131.61, $r^2$=0.97) and mean temperature data ($y$=10.807$x$+106.24, $r^2$=0.92) of January in 2011 (Fig. 7). Therefore, we drawn up the table on hatching rate (%) resulted in monthly minimum and

mean temperature in January (Table 3). As a result of the validation, deviation between observed and expected temperature ranged from -1.97 ℃ to 0.97 ℃ at daily minimum temperature and -0.75 ℃ to 1.28 ℃ at daily mean temperature during four years (Table 4). As a result, monthly temperature in January was well explained the density of 1st nymph of *L. delicatula* following spring season in Korea.

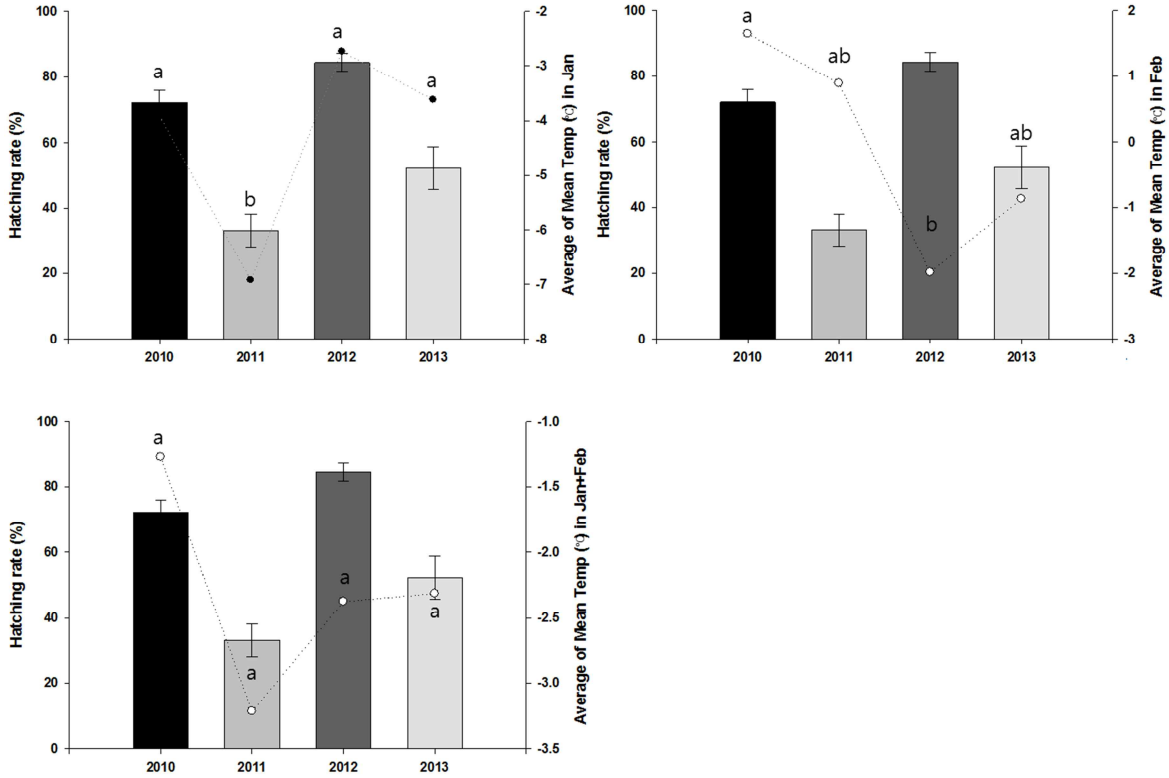



Fig. 5. Interaction of hatching rate (Mean±SE) and mean air temperature for winter season in CA (a) January (b) February (c) January and February. Points bearing the different letters were significantly different (Tukey HSD, α=0.05).

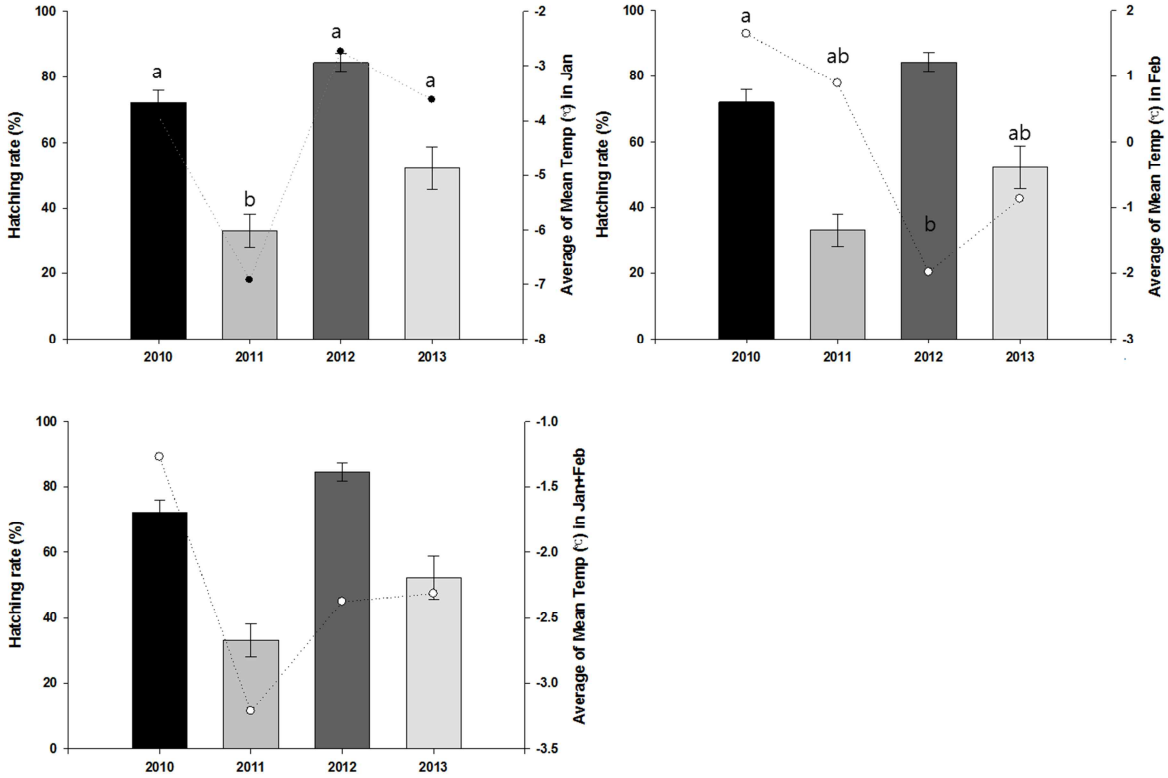



Fig. 6. Interaction of hatching ability (Mean±SE) and minimum air temperature for winter season in CA (a) January (b) February (c) January and February. Points bearing the different letters were significantly different (Tukey HSD, α=0.05).

Table 2. Hatching rate (%, Mean±SE) from six sampling sites in 2011. Daily minimum and mean temperature (°C) in January at each collected site. Different letters were indicated significantly different (Tukey HSD, α=0.05).

| Sites / Temp (℃) | DG | GS | CA | SW | YD | CC |
|---|---|---|---|---|---|---|
| Daily Min Temp (℃) | -6.629$^{a}$ | -8.974$^{b}$ | -12.757$^{c}$ | -12.445$^{c}$ | -13.871$^{c}$ | -16.013$^{d}$ |
| Daily Mean Temp (℃) | -2.503$^{a}$ | -4.539$^{b}$ | -6.926$^{c}$ | -7.255$^{c}$ | -7.296$^{c}$ | -9.503$^{d}$ |
| Hatching rate (%) (Mean±SE) | 79±3.75$^{a}$ | 55.6±5.98$^{b}$ | 30.82±4.56$^{bc}$ | 41.8±4.01$^{cd}$ | 17.28±4.01$^{de}$ | 1.76±1.47$^{e}$ |

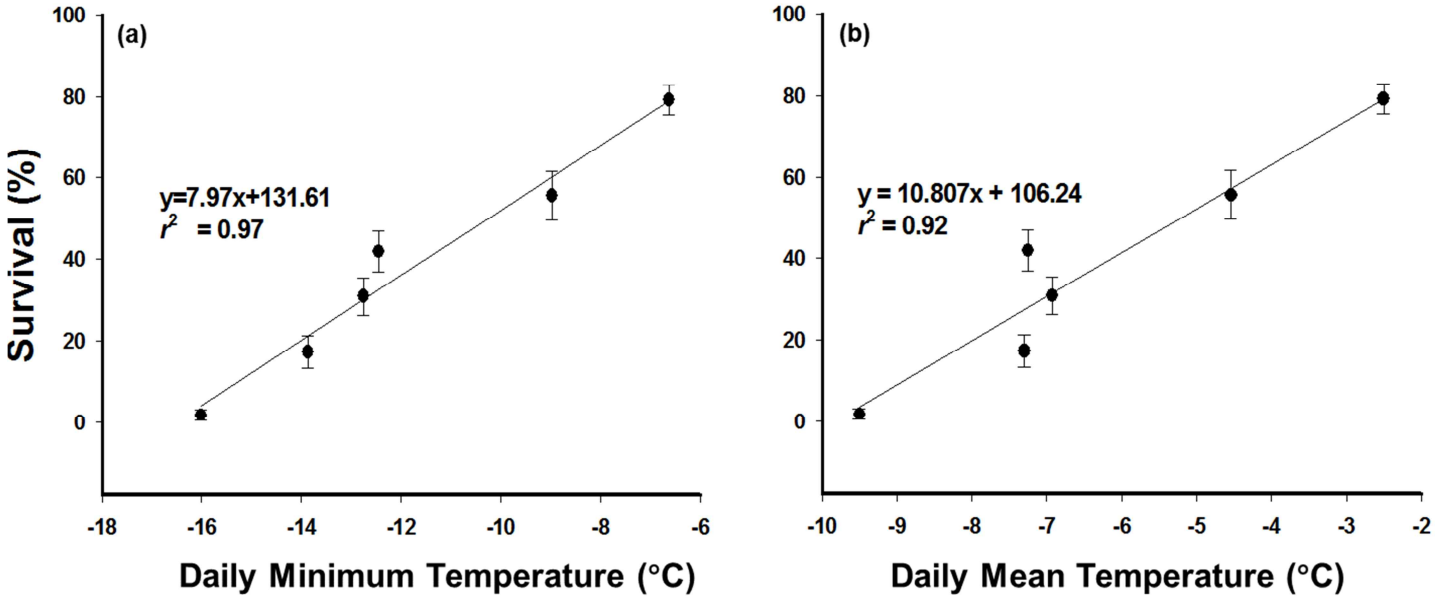



Fig. 7. Linear regressions of hatching rate and (a) average of daily minimum temperature (°C) in January (b) average of mean temperature (°C) in January from six sampling sites in 2011.

Table 3. Hatching rate (%) of *L. delicatula* eggs according to the minimum and mean temperature (°C) in January.

| Hatching Rate (%) | | 100 | 90 | 80 | 70 | 60 | 50 | 40 | 30 | 20 | 10 | 0 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Daily Temp (°C) in Jan | Min | -3.97 | -5.22 | -6.48 | -7.73 | -8.98 | -10.24 | -11.49 | -12.75 | -14.0 | -15.26 | -16.51 |
| | Mean | -0.58 | -1.5 | -2.43 | -3.35 | -4.28 | -5.2 | -6.13 | -7.05 | -7.98 | -8.91 | -9.83 |

Table 4. Hatching rate (%) of eggs collected in Cheonan using field temperature (°C) in January from 2010 to 2013.

| Year | Sample Size[1] | Hatching Rate (%)[2] | Daily Minimum Temp (°C) | | | Daily Mean Temp (°C) | | |
|---|---|---|---|---|---|---|---|---|
| | | | Obs. | Est. | Dev. | Obs. | Est. | Dev. |
| 2010 | 56 | 72.1[a] | -9.44 | -7.47 | -1.97 | -3.91 | -3.16 | -0.75 |
| 2011 | 59 | 33.1[b] | -12.75 | -12.36 | -0.39 | -6.93 | -6.77 | -0.16 |
| 2012 | 78 | 84.3[a] | -7.85 | -5.93 | -1.92 | -2.74 | -2.03 | -0.70 |
| 2013 | 50 | 52.3[c] | -8.98 | -9.95 | +0.97 | -3.62 | -4.90 | +1.28 |

[1] The number of collected egg masses at each sampling year.
[2] Same letters were indicated not significantly different (Tukey HSD, α=0.05)

## 1-4. Discussions

Cold response of an organism was assessed in three ways; with freeze-tolerance, freeze-avoidance or intolerance, and chill intolerance (Lee, 1991). The lower lethal temperature can evaluate an insect's response by exposing to low temperature. Duration of exposure at low temperature can affect the mortality. Therefore, temperature-time interactions account for more explicitly on ecologically relevant assessment describing the response to cold stress (Bale, 1991).

While winter diapause frequently extends an insect's tolerance to cold temperatures, cold hardening can be achieved independently of diapauses by acclimation at a cooler temperature (Fields *et al*., 1998). Acclimation by cool temperature affected their survival by increasing adaptability to low temperature. In this study, survival was significantly different between AC and NAC groups. Acclimation duration and temperature (for 7 days at 1 °C in this study) were sufficient to influence its cold tolerance capacity on overwintering *L. delicatula* eggs. Effects of acclimation showed the reversed results among treated temperatures after 24 hours exposure (Table 1). This result showed the differences of cold tolerance capacity in level of experienced temperature, however, its

physiological mechanism was unknown. Therefore, biochemical assay (e.g. *myo*-inositol) may support this obscure (Watanabe, 2002).

Seasonal change of cold tolerance capacity was reported on overwintering insect in temperate regions (Watanabe, 2002). In this study, seasonality of hatchability was shown in *L. delicatula* eggs, which may imply association with the physiological process (e.g. diapause) of cold tolerance capacity (Fig. 3). No response to ambient temperature was assumed as diapause, which probably maintaining the diapause depth resulting in arrested development endogenously. Also, acclimation by cooler temperature prior to cold temperature exposure may indicate the phenotypic plasticity, which showed the difference of hatchability between NAC and AC (Fig. 3). Also, February was not explained temporal patterns of hatching ability, which showed high hatching rate despite the low temperature of February in 2012, in field study (Fig. 5, 6). This phenomenon was associated with increased diapause intensity, therefore, this time was inadvisable to forecast the population dynamics following 1st nymph stage in spring.

Statistical methods can provide the cold tolerance indices (e.g. $LTime_{50}$ and $LTemp_{50}$) analyzing the time and temperature required for 50% of samples through fixed examined time and temperature (Bale, 1991). In

this study, estimation of $LTime_{50}$ and $Ltemp_{50}$ was failed to low statistical power results from unstable hatching rate of eggs. Survival ability was increased toward the cumulative cold effects; therefore, it was probably classified chill tolerance by criterion suggested by Bale (1987). Also, high variable of hatching ability of *L. delicatula* was shown among /within egg masses through observation of laboratory and natural field populations. Therefore, larger sample size was required to reduce the sampling errors and improving statistical power. In this study, we indirectly assumed -10.24 °C of $LTemp_{50}$ and -16.51 °C of $LTemp_{100}$ using the monthly minimum temperature in January (Table 3, Fig.7). Lee *et al*., (2011) suggested at -12.72 °C of $LT_{100}$, which showed gap *c.a*. 4 °C between our results. Validation indicated well-constructed models showing narrow deviation ranges between estimated and observed hatching rate (%) in field (Table 3).

Although, it is now widely accepted that SCP cannot be considered as single sufficient descriptor of cold tolerance in freeze-avoiding insects, it is also known that SCP corresponds well to a lower limit for survival during relatively short exposures to low temperatures (minutes to hours) in many insects (Bale, 1989; Ma *et al*., 2006; Hiiesaar *et al*., 2009). In this study, low lethal temperature, which indicated 100% mortality, was

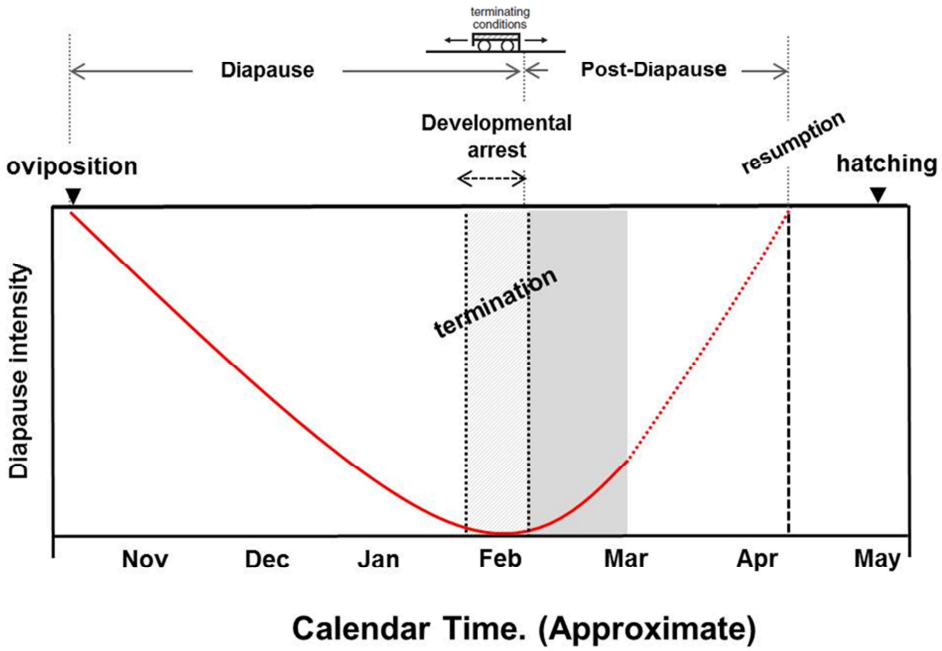
considered around -20 °C, because all eggs were failed at -20 °C. Recorded minimum temperature by KMA was -19.5, -18.5, -17.8, and -19.2 °C in 2010, 2011, 2012, and 2013, respectively. Shortly exposed low lethal temperature did not affect the survival of eggs *L. delicatula* in field. Low lethal temperature was essential component for the construction of species distribution map to assess; (1) risk of invasive species for establishment probability in worldwide range (2) spreading pathway and boundary.

Cold tolerance has been applied in the pest management concept as “thermal biology” in wide range ; forecasting systems for pest outbreak (Werker *et al*., 1998), and analyzing the establishment potential of exotic species in the new environment (Bales and Walters 2001). Also, thermal data is useful tool for assessing the risk, which is first step for screening introduced exotic species like a biological control agents (e.g. natural enemy) and pollinators in agriculture (Hatherly *et al*., 2005).

We suggested the monthly temperature in January is strong predictor for hatching ability of *L. delicatula* following spring season (Table 4). The low mortality of overwintering eggs may result in high population density and serious crop damage following season. Economic injury level (EIL) of 1st nymph of *L. delicutla* was not determined in agricultural of

Korea until now. Therefore, control thresholds may helpful for quick and effective decision making of *L. delicatula* in advance. In this study, we developed the forecasting system of *L. delicatula* egg stage. This information is useful for control.

Appendix 1. Schematic was depicted on changes of cold tolerance and hatching in the diapause phase of egg stage *L. delicatula*. This diapause phase and definition was based on the Eco-physiological phases of insect diapause Koštál (2006). Gray box indicated the portion for post-diapause of hatching model of *L. delicatula*. Dotted line indicates the released diapause intensity during post-diapause phase, which is exogenously imposed inhibition until favorable for resumption of direct development.



## Chapter 2. Egg hatching models and its spring emergence


## Abstract

Diapause development was studied for hatching model of overwintering *L. delicatula* eggs. Egg hatching rate was significantly different both control and 15 days at 5 °C for winter season. Also, hatching rate and hatching periods were changed between NCH (kept at 25 °C) and CH (pre-chilled for 15 days at 5 °C) conditions. As drawn near the spring, hatching periods were shortened; but, hatching rate was changed inconsistently at NCH. Egg development was investigated for post-diapause. The relationship between egg developmental rate and temperature was described by a linear model and Brière 2 equation for a non-linear model. The lower developmental threshold temperature was 11.13 °C, and thermal constant was 293.26 degree days. The variation in egg development was described by the two-parameter Weibull function ($r^2$=0.979). Hatching patterns of overwintering egg masses in fields were validated for the samples collected from 4 locations. In addition, spring emergence was observed in CA and SW.



Keywords: diapause development, hatching model, post-diapause development, model validation

## 2-1. Introduction

Insect diapause has developed the physiological capacity for survival to overcome the ambient harsh environment (Denlinger, 2002). Diapause status gradually changed by induction, preparation, initiation, maintenance and termination, occasionally post-diapause quiescence as physiologically graded series (Koštál, 2006). But, direct observation has a difficulty without visible morphogenesis features by performing a certain diapause phase. Therefore, thermal response (e.g. time to hatch, distribution and hatching rate) from field populations with time interval can interpret the diapause states, indirectly (Tauber *et al*., 1990).

Exposure to low temperature (generally 5 or 10 °C) can accelerate the diapause development and hatching incidence in temperate zone (Tauber *et al*., 1990). Comparison between pre-chilled and natural conditions may complement the ambiguous responses, in particular, stepwise and discrete field sampling. Also, optimal chilling condition can stimuli the diapause termination, which is a prerequisite for winter diapause completion in some insect species (Xiao *et al*., 2013). Even though diapause development was important for construction of the forecasting model, it has not been evaluated for *L. delicatula* eggs in winter season until now.

Identify the key stage of target species is essential for effective control. *L. delicatula* oviposition occurs in late September in Korea. Therefore, eggs overwintered approx. 6~7 months a year, until it hatch in following spring. After becoming adult, *L. delicatula* disperse into the grapevine yards, where they cause the damage by sucking the sap and producing the honey dew which results in sooty mold disease on grape fruits. Also, it lays egg masses around the grape vine yard including non-host plant and artefacts. Grape growers often destroy egg masses mechanically, but it can be effective in reducing the density. Therefore, it is important to forecast the correct occurrence time of the 1st instar nymphs of *L. delicatula* to synchronize insecticide application in spring. Also, chemical spraying is allowed in spring before the development of fruit during the season when grape grows.

Insect development model assumed that development of immature stage is dependent on a given temperature (Gray *et al*., 1991). Sometimes, insect egg develop in dynamic process in which the response to temperature gradually changes over the course of diapause phases (Sawyer *et al*., 1993). Therefore, diapaused eggs may have low accuracy in the prediction of developmental models without knowing egg phenology. Development time of each life stage is key component to construct the

temperature dependent models of poikilothermal organisms. Alternatively, time interval sampling was required under natural field condition in winter season, diapauses information on *L. delicatula* is unknown. Also, evaluating the constructed model is essential for reliable prediction in field.

In this study, we investigated the effective chilling days at 5 °C and thermal response of *L. delicatula* eggs to know the winter diapause phases. In addition, we developed an overwintering model of *L. delicatula* eggs for the prediction of 1st nymph occurrence in spring. Also, considered its egg phenology, overwintering eggs were collected with time interval from field in 2010 and 2011. And, hatching model was validated in spatial and temporal scales in Korea.

## 2-2. Material and Methods

### Diapause development

We collected the egg masses by one month interval in Cheonan (CA, N36°52´, E127°10´), major grape vine yard producing area in South Korea, from November in 2011 to April in 2012. *L. delicatula* was released to lay eggs on the bark of *Vitis vinifera*, *Prunus serrulata* and *Morus alba* in field. Then, we detached the egg masses from bark, gently.

To identify the optimal chilling time, we incubated the egg masses for the different chilling days (0, 7, 15 and 30 days) at 5 °C by each sampling date. A previous study indicated that 5 °C was effective temperature for the diapause determination of *Lymantria dispar* egg in temperate zone (Tauber *et al*., 1990). Also, exposure to 5 °C was effective for the termination of overwintering eggs of *L. delicatula* in the preliminary experiment. Egg masses were carefully separated by one individual, and then it put on the petri dish (Ø 50 mm, height 15 mm) with dry cotton in the bottom. Sample size was 30 individuals in each treatment. After treatment, it was kept in insect rearing laboratory (25±1 °C, 60~70 % RH), and observed until hatching to 1st nymph. We analyzed the ANOVA with post hoc analysis using Tukey HSD for variance of hatching rate (%) according to chilling days and collecting time.

Collected egg masses were moved in laboratory, and then it was set on the insect breeding dish (Ø 12 cm, height 8 cm, SPL Life Science). Water saturated cottons were put on the bottom of dish for supplying the moisture, and egg masses were attached the rid by avoiding direct contact to water. And then, we provided the two conditions (1) NCH; incubated under 25 °C after collecting (2) CH; pre-chilled for 15 days at 5 °C, and then it kept under at 25 °C. In this study, 50 egg masses were tested at each treatment, respectively. For each collected time, requiring the periods first to hatching and hatching rate were observed in one day interval until hatching completely. And then, we compared the time to first hatch and hatching rate (%) between NCH and CH groups at each sampling time, respectively. Also, cumulative distribution curves were drawn at each sampling date.

**Egg hatching models for post-diapause**

In this study, we objected to construct the egg hatching model in post-diapause of *L. delicatula*. To determine the post-diapause, egg masses were collected at four times on 1 and 17 February, and 4 March, 2010, and 22 February, 2011 in CA. All eggs were chilled for 15 days at 5 ± 0.5 °C in the growth chamber prior to temperature treatment, to ensure

the termination of diapause. Development was investigated at 15, 19, 23, 27, 31, and 35 ± 0.5 °C in the 2010 experiment, and at 12, 15, 18, 21, 24, 27, 30 and 33 ± 0.5 °C in the 2011 experiment. They were all kept under the photoperiod of 16:8 (L:D) h. For each temperature treatment and sampling date group, ten egg masses were tested. Egg hatching was monitored by one day interval.

After exposing the temperatures treatment, we calculated the development time for eggs of each sampling date. Development time was converted to development rate (1/day). Development rates were fitted to the Brière 2 model (Brière *et al*., 1999) against the temperatures. The Brière 2 model is:

$$\boldsymbol{\gamma(T) = \alpha T(T - T_0)(T_L - T)^{1/m}} \qquad \text{(eq. 1)}$$

where, $T$ is the temperature (°C), $T_L$ is the high temperature threshold, $T_0$ is the low temperature threshold. $\alpha$ and $m$ were fitted parameters.

The estimates of model parameters for the regression models were calculated by Table Curve 2D (Jandel Scientific, 1996). Then we

calculated the physiological age (*p*x) by adding the development rate for each sampling date (Curry and Feldman 1987):

$$\boldsymbol{p}\mathbf{x} = \sum_{\boldsymbol{i}=\mathbf{1}}^{\boldsymbol{n}} \boldsymbol{r}(\boldsymbol{T_i}) \qquad \text{(eq. 2)}$$

where, *px* is the physiological age at *n* th day, and $r(T_i)$ is the development rate at temperature (°C) at day *i*.

And then, we compared the emergence patterns against the physiological age in each sampling date, to determine post-diapause progress in late winter.

After comparing the cumulative distribution patterns, we excluded the data on 1st Feb which showed delayed hatching pattern in this study. Therefore, we analyzed the pooled data, which is combined at three sampling times on 17th Feb, 4th Mar in 2010 and on 22nd Feb in 2011(Fig. 10).

Development time was converted to development rate (1/day) for pooled data. Development rate was fitted against temperatures, and then the lower developmental threshold and thermal requirement were calculated by $-\beta/\alpha$, and $1/\alpha$, respectively (Arnold, 1959). The linear model is:

$$r(Tc) = \alpha Tc + \beta \quad \text{(eq. 3)}$$

where *r* is the rate of development at temperature (*Tc*), *Tc* is the temperature *α* is the slope, and *β* is the intercept (Campbell *et al*., 1974).

Physiological age (*px*) was calculated with pooled data (eq. 2). And then, variations in the development completion of the egg hatching were fitted with two-parameter Weibull function (Cockfield *et al*., 1994):

$$f(x) = \left[1 - \exp\left(-(x/\alpha)^{\beta}\right)\right] \quad \text{(eq. 4)}$$

where *f(x)* is the cumulative proportion of egg hatch at the physiological age *x*, and $\alpha$ and $\beta$ are parameters.

Eggs survival was examined individually and number of exposed eggs for survival model was indicated in Table 7. Therefore, egg survival rate (%) was calculated by dividing the number of hatched eggs by the total number of exposed eggs in each temperature. Relationship between egg survival rate (%) and temperatures (°C) was described using a Gaussian function (Taylor, 1981).

$$S(T) = R_m exp\left[\frac{1}{2}\left(\frac{T-T_m}{k}\right)^2\right] \quad \text{(eq. 5)}$$

where, *S(T)* is the survival rate (%) at temperature (°C), $R_m$ is maximum survival rate, $T_m$ is temperature at the maximum survival rate, and *k* is fitted parameter.

**Model validation**

Models of egg development were constructed using egg masses collected in the field Cheonan. Therefore, this model was validated in field condition for the application in whole ranges of Korea. Egg masses of *L. delicatula* were collected at six locations (Fig 2). Fifty egg masses were examined at each location. Collected egg masses were brought into the laboratory and chilled for 15 days at 5 °C. Then egg masses were treated at 23 ± 0.5 °C and the photoperiod of 16:8 (L:D). Egg hatching was monitored at one day interval. Egg hatching model was simulated the accumulated developmental rate and applied as input the distribution functions using the constructed models in CA, previously. The emergence proportions were measured at a given physiological age from four

overwintering local populations. And then, the emergence patterns were compared with simulated the egg hatching model.

In addition, the egg hatching model was validated with the field occurrence data for first instar nymph of *L. delicatula.* The occurrence was monitored between CA and SW in grape vine yard and *Ailanthus altissima* community, respectively. Blue sticky traps (Green Agrotech, Korea) were installed closely near the egg masses, to catch the nymphs shortly after hatching from 1st May to 10th June. Traps were changed in one week interval in 2011 and 2012 in CA, and in 3~4 days interval in 2012 in SW. Cumulative proportions was calculated by dividing the number of the first instar nymph at each observation date by total number of first instar nymphs. For model simulation, developmental rate was calculated using the daily temperature in the field starting on 1 April. Temperature data were obtained from the CA and SW weather stations of the Korea Meteorological Administration (KMA). Simulation process was similar in above mentioned. Then, these occurrence data were compared against the simulated hatching model.

## 2-3. Results

### Effective Chilling days at 5 °C

In this study, hatching rate varied with chilling days at 5 °C (Table 5). Hatching rates among chilling days was not significantly different in November and January (22 Nov: $F_{3,8}$=3.778, $p$=0.059 ; 2 Jan: $F_{3,8}$=2.424, $p$=0.141). But, following mid-winter season showed significantly difference of hatching rate (1 Feb: $F_{3,8}$=5.944, $p$=0.020 ; 27 Feb: $F_{3,8}$=8.733, $p$=0.007 ; 1 Apr: $F_{3,8}$=4.44, $p$=0.041).

Also, hatching rate was not significantly affected by sampling date in chilling duration of days at 7 days ($F_{4,10}$=2.921, $p$=0.078) and 30 days ($F_{4,10}$=1.909, $p$=0.185) in full winter season. Otherwise, egg hatching was significantly affected by sampling date in both control ($F_{4,10}$=3.583, $p$=0.046) and 15 days ($F_{4,10}$=11.083, $p$=0.001) at 5 °C (Table 5). In this study, winter diapause of *L. delicatula* egg hatched without the chilling treatment. But, pre-chilled treatment for 15 day at 5 °C was effective conditions on overwintering eggs *L. delicatula* in subsequent to mid-winter season in Korea.

Table 5. Hatching rate (Mean±SE) of *L. delicatula* eggs for chilling days (0, 7, 15 and 30 days) at 5 °C.

| | 23 Nov | 2 Jan | 1 Feb | 27 Feb | 1 Apr |
|---|---|---|---|---|---|
| Control | $0_a$[3] | $16.67 \pm 6.7_b$ | $0_a$[a4] | $3.33\pm3.33_a$[ab] | $1.11\pm0.18_a$[a] |
| 7days | $3.33 \pm 3.33$ | $23.33 \pm 3.33$ | $16.67\pm3.33$[ab] | $23.33\pm6.67$[b] | $15.56\pm1.86$[a] |
| 15days | $13.33 \pm 3.33_{abc}$ | $30.0 \pm5.77_b$ | $23.33\pm3.33_{ba}$[b] | $0_c$[a] | $2.22\pm0.19_{bc}$[a] |
| 30days | $16.67 \pm 6.67$ | $10.0\pm5.77$ | $16.67\pm6.67$[ab] | $0$[a] | $3.33\pm0$[a] |

[3] Chilling days for control and 7 days were significantly different ANOVA test. In a row, same letters means not significantly different by Tukey HSD at $p$=0.05.

[4] Means with same letters in a column are not significantly different by Tukey HSD at $p$=0.05.

**Thermal response**

The duration for egg hatching and hatching rate were examined between NCH and CH conditions for the overwintering eggs of *L. delicatula*. Hatching rate was 60.3 ± 3.75 % in Jan, following sharply decrease to 3.09 ± 1.06 % in Feb, and then it increased in Apr (24.61 %) at NCH. After pre-chilled treatment, it showed different hatching rate, following 67.8 ± 3.89 % on 2 Jan and 29.72 ± 3.64 % on 1 Feb. Also, eggs were not hatched on 27 Feb in CH, and then the hatching rate reached 33.93±4.08 % on 1 Apr (Table 6). The longest days of first hatching were 23.12±5.06 days (Mean ± SE) on 2 Jan and the shortest was on 1 Apr (16 ± 1.24 days) at NCH. Otherwise, hatching days was the longest in Feb (22.03 ± 2.0 days) following in Jan (20.63 ± 0.05 days) and in Apr (15.84 ± 1.1 days) at CH (Table 6).

The duration of hatching was lengthened to 2.5 days and delayed of hatching pattern above 60 % of cumulative proportion in January at NCH. On the other hand, delayed hatching pattern were not shown by chilled for 15 days at 5 °C in the same sampling time. And, hatching days was different by 1 day between NCH and CH, and hatching rate of NCH (3.1 ± 1.06 %) was significantly lower than CH (29.9 ± 3.64 %) in February (Table 6). And, hatching was delayed at CH caused by incompletely

developed eggs in level of 90 % above of cumulative proportion (Fig. 8). Otherwise, hatching patterns were synchronized and no difference of hatching days between two groups in April (Fig. 8, Table 6). Therefore, hatching days were shortened, and proportion of fully developed eggs became higher near the spring. Therefore, changed thermal response partially implied diapause phase of overwintering eggs *L. delicatula* in Korea.

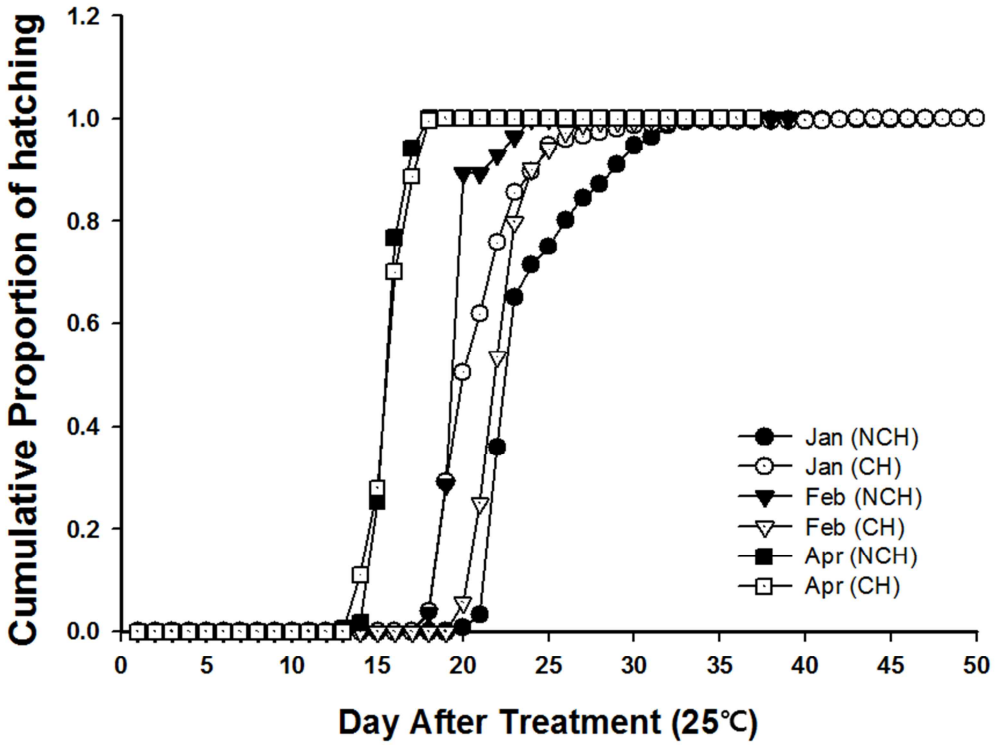



Fig. 8. Cumulative proportions between NCH (without chilling) and CH (pre-chilled for 15 days at 5 °C) at each collecting date.

Table 6. Thermal responses between NCH (25 °C) and CH (5 °C, 15days->25 °C) conditions at each sampling date in 2012.

| Date | Treatment | n[5] | Hatching rate (%) (Mean±SE) | Duration days (Mean±SE) | Median |
|---|---|---|---|---|---|
| 2 Jan | NCH | 48 | 60.3 ± 3.75 | 23.12 ± 5.06 | 22.5 |
| | CH | 48 | 67.8 ± 3.89 | 20.63 ± 0.05 | 20 |
| 1 Feb | NCH | 13 | 3.1 ± 1.06 | 21 ± 1.92 | 21 |
| | CH | 39 | 29.7 ± 3.64 | 22.03 ± 2.0 | 22 |
| 1 Apr | NCH | 32 | 24.6 ± 4.12 | 16 ± 1.24 | 16 |
| | CH | 41 | 33.9 ± 4.08 | 15.84 ± 1.1 | 16 |

[5] Number of egg masses at each treatment.

**Egg hatching Model**

We obtain the cumulative distribution patterns with fitted Weibull function for all sampling times. After comparing the patterns, we excluded the data on 1st Feb which showed delayed hatching pattern in this study. Therefore, it was pooled at three sampling times on 17 Feb and 4 Mar in 2010 and on 22 Feb in 2011(Fig. 9). All analyses were used by the pooling data.

Development time and hatching rate (%) from overwintering egg to 1st lymph was shown in different temperatures (Table 7). The relationship between the developmental rate and temperature was described with linear and nonlinear developmental model (Fig. 10a). The estimated base temperature ($T_0$) for overwintering egg masses was 11.13 °C, and thermal constants was 293.26 DD (Y=0.00341X-0.03795, $r^2$=0.98). The nonlinear models, by using Brière 2 equation, fitted well to describe the significant relationship between temperature and development rate ($r^2$=0.992).

The survivorship of overwintering eggs varied at different temperatures; thereafter sharply decreased at 31 °C (Table 7). Survival rate (%) was well described with gauss function; survivorship was the highest at ≈21 °C in predicted survival model (Fig. 10b). Developmental variation was well described with the cumulative Weibull distribution model

($r^2$=0.979) (Fig. 10c). We estimated the parameter values of temperature-dependent developmental models in post-diapause of *L. delicatula* eggs (Table 8).

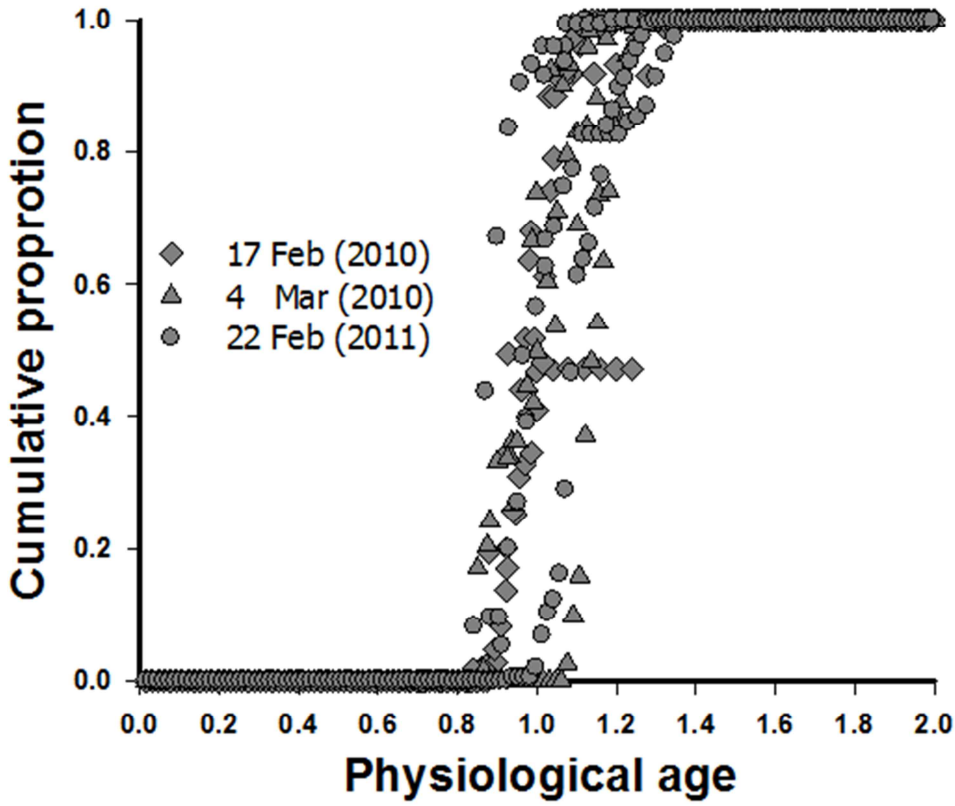



Fig. 9. Cumulative proportions of *L. delicatula* eggs as a given physiological age at each sampling date between 2010 and 2011 in CA.

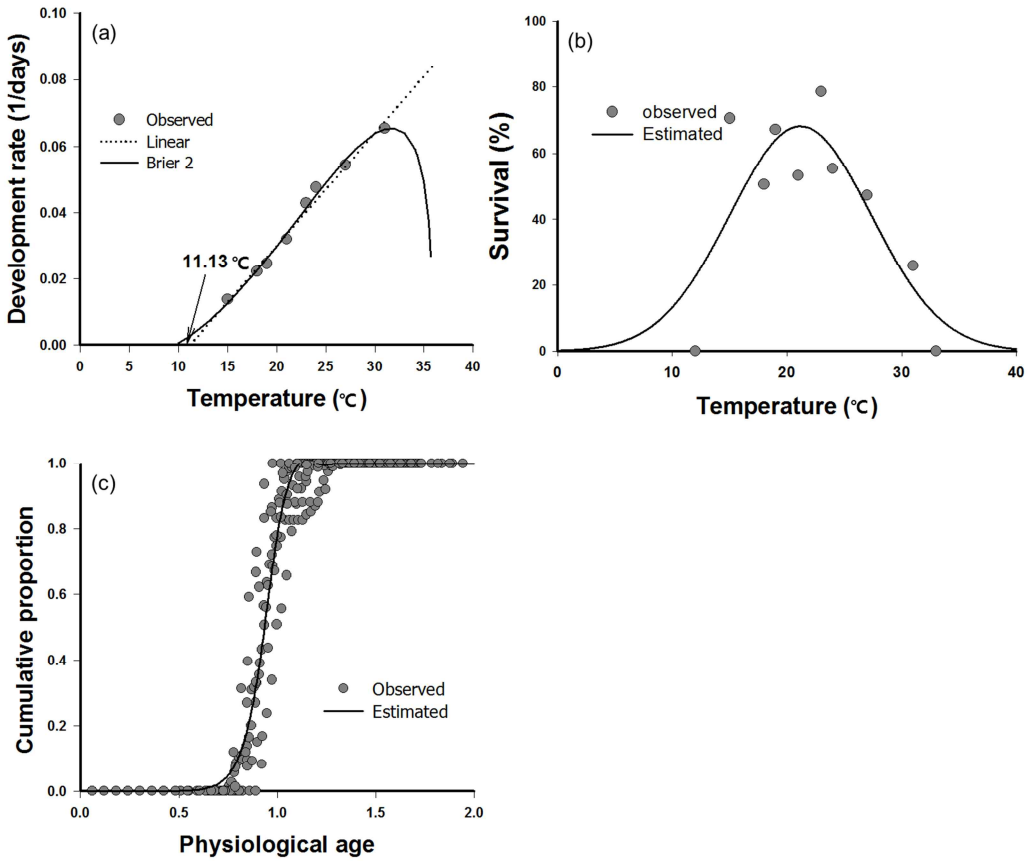



Fig. 10 (a) Temperature-dependent developmental rate (1/day) for egg stage *L. delicatula* fitted by linear and non-linear models (solid line). Brière 2 model was applied. (b) Temperature-dependent survival rate (%) of *L. delicatula* eggs predicted by Gauss model (solid line) (c) Cumulative proportions of development completion for egg stage of *L. delicatula* as a given physiological age. Two-parameter Weibull function was applied (solid line).

Table 7. Developmental period (day) and survival rate (%) for post-diapause development of *L. delicatula* eggs collected from Cheonan, in 2010 and 2011.

| Temp. (°C) | Sampling year | Developmental Period (Mean±SD) | No. of eggs exposed / No. of nymph hatched | Survival rate % |
|---|---|---|---|---|
| 12 | 2011 | -[6] | - | - |
| 15 | 2010,2011 | 72.6±5.93[7] (71.9±6.04,75.0±4.59)[8] | 1064/751(401/220, 663/531) | 70.6 (54.9,80.1) |
| 18 | 2011 | 45.0±5.85 | 227/115 | 50.7 |
| 19 | 2010 | 40.7±3.45 | 776/521 | 67.1 |
| 21 | 2011 | 31.3±1.91 | 274/146 | 53.3 |
| 23 | 2010 | 23.4±3.72 | 686/539 | 78.6 |
| 24 | 2011 | 21.0±0.96 | 227/126 | 55.5 |
| 27 | 2010,2011 | 18.4±1.88 (18.3±2.2,18.6±0.83) | 983/689 (290/224, 693/465) | 70.1(76.2,67.1) |
| 31 | 2010 | 15.3±1.15 | 707/183 | 25.9 |
| 33 | 2011 | - | - | - |
| 35 | 2010 | - | - | - |

[6] Eggs were not hatched.
[7] The average of the developmental periods and survival rate (%) in 2010 and 2011.
[8] The values indicated the developmental periods, number of eggs exposed/hatched and survival rate (%) in 2010 and 2011, respectively (in parentheses).

Table 8. Parameters of egg hatching models describing the relationship between temperature (°C) and development rates (1/day, Mean±S.E)

| Models | | Linear | | Brière 2 | | Gauss | | Weibull |
|---|---|---|---|---|---|---|---|---|
| Parameters | *A* | 0.038±0.004 | *α* | 0.0000597±0.72 | *Rm* | 0.34±0.66 | *α* | 0.96±396.72 |
| | *B* | 0.0034±0.0002 | $T_O$ | 9.66±3.87 | *Tm* | 41.27±2.25 | *β* | 12.02±26.82 |
| | LT | 11.13 | $T_L$ | 35.8±3.38 | *k* | 38.01±15.31 | | |
| | DD | 293.26 | *m* | 3.13±0.62 | | | | |
| $r^2$ | | 0.98 | | 0.992 | | 0.7 | | 0.979 |

**Model Validation**

These developmental rate and distribution models were used for simulation process of first instar nymph occurrence of *L. delicatula* in field.

The emergence proportion appeared similar patterns from four populations (DG, CA, SW and GS) against the simulated model (Fig. 11). Furthermore, the observed and predicted occurrences for first instar nymph were compared in Fig. 12. The observed data was well described with simulated model both 2011 and 2012 in CA, respectively (Fig. 12a, Fig 12b). But, the discrepancy was shown by appearing the early emergence of first instar nymph against the prediction model in SW (Fig. 12c).

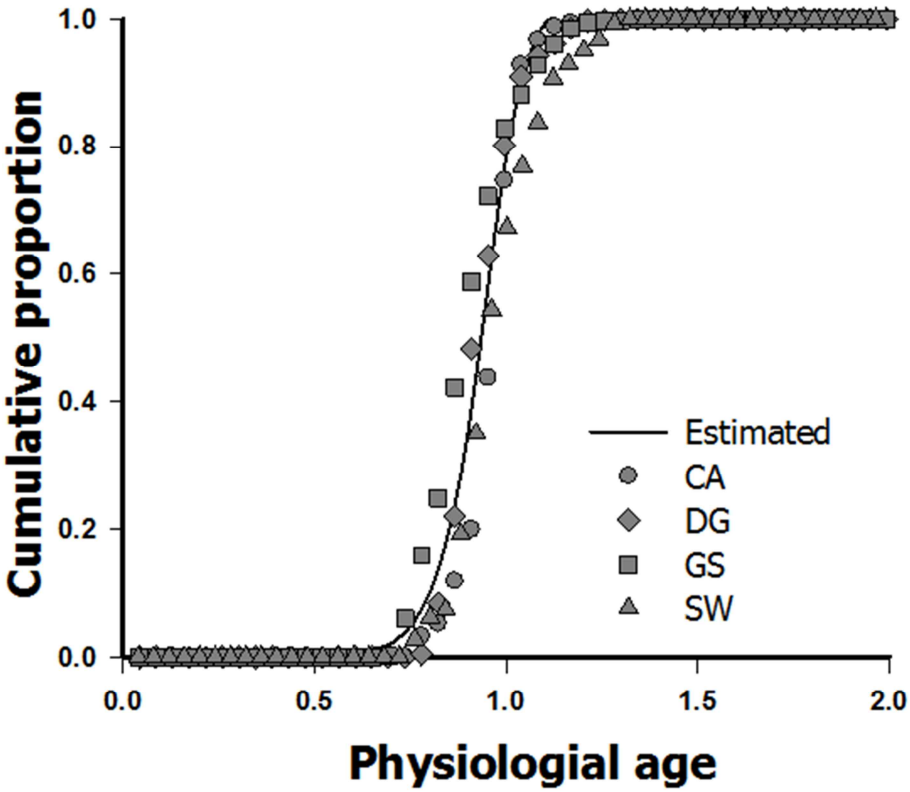



Fig. 11. Cumulative proportions (%) for emergence of first instar nymph *L. delicatula* from four overwintering local populations in 2011 against the simulated hatching model (solid line). Hatching rate (%, Mean±SE) was 79.0±3.75, 55.6±5.98, 30.8±4.56, and 41.8±4.01 %, in DG, GS, CA, and SW, respectively.

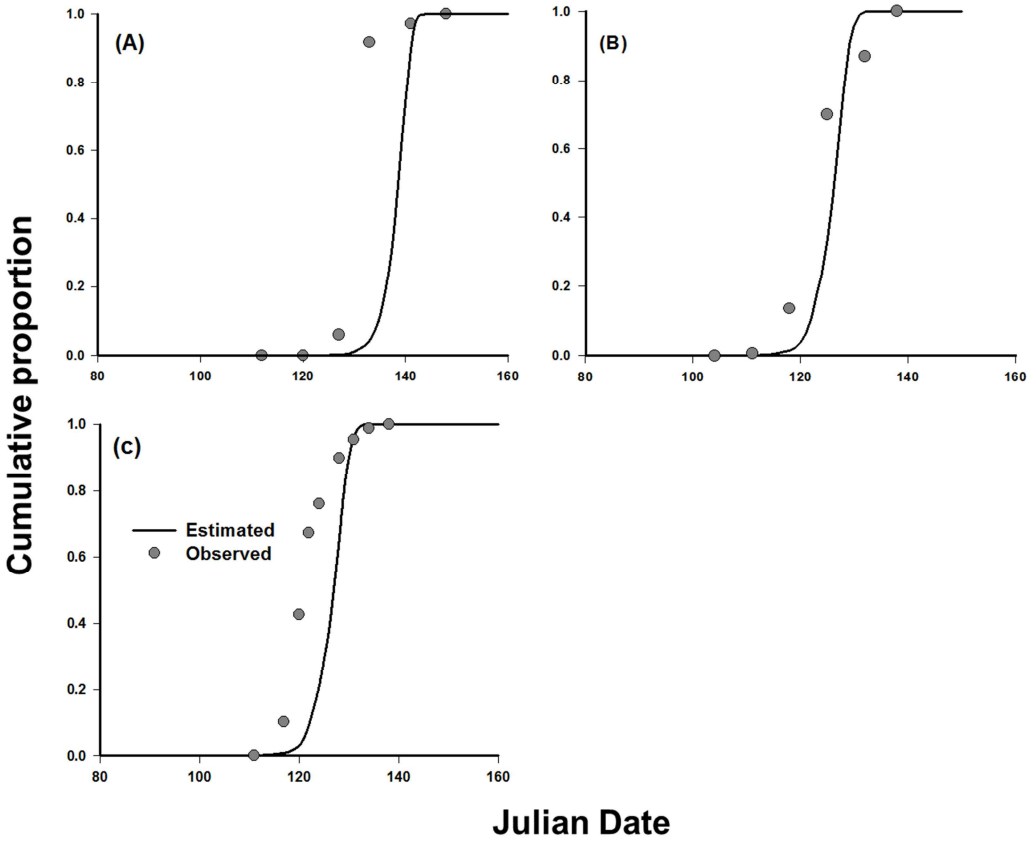



Fig. 12. Egg hatching model was compared with field observation (a) 2011 in CA (b) 2012 in CA (c) 2012 in SW. Egg hatching model was simulated using the daily temperature from on 1 April.

## 2-4. Discussions

In this study, winter diapause phases of eggs *L. delicatula* were investigated by thermal response with time intervals field sampling in Korea. Also, effective chilling days were identified for diapause termination. As a result, chilling days affected hatching ability at 5 °C (Table 5). Effective chilling condition for the completion of the diapause was 15 days at 5 °C after mid-winter. But, this chilling condition was not effective in early winter season. Namely, early diapause phase was required more intensity (lower temperature) and quantity (prolonged chilling days) factors for optimal chilling of *L. delicatula* eggs. Therefore, detailed study was required by providing the various exposed temperatures (0~10 °C) and durations for diapause termination in full overwintering season of *L. delicatula*. In this study, overwintering eggs of *L. delicatula* were not required the obligated condition for winter diapause termination; eggs hatched without chilling treatment. But, this information was useful to make artificial termination for diapause (e.g. mass rearing). Although, suggested chilling condition was limited only experimented at 5 °C, it was useful for post-diapause development of overwintering *L. delicatula* eggs.

Insect diapause has a seasonality including the diapause maintenance, diapause termination, and post diapause development in

general (Tauber and Tauber, 1979; Koštál, 2006). In present study, hatching rate and days was changed in U shape in winter season. Also, this result may explain the diapause depth in early Feb when no hatch was observed (developmental arrest) following post-diapause development. Shin *et al*. (2010) investigated the hatching rate and period of *L. delicatula* eggs from field samples with one month interval in Cheongju, from 2009 to 2010. They showed successively increased hatching rate (%) by spring; but, hatching day was significantly shortened after late February. This result was considered seasonally changed winter diapause phases of overwintering *L. delicatula* eggs. Also, the timing of diapause phases may be different among locations and years.

Morphological characters are useful indicators to verify the embryonic development of insect diapause (Ingrisch, 1984). But, it is difficult to observe the diapause transition using the naked eye, because of the opaque and hard egg shell of *L. delicatula*. Alternatively, diapause states can be identified through assessing thermal response after the treatment of environmental stimuli (e.g. temperature and photoperiod). Successive and dynamic progressed diapause was not distinguished by the discrete sampling method. Therefore, continuous measurement has been proposed on the biochemical process like a respiration (Zaslavski,

1988; Gray *et al*., 1991), protein synthesis (Venkatesh and Chippendale, 1986), and concentration of the polyhydric alcohol (Nordin *et al*., 1984).

Rates of diapause termination and post-diapause development can be used to predict the hatching (or emergence) timing in the field (Xiao *et al*., 2013). Lower developmental threshold ($T_o$) and effective thermal requirement were based on the estimation of developmental days of target stages. Previously, temperature-dependent model of the *L. delicatula* eggs were suggested by Choi *et al*. (2012). Identifying the post-diapause development was critical for more reliable forecasting of hatching time in the spring (Logan *et al*., 1979; Tauber *et al*., 1990). Without information of diapause process, it has a possibilities over- and under-estimation of the modeling parameters.

For population modeling, population dynamics over the time is important rather than individual transition of diapause process. Therefore, we conducted the field sampling with the time interval to verify the population progress for winter season.

Temperature-dependent development model was constructed on post-diapause stage for overwintering egg masses of *L. delicatula* to predict the hatching in spring. Lower thermal thresholds ($T_L$) and heat units (Degree Days) were required after post-diapause termination for hatching

of *L. delicatula* eggs. Also, we described the quantitative information on the development variation of *L. delicatula* by observing population response under field conditions. Egg development showed the similar patterns at the four locations against predicted model (Fig. 11). This result suggested the possibility of applying the whole range in Korea, even if it was conducted based on CA population. In field validation, observed and predicted through the distribution model with Weibull function in CA was well described (Fig. 12a, Fig. 12b). In later case, mainly two probabilities, depending on intrinsic and/or extrinsic factor, were raised for discrepancy of model prediction. First, it has potential which requires the different heating thermal according to region divided by the ecological race. In case of *Thecodiplosis japonensis,* according to North and South regions in Korea, it was differentiated the development of biology by overwintering eggs (Lee and Woo, 1987). But, it could be clearly excluded since it has a short invading periods (around 10 years) and univoltine life cycle in Korea. In this study, we did not apply the on-site weather data for field validation, only used the weather station data near the study sites provided the KMA. Because survey site was *A. altissima* community within urban habitat, temperature data was improper for field validation in SW. Therefore, it will need to quantify the climate element in habitat characters (e.g. coverage)

and types (e.g. agro- and urban) for more reliable prediction in heterogeneous landscape of Korea. This information will promote the effective management by providing the information of the parameters of the egg stage.

## Chapter 3. Phenology and age structure among host plants


## Abstract

*L. delicatula* is polyphagous pest, which has different host plants between nymph and adult stages. Seasonal occurrence of *L. delicatula* was investigated among three host plants (*Vitis vinifera, Ailanthus altissima,* and *Morus alba*). 1st nymph emergence begins in early May among three host plants. Adult emergence began in late July or early August with different peak time among host plants. Sex ratio by female was ranged from 35 to 45 % sampled from *A. altissima*. Adults immigration into grape vine yard occurred every year and *A. altissima* was estimated as potential source of nymph of *L. delicaula*. The occurrence data were fitted by logistic model based on the degree days (base temp. 11.13 °C) on *A. altissima*. Model fitting result was shown that accumulated degree days (DD) were calculated as 271, 492, 620, and 908 at 1, 2, 3, and 4th instar nymph in the peak time, respectively. This model was not applied to adult stage of *L. delicatula* due to its dispersal behavior on *A. altissima*. This model would be help for the prediction of effective control timing of *L. delicatula* in agriculture crops in Korea.



Key words: host plants, phenology, age structure, sex ratio

## 3-1. Introduction

*L. delicatula* was known to polyphagous insect both China and Korea. In China, serious damage of *L. delicatula* was known on *Ailanthus altissima*, especially nymph stage. Different damage degrees were reported among 41 host plants between nymph and adult stages (Park *et al*., 2009). Adult dispersal into grape vine yard cause significant economic loss in Korea. Although, the factor for dispersal ability was critical, this was not revealed clearly in agricultural system until now.

*L. delicatula* appears the falling-ascending behavior on the host plants, which probably possible to short range dispersal. And, increased cycle course were improved more long distance dispersal of adult than nymph stages. Host plant preferences were changed along its growth, with broad range in the early nymph and a narrower, mainly *A. altissima*, in the adult stage. But, they couldn't describe the dispersal ability in agricultural environment. Therefore, studying the seasonal occurrence among host plants was required, in particular, grape vine, which serious damaged by *L. delicatula.*

Quantifying occurrence may help the comprehensive understanding population dynamics for *L. delicatula* populations. Comparing the seasonal occurrence among host plants may provide the

insight the dispersal pathway, furthermore, critical information for implement of control strategies. Also, age structure was able to more precise prediction, by revealing the valuable stages within full season. Animal dispersal seldom occurs randomly, and it depends on actual environmental conditions and the developmental stage and sex-biased (Ims and Hjermann, 2001, Gros *et al*., 2008).

Predicting the time of peak occurrence of severe developmental stages for a crop is important to pest management. Well-timed control is critical point for optimum timing of chemical spray and releasing natural enemy in perspective IPM (Integrated Pest Management). Therefore, phenology models are useful to explain the timing of insect developmental stage events, moreover, applied agricultural practice in the field.

Stochastic model was widely used on insect for a long time, using the evaluated developmental rate across a variety of experimental constant temperatures in laboratory (Dennis *et al*., 1986 ; Murtaugh *et al*., 2012). But, this procedure needs mass rearing of target species in laboratory condition, which causes higher mortality and time consumption, especially sporadically occurring pest as *L. delicatula* without standard rearing information.

Therefore, we aim to characterize the seasonal occurrence of *L. delicatula* among host plants, which is different preference between nymph and adult stages, in agriculture of Korea. We also investigated the sex ratio of adults on *A. altissima. L. delicatula* has morphological sexual characteristics only in adult stage, by the red color in postero-caudal end of abdomen in female. And, phenology model of *L. delicatula* was developed from field observation data in Cheonan. Also, this model was validated from Suwon population in 2012.

## 3-2. Material and Methods

### Age structure among three host plants

*L. delicatula* was monitored in field in in CA, from 2010 to 2012. Both *V. vinifera* (Kyoho) and *A. altissima* were monitored at two sites in Seonggeo-eup, Cheonan. CA1 was conventional applied grape vine yard, which distributed of *A. altissima* nearby orchard. This area was serious damaged by *L. delicatula* in 2008. But, we investigated the occurrence on *V. vinifera* in CA 1 from 2010 to 2011, because grape vine yard had been destroyed by farmers after 2011. CA 2 was experimental field managed by Cheonan Agricultural Technology Service Center. *V. vinifera* (Kyoho) and *M. alba* was monitored, from 2010 and 2011 in CA2, respectively. In addition, *L. delicatula* was monitored on isolated *A. altissima* community in Suwon, Gyeonggido in 2012 (SW). Site in SW does not have grape vine yard, within the perimeter of 15 km. This area is not major district of grape production in Korea.

The number and life stage of individuals of *L. delicatula* was recorded at each sampling site. *V. vinifera* were not possible to install the sticky traps, because it has a unique structure having a narrow branches and twigs in the tree. Therefore, we counted the numbers using the direct observation method. Distinct morphological characters at each stage were

able to naked eye recorded, easily. *A. altissima* and *M. alba* were not possible to the naked eye method, because of the high DBH. Blue color was effective radiation to attract the *L. delicatula* for all stage, nymph to adults, and both sex (Choi *et al*., 2011). Therefore, we installed the blue sticky trap around the trunk closely in the approximately 1.5 m height. Blue sticky trap was manufactured by Green AgroTech.

**Phenology model and validation on *A. altissima***

Developmental threshold was estimated in the egg stage of 11.13 °C (Chapter 2). Developmental threshold was not available of *L. delicatula* except for egg stage, because insect rearing was failed by high mortality in laboratory. Degree-days (DD) were calculated using DDU (Degree-Day Utility, University of California) by single sine wave (Allen, 1976). Therefore, we assumed that there is no difference in developmental thresholds between eggs and further stages. All degree-day values were based on accumulated heat units (lower developmental threshold of 11.13 °C) at each sampling year.

$$\sum_{1}^{n} \boldsymbol{DD(n)} = \int_{1}^{n} (\boldsymbol{Ta - LT})\boldsymbol{dT}$$

when DD (*n*) is daily DDs, Ta is mean daily air temperature, and LT is the lower developmental threshold. The biofix for DD accumulation was set as April 1.

Phenology model (Dennis *et al*., 1986; Dennis and Kemp, 1988) was developed to provide a more generalized and quantitative representation of the occurrence of nymph and adult stages through time. For phenology modeling, we used the field sampling data. Density was recorded at each stage (first nymph to adult) on *A. altissima* in CA, from 2010 to 2012. Detailed insect sampling method was explained in Chapter 2. Only *L. delicatula* population from *A. altissima* explained well the age structured density of *L. delicatula.* Meteorological data were obtained by Cheonan weather station.

The model is based on a logistic probability distribution that changes as a function of time *t*, here measured through the use of accumulated degree-days. The model assumes that mortality rates are equal among the developmental stages and that developmental rates are homogenous among individual insects. We fitted the frequency data according to logistic probability distribution using Table Curve 2D.

$$f(T) = \alpha + \frac{4\beta \, exp\,[-(t-\mu/\sigma)]}{\left[1 + exp\,[-(t-\mu)/\sigma]\right]}$$

where $\mu$ is units of accumulated degree days $\alpha$, $\beta$, $\sigma$ are fitted parameters.

Evaluation was important to improve the efficiency in spatial scales for predicting tools, especially newly suggested model. Also, this model is a probable for applicability throughout the Korea, because it was constructed only rely on the field occurrence data in one region. Therefore, we validated this model comparing the occurrence on *A. altissima* of SW in 2012.

## 3-3. Results

### Age structures among host plants

In 2010, 1st nymph occurrence begins in late May, and it was not different between *V. vinifera* and *A. altissima*. Density was fluctuated with chemical spray events in grape vine yard. Despite the chemical control, 4th instar nymph and adults were increased from early July, in vine yard. Peak timing of adults was double in early August and October in grape vine yard (Fig. 13). Similar seasonal pattern was observed between *V. vinifera* and *A. altissima* in 2011 (Fig. 14). Even though high level of 4th nymph population was detected, adult was not observed from *M. alba* in 2011 and 2012 (Fig. 14, Fig. 15). In only *A. altissima,* consistent demographic structure of *L. delicatula* was observed in Suwon (Fig. 16). Seasonal occurrence was opposite patterns between *V. vinifera* and *A. altissima,* in particular 4th nymph and adult stage. Totally, 1st nymph was occurred in late May and then adult emergence began in late July or early August with different peak time at each stage among host plants. Therefore, influx of adults was implied in grape vine yard in every year and *A. altissima* and *M. alba* were estimated as potential source of *L. delicatula* nymph.

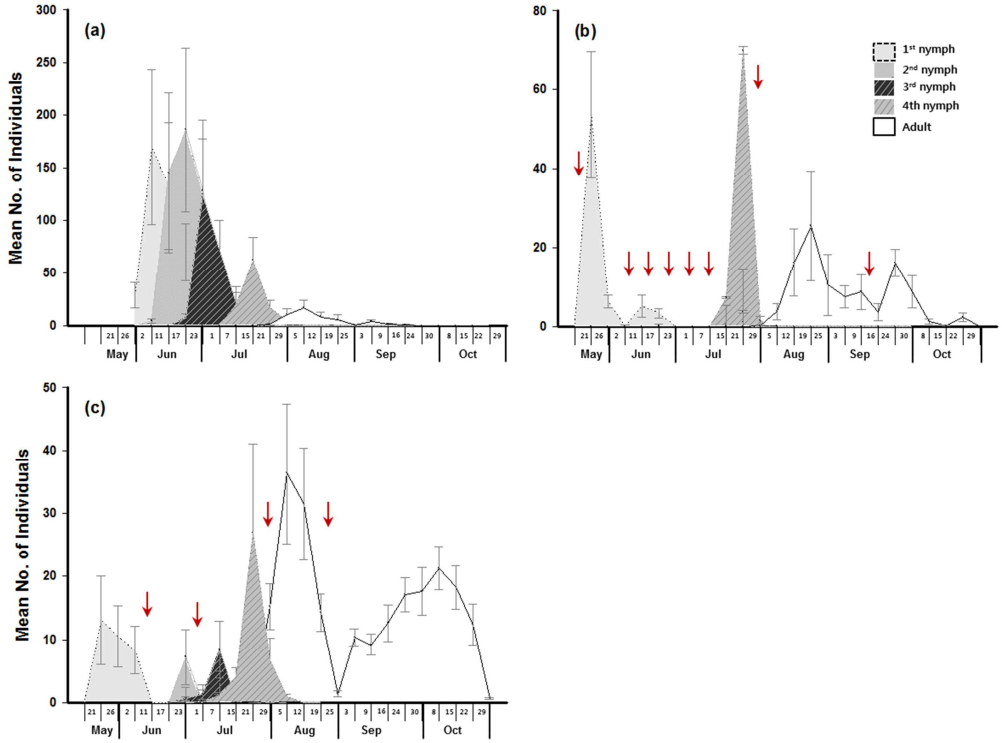



Fig. 13. Age structure of *L. delicatula* in 2010 (a) *A. altissima* in CA 1 (b) *V. vinifera* in CA1 (c) *V. vinifera* in CA 2. Area curves for each stage are based on mean numbers (±SE) at each sampling date. ↓ indicates the pesticide spray time.

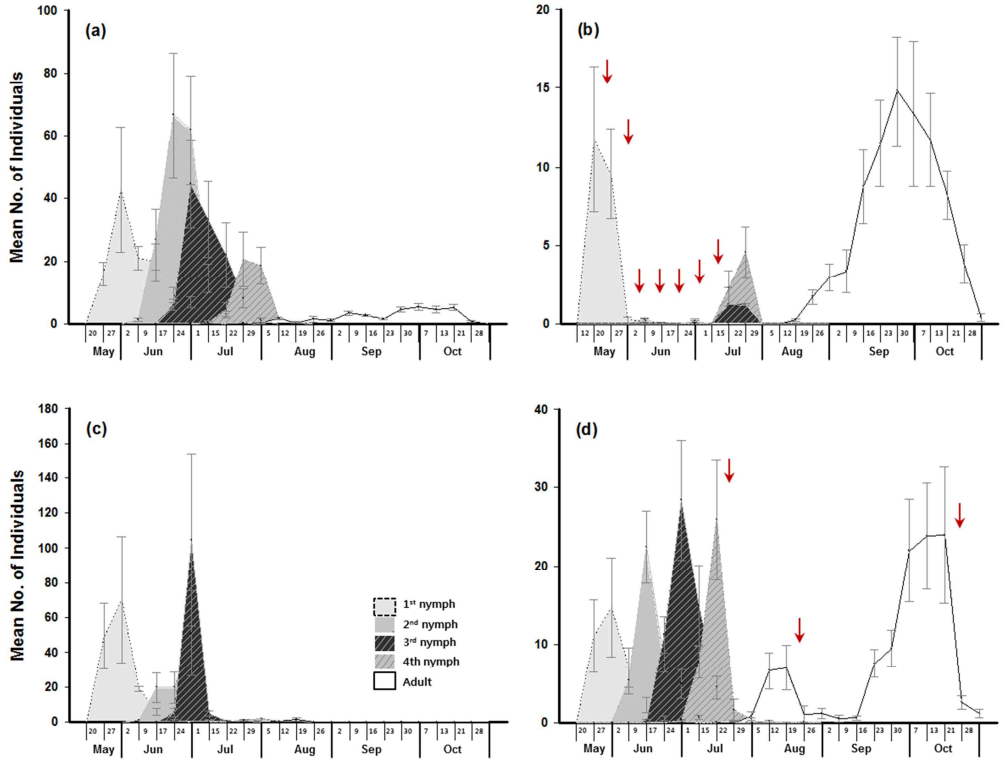



Fig. 14. Age structure of *L. delicatula* in 2011 (a) *A. altissima* in CA 1 (b) *V. vinifera* in CA 1 (c) *M. alba* in CA 2 (d) *V. vinifera* in CA 2. Area curves for each stage are based on mean numbers (±SE) at each sampling date. ↓ indicates the pesticide spray time.

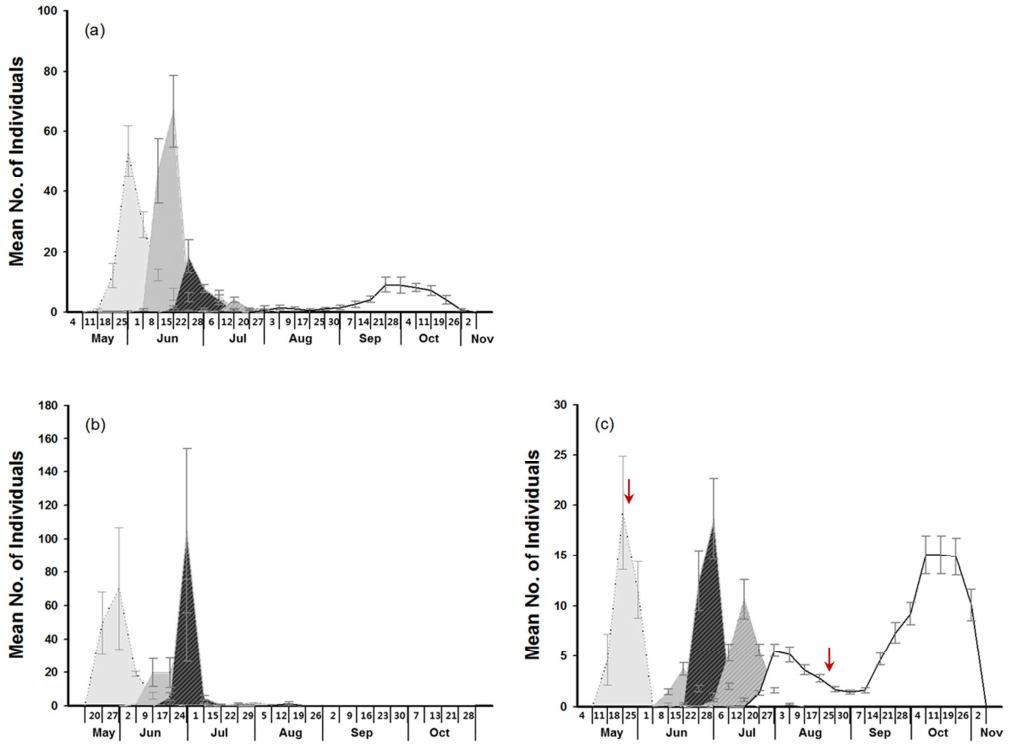



Fig. 15. Age structure of *L. delicatula* in 2012 (a) *A. altissima* in CA 1 (b) *M. alba* in CA 2 (c) *V. vinifera* in CA 2. Area curves for each stage are based on mean numbers (±SE) at each sampling date. ↓ indicates the pesticide spray time.

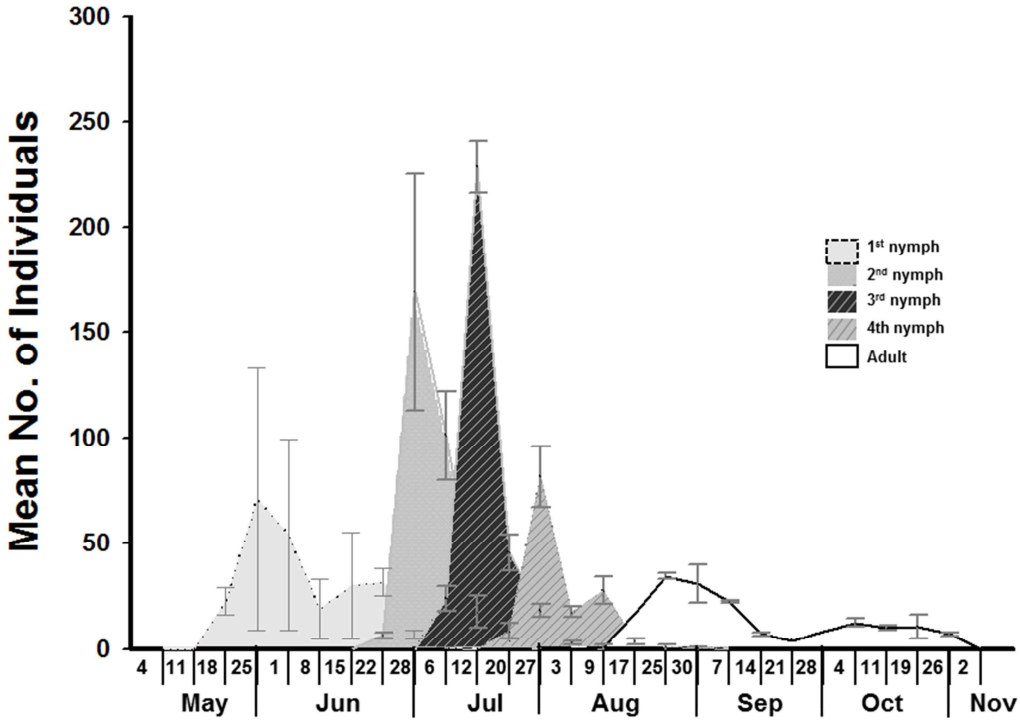



Fig. 16. Age structure of *L. delicatula* from isolated *A. altissima* community of SW, in 2012. Area curves for each stage are based on mean numbers (±SE) at each sampling date.

**Sex ratio of female with seasonal changes**

Sex ratio of female was 39 and 43 % in 2010 and 2011 in CA 1, respectively. Also, it was 35 and 45 % at CA 1 and SW in 2012, respectively. All adult samples were derived from *A. altissima*. No significance was shown between male and female individuals in four samples. Female individuals were lower than males around late September to early October on *A. altissima* (i.e. presumably female immigrated from *A. altissima* (Fig. 17a, Fig. 17b). Therefore, these results were explained partially that sex-biased dispersal by female of *L. delicatula*. Female density of *V. vinifera* was required for more strong evidence of its dispersal patterns.

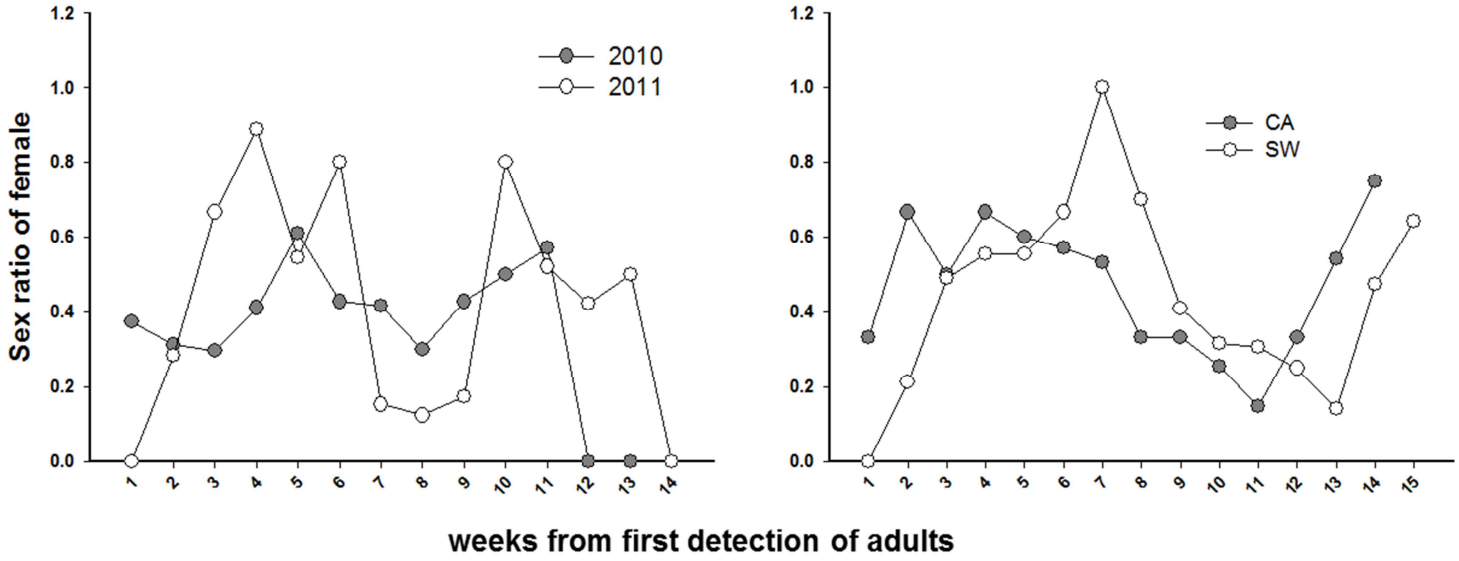



Fig. 17. Seasonal patterns of female sex ratio of *L. delicatula* collected from *A. altissima* (a) CA 1 in 2010 and 2011 surrounding the grape vine yards (b) CA 1 between CA and SW populations in 2012 without grape vine yards.

**Phenology model on *A. altissima***

The phenology model was estimated proportion of *L. delicatula* in development stage, inhabited on *A. altissima*. This was plotted as a function of temperature-dependent time (degree days by single sine wave) using lower threshold temperature of 11.13 °C (Fig. 18). Required Degree Day for peak occurrence time were *ca*. 271, 492, 620 and 908 DD, at 1, 2, 3, and 4th instar nymph, respectively (Table 9). The gap between estimated and calculated DD (293.26) was *ca*. 20 DD in 1st nymph stage. Distribution from eggs to 1st nymph showed dispersive pattern in laboratory, therefore, occurrence was variable and lasted long in field. Also, this model was not applied to adult stage ($r^2$=0.44) due to its dispersal behavior on *A. altissima* in agricultural system. But, the model was able to well predict the peak time for each stage, generally.

Developed phenology models were validated using samples collected in SW. Estimated plots were accorded with peak time for observed occurrence in nymph stages (Fig. 19).

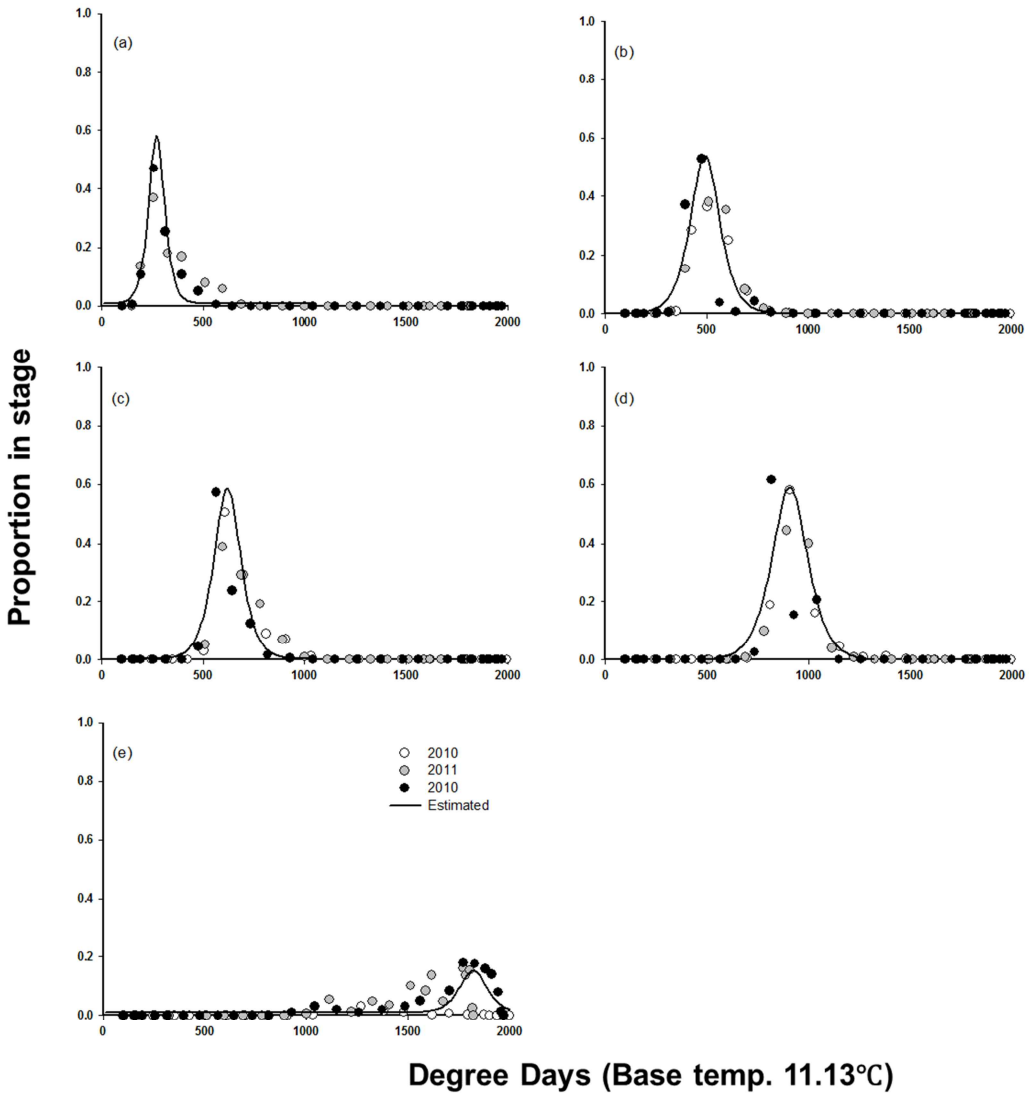



Fig. 18. Comparison of field data (plotted points) and estimated results (–) for the proportion of the population in each life stage (a) 1st nymph (b) 2nd nymph (c) 3rd nymph (d) 4th nymph (e) adult as a physiologically accumulated Degree Days. Sampling was conducted on *A. altissima* in CA.

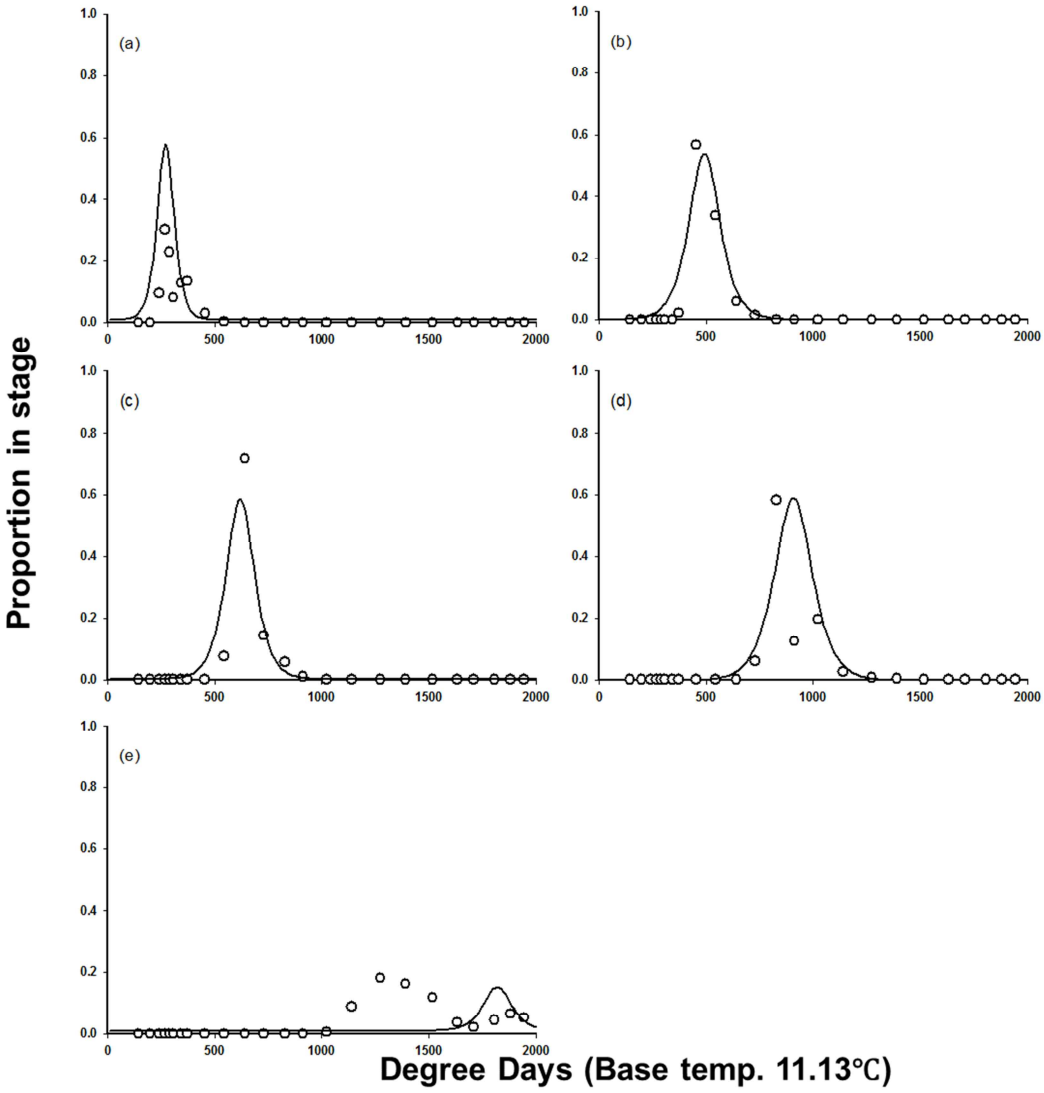



Fig. 19. Validation for developed phenology model between field occurrence data (plotted points) and estimated results ( – ) for the proportion of the population in each life stage (a) 1st nymph (b) 2nd nymph (c) 3rd nymph (d) 4th nymph (e) adult as a physiologically accumulated Degree Days. Sampling was conducted on *A. altissima* in SW.

Table 9. Degree days of peak occurrence time at each stage on the *A. altissima* from 2010 to 2012, in CA

| Stage | Degree Days | $r^2$ |
|---|---|---|
| 1st | 270.71±3.38 | 0.89 |
| 2nd | 491.98±7.15 | 0.78 |
| 3rd | 619.31±6.15 | 0.82 |
| 4th | 907.60±9.72 | 0.75 |
| Adult | 1820.65±14.21 | 0.44 |

## 3-4. Discussions

To know the longevity among host plants, we conducted cage study of three host plants (1) *A. altissima* (2) *V. vinifera* (3) *M. alba (*4) mixed the above mentioned three plants in semi-field condition. We used three cages per plot, 10 individuals of 1st nymph *L. delicatula* (3-5 days old) were provided at each cage. The square cage size was (1m width and 1m height, covered by white mesh. As a result, *L. delicatula* could survive until adult stage, but not laid the eggs on *A. alissima*. On the other hand, high mortality of 1st nymph was shown on *V. vinifera* and *M. alba*. But, it couldn't provide significant evidence of survival abilities on the host plant, following the high mortality rate of 1st nymph in cage (Appendix 2). These results may infer the *V. vinifera* and *A. altissima* were not sufficient host plants for complete the full life cycle of *L. delicatula.* Li and Tao (1980) and Yao and Liu (1993) insisted that this insect is a generalist and does not live solely on *A. altissima,* consistent with our results.

Standing traps, attached sticky traps in both sides at 50, 150, 250 m, and modified pitfall traps were installed to catch the adults of *L. delicatila* in a boundary line between *V. vinifera* and *A. altissima* in Cheonan, in 2011. These traps were aimed to identify the direction of movement of adult stage among host plants and dispersal ability along the height. There

are no difference between side and height. Low flight activity of adults *L. delicatula* was reduced the efficiency of standing traps.

Age structure was provided evidence of dispersal of *L. delicatula* among host plants. Seasonal occurrence of *L. delicatula* showed the different peak timing among host plants. Therefore, *A. altissima* and *M. alba* were estimated as alternative resource of nymph *L. delicatula* in this study. Also, nymph preferred host plants was considered as source, providing the adults population surrounding the vine yard. Therefore, identifying the host plants infested by nymph is first step for control. And then, we suggested the control timing in late May (peak occurrence of 1st nymph after hatching) and late September (before oviposition time) (Appendix 3). But, timing in late September was paid more attention to reduce the fecundity, sometimes, because farmers not conducted chemical spray after harvest the grape.

Sex biased dispersal defined that individuals of one sex stay or return to their natal site or group to breed while the other is more prone to disperse (Pusey, 1987). In this study, field observation indicated weak evidence for sex biased dispersal of *L. delicatula* adults. Because, comprising the all possibilities for dispersal was impossible by direct method (e.g. field observation, capture-release-recapture), genetic

techniques were suggested for interpretation of mating system (Prugnolle and Meeus, 2002). Because, we only observed seasonal patterns of sex ratio on *A. altissima,* adult census by sex on *V. vinifera* was required to provide the sex-biased dispersal of *L. delicatula* in agricultural system.

Recently, introduction of egg parasitoid (*Anastatus orientalis*) from China has been tried for biological control of egg stage *L. delicatula* in Korea (Choi *et al*., 2014). Lower parasitism rate was expected in Korea than China due to the environmental differences between them. Preventing the introduction of reproductive female is best action using the physical and chemical controls in grape vine yard. Therefore, develop the deterrent materials is more useful to control the *L. delicatula* populations in timing of oviposition. Profiling the life history was needed for invasive population control, moreover, linked by environmental factors in the field.

These day, it has trouble with invasive pest such as citrus *flatid planthopper*, *Metacalfa pruinosa* (Flatidae: Hemiptera) and *Ricania* sp. (Hemiptera: Ricaniidae). These species was similar ecological characters to *L. delicatula*, which having wide range host plants and dispersal to crop at oviposition stage from inhabited surrounding hill (Kang *et al*., 2013; RDA, 2011). Therefore, age structure was critical to know the dispersal pathway and control making decision among their host plants.

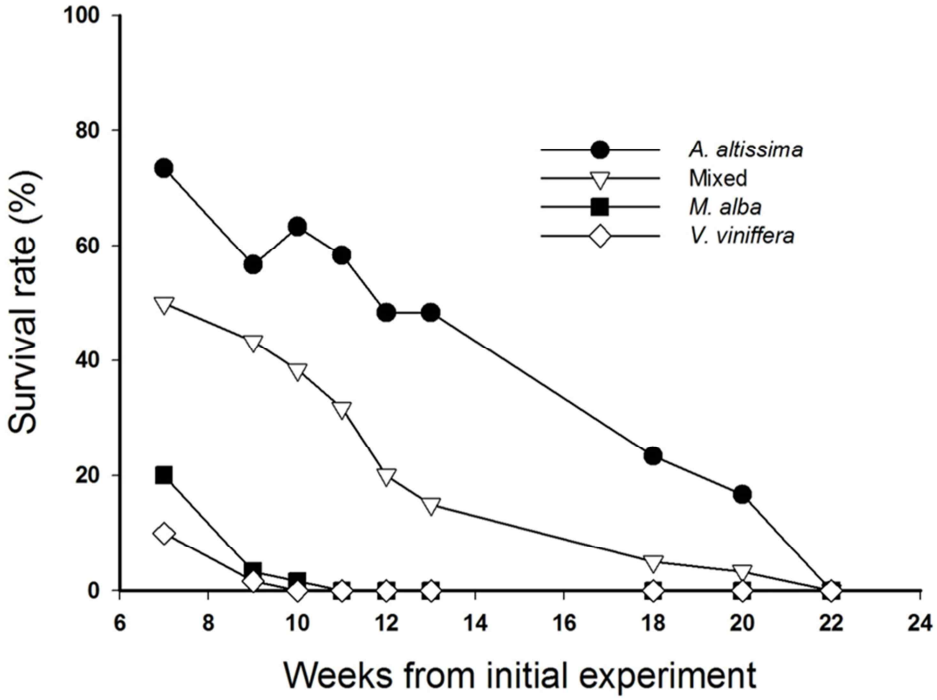
The difference of occurrence was shown at adult populations between CA and SW. Even though it was conducted at same host plant, different environmental conditions whether existing grape vine yard or surroundings *A. altissima* might occurrence discrepancy of adult stage. But, it generally predicted well temperature-dependent phenology in nymph stages across the regions. *A. altissima* may be critical factors to be considered for the management of adult *L. delicatula* in Korea. Therefore, peak occurrence time of 4th nymph may be important period to prevent inflow to grape vine yard.

The phenology data presented here is the first empirical data to suggest that three year observation. But, it may not functionally materialize across the host plants. Observed occurrence of *V. vinifera* also fluctuated by the insecticide spray in grape vine yard. Different developmental rate was reported in polyphagous insect depending on the host plants (Betplhke *et al*., 1991). Therefore, observation of occurrence pattern on major host plants of *L. delicatula* is needed to implement effective control along its dispersal pathway.

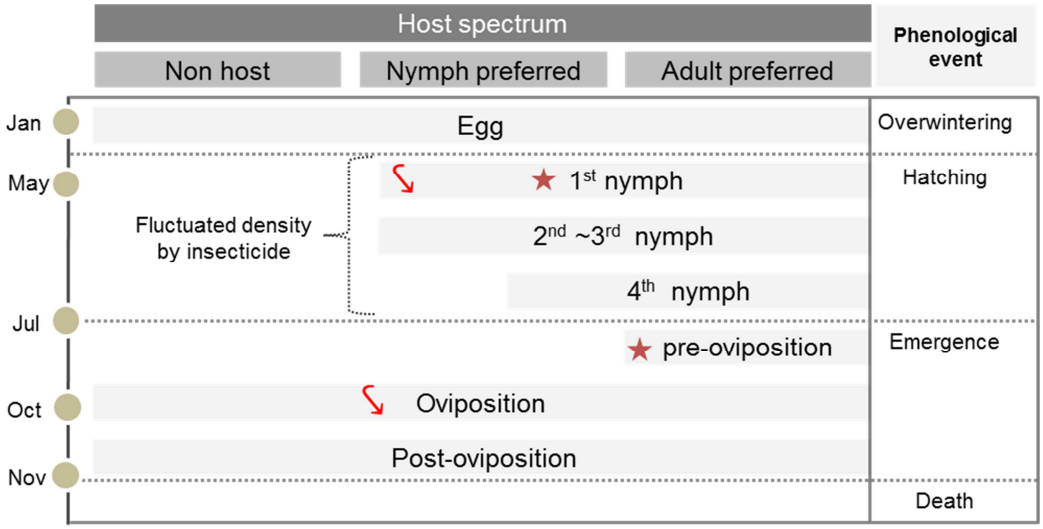
In recent years, developmental database was constructed to suggest universal thermal requirement in levels of Genus, Family and Order (Jarošìk *et al*., 2011). Thermal requirement information for

Fulgoridae has not been known. Risks of exotic species should be assessed in terms of potential establishment and spreading in introduced site. Therefore, understanding the effects of temperature on *L. delicatula* may help predict their seasonal occurrence and pest management, also risk assessment modeling.

Appendix 2. Longevity of *L. delicatula* among four host plants conditions in semi-field of Suwon, in 2011.



Appendix 3. Schematic of short distance dispersal of *L. delicatula* based on the demographic data among host plants. ↘ and * indicated occurred dispersal events and critical control timing in grape vine yard , respectively.



Appendix 4. Observed data of phenological change of *L. deliatula* in the *A. altissima (*CA, 2010).

| Sampling Date | 1st instar | 2nd instar | 3rd instar | 4th instar | Adult |
|---|---|---|---|---|---|
| 5-26 | 0 | 0 | 0 | 0 | 0 |
| 6-2 | 176 | 0 | 0 | 0 | 0 |
| 6-11 | 1,019 | 27 | 0 | 0 | 0 |
| 6-17 | 796 | 872 | 0 | 0 | 0 |
| 6-23 | 419 | 1,117 | 45 | 0 | 0 |
| 7-1 | 182 | 768 | 745 | 0 | 0 |
| 7- 7 | 30 | 238 | 429 | 3 | 0 |
| 7-15 | 3 | 27 | 132 | 121 | 0 |
| 7-21 | 0 | 9 | 105 | 372 | 0 |
| 7-29 | 0 | 3 | 19 | 103 | 8 |
| 8-5 | 0 | 0 | 0 | 29 | 64 |
| 8-12 | 0 | 0 | 0 | 5 | 101 |
| 8-19 | 0 | 0 | 1 | 6 | 51 |
| 8-25 | 0 | 0 | 0 | 1 | 36 |
| 9-3 | 0 | 0 | 0 | 0 | 7 |
| 9-9 | 0 | 0 | 0 | 0 | 24 |
| 9-16 | 0 | 0 | 0 | 0 | 10 |
| 9-24 | 0 | 0 | 0 | 0 | 7 |
| 9-30 | 0 | 0 | 0 | 0 | 2 |
| 10-8 | 0 | 0 | 0 | 0 | 0 |
| 10-15 | 0 | 0 | 0 | 0 | 0 |
| 10-22 | 0 | 0 | 0 | 0 | 0 |
| 10-29 | 0 | 0 | 0 | 0 | 0 |

Appendix 5. Observed data of phenological change of *L. deliatula* in the *A. altissima (*CA, 2011)

| Sampling Date | 1st instar | 2nd instar | 3rd instar | 4th instar | Adult |
|---|---|---|---|---|---|
| 5-20 | 0 | 0 | 0 | 0 | 0 |
| 5-27 | 79 | 0 | 0 | 0 | 0 |
| 6-2 | 214 | 0 | 0 | 0 | 0 |
| 6-9 | 105 | 7 | 0 | 0 | 0 |
| 6-17 | 98 | 135 | 0 | 0 | 0 |
| 6-24 | 46 | 333 | 30 | 0 | 0 |
| 7-1 | 34 | 309 | 223 | 0 | 0 |
| 7-8 | 3 | 73 | 167 | 2 | 0 |
| 7-15 | 0 | 15 | 110 | 23 | 0 |
| 7-22 | 0 | 1 | 40 | 103 | 0 |
| 7-29 | 0 | 0 | 6 | 93 | 1 |
| 8-5 | 0 | 0 | 0 | 9 | 9 |
| 8-12 | 0 | 0 | 0 | 2 | 3 |
| 8-19 | 0 | 0 | 0 | 0 | 16 |
| 8-26 | 0 | 0 | 0 | 0 | 12 |
| 9-2 | 0 | 0 | 0 | 0 | 17 |
| 9-9 | 0 | 0 | 0 | 0 | 14 |
| 9-16 | 0 | 0 | 0 | 0 | 8 |
| 9-23 | 0 | 0 | 0 | 0 | 23 |
| 9-30 | 0 | 0 | 0 | 0 | 27 |
| 10-7 | 0 | 0 | 0 | 0 | 23 |
| 10-13 | 0 | 0 | 0 | 0 | 26 |
| 10-21 | 0 | 0 | 0 | 0 | 4 |
| 10-28 | 0 | 0 | 0 | 0 | 0 |

Appendix 6. Observed data of phenological change of *L. deliatula* in the *A. altissima* (CA, 2012)

| Sampling Date | 1st instar | 2nd instar | 3rd instar | 4th instar | Adult |
|---|---|---|---|---|---|
| 5-4 | 0 | 0 | 0 | 0 | 0 |
| 5-11 | 4 | 0 | 0 | 0 | 0 |
| 5-18 | 73 | 0 | 0 | 0 | 0 |
| 5-25 | 321 | 1 | 0 | 0 | 0 |
| 6-1 | 174 | 4 | 0 | 0 | 0 |
| 6-8 | 74 | 281 | 0 | 0 | 0 |
| 6-15 | 35 | 400 | 9 | 0 | 0 |
| 6-22 | 4 | 29 | 111 | 0 | 0 |
| 6-28 | 0 | 5 | 46 | 0 | 0 |
| 7-6 | 0 | 32 | 24 | 1 | 0 |
| 7-12 | 0 | 4 | 3 | 24 | 0 |
| 7-20 | 0 | 0 | 1 | 6 | 0 |
| 7-27 | 0 | 0 | 0 | 8 | 3 |
| 8-3 | 0 | 0 | 0 | 0 | 9 |
| 8-9 | 0 | 0 | 0 | 0 | 6 |
| 8-17 | 0 | 0 | 0 | 0 | 3 |
| 8-25 | 0 | 0 | 0 | 0 | 6 |
| 8-30 | 0 | 0 | 0 | 0 | 9 |
| 9-7 | 0 | 0 | 0 | 0 | 15 |
| 9-14 | 0 | 0 | 0 | 0 | 25 |
| 9-21 | 0 | 0 | 0 | 0 | 54 |
| 9-28 | 0 | 0 | 0 | 0 | 53 |
| 10-4 | 0 | 0 | 0 | 0 | 48 |
| 10-11 | 0 | 0 | 0 | 0 | 42 |
| 10-19 | 0 | 0 | 0 | 0 | 24 |
| 10-26 | 0 | 0 | 0 | 0 | 4 |

Appendix 7. Observed data of phenological change of *L. deliatula* in the *A. altissima* (SW, 2012)

| Sampling Date | 1st instar | 2nd instar | 3rd instar | 4th instar | Adult |
|---|---|---|---|---|---|
| 5-11 | 0 | 0 | 0 | 0 | 0 |
| 5-18 | 45 | 0 | 0 | 0 | 0 |
| 5-21 | 142 | 0 | 0 | 0 | 0 |
| 5-23 | 108 | 0 | 0 | 0 | 0 |
| 5-25 | 38 | 0 | 0 | 0 | 0 |
| 5-29 | 60 | 0 | 0 | 0 | 0 |
| 6-1 | 63 | 13 | 0 | 0 | 0 |
| 6-8 | 14 | 338 | 0 | 0 | 0 |
| 6-15 | 1 | 202 | 48 | 0 | 0 |
| 6-22 | 0 | 35 | 457 | 0 | 0 |
| 6-28 | 0 | 8 | 91 | 17 | 0 |
| 7-6 | 0 | 0 | 36 | 163 | 0 |
| 7-12 | 0 | 0 | 6 | 35 | 0 |
| 7-20 | 0 | 0 | 0 | 55 | 2 |
| 7-27 | 0 | 0 | 0 | 7 | 34 |
| 8-3 | 0 | 0 | 0 | 2 | 69 |
| 8-9 | 0 | 0 | 0 | 1 | 62 |
| 8-17 | 0 | 0 | 0 | 0 | 45 |
| 8-25 | 0 | 0 | 0 | 0 | 14 |
| 8-30 | 0 | 0 | 0 | 0 | 8 |
| 9-7 | 0 | 0 | 0 | 0 | 17 |
| 9-14 | 0 | 0 | 0 | 0 | 25 |
| 9-21 | 0 | 0 | 0 | 0 | 20 |
| 9-28 | 0 | 0 | 0 | 0 | 30 |
| 10-4 | 0 | 0 | 0 | 0 | 12 |
| 10-11 | 0 | 0 | 0 | 0 | 8 |
| 10-19 | 0 | 0 | 0 | 0 | 21 |
| 10-26 | 0 | 0 | 0 | 0 | 14 |

# Part II

## Chapter 1. Isolation and characterization of microsatellite markers

## Abstract

Polymorphic DNA markers like microsatellites are widely used for characterizing dispersal patterns and capacity of invasive insect pests which can contribute to designing effective management of the species. To facilitate such population genetic studies of *L. delicatula* in Korea, we isolated and characterized eight microsatellite loci for *L. delicatula* using a hybridization-biotin enrichment method. We further used these novel microsatellite loci to determine population genetic parameters for 33 *L. delicatula* specimens collected from Cheonan, South Korea where outbreaks of this species were first reported in Korea. The number of alleles per locus ranged from three to ten, with an average of 6.25. The mean expected ($H_E$) and observed heterozygosities ($H_O$) were 0.575 and 0.626, respectively. The eight loci showed no deviation from Hardy–Weinberg equilibrium according to the adjusted significance threshold ($P$=0.00625), and there was no linkage disequilibrium between each pair of these eight markers. Bayesian cluster analysis using the program structure revealed no evidence of genetic structuring in *L. delicatula* samples from Cheonan. These new microsatellite markers will be widely

applicable to future ecological genetic studies of *L. delicatula*, including assessment of the level of gene flow and genetic connectivity among populations that are necessary for effective management and monitoring of the species.

Keywords : Microsatellite, Biotin-enrichment method, Population genetics

## 1-1. Introduction

The gaps existed between observation and invisible phenomenon, when ecological study was conducted. Field studies were providing the knowledge on the interaction among *L. delicatula* population and their environmental conditions. But, the questions have been raised unsolved invisible phenomenon (e.g. insect behavior). Therefore, molecular method has been used widely in ecological study by providing the evidence both natural and laboratory studies, so-called molecular ecology. To achieve its objective, selecting appropriated molecular markers is important to gain the precise answers on the raised questions.

Microsatellite consists of short runs of usually di, tri, or tetra nucleotide repeats that are scattered throughout the genome (Goldstein and Schlotteter, 1999). PCR-based analyses and species specific primers were easily identified both homozygotes and heterozygotes on the polycrylamide gels. And, fluorescent labeled PCR product can analyze the allele using the automated DNA sequencers. The good markers for molecular population genetics would be cheap and easy to develop and to use, high polymorphic and neutral with respect to natural selection.

Codominant marker such as microsatellite is the most powerful tools to investigating the genetic properties of populations. Also, it can

evaluate DNA variability and differentiation among closely related populations in the field. High polymorphic DNA markers such as microsatellites have been successfully used to characterize the genetic structure and level of genetic diversity among populations of migratory insects (Kim *et al*., 2009; Kim *et al*., 2006; Kim *et al*., 2008).

To understand the invasion ecology of *L. delicatula* in Korea, it is important to determine the geographical location source populations and to understand patterns of dispersal and gene flow in regions where outbreaks of the species have been reported.

In this study, we report the isolation and characterization of microsatellite markers from *L. delicatula* using the bio-enrichment method. Also, the genetic variability was evaluated from Cheonan population in Korea.

## 1-2. Material and methods

### Microsatellite marker development

Polymorphic microsatellites were isolated from *L. delicatula* genomic DNA based on the biotin-enrichment methods described by Ronald *et al*. (2000) and Kim and Sappington (2004). Genomic DNA was extracted using a Puregene Core Kit (QIAGEN, Germany). Extracted genomic DNA was digested with NdeII (Promega, USA), and fragments larger than 400 bp were screened using Chroma Spin-400 columns (Clontech, USA). One microgram of the NdeII linkers EP-1 (CCC CCA CCT CCT GCC CAT CAT AAA AAA TC) and EP-2 (GAT CGA TTT TTT ATG ATG GGC AGG AGG TGG GGG, 5'-phosphorylated, for NdeII), described in Ronald *et al*. (2000), were ligated to DNA fragments by adding 20 μl of T4 DNA ligase (Promega, USA)(Fig. 1(a)). These attached linkers provide a priming site for EP-3 (CCC CCA CCT CCT GCC ATC AT) in the initial polymerase chain reaction (PCR) amplification. PCR reaction mixtures contained 1 X PCR buffer, 2 μM MgCl2, 0.2 μM each dNTP, 1.6 μM EP-3 primer, 1.5 U i-Star Taq DNA polymerase (iNtRON Inc. Korea), and 20 ng of genomic DNA template in a final volume of 30 μl. PCR was performed with an initial denaturation for 2 min at 94 °C, followed by 30 cycles of 1 min at 65 °C, 30 sec at 72 °C, 2 min at 72 °C; this was followed

by a final extension step of 5 min at 72°C(Fig.1(b)). Unattached residual linkers less than 100 bp in size were discarded by running the completed PCR reactions through Microcon 100 columns (Millipore Corporation, USA). One microliter each of biotinylated capture probe, 5'biotin (CA)15, 5'biotin (CT)15, and 5'biotin (AGC)7 was annealed to 10 µl of linker-ligated DNA in 89µlof 5 X SSC, and then this mixture was heated at 95 °C for 10 min, cooled on ice for 30 sec, and incubated for 5 min at room temperature. To bind the biotinylated probes, we added 100µlof washed magnetic beads (1 mg-µl) to the DNA, followed by a15min incubation at room temperature. Residual unattached fragments were removed through three washes with 200 µl 2 X SSC at room temperature and then three washes with 200µlof 1 X SSC at an optimized temperature for 3 min (65 °C for (CA)15, 61 °C for (CT)15, and 67 °C for (AGC)7). The DNA was eluted from the beads into 50 µl of water after incubation for 5 min at 95 °C. The repeat sequences were amplified with the EP-3 primer. PCR products were ligated into the pGEM®-T Vector (50 ng-µl) and transformed into Escherichia coli JM109 competent cells (Promega, USA). Positive clones were screened with M13 forward and reverse primers using the method described in Schuelke 2000. Amplification of the presumptive microsatellite markers was conducted in a 10µl reaction volume containing

2.5 μM MgCl2, 0.2 μM dNTPs, 1 μM each primer (M13 forward, M13 reverse, one of the internal repeat primers; (CA)12, (CT)12, (AGC)6), 0.25 U of i-Star Taq DNA polymerase (iNtRON Inc., Korea), and 10-50 ng of template. The PCR was performed under the following conditions: initial denaturation for 5 min at 96 °C, followed by 35 cycles of 96°C for 1 min, 50 °C for 1 min, and 72 °C for 1 min; a final extension step was performed at 72 °C for 1 min. PCR products were electrophoresed on agarose gels to select repeated sequence inserts (products with a smeared band pattern (Fig. 1(c)), and these products were sequenced using either a forward or reverse M13 primer. Primers for microsatellite amplification were designed using the program Primer 3 (Rozen and Skaletsky, 2000). To screen for microsatellite markers, the primers used to PCR amplify template genomic DNA from 3~4 individuals from each of four geographic locations: Gwang-Ju (N 35° 8′ 33″ E 126° 54′ 46″), Yeong-Gwang (N 35° 18′ 21″ E 126° 32′ 49.9″), Cheonan (N 36° 53′ 50.2″ E 127° 10′ 55.6″), and Suwon (N 37° 16′ 43.1″ E 126° 58′ 53.5″). PCR reaction mixtures contained 6.15 distilled water, 1.0 μM MgCl2, 0.8 μM each dNTP, and 0.05 U of i-Star Taq DNA polymerase (iNtRON Inc., Korea). The PCR was performed under the following conditions: initial denaturation for 5 min at 94 °C, followed by 20 cycles of 94 °C for 20 sec, annealing at 60 °C to 50 °C decreased by

0.5 °C per cycle, 72 °C for 20 sec, followed by 20 cycles of 94 °C for 20 sec, 50 °C for 20 sec, 72 °C for 20 sec, a final extension was performed at 72 °C for 7 min. PCR products were electrophoresed on agarose gels to verify the presence of PCR bands, indicating successful amplification of genomic template across all samples tested. The Forward primer of selected primer sets was labeled with the fluorescent dye for further study.

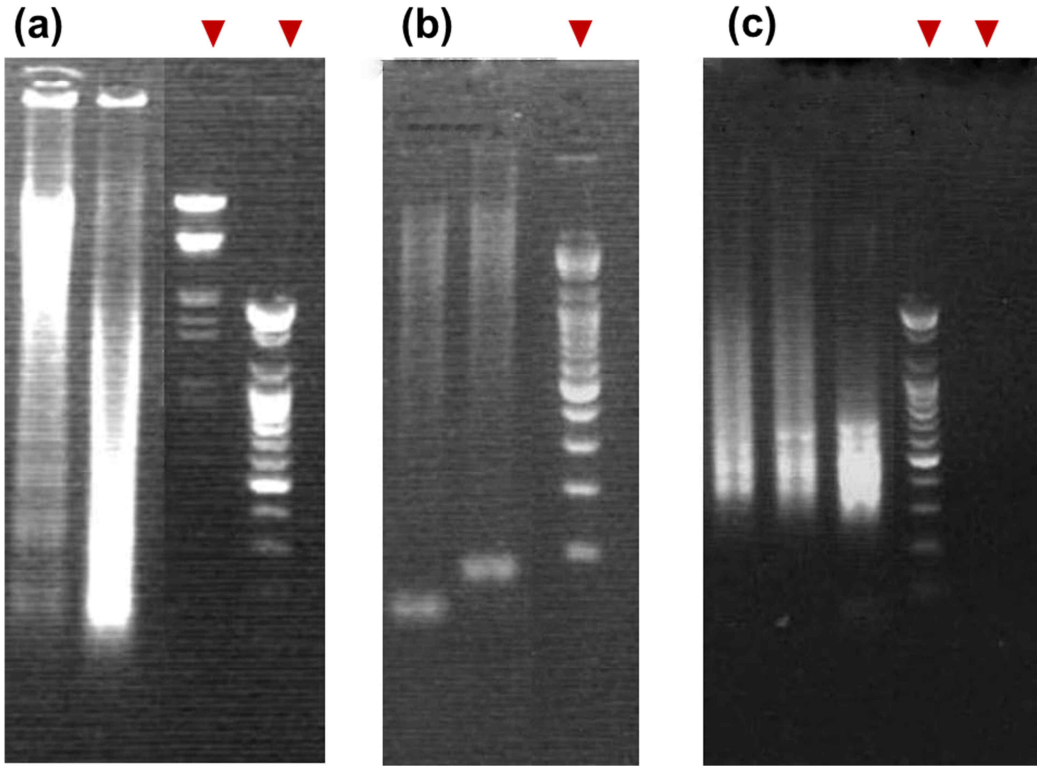



Fig. 1. Electrophoresed on agarose gels of PCR products (a) DNA and ligated DNA fragments with *Nde*II. ∇ indicated Lamda Hind and 100 bp ladder(b) before and after attached the linkers. ∇ indicated 100 bp ladder (c) amplified with M13 and repeat primers $(CA)_{12}$, $(CT)_{12}$, $(AGC)_6$. ∇ indicated 100 bp ladder and negative control.

**DNA samples and genotyping**

All *L. delicatula* samples (thorax, n=33) were collected from Cheonan, Korea in 2010 and were frozen at -20 °C until used for DNA extraction. Genomic DNA was extracted from the thoraces of individuals using the Puregene Core Kit (QIAGEN, Germany).

To genotype the samples, genomic DNA was amplified using the microsatellite primers following the touchdown PCR amplification profile mentioned above with only the change of annealing at 65 °C to 55 °C. PCR reaction mixtures contained 1.0 μM $MgCl_2$, 0.8 μM each dNTP, 0.05 U of i-Star *Taq* DNA polymerase (iNtRON Inc., Korea), 0.5 μM each of the fluorescently labeled forward primer and unlabeled reverse primer, and 10-50 ng of template DNA. For fluorescent detection, the forward primer of each primer pair was labeled with either Hex, 6 Fam or Tamra dyes. Amplified PCR products (alleles) were separated and electrophoresed on an ABI Prism 3730 XL DNA Analyzer (Applied Biosystems Inc., USA) using the GENESCAN-500 [Rox] size standard, and the genotype data were analyzed using GeneMapper version 3.7 (Applied Biosystems Inc., USA).

**Data analysis**

Three measures of genetic diversity, the number of alleles (*A*) per locus, observed heterozygosity ($H_O$), and expected heterozygosity ($H_E$), were calculated using the program GenAlEx version 6.1 (Peakall and Smouse, 2006). $F_{IS}$, the inbreeding coefficient at each locus, represents the reduction of observed heterozygosity from heterozygosity expected under Hardy-Weinberg equilibrium. A significant difference between observed and expected heterozygosity results in a significant $F_{IS}$ value, and may indicate non-random mating, the presence of null alleles, the Wahlund effect or some other anomaly. Deviations from Hardy-Weinberg equilibrium (HWE) and linkage equilibrium between loci were tested using GENEPOP version 4.0.10 (Raymond and Rousset, 1995). The sequential Bonferroni correction was applied to the significance level for multiple comparisons (Rice, 1989). The plausible occurrence of null alleles was tested using the program MICROCHECKER (Oosterhout *et al*., 2004). The presence of null alleles is expected when excess homozygotes are evenly distributed across all alleles at a locus. The population structure of *L. delicatula* in Cheonan was investigated via structure simulation using the Bayesian clustering procedure implemented in Structure 2.3.2 (Pritchard *et al*., 2000). Assuming that the data could be represented as *K* separate

clusters, the log posterior probability of the data for a given *K*, Ln Pr(*X*-*K*), was generated for each of the five STRUCTURE runs at *K* values of 1-5 for the *L. delicatula* sampled. The initial burn-in period was 100,000, followed by 200,000 replications. The mean posterior probability for each K was also calculated. The software was further used for the graphical display of Q-matrix output data from the analysis.

## 1-3. Results

A total of 541 positive (white) colonies 214 for the CA repeat probe, 177 for the AGC repeat probe, and 150 for the CT repeat probe - were obtained after microsatellite cloning. Of these, a total of 195 (36 %) colonies [99-CA (46 %), 49-AGC (28 %) and 47-CT (31 %)] were considered to have inserts of repeat units as determined by a smearing band pattern on agarose gels. Out of 195 colonies, 150 were sequenced with the M13 forward primer and at least 96 (64 %) were confirmed to have repeat sequences. We selected 36 unique sequences (21 CA, 12 AGC, and three CT repeat sequences) with more than five repeats for each sequence unit to design primers for PCR amplification. Of these, 20 primer pairs (12 CA- and eight AGC-targeting primer pairs) produced positive PCR results based on the test panel of four *L. delicatula* DNA samples. Among these, 16 primers pairs that amplified discernable PCR products were further selected, and the forward primer of each primer pair was labeled with one of three different fluorescence dyes (6Fam, Hex or Tamra) for multiplex PCR analysis to reduce the time and cost of genotyping. Finally, eight polymorphic microsatellite markers for *L. delicatula* were selected. Two subsets of microsatellite markers, LD-T1,

LD-T3, LD-D4, LD-5 and LD-D1, LD-D2, LD-T2, LD-D3 were successfully amenable to multiplexing conditions.

A total of 33 adult individuals collected from Cheonan were genotyped with the eight microsatellite loci developed in this study. All microsatellite markers were polymorphic, with the number of alleles per locus ranging from three to ten (mean = 6.25) (Table 1). The mean observed and expected heterozygosity values were 0.575 (0.267-0.903) and 0.626 (0.369-0.837), respectively (Table 1). The Inbreeding coefficient ($F_{IS}$) ranged from -0.242 in LD-T1 to 0.405 in LD-T2, with a mean of 0.103 across loci. The eight loci did not show significant deviation from Hardy-Weinberg proportions at the adjusted significance threshold ($P$ = 0.00625) for multiple testing. However, when the unadjusted significant threshold ($P$< 0.05) was applied, three of eight loci, LD-D2, LD-D3, and LD-T2, showed significant deviation from HWE in the direction of heterozygote deficiency. Of these, two loci, LD-D2 and LD-T2, appeared to harbor a null allele as confirmed by the program Microchecker (Oosterhout's null allele frequency; 0.188 for LD-D2, 0.163 for LD-T2). No significant linkage disequilibrium was found among any of the loci pairs. Structure 2.3.3 software was employed to determine the population structure of the 33 *L. delicatula* individuals sampled from Cheonan in 2010. The highest

likelihood values in all runs were obtained for *K*=1 (Table 2), implying that the *L. delicatula* from Cheonan constitute a single genetic population. Therefore, it is concluded that the 33 *L. delicatula* specimens collected from “Cheonan” where the outbreak of the species was reported in Korea have genetically similar background with no apparent genetic structuring among them.

Table 1. Characteristics of the eight *L. delicatula* microsatellite loci tested in 33 *L. delicatula* specimens from Cheonan, South Korea. Microsatellite primer sequences with fluorescent labeled dyes, repeat motifs, number of individuals (*N*), number of alleles (*A*), size of PCR products in base pairs (bp), expected heterozygosity ($H_E$), observed heterozygosity ($H_O$), *P*-value of the HW test, inbreeding ($F_{IS}$) and GenBank accession numbers are shown.

| Locus | Primer sequence (5'-3') | Repeat motif | N | No. alleles | Size range (bp) | $H_O$ | $H_E$ | *P*-value | $F_{IS}$ | Genbank Accession |
|---|---|---|---|---|---|---|---|---|---|---|
| LD-D1 | F:(6FAM)-CCCAACATATGTCAGCTCCA<br>R:CCCCTGAGTGAATTTTCCAA | **CA** | 31 | 5 | 266-276 | 0.839 | 0.773 | 0.8188 | -0.0692 | JF913272 |
| LD-D2 | F:(6FAM)-GAAACCCAACAAATCGGAAG<br>R: CGGTTTAGTGAGTCTTACACCAA | **CA** | 29 | 10 | 108-142 | 0.517 | 0.837 | 0.0063 | 0.3966 | JF913273 |
| LD-D3 | F:(TAMRA)-GGTCAAAACCGGTCCAGTAG<br>R:AAGGAATCCAGAAAACCGGA | **CA** | 30 | 6 | 122-168 | 0.267 | 0.369 | 0.0313 | 0.2938 | JF913274 |
| LD-D4 | F:(6FAM)-TTAAATCATCAGCCTTATCCACT<br>R:CGGGTAGTTCGGGGATATTT | **CA** | 28 | 8 | 96-158 | 0.786 | 0.819 | 0.3188 | 0.0586 | JF913275 |
| LD- D5 | F:(HEX)-CCTGGAGGTAGGTGATTCCA<br>R:TTGATAGTGTTCATGAGAATGCG | **CA** | 31 | 8 | 168-236 | 0.903 | 0.76 | 0.9688 | -0.1724 | JF913276 |
| LD-T1 | F:(6FAM)-CCGACCTCTACCACTCCTCA<br>R:CTTGTGGCTCCGGTGTATTT | **AGC** | 32 | 3 | 202-211 | 0.500 | 0.398 | 0.9563 | -0.2416 | JF913277 |
| LD-T2 | F:(6TAM)-GAATCCAGACCTCTGCTGGT<br>R:CGGGTAGTTCGGGGATATTT | **AGC** | 31 | 4 | 181-190 | 0.290 | 0.477 | 0.0125 | 0.4053 | JF913278 |
| LD-T3 | F:(TAMRA)-GCACGCGTGAGTGTTATGAT<br>CGCGCTACACTCAACCGTA | **AGC** | 30 | 6 | 155-218 | 0.500 | 0.576 | 0.1688 | 0.1487 | JF913279 |
| Across loci | | | | 6.25 | | 0.575 | 0.626 | 0.4102 | 0.1025 | |

HW test: Hardy–Weinberg exact test (Raymond and Rousset, 1995) with a sequential Bonferroni correction (*P*=0.00625)

Table 2. Likelihood values, Ln Pr(X-K), from Structure analyses (Pritchard *et al*., 2000) to determine the genetic structure of the 33 *L. delicatula* specimens sampled from Cheonan in 2010. The highest mean likelihood value (over five runs at 200,000 replications per run) was for *K*=1 indicating the sample of individuals most likely represents a single genetic population.

| Run | K=1 | K=2 | K=3 | K=4 | K=5 |
|---|---|---|---|---|---|
| 1 | -655.6 | -694.8 | -679.3 | -727.2 | -771.2 |
| 2 | -655.9 | -656.9 | -670.4 | -742.8 | -728.8 |
| 3 | -655.7 | -695.6 | -656.5 | -752.2 | -767.7 |
| 4 | -655.7 | -696.2 | -690.7 | -697 | -748.1 |
| 5 | -655.9 | -657 | -698.1 | -717 | -718.7 |
| *Mean* | -655.76 | -680.1 | -679 | -727.24 | -746.9 |

## 1-4. Discussions

Eight polymorphic microsatellite markers developed in this study have been successfully applied to obtain preliminary population genetics parameters for 33 *L. delicatula* specimens from a location in Korea. Although three of eight loci showed significant deviation from HW proportion at the unadjusted significant threshold with the plausible occurrence of a null allele at two of these loci, these markers can still be used for population genetics studies if analytical methods are used to correct for null alleles (Kim *et al*., 2009).These new microsatellites will facilitate the study of the population and ecological genetics of *L. delicatula* and closely related species in Korea. For example, these microsatellites will be available to trace the unknown origin of this species in Korea using a population assignment strategy successfully employed to identify the origin of cotton boll weevils captured in eradication zones of North America (Kim *et al*., 2006; Kim *et al*., 2008). These markers also could be applicable to elucidate invasion routes of the insects from China to Korea using the Approximate Bayesian Computation method as was done to reveal frequent and ongoing introductions of western corn rootworm from North America to Europe (Miller *et al*., 2005). In addition, these markers will be useful to characterize the dispersal patterns, genetic

connectivity among populations, effective population size, population dimensions, and spatial and temporal variation among geographic populations of *L. delicatula* in Korea and adjacent countries, as has been done with European corn borer (Kim *et al*., 2009). These studies eventually will contribute to designing effective strategies for controlling and monitoring strategies for this species.

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## Chapter 2. Genetic structure of *L. delicaula* populations in Korea:implication for invasion processes in heterogeneous landscapes

## Abstract

To investigate the population structure and dispersal pattern of *L. delicatula* in South Korea, we estimated the population genetic structure and gene flow among nine locations across the country using seven microsatellite markers. Although *L. delicatula* spread throughout most of its geographic range in South Korea within 5–7 years following invasion, its populations show evidence of genetic structuring across the range with a low but significant global $F_{ST}$ (Genetic differentiation across all populations) of 0.0474. Bayesian-based clustering analysis indicates the presence of at least three genetically unique populations in South Korea, including populations in northeastern South Korea, which show a distinct genetic background. However, isolation by distance suggests that populations in South Korea have not yet reached genetic equilibrium. Estimates of the historical rate of gene flow ($N_em$) indicate that relatively high rates of flow have been maintained among populations within the western region, which may indicate recent range expansion. A population assignment test using the first generation migrant detection method

suggest that long-distance dispersal of *L. delicatula* may have occurred over large areas of South Korea. More complex dispersal patterns may have occurred during *L. delicatula* invasion of heterogeneous landscapes in South Korea.

Keywords: invasive pest, microsatellite, genetic structure, dispersal pathway

## 2-2. Introduction

Biological invasion of non-indigenous species to agricultural areas can negatively impact regional crop production as well as other native biota. Biological invasions can occur by long-distance movements that are aided of air currents, but also by human transportation and global trade activities (Hulme, 2009). Biological invasions can be characterized by three key phases: (1) initial introduction into a new habitat, (2) colonization and successful establishment, and (3) dispersal and secondary spread into new habitats or ecological niches (Sakai *et al*., 2001). After the establishment of an invasive species in the recipient country, invasion ecologists focus on features influencing the third invasion phase such as the dispersal mode and subsequent colonization spreading in the recipient regions.

*L. delicatula* has spread from western parts, mainly in Cheonan, and become abundant across South Korea (Han *et al*., 2008), where it causes crop damage, particularly vine yards. The geographic expansion of *L. delicatula* has continued to the eastern region of South Korea, but it remains unclear where the initial introduction occurred and what is the source location of spread to other regions. Range expansion by *L. delicatula* is speculated to have been asymmetric from west to east, which

may be the result of geographical barriers including the mountain range dividing the western and eastern regions of South Korea.

The first instar nymphs of *L. delicatula* appear in May; these molt four times, becoming adults in late July. Mating, ovipositing, and the death of adults occur prior to winter, and the eggs overwinter (Park *et al*., 2009). Successful establishment of this species is thought to be associated with its overwintering abilities and a recent increase in Korean winter temperatures in Korea (Lee *et al*., 2011). Because the range of *L. delicatula* increased rapidly within South Korea, it is thought that movement occurs by both short-range expansion into adjacent areas and also by long-distance dispersal among distant sites, reflecting a stratified dispersal pattern. Short-distance dispersal of *L. delicatula* may be related to different host plant preferences between nymphs and adults. The host plant preferences of *L. delicatula* change during its growth cycle, with a broad range of host plants being fed upon during the nymph stages, but only a few plant species, including *Ailanthus altissima*, acting as food sources in the adult stage (Kim *et al.*, 2011). Therefore, the short-range dispersal behavior of *L. delicatula* may be influenced by the spatial distribution of available host plants. However, its long-distance dispersal ability and the pattern of *L. delicatula* range expansion remain unknown.

Knowledge of the rate of range expansion and the mode of dispersal is required to enable mitigation and pest control strategies to be devised.

Molecular genetic markers enable estimation of the genetic diversity, movement of individuals (Kim *et al*., 2008), inbreeding, and historical patterns of dispersal (Miller *et al*., 2005). Investigations of population demography using molecular genetic marker data have been facilitated by the development of cost-effective methods of data acquisition, as well as the development of statistical approaches that have improved the capacity to estimate the proportion of a population that has moved various distances (Hastings *et al*., 2005). Multilocus genotyping techniques using microsatellite markers have proven to be useful tools for understanding the biology of invasive species. Using microsatellite markers to estimate gene flow is a powerful alternative population assignment technique that has the potential to complement direct methods for measuring contemporary migration (Kim and Sappington, 2006). Using analogous methods, we conducted a population genetics study of *L. delicatula* in South Korea, analyzing microsatellite marker data to estimate gene flow and genetic structuring.

## 2-2. Material and methods

### Study insect and sample collection

*L. delicatula* was collected in 2011 from nine locations throughout their current distributional range in Korea, including the initial occurrence locations (Table 3, Fig. 3). The sedentary behavior of *L. delicatula* nymphs on host plants enabled collection of one individual *L. delicatula* specimen from each host plant, *Vitis vinifera* or *Ailanthus altissima*. Sampled plants were at least 5 m apart to avoid collection of full siblings. The collected specimens were placed in 95 % ethanol and stored at –20 °C until DNA extraction was performed.

### Microsatellite genotyping

DNA was extracted from the stored *L. delicatula* specimens using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA), and the eluted DNA template was diluted ten-fold with deionized water. Seven microsatellite loci previously developed for *L. delicatula* by Park *et al*. (2012) were used for genotyping. Multiplex polymerase chain reaction (PCR) was conducted in two separate reactions: (1) for markers LD-D4, LD-D5, LD-T1, and LD-T3; and (2) for markers LD-D1, LD-D2, and LD-T2. For these reactions we used the i-star Taq PCR Kit (Qiagen) in a total volume of 10 μL, which contained 5.55 μL distilled water, 1.0 μL 10× buffer,

0.8 μL dNTPs, 0.2 μL of each primer, 0.05 Taq, and 1 μL template DNA. The PCR profiles followed a 'touchdown' protocol (Don *et al*., 1991), whereby an initial denaturation of 15 min at 95 °C was followed by 7 cycles of PCR, each consisting of 30 s denaturation at 94 °C, 90 s annealing at 67 °C, 60 s extension at 72 °C, and a 0.5 °C decrease per cycle. A total of 25 cycles were then run with 1 min denaturation at 60 °C.

Table 3. Sampling information for *L. delicatula* specimens collected in South Korea during 2011.

| Sample site | Sample ID | First detection | Sampling year | Coordinates |
|---|---|---|---|---|
| Seoul | SE | 2006 | 2011 | N37°27′ E126°56′ |
| Suwon | SW | 2008 | 2011 | N37°15′ E126°59′ |
| Samcheok | SC | 2010 | 2011 | N37°26′ E129°09′ |
| Chuncheon | CC | 2008 | 2011 | N37°52′ E127°44′ |
| Cheonan | CA | 2004 | 2011 | N36°52′ E127°10′ |
| Okcheon | OC | 2006 | 2011 | N36°18′ E127°34′ |
| Gunsan | GS | 2010 | 2011 | N35°57′ E128°30′ |
| Gwangju | GJ | 2010 | 2011 | N35°09′ E126°55′ |
| Daegu | DG | 2010 | 2011 | N35°52′ E128°30′ |

## Statistical Analysis

### *Genetic variation and genetic structure*

Micro-Checker (Van Oosterhout *et al*., 2004) was used to evaluate potential scoring errors resulting from stuttering, large allele drop-out, and null alleles in the *L. delicatula* microsatellite genotypes. The mean number of alleles per locus and the observed ($H_O$) and expected heterozygosities ($H_E$) were calculated using the Microsatellite Toolkit (Park, 2001). Multiple comparisons were made after applying the sequential Bonferroni correction (Rice, 1989). The Genepop program (Raymond and Rousset, 1995) was used to test deviations from Hardy–Weinberg equilibrium (HWE) conditions.

The program Structure v. 2.3.1 (Pritchard *et al*., 2000) was used to estimate the most likely number of clusters for the datasets by determining the change in the marginal likelihood of the data Pr (X-*K*), where *K* was fixed at different values. The range of possible clusters (*K*) tested was set from 1 to 10, with five iterations. The lengths of the Marcov Chain Monte Carlo (MCMC) iteration and burn-in were set at 100,000 and 200,000, respectively. We used an ancestry model allowing for admixture and correlated allele frequency among populations. The *K* value was estimated using the maximal value of the log-likelihood [Ln Pr (X-*K*)] of the posterior

probability of the data for a given *K* (Pritchard *et al*., 2000). The second order rate of change in the log probability of the data between successive values of Δ*K* (the 'true' number of *K* within the *L. delicatula* sample dataset) was also calculated using $\Delta K = m\,|L''(K)|\text{-}s[L(K)]$ (Evanno *et al*., 2005). We carried out a principal coordinate analysis (PCoA) using the GenAlex program (Peakall and Smouse, 2006). A scatter diagram was plotted based on factor scores along the two PCo axes accounting for most variation.

***Gene flow measures***

Indirect estimates of the historical rates of gene flow between populations ($N_em$) were calculated according to the relationship $N_em = (1 - F_{ST})/4F_{ST}$ (Wright, 1931), where $N_em$ is the effective number of migrants per generation, $N_e$ is the effective population size, and $m$ is the migrant rate. Pairwise estimates of the genetic differentiation ($F_{ST}$) between populations were made using FSTAT v. 2.9.3 (Goudet, 2001). As Micro-Checker revealed the potential occurrence of null alleles on at least one locus for each population (see Table 2), the FreeNA program (Chapuis and Estoup, 2007) was used to estimate $F_{ST}$, which was adjusted for null

alleles (excluding null alleles), and the result was compared to that of $F_{ST}$ assuming no null alleles.

Isolation by distance (IBD) was tested by regressing pairwise population estimates of linearized $F_{ST}/(1 - F_{ST})$ (Rousset, 2000) on the natural log of the geographic distance between all pairs of sample locations, using the Mantel test implemented with Genalex software. Hierarchical partitioning of genetic variation was assessed using analysis of molecular variance (AMOVA) for populations and individuals. AMOVA provides an estimate of the proportion of genetic variation within and between populations.

Population assignment-exclusion tests were conducted by direct and simulation methods using the Geneclass2 program (Piry *et al*., 2004), to detect genetic signatures of dispersal and immigration (Rannala and Mountain, 1997). The direct assignment test allocates an individual to one of the reference populations without probability computation. The test calculates the proportion of individuals correctly assigned to the most likely population of origin, even though the true population of origin is not among the reference populations. In contrast, the exclusion method uses a simulation approach in which the likelihood of a genotype occurring in the population is computed by simulating multilocus genotypes based on the

allele frequencies of each reference population. In this method the likelihood of the genotype of an individual is compared to the distribution of likelihoods of simulated genotypes for each reference population. If the genotype likelihood (a) of an individual is below a predetermined threshold (e.g. a = 0.01), the population is excluded as a possible origin of the individual (Cornuet *et al*., 1999). Unlike the direct assignment method, the exclusion method does not assume that the true population of origin has been sampled because each population is treated independently (Cornuet *et al*., 1999). Frequency probabilities of multilocus genotypes in each reference population were determined in the exclusion test using Monte Carlo simulations of 10,000 independent individuals for the population (Paetkau *et al*., 2004). We followed a Bayesian statistical approach (Rannala and Mountain, 1997) using a Monte Carlo resampling method (Paetkau *et al*., 2004).

To infer contemporary migration of individuals between populations, we employed the *detection of first generation migrants* criterion implemented in Geneclass2 (Piry *et al*., 2004), which assigns each potential individual that traveled from Site A to Site B in year X, or individuals born in year X to a gravid female that moved from Site A to B in year X – 1. Because we do not know whether all source populations for

immigrants were sampled in the current study, two test statistics ($L_{home}$ and the ratio $L_{home}$-$L_{max}$) were used to compute the likelihood of migrant detection (L) (Paetkau *et al*., 2004). The analysis was conducted using a simulation of 10,000 independent individuals at thresholds of *a* = 0.05 and *a* = 0.01. Because the method of Paetkau *et al*. (2004) is intended to measure real time migration between populations, it assumes that all populations are sampled in the same year.

***Bottleneck tests***

The Bottleneck (Cornuet and Luikart, 1996) program was used to assess the evidence for past bottlenecks. We used both a strict stepwise mutation model (SMM) (Ohta and Kimura, 1973) and a two-phase model (TPM) (Di Rienzo *et al*., 1994). The bottleneck test analyzed the heterozygosity excess by comparing the observed number of alleles at each locus, assuming mutation-drift equilibrium. Estimated values were determined using a two-phase model (Piry *et al*., 1999) with an 80% single step mutation proportion, a variance among multiple steps of 12 and 5,000 iterations. The probability of significant heterozygosity excess was determined using the Wilcoxon signed rank test. We also used a model-shift in allele frequency distribution as a qualitative indicator of population

bottlenecks (Luikart *et al*., 1998). The M-ratio of Garza and Williamson (2001), which is the mean ratio of the number of alleles to the range of allele size, was calculated using the AGARST program (Harley, 2001). The M-ratio has a long recovery time following a decline in population size (e.g. > 100 generations), and so enables recent population reductions to be distinguished from those occurring a long time ago. Garza and Williamson (2001) suggested that the M value and its variance across loci could be used as an alternative test for detecting reductions in population size over a much longer time frame.

## 2-3. Results

### Genetic variability

A total of 86 alleles were detected across seven microsatellite loci for 260 *L. delicatula* individuals from among the nine locations in South Korea. Scoring errors resulting from large allele drop-out were not detected in any *L. delicatula* population or locus, but Micro-Checker identified possible stuttering for the marker LD-D4 in population DG and marker LD-T2 in population GS. The presence of potential null alleles was indicated by a general excess of homozygotes for most allele size classes for one or two loci within at least one population (Table 4). The genetic variability estimates for each *L. delicatula* population deduced from the seven microsatellite loci included allelic diversity, the observed ($H_o$) and expected ($H_E$) heterozygosity, the inbreeding coefficient ($F_{IS}$), and the *P* values for deviations from the HWE. Allelic richness varied from 4.904 to 6.709, and $H_E$ ranged from 0.654 to 0.757. Six of nine populations exhibited a significant deviation from HWE following sequential Bonferroni correction for multiple testing. These six populations had positive $F_{IS}$ values across loci with an excess of observed homozygotes (Table 4).

Table 4. Genetic variability estimates for each *L. delicatula* population, inferred from seven microsatellite loci. Number of alleles, expected heterozygosity ($H_E$) at HWE, observed heterozygosity ($H_O$), inbreeding coefficient ($F_{IS}$), probability (*P*-value) of being in HWE, and loci showing potential null alleles.

| Population ID | Sample size | No. of Alleles | Allelic richness | $H_O$ | $H_E$ | $F_{IS}$ | P-value[9] | Loci with null alleles |
|---|---|---|---|---|---|---|---|---|
| SW | 30 | 6.71 | 6.443 | 0.637 | 0.709 | 0.119 | 0.0024 | LD-D2, LD-T3 |
| DG | 27 | 5.29 | 5.206 | 0.603 | 0.711 | 0.155 | 0.0008 | LD-D4 |
| GS | 28 | 7 | 6.709 | 0.63 | 0.725 | 0.133 | 0.0016 | LD-T2 |
| OC | 31 | 6.57 | 6.021 | 0.695 | 0.707 | 0.017 | 0.3183 | LD-T3 |
| SE | 30 | 6.86 | 6.37 | 0.692 | 0.731 | 0.053 | 0.1103 | LD-D2 |
| GJ | 29 | 5.86 | 5.661 | 0.589 | 0.668 | 0.12 | 0.004 | LD-D4, LD-T3 |
| SC | 30 | 5 | 4.904 | 0.532 | 0.654 | 0.189 | 0.0016 | LD-D2 |
| CC | 26 | 6.71 | 6.599 | 0.599 | 0.757 | 0.212 | 0.0008 | LD-D2, LD-T2 |
| CA | 29 | 5.86 | 5.59 | 0.596 | 0.662 | 0.101 | 0.0135 | LD-D2 |

[9] Hardy–Weinberg exact test (Raymond and Rousset, 1995) with Bonferroni correction ($P = 0.00079$).

**Genetic structure within and among populations**

The genetic differentiation between each pair of populations (uncorrected and corrected pairwise $F_{ST}$) and the effective number of migrants exchanged per generation ($N_em$) are shown in Table 5. Uncorrected estimates of pairwise $F_{ST}$ values ranged from −0.0008 for the SW and OC populations (ENA corrected $F_{ST}$ = 0.0009; SE and OC populations) to 0.1294 for the SC and CA populations (ENA corrected $F_{ST}$ = 0.1365; CA and SC populations). Both estimates of $F_{ST}$ ($F_{ST}$ adjusted for null alleles and $F_{ST}$ assuming no null allele results) were similar. Global estimates of $F_{ST}$ across all loci and all populations were low but significant (uncorrected $F_{ST}$ = 0.0474, 95 % CI = 0.0327–0.0634; ENA corrected $F_{ST}$ = 0.0477, 95 % CI = 0.0338–0.0631). The $N_em$ calculated from the uncorrected $F_{ST}$ ranged from 1.68 (CA and SC) to infinity (OC and SW, SE and OC), implying an intermediate to very high level of gene flow.

The M-ratio values, which are used to detect long-term bottleneck events, were generally low in all populations, ranging from 0.497 to 0.578 (Table 6). The results indicated a significant bottleneck event for all populations of *L. delicatula* as recently invading species. However, deviation from mutation-drift equilibrium (under the TPM model) and

mode-shift revealed a signature of recent population reduction only for the DG population ($p$ = 0.0078).

AMOVA analysis among the *L. delicatula* samples revealed that most of the genetic variation was partitioned to among populations and individuals within populations. More than 93 % of the total genetic variation was accounted for by individuals within a population and, correspondingly, 7 % of the total genetic variation was among populations (Table 7). No significant correlation was found between genetic distance and geographic distance among the populations, as evidenced by the Mantel tests of IBD over all samples ($r^2$ = 0.049, $p$ = 0.260), indicating recent range expansion and-or frequent gene flow among populations in South Korea (Fig. 2).

In the PCoA, the mean factor scores for the nine populations were plotted along the first two principal component axes, which together accounted for 72 % of the total variance (40.2 % for axis 1 and 31.97 % for axis 2; Fig. 4). This analysis showed conspicuous divergence of the SC and CA populations from the other populations in South Korea.

Bayesian clustering revealed three clusters. The value of $\Delta K$ calculated from Ln P (D) of the Structure output revealed a maximum value of 23.41 for $K$ = 3 among the genotypes. The average distances among individuals in the same cluster were 0.38 for cluster 1, 0.30 for

cluster 2, and 0.32 for cluster 3; the variance in the mean individual membership within each cluster was accounted for by between-sample site differences (Table 8). This showed that co-ancestry of genotypes provides evidence for three distinct populations (Fig. 3, Fig. 5); 1) one encompassing most of Korea (populations SE, SW, GJ, GS, DG, and OC); 2) population CA; and 3) population SC. Population CA has a genetic structure that differs from that of the other western population.

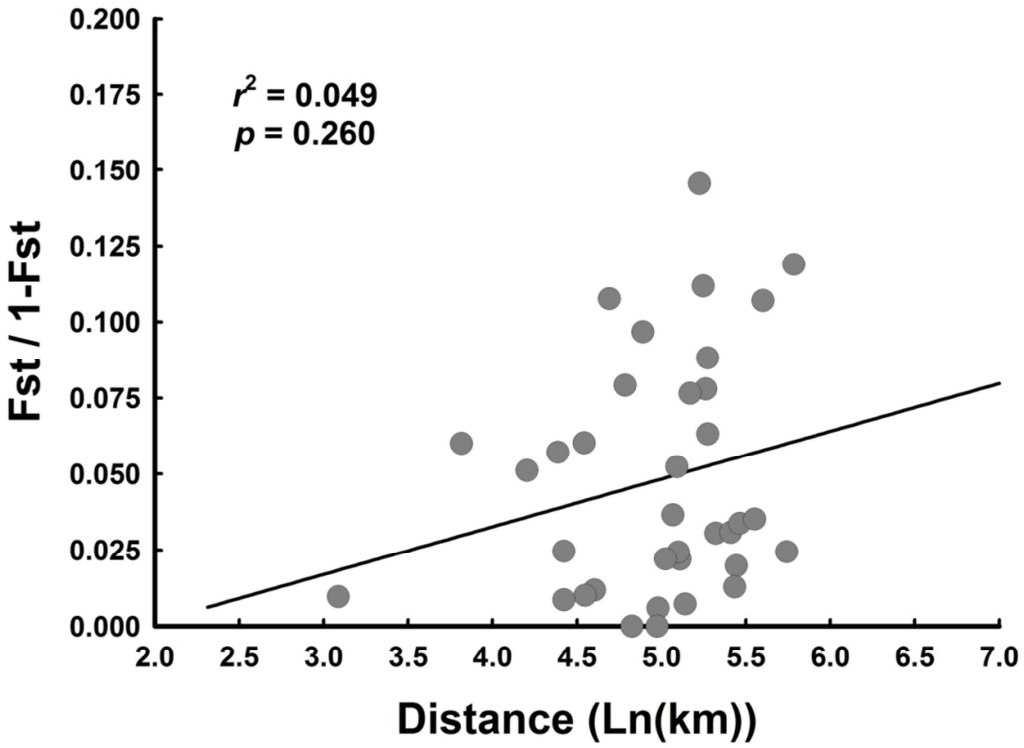


Fig. 2. Geographic distance versus genetic distance ($F_{ST}$ / 1 – $F_{ST}$) for populations of *L. delicatula* using pairwise $F_{ST}$. Correlations and probabilities were estimated from a Mantel test with 10,000 bootstrap repeats.

Table 5. Pairwise estimates of genetic differentiation ($F_{ST}$) (below the diagonal) between *L. delicatula* populations, and gene flow ($N_em = (1 - F_{ST})/4F_{ST}$) inferred from each estimate (above diagonal)

| | SW | DG | GS | OC | SE | GJ | SC | CC | CA |
|---|---|---|---|---|---|---|---|---|---|
| SW | - | 7.0599 | 39.4325 | infinity | 29.8705 | 7.6364 | 3.0137 | 34.9613 | 4.1283 |
| DG | 0.0342*[10] | - | 5.564 | 8.6786 | 7.4187 | 9.75 | 2.0186 | 10.1234 | 4.681 |
| GS | $0.0063^{NS}$ | 0.043* | - | 17.9982 | 10.1234 | 3.9922 | 2.1219 | 13.5622 | 2.2252 |
| OC | $-0.0008^{NS}$ | 0.028* | $0.0137^{NS}$ | - | infinity | 11.3241 | 3.0439 | 41.4167 | 4.2383 |
| SE | $0.0083^{NS}$ | 0.0326* | 0.0241* | $-0.0007^{NS}$ | - | 6.7332 | 2.801 | 34.9613 | 4.8416 |
| GJ | 0.0317* | 0.025* | 0.0605* | 0.0216* | 0.0358* | - | 2.03519 | 11.2179 | 3.835 |
| SC | 0.0766* | 0.1102* | 0.1054* | 0.0759* | 0.0817* | 0.1094* | - | 2.4011 | 1.682 |
| CC | $0.0071^{NS}$ | 0.0241* | $0.0181^{NS}$ | $0.006^{NS}$ | $0.0071^{NS}$ | $0.0218^{NS}$ | 0.0943* | - | 3.1983 |
| CA | 0.0571* | 0.0507* | 0.101* | 0.0557* | 0.0491* | 0.0612* | 0.1294* | 0.0725* | - |

[10] Probability of being different from zero following correction for multiple comparisons. $^*P < 0.05$; NS: not significant. The adjusted nominal level (5 %) for multiple comparisons was 0.001389.

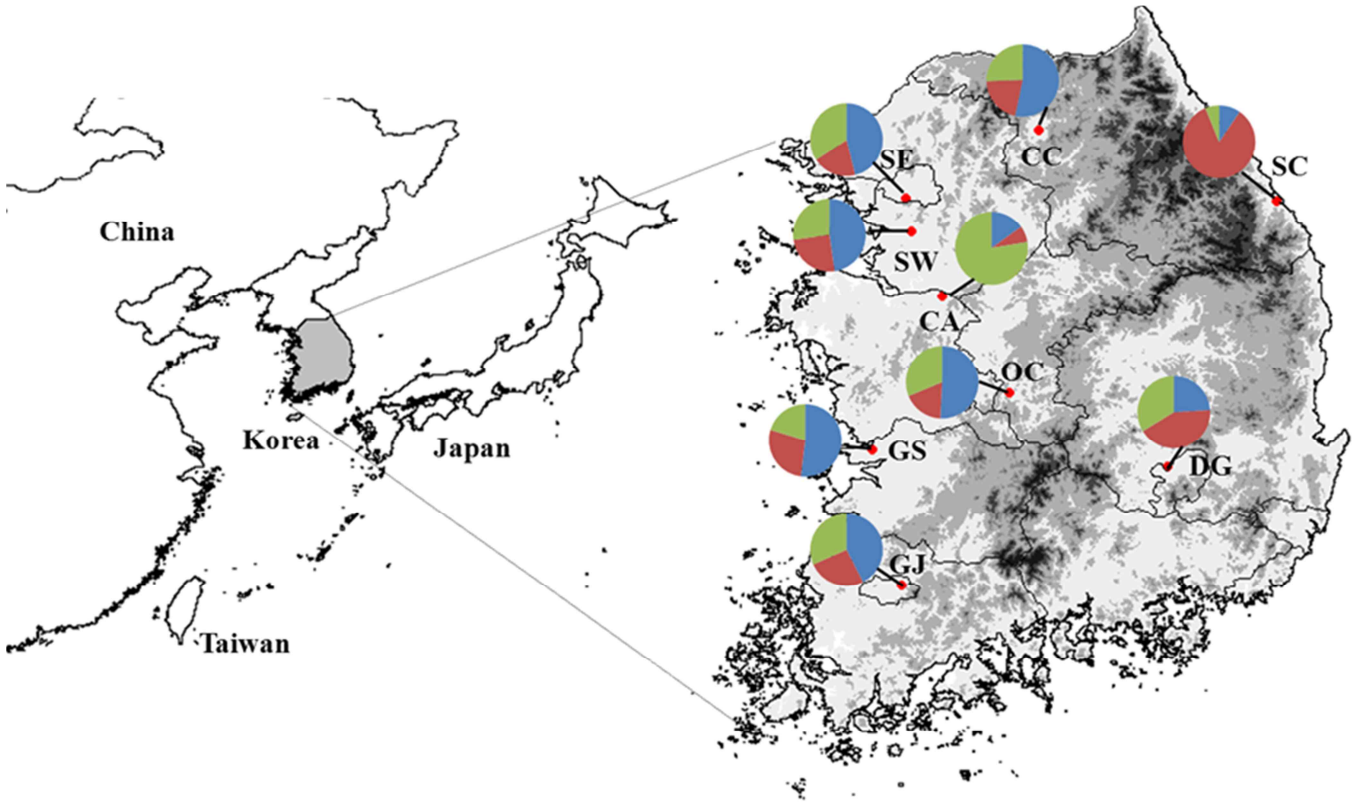


Fig. 3. The pie graphs show the results of a Bayesian cluster analysis of multilocus microsatellite genotypes. Each location is partitioned into $K = 3$ components.

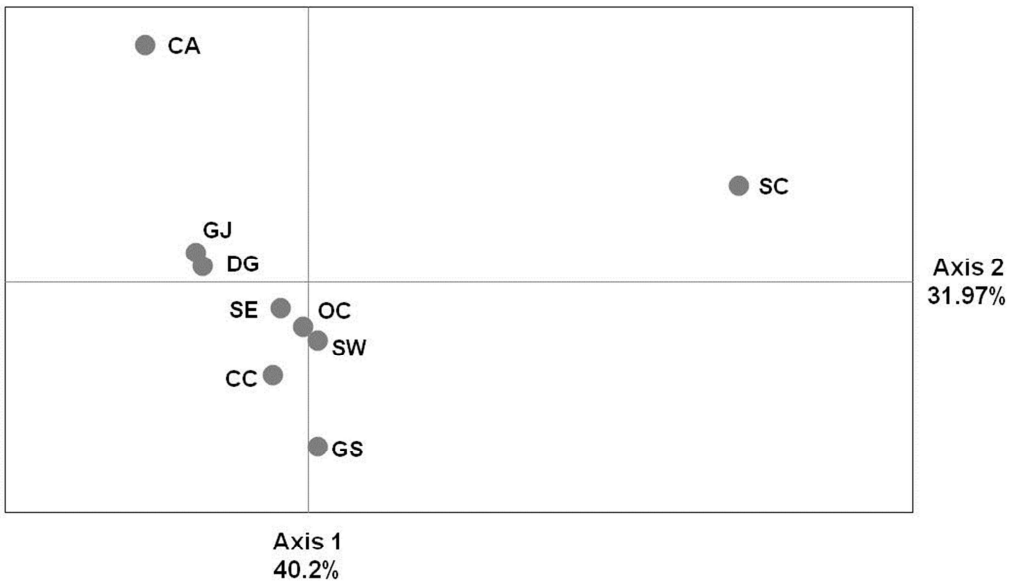


Fig. 4. Scatter diagram of factor scores from a principal coordinate analysis of genotype data for seven microsatellite loci in samples of *L. delicatula* collected from nine locations in South Korea (see Fig. 3). The percentage of total variation attributed to each axis is indicated.

Table 6. M-ratio test results using the stepwise mutation model (SMM) (Ohta and Kimura, 1973) and a two-phase model (TPM) (Di Rienzo *et al*., 1994) to detect a recent population bottleneck event within each *L. delicatula* population. Significance tested using the Wilcoxon sum-rank test ($\alpha$ = 0.05).

| Population | WILCOXON tests[11] | | Mode-shift | M[12] |
|---|---|---|---|---|
| | TPM | SMM | | |
| SW | 0.7109 | 0.9609 | Normal | 0.499 (0.050) |
| DG | 0.0078 | 0.1875 | Shifted | 0.497 (0.097) |
| GS | 0.8556 | 0.9609 | Normal | 0.578 (0.050) |
| OC | 0.9726 | 0.9961 | Normal | 0.559 (0.067) |
| SE | 0.7656 | 0.9804 | Normal | 0.539 (0.053) |
| GJ | 0.7656 | 0.9726 | Normal | 0.540 (0.055) |
| SC | 0.2891 | 0.8516 | Normal | 0.537 (0.055) |
| CC | 0.2891 | 0.8516 | Normal | 0.503 (0.030) |
| CA | 0.9563 | 0.7656 | Normal | 0.537 (0.047) |

[11]One-tail probability for an excess or deficit of observed heterozygosity relative to the expected equilibrium heterozygosity, computed from the observed number of alleles under mutation-drift equilibrium.

[12]M = mean ratio of the number of alleles to the range of allele size (Garza and Williamson, 2001); variance in parentheses.

SMM: stepwise mutation model; TPM: two-phase model of mutation.

Table 7. Analysis of molecular variance (AMOVA) among *L. delicatula* samples from nine locations in South Korea.

| Source of variation | d.f. | Sum of squares | Mean sums of squares | Estimated variance | % |
|---|---|---|---|---|---|
| Among Populations | 8 | 157.11 | 19.638 | 0.476 | 7 |
| Individuals within populations | 251 | 1479 | 5.892 | 5.892 | 93 |
| Total | 259 | 1636.1 | | 6.368 | 100 |

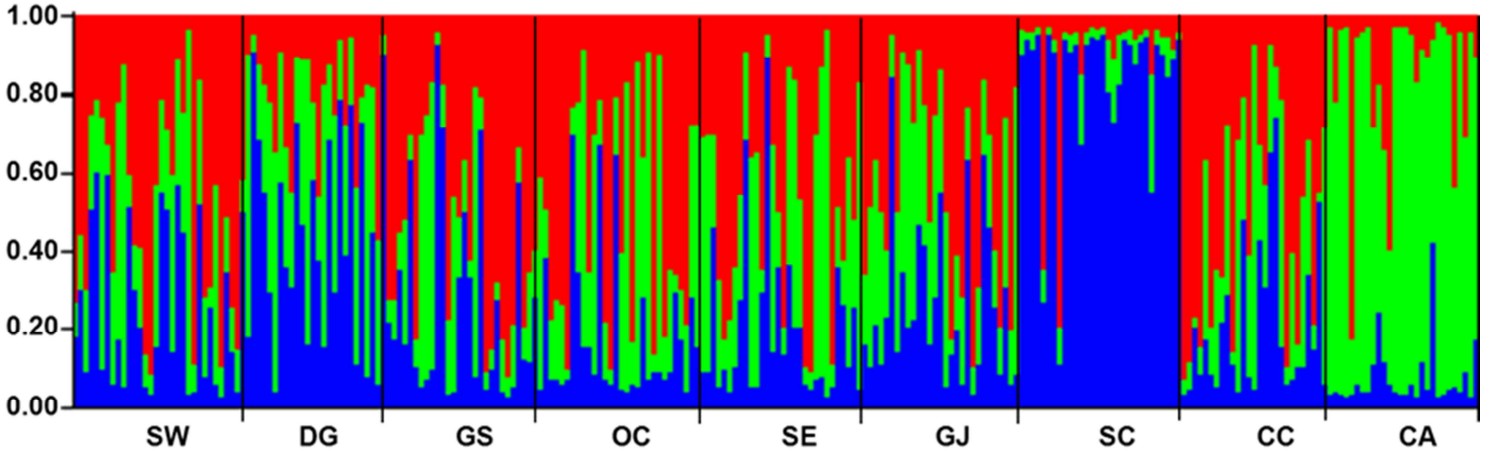


Fig. 5. Bar plot of population structure estimates for 260 *L. delicatula* specimens collected from nine locations in Korea, generated by Structure. The maximum value among genotypes was 23.41 at $\Delta K = 3$, using $\Delta K = m|L''(K)|\text{-}s[L(K)]$ (Evanno *et al*., 2005).

Table 8. Average coefficient of ancestry obtained from a structure analysis with *K* = 3 for 260 *L. delicatula* specimens collected from nine sampling locations in South Korea.

| Sample | Cluster | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| SW | **0.477**[13] | 0.25 | 0.272 |
| GS | **0.52** | 0.278 | 0.203 |
| OC | **0.509** | 0.179 | 0.313 |
| SE | **0.462** | 0.195 | 0.343 |
| GJ | **0.425** | 0.259 | 0.316 |
| CC | **0.534** | 0.21 | 0.256 |
| CA | 0.152 | 0.072 | **0.776** |
| SC | 0.096 | **0.843** | 0.062 |
| DG | 0.24 | **0.423** | 0.337 |

[13]The highest value of co-ancestry for each population in a cluster is shown in bold.

**Assignment-exclusion test and detection of first generation migrants**

We calculated the percentage of *L. delicatula* individuals from each population and excluded as potential immigrants at a threshold of *a* = 0.01, and the mean assignment log-likelihood for each possible donor population (Table 9). Populations from most locations contained members whose potential origins in other populations could be excluded with ≥99 % certainty. Individuals from the SC population could be assigned to their own population with >90 % certainty, and individuals from DG and CA populations were assigned to their own population with 74.1 % and 65.5 % certainty, respectively. In the exclusion test the SC population could be excluded with 34.5 –75.9 % certainty (0.01 threshold) as a putative origin of all populations. The assignment and exclusion values were evenly distributed among the other populations. The mean estimated individual assignment likelihood indicated that the highest assignment likelihood of individuals of the OC population (apart from itself) come from the SW population (mean assignment log-likelihood = –7.68). Similarly, the highest assignment likelihood of SW individuals was from the OC population (–8.20) (Table 9).

The number of immigrant individuals estimated for the current generation is summarized in Table 10. When the $L_{home}$-$L_{max}$ ratio was

considered for the detection of first generation migrants between sample locations, a total of 36 and 14 individuals were detected as probable first generation migrants at thresholds of *a* = 0.05 and *a* = 0.01, respectively. This result suggests that the OC population received individuals from each of the SE, CC, and CA populations. The proposed dispersal pathway among populations is shown in Figure 6, based on the result of 'detection of first generation migrants'. The results indicate that there was movement of *L. delicatula* to adjacent locations and, unexpectedly, that long-distance dispersal beyond the geographical barrier also occurred (Fig. 6).

Table 9. Percentage of *L. delicatula* individuals assigned to and excluded from (i.e. determined to not be a potential immigrant from) each reference population, and the mean assignment log-likelihood for individuals from each geographic population to possible source populations.

| Sample location | Method | Potential source (reference) population | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | SW | DG | GS | OC | SE | GJ | SC | CC | CA |
| SW | Assignment[14] | 6.67 (2) | 10 (3) | 16.7 (5) | 33.3 (10) | 13.3 (4) | 3.3 (1) | 0 (0) | 13.3 (4) | 3.3 (1) |
| | Exclusion[15] | 3.33 (1) | 23.3(7) | 3.3 (1) | 3.3 (1) | 0 (0) | 16.7 (5) | 40 (12) | 3.3 (1) | 33.3 (10) |
| | -LOG(L)[16] | 8.22 | 10.28 | 8.48 | **8.20** | 8.60 | 8.81 | 11.23 | 8.42 | 9.76 |
| DG | Assignment | 0 (0) | 74.1 (20) | 7.4 (2) | 0 (0) | 3.7 (1) | 11.1 (3) | 3.7 (1) | 0 (0) | 0 (0) |
| | Exclusion | 11.1 (3) | 3.7 (1) | 7.4 (2) | 7.4 (2) | 18.5 (5) | 29.6 (8) | 55.6 (15) | 14.8 (4) | 51.9 (14) |
| | -LOG(L) | 9.60 | 7.45 | 9.55 | 9.68 | 10.06 | **9.41** | 12.40 | 9.66 | 11.33 |
| GS | Assignment | 14.3 (4) | 7.1 (2) | 42.9 (12) | 21.4 (6) | 0 (0) | 0 (0) | 3.6 (1) | 10.7 (3) | 0 (0) |
| | Exclusion | 3.6 (1) | 21.4 (6) | 0 (0) | 3.6 (1) | 10.7 (3) | 25 (7) | 57.1 (16) | 3.6 (1) | 50 (14) |
| | -LOG(L) | 8.81 | 10.23 | 8.35 | **8.66** | 9.41 | 9.82 | 12.24 | 9.07 | 11.03 |
| OC | Assignment | 25.8 (8) | 3.2 (1) | 13.0 (4) | 22.6 (7) | 13.0 (4) | 6.5 (2) | 0 (0) | 6.5 (2) | 9.7 (3) |
| | Exclusion | 0 (0) | 6.5 (2) | 0 (0) | 0 (0) | 6.5 (2) | 6.5 (2) | 45.2 (14) | 0 (0) | 22.6 (7) |
| | -LOG(L) | **7.68** | 9.28 | 7.94 | 7.76 | 8.06 | 8.53 | 11.41 | 8.18 | 9.17 |
| SE | Assignment | 10 (3) | 3.3 (1) | 6.7 (2) | 16.7 (5) | 36.7 (11) | 6.7 (2) | 3.3 (1) | 13.3 (4) | 3.3 (1) |
| | Exclusion | 6.7 (2) | 20 (6) | 6.7 (2) | 9.7 (3) | 3.3 (1) | 26.7 (8) | 53.3 (16) | 6.7 (2) | 30 (9) |
| | -LOG(L) | 8.94 | 10.64 | 9.50 | **8.78** | 8.43 | 9.71 | 12.49 | 8.95 | 10.28 |
| GJ | Assignment | 6.9 (2) | 6.9 (2) | 0 (0) | 6.9 (2) | 6.9 (2) | 51.7 (15) | 0 (0) | 20.7 (6) | 0 (0) |
| | Exclusion | 0 (0) | 6.8 (2) | 0 (0) | 0 (0) | 0 (0) | 3.4 (1) | 34.5 (10) | 0 (0) | 17.2 (5) |
| | -LOG(L) | 8.72 | 9.47 | 9.06 | 8.58 | 8.89 | 7.46 | 11.45 | **8.27** | 9.66 |
| SC | Assignment | 0 (0) | 0 (0) | 6.7 (2) | 3.3 (1) | 0 (0) | 0 (0) | 90 (27) | 0 (0) | 0 (0) |
| | Exclusion | 10 (3) | 36.7 (11) | 6.7 (2) | 23.3 (7) | 16.7 (5) | 46.7 (14) | 0 (0) | 16.7 (5) | 63.3 (19) |
| | -LOG(L) | **10.15** | 11.78 | 10.34 | 10.61 | 10.17 | 10.40 | 6.76 | 10.70 | 12.43 |
| CC | Assignment | 11.5 (3) | 3.8 (1) | 15.4(4) | 11.5(3) | 15.3 (4) | 19.2 (5) | 0 (0) | 19.2 (5) | 3.8 (1) |
| | Exclusion | 3.8 (1) | 38.5 (10) | 0 (0) | 3.9 (1) | 0 (0) | 30.8 (8) | 73.1 (19) | 0 (0) | 65.4 (17) |
| | -LOG(L) | **9.10** | 10.89 | 9.32 | 9.26 | 9.16 | 9.23 | 13.01 | 8.80 | 11.31 |
| CA | Assignment | 10.3 (3) | 3.4 (1) | 0 (0) | 10.3(3) | 0 (0) | 6.9 (2) | 0 (0) | 3.4 (1) | 65.5 (19) |
| | Exclusion | 0 (0) | 27.6 (8) | 24.1 (7) | 13.8 (4) | 6.9 (2) | 44.8 (13) | 75.9 (22) | 6.9 (2) | 3.4 (1) |
| | -LOG(L) | **9.29** | 11.42 | 11.62 | 9.96 | 9.61 | 10.98 | 13.87 | 10.77 | 7.70 |

[14] The number of individuals assigned to the most likely population is shown in parentheses.

[15] The number of individuals excluded from the reference population $p$ =0.01 is shown in parentheses.

[16] Mean assignment –log likelihood (L) value for individuals from a given sample population. Bolding indicates the value most similar to that of the sample population, and therefore represents the population from which it most likely originated, under the assumptions of the test.

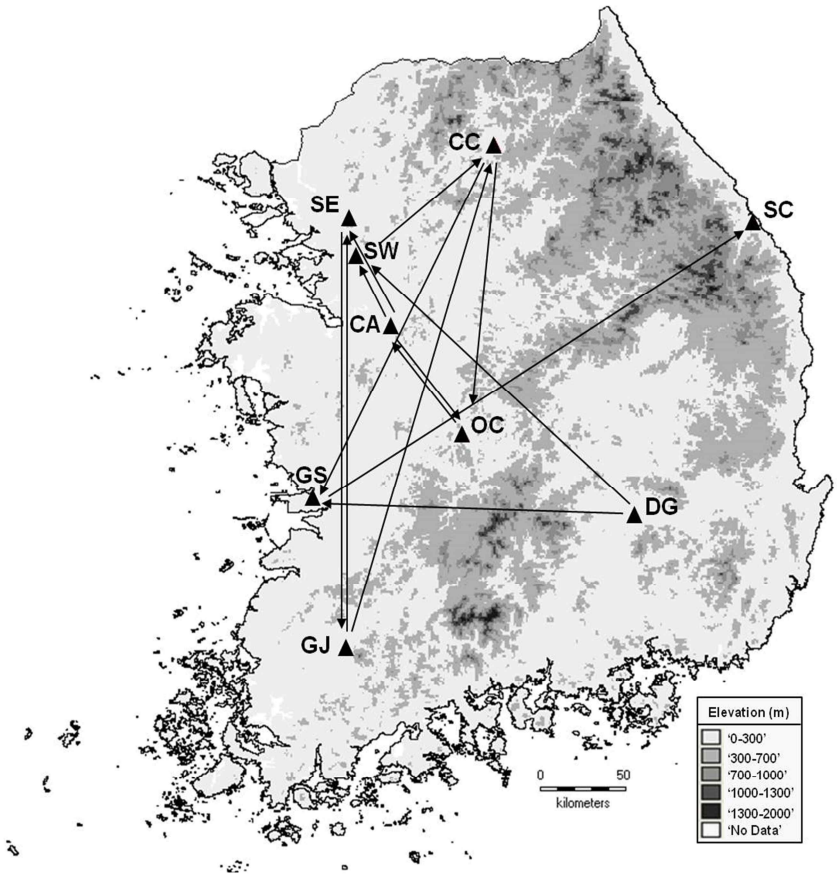


Fig. 6. Dispersal pathway of *L. delicatula* populations in South Korea. The arrows indicate the probable source and recipient populations of first generation migrants detected using the $L_{home}$-$L_{max}$ statistic.

Table 10. Number of probable first-generation migrants identified in each population of *L. delicatula*, and its putative source population at thresholds of $a$ = 0.05 and $a$ = 0.01 (in parentheses).

| Population | Test statistic[17] | Putative source population | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | SW | DG | GS | OC | SE | GJ | SC | CC | CA |
| SW | $L_{home}$ | - | | | | | | | | |
| | $L_{home}$-$L_{max}$ | - | 1(1) | 1(0) | | | | | | 1(1) |
| DG | $L_{home}$ | | - | | | | | | | |
| | $L_{home}$-$L_{max}$ | | - | 1(0) | | | | | | |
| GS | $L_{home}$ | | | - | | | | | | |
| | $L_{home}$-$L_{max}$ | 2(0) | 1(1) | - | | | | | 1(1) | |
| OC | $L_{home}$ | | | | - | | | | | |
| | $L_{home}$-$L_{max}$ | 2(0) | 1(0) | | - | 2(0) | | | 1(1) | 1(1) |
| SE | $L_{home}$ | | | | | - | | | | |
| | $L_{home}$-$L_{max}$ | 1(0) | | | 2(0) | - | 1(1) | | 1(0) | 1(1) |
| GJ | $L_{home}$ | | | | | | - | | | |
| | $L_{home}$-$L_{max}$ | | | | | 2(1) | - | | 1(0) | |
| SC | $L_{home}$ | | | | | | | - | | |
| | $L_{home}$-$L_{max}$ | | | 2(2) | | | | - | | |
| CC | $L_{home}$ | | | | | | | | - | |
| | $L_{home}$-$L_{max}$ | 1(1) | | 2(0) | | | 1(1) | | - | |
| CA | $L_{home}$ | | | | | | | | | - |
| | $L_{home}$-$L_{max}$ | 2(0) | 1(0) | | 1(1) | | 2(0) | | | - |

The analysis used the assignment criterion described by Rannala and Mountain (1997) and the Monte Carlo resampling method reported by Paetkau *et al*. (2004).

[17]L=likelihood of migrant detection (Paetkau *et al*., 2004).

## 2-4. Discussions

Evidence from this study suggests that there is no significant difference in genetic diversity, allelic richness, or $H_E$ among the *L. delicatula* populations in South Korea. In contrast, a low but significant level of genetic differentiation, based upon $F_{ST}$ estimates, was observed among the populations. A general trend of low level but significant population differentiation has previously been reported among populations of the Chinese mitten crab that have recently established in Europe (Herborg *et al*., 2007). Inbreeding coefficients ($F_{IS}$) indicate a significant difference between observed and expected heterozygosity as a consequence of non-random mating, the presence of a null allele, and/or the Wahlund effect (Wahlund, 1928). Analogously, a non-significant IBD relationship is expected among populations recently introduced to South Korea (such as *L. delicatula*), which show little evidence of genetic divergence. The absence of IBD is indicative of a recent range expansion-high gene flow, or a lack of gene flow coinciding with extensive genetic differentiation among all populations. The latter can be excluded as a possible explanation in relation to the South Korean *L. delicatula* populations, as low levels of genetic differentiation were estimated among the sample populations (Table 5). These analyses further suggest that the

*L. delicatula* populations in South Korea have not reached equilibrium since colonization.

Although the IBD test has been widely used to investigate the spatial patterns of gene flow and genetic relatedness between populations (Wright, 1943), the presence of geographic barriers to dispersal can also limit gene flow. McRae (2006) suggested that the resistance distance of the two-dimensional geographical distances might be a more realistic reflection of the underlying barriers to dispersal, and that geographic distances that integrate heterogeneities in dispersal pathways might be more relevant. For heterogeneous landscapes, use of the resistance distance may help to reveal patterns of IBD that are absent from Euclidean distance estimates. For the spread stage of invading populations the major outstanding study aspects include linking the traits of species to their movement ability, and exploring the impacts of landscape heterogeneity on dispersal success (Puth and Post, 2005). Consequently, to gain a greater understanding of gene flow among populations of *L. delicatula*, our future studies (both laboratory and field) will focus on the dispersal abilities of the species.

The genetic structure analysis, based on PCoA and Structure, indicated that three genetically divergent *L. delicatula* populations are

present in South Korea. The results suggest that simultaneous establishment, rather than point or independent introduction, probably occurred in western South Korea, and that the subsequent outbreak can in part be attributed to colonization of the eastern region (populations SC and DG). It is possible that two temporally separated incursions of *L. delicatula* into the eastern regions occurred. While the CA population (which has an apparently different genetic structure) may have been introduced independently, additional studies are needed to clarify the mode of introduction; such studies should include samples from eastern China.

Assessing bottlenecks is important in determining founder effects underpinning demographical events, especially recent species invasions. Microsatellites are particularly informative in the study of recent population phenomena. A combination of approaches was applied to *L. delicatula*, based on a range of microsatellite characteristics. The M values for the studied populations were less than those expected from historically stable populations (0.82), and also below the M-ratio range (0.599–0.693) for populations that have undergone an historical reduction in population size or recently founding population (Garza and Williamson, 2001). Although the M-ratio for a stable population of *L. delicatula* needs to be established, the results indicate that in Korea this species was subject to a bottleneck

event at some time in the past, as is expected for invasive species. Our mutation-drift equilibrium (under the TPM model) and mode-shift analyses indicated that a bottleneck occurred in the DG population (Table 6). More reliable demographic evidence will require investigations of longer time scales of biological invasion, because short-term population structures may provide only weak and-or incorrect indications of demographic events associated with invasion processes (Fitzpatrick *et al*., 2012).

Although gene flow can serve as a surrogate for dispersal ability (Bohonak, 1999), it has limitations when movement cannot be verified because of insufficient genetic information. Moreover, high gene flow yields within open landscapes can produce populations that are genetically homogenous over great geographic distances. Our assignment-exclusion tests showed similarity of the –log (L) estimates (excluding the highest values) in the potential source population (Table 9), suggesting that the majority of *L. delicatula* sampled in this study were not representative of the initial outbreak source population in South Korea. In a previous study the possible source population of the introduced insect species *Ostrinia nubilalis* in North America was investigated by sampling each geographic population and assessing the qualitative information on gene flow (Kim *et al*., 2009). However, there were limitations in the method

used, as the source population could not be identified because of the high rates of dispersal among populations in the range expansion phase.

First-generation migrant detection can enable inferences to be made regarding the point origin of individuals, and provides the potential to estimate real-time dispersal through detection of immigrant individuals (Paetkau *et al*., 2004; Rannala and Mountain, 1997). The probable source and recipient populations of first generation *L. delicatula* migrants were investigated using the $L_{home}$-$L_{max}$ statistic, which revealed that dispersal probably occurred over long distances as well as among adjacent locations (Fig. 6). It is notable that more frequent dispersal may occur in the western region compared with the eastern region, and that long-distance dispersal may be occurring beyond the mountain range.

Since its first appearance in Cheonan (western Korea) in 2004, the distribution of *L. delicatula* has expanded across South Korea in only 5–7 years. In this study we applied a combination of population genetics analyses to study the invasion dynamics. The presence of a mountain chain appears to have caused only a minor delay in the dispersal of *L. delicatula* from west to east across the country. The dispersal patterns may have influenced the invasion dynamics with naturogenic deceleration (heterogeneous topography) and anthropogenic acceleration (human

activity and ground transportation) of gene flow during the spread phase of *L. delicatula* in South Korea.

Understanding of the invasion process and dispersal pathways is important in the development of effective quarantine measures and for improving biosecurity in relation to invasive insect pests. Genetics should play a greater role in the development of policy to manage and control invasive species because it is important in understanding the invasion process and developing defense mechanisms against invading pests (Allendorf and Lundquist, 2003). Our study indicates that the Korean Peninsula is susceptible to invasive by insects from adjacent countries. Following species invasions, subsequent establishment and spread within South Korea can occur if conductive climatic and ecological conditions are present. For example, the rice water weevil *Lissorhoptrus oryzophilus* Kuschel spread throughout the paddy fields of Korea in a relatively short time period. Rapid dispersal was possible because the host plant is widely distributed, and long-distance dispersal was facilitated by ground transport and human activity in Korea (Lee and Uhm, 1993).This example underscores the importance of a specific framework for preventing the range expansion of invasive pests, based on knowledge of the invasion dynamics in the heterogeneous landscape of Korea.

Invasion is often cryptic in terrestrial habitats during the initial phase of introduction, and detection of pests typically occurs during the dispersal stages, when the species begins to damage native biota. Generalizations about the dynamics of establishment and spread of invasive species are difficult, and consequently assessment of population structure and movement are fundamental to understanding the potential effects of pest introductions within specific regions.

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# Chapter 3. Genetic analysis for dispersal ability in spatiotemporal scales in Korea


## Abstract

Life stage of *L. delicatula* was split into sedentary (nymph) and dispersive (adult) phase within generation. Therefore, we compared the genetic structure between/within generation for dispersal ability. First, we evaluated the sink-source metapopulations between two generations in Korea. Cheonan have a distinct genetic background and relatively larger effective population size *($N_e$)* and lower immigration rate (*m*) than other regions. By comparison of life stages, isolation by graph distance (IBgD) revealed genetic distance was significantly correlated to the geographic distance ($r^2$=0.237, *p*=0.01). AMOVA analysis showed different genetic variation between nymph and post-oviposition populations. Also, subgraph was constructed by post-oviposition in full graph with 29 edges. Totally, genetic difference was revealed between nymph and post-oviposition stage of *L. delicatula* in Korea. Therefore, genetic parameters were partially implied the oviposition is trigger for active dispersal of *L. delicatula*.



Key words: genetic structure, active dispersal, oviposition

## 3-1. Introduction

Life systems of populations was defined what is the determined existence, abundance and evolution of a particular population in their ecosystem (Clark *et al*., 1967). Resource available was major component for constructing the life system of subject population, in particular polyphagous pest which different preference among host plants in agroecosystem (Kennedy and Storer, 2000).

For optimized feeding, mating and reproduction, insect behavior was progressed directly related its development of individual level. Host-habitat fidelity was critical to determine its existence varying in availability and suitability habitats, which genetically well suited to one habitat has relatively poor survivorship on others (Berlocher and Feder, 2002). Also, insect phenology may form sequentially to maximize the probability of survival abilities occurring at an appropriate host plant. Relative abundance among host plants may provide the evidence of the fidelity in nature, especially polyphagous insect with hierarchal preference. Sometimes, environmental factors (e.g. resource availability, habitat permanent) were affected on insect dispersal, which is act as ‘surrogates’ of the host/habitat preference (Loxdale and Lushai,1999).

Life history of *L. delicatula* was split into sedentary (nymph) and dispersive (adult) phase within generation in Korea. When it nymph, they sucked many plants, recorded 27 plants only nymph stage, as gregarious and sedentary, otherwise, it was move to a few plants mainly *V. vinifera* and *A. altissima* (commonly known to tree-of-heaven) in adult stage (Kim *et al*., 2011). Therefore, the discrepancy of mobility may create the different gene flow between life stages of *L. delicaula*. The relative strength of gene flow may be influenced by host specialization, habitat persistence and habitat structure within the spatiotemporal scale (Peterson and Denno, 1998). *A. altissima* and *V. vinifera*. were known to primary host plant of *L. delicatula*, both China (Hua, 2000) and Korea (Park *et al*., 2009). After establishment, adults of *L. delicatula* significant damage to *V. vinifera,* otherwise, *A. altissima* was considered as nursery host for nymph stages in Korea. Also, they cannot survive in solely existed *A. altissima* in China (Li and Tao, 1980; Yao and Liu, 1993). Difference of ecological pressure may display the different preference of host utilization for polyphagous insect among host plants. Biological invasion can provide the enemy and/or parasitoid free and resource-rich circumstances in natural population, what is possible to easily be observed and measured forced its host-choice polymorphism.

The oviposition of *L. delicatula* was considered specific time for survival strategy in that strongly preferred only few plants (Kim *et al*., 2011). Also, demographic structures across the life stages were partially implied the oviposition-mediated dispersal of *L. delicatula* in agricultural environments (detailed descripted in Chapter 3). Therefore, the habitat structure may act critical factor for determination of its distribution and abundance in Korea. Interaction between more and less favorable habitats has promoted comprehend the dispersal ability in spatial scales, from perspective of sink-source metapopulations (Hanski, 1998). Genetic studying has supported the ecological questions by solving the invisible phenomenon, so-called by ecological genetics (Sara, 1990). Molecular markers are versatile method for monitoring the dispersal at a variety of spatial and temporal scales, with different degrees of resolution. Microsatellite markers, largely selective-neutral markers, strongly indicated that migration characteristics of insect field populations (Endersby *et al*., 2006). Previously, invasion dynamics with dispersal pathway of *L. delicatula* was revealed using the microsatellite throughout the Korea across the topographical barrier, mediated by anthropogenic force like a transport (Park *et al*., 2013).

Effective population sizes ($N_e$) and migration rates ($m$) are critical parameters that population survival and reproduction success also determine the relative influence of selection and genetic drift (Watt *et al*., 2007). Also, population persistence was correlated with the inbreeding level and loss of allele frequent passed by the generations (Keller and Waller, 2002).These finding may facilitate the management strategy, by designated the source populations which is relatively wide effective population size and high levels of immigration rate, for invasion populations of *L. delicatula* in Korea.

In this study, we were aimed to reveal the relationship of sink-source metapopulations for recently invaded *L. delicatula* in Korea. And, we focused on the active dispersal ability among life stages in wild populations. Therefore, we evaluated (1) effective population size and migration rate revealing temporal variation in allele frequencies between two generations (2010 and 2012) of four local populations of Korea. And, we estimated (2) the genetic structure and gene flow among life stages (nymph, pre-oviposition and post-oviposition adults) of *L. delicatula* across the habitats in Korea. In addition, geographical distance and temporal dimension was considered assessing the potential factors affecting the dispersal of *L. delicatula* in Korea.

## 3-2. Material and Methods

### Study insect and sample collection

In 2010*, L. delicatula* was collected in four sites in Korea (Fig. 7). For temporal analyses between generations, we resampled in same locations in 2012. Also, we collected the 1st and 2nd nymphs in mid-May which low movement ability and high fidelity to their natal habitats from all sites in 2012. Adult of *L. delicatula* occurred in late July and laid egg masses were discovered in early October. Adult of *L. delicatula* was collected two times before and after oviposition. Because sampling site was mainly consisted of *A. altissima* in DG, SW and GJ, samples of post-oviposition stage were not obtained in same site. To find out the genetic structure between life stages in file scale, we collected the adults after laying the eggs in four sites within Cheonan. CA2 and CA3 were grape vine yard surrounding *A. altissima* and other plants preferred by nymph of *L. delicatula*. CA 1 and CA 4 was hillock covered with *A. alittisima*. And CA 4 was geographically separated from other sites by the road in Cheonan (Fig. 7). Collecting information was in Table 11.

The sedentary and aggregation behavior of *L. delicatula* nymphs on host plants enabled collection of one individual *L. delicatula* specimen. Also, the distance between sampling plants was placed from each other

by at least 5 m to avoid collection of its full siblings. The collected *L. delicatula* specimens were placed in 95% ethanol and stored at -20 °C until DNA extraction.

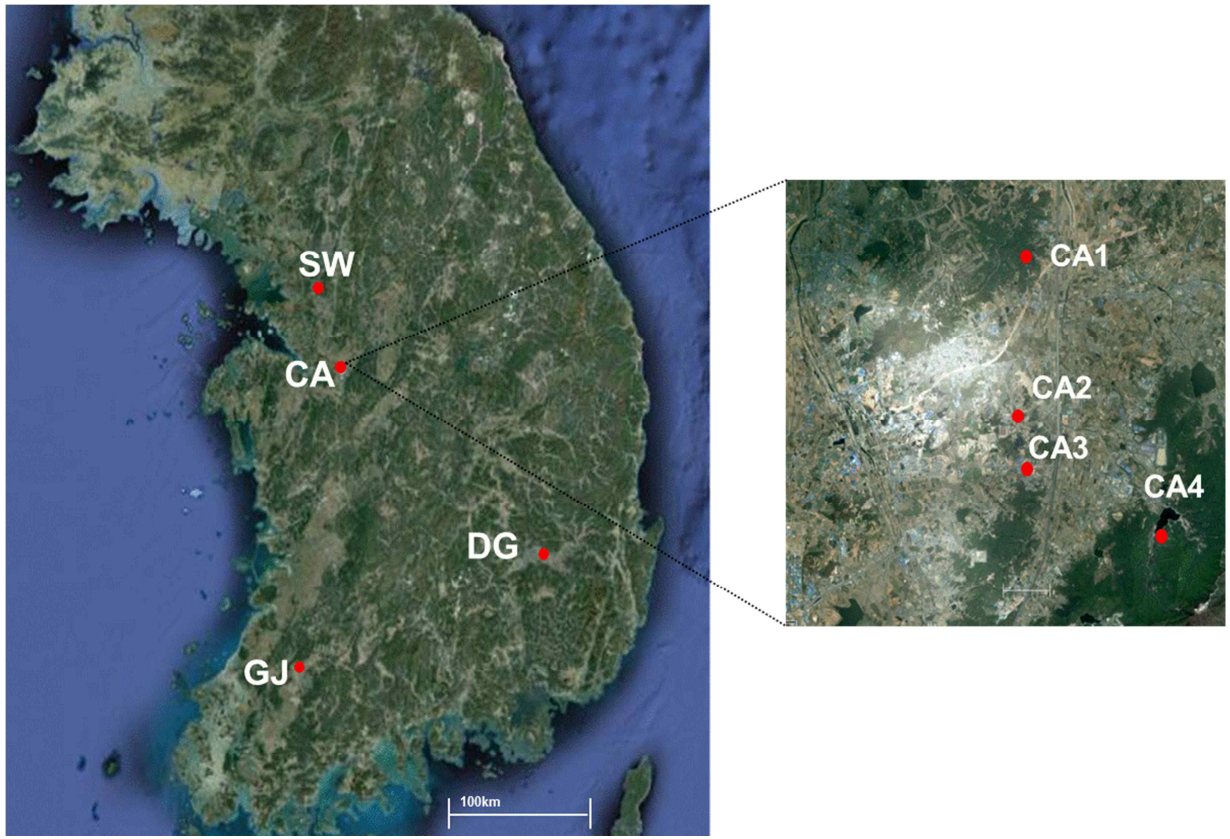



Fig. 7. Map showing the locations of the collection sites of *L. delicatula*.

Table 11. Sampling information for *L. delicatula* specimens collected both 2010 and 2012 in South Korea.

| Collection Sites | Habitat Type | Sampling Date | | |
|---|---|---|---|---|
| | | 2010 | 2012 | |
| | | | Nymph | Adult |
| DG | *A. altissima* in the hill without grape vine | 9-13 | 5-25 | 8-20 |
| SW | *A. altissima* in the hill without grape vine | 7-29, 9-24 | 5-24 | 8-25 |
| GJ | *A. altissima* in the hill without grape vine | 7-10 | 5-25 | 8-19 |
| CA1 | *A. altissima* in the hill far from grape vine | - | 6-22 | 10-26 |
| CA2 | Grapevine nearby *A. altissima* | 7-15 | 5-25 | 8-24 , 10-26 |
| CA3 | Grapevine nearby *A. altissima* | - | 6-22 | 10-26 |
| CA4 | *A. altissima* in the hill far from grape vine | - | 6-22 | 10-26 |

## DNA extraction and Genotyping

We used six microsatellite loci for genotyping previously developed for *L. delicatula* (Park *et al*., 2012). Multiplex polymerase chain reaction (PCR) was conducted in two separate reactions, (1) markers LD-D4, LD-T1 and LD-T3 and (2) markers LD-D1, LD-D2 and LD-T2. Process for DNA extraction, PCR and genotyping were descripted in Chapter 1.

## Statistical analysis

### *Genetic variation and genetic structure*

At each population, the mean number of alleles per locus and observed ($H_O$) and expected heterozygosities ($H_E$) were calculated using the Microsatellite Toolkit (Park, 2001). Hardy-Weinberg equilibrium (HWE) and their significance of multiple *P*-value were tested using Fisher's method in GENEPOP program (Raymond and Rousset, 1995). Micro-Checker (Oosterhout *et al*., 2004) was used to evaluate the homozygote excess by potential scoring errors or presence of a null alleles. Pairwise genetic differentiation ($F_{ST}$) was estimated between populations using FSTAT v.2.9.3 (Goudet, 2001).

***Effective population size and migration rate***

We used MLNE ver. 2.3 (Wang and Whitlock, 2003) to calculated the maximum-likelihood of effective population size ($N_e$) and immigration rate ($m$) jointly with 95 % CIs. This method enables the identification of $N_e$ and $m$ to measure the short-term consequences of gene flow and can be applied to a finite source population composing one or more small subpopulations (Wang and Whitlock, 2003). Therefore, we analyzed the $N_e$ and $m$ between two generations (2010 and 2012) in same site by pooling the sample for potential source from each three samples to know the bidirectional gene flow under island model. Values for $m$ indicate the immigrants from potential source populations. The maximum possible $N_e$ value was set to 10,000.

***Genetic structure across habitats and among life stages***

The levels of genetic strata were tested to carry out the genetic variation within and between stage groups (1) nymph vs. pre-oviposition throughout Korea (DG, SW, GJ and CA) (2) nymph vs. post-oviposition in Cheonan (3) nymph vs. pre- vs. post- oviposition in CA2. And then, we evaluated the distribution of genetic variability by means of molecular variance (AMOVA) as executed in GeneAlex 6.5 software (Peakall and

Smouse, 2012). The Significance of the fixation indices calculated at each level is determined by 999 permutations.

The networks were constructed using POPULATION GRAPH and the analyses were performed with Genetic Studio (Dyer, 2009). Population graph analysis was conducted to evaluate genetic variation is distributed amongst populations. The network topology was used to infer which population may have related with the gene flow. For the graph construction, we retained the minimal edge set that sufficiently described the total among population covariance stricture. And, we conducted STRUCTURE v. 2.3. (Pritchard *et al*., 2000) and assignment test using the Geneclass 2 program (Piry *et al*., 2004),

Two sites will share an edge if there is significant genetic covariance between the sites after removing the covariance each population has with all the remaining populations in the network. Significance was evaluated using edge exclusion deviance, which identifies the most important edges for each node in terms of genetic covariance. A binomial test for the existence of sub-graphs within the data set was used for each network to determine whether there was restricted gene flow between the categories determined by the clustering analysis. The possibility of population structure was constituted by distinct genetic

groups according to life stage, the null hypothesis stated that the possibility of obtaining an edge connecting both sub-graphs using binomial test in the graph.

Across the entire Population Graph, conditional genetic distance (*c*GD) was estimated as the length of the shortest path connecting pairs of sites (nodes) to test for isolation by graph distance (IBgD). Correlations between measured distances were determined using Mantel tests and considered significant after 999 randomizations using the GeneAlex 6.5 (Peakall and Smouse, 2012).

## 3-3. Results

### Overall Genetic variability for all populations

Allelic diversity with alleles identified across all 19 populations using six microsatellite loci. Inbreeding coefficients, $F_{IS,}$ ranged from -0.045 to 0.273. Also, values across loci with an excess of observed homozygotes, thus indicating that the Hardy-Weinberg Equlibrium (HWE) after correction for multiple test, was detected at five populations (sample ID: 1, 2, 5, 6 and 14) (adjusted significance [5 %] threshold =0.0004). MicroChecker not detected scoring errors resulting from large allele drop-out in any *L. delicatula* population or locus, in this study. The presence of potential null alleles was indicated by a general excess of homozygotes for most allele size classes for no or one more loci within at each population (Table 12).

Bottleneck was evaluated for all populations. Deviation from mutation-drift equilibrium (under the TPM model) was revealed in DG_10 ($p$=0.00781), DG_12_N ($p$=0.01563) and DG_12_A ($p$=0.02344). And bottleneck was not detected all populations by mode-shift analysis. The M-ratio ranged from 0.39 (CA_10) to 0.571 (SW_10), which provided the evidence for bottleneck events in all populations (Table 13). All populations has experienced a historically bottleneck by M-ratio values while DG was occurred continuous reduction of population size in Korea.

Table 12. Genetic variability estimates for each *Lycorma delicatula* population, inferred from seven microsatellite loci. Number of alleles, expected heterozygosity ($H_E$) at HWE, observed heterozygosity ($H_O$), inbreeding coefficient ($F_{IS}$), probability (*P*-value) of being in HWE, and loci showing potential null alleles.

| Sample ID | Sample Name | Sample size | No Alleles | Genetic Diversity | $H_O$ | $H_E$ | $F_{IS}$ | *P*-value[18] | Loci with Null allele |
|---|---|---|---|---|---|---|---|---|---|
| 1 | DG_10 | 29 | 5.33 | 0.6905 | 0.502 | 0.674 | 0.273 | 0.0004 | LD1, LD15, LD22 |
| 2 | SW_10 | 21 | 5.5 | 0.7142 | 0.576 | 0.693 | 0.193 | 0.0004 | LD32 |
| 3 | GJ_10 | 11 | 4.5 | 0.6782 | 0.494 | 0.636 | 0.272 | 0.0018 | LD32 |
| 4 | CA_10[19] | 30 | 6 | 0.6313 | 0.569 | 0.619 | 0.099 | 0.0206 | LD15, LD31 |
| 5 | DG_12_N[20] | 29 | 6 | 0.7388 | 0.604 | 0.723 | 0.183 | 0.0004 | LD15, LD22, LD25 |
| 6 | SW_12_N | 26 | 7.5 | 0.7152 | 0.571 | 0.698 | 0.202 | 0.0004 | LD15, LD22, LD32 |
| 7 | GJ_12_N | 25 | 6.67 | 0.7148 | 0.619 | 0.697 | 0.133 | 0.0026 | LD32 |
| 8 | CA1_12_N | 30 | 5.67 | 0.6732 | 0.543 | 0.600 | 0.061 | 0.1061 | LD15 |
| 9 | CA2_12_N | 27 | 6 | 0.713 | 0.632 | 0.658 | 0.192 | 0.0009 | LD15 |
| 10 | CA3_12_N | 28 | 5.83 | 0.6262 | 0.506 | 0.612 | 0.115 | 0.0061 | LD15, LD22 |
| 11 | CA4_12_N | 28 | 6 | 0.6127 | 0.685 | 0.699 | 0.039 | 0.1768 | no |
| 12 | DG_12_A | 32 | 6.17 | 0.7398 | 0.642 | 0.726 | 0.132 | 0.0013 | LD15, LD32 |
| 13 | SW_12_A | 15 | 6 | 0.7128 | 0.674 | 0.687 | 0.055 | 0.1759 | LD32 |
| 14 | GJ_12_A | 32 | 7.67 | 0.7512 | 0.642 | 0.737 | 0.146 | 0.0004 | LD15 |
| 15 | CA1_12_O | 32 | 8 | 0.6625 | 0.646 | 0.684 | 0.074 | 0.0456 | LD15 |
| 16 | CA2_12_A | 32 | 6.67 | 0.6368 | 0.614 | 0.651 | 0.138 | 0.0026 | LD15 |
| 17 | CA2_12_O | 23 | 6 | 0.7017 | 0.549 | 0.621 | 0.111 | 0.0053 | LD15 |
| 18 | CA3_12_O | 32 | 7 | 0.692 | 0.615 | 0.679 | 0.072 | 0.0373 | LD15 |
| 19 | CA4_12_O | 32 | 8.3 | 0.6955 | 0.733 | 0.691 | -0.045 | 0.8531 | no |

[18] Significant was determined after adjusted nominal level (5 %) : 0.00044
[19] 10 and 12 indicated the sampling year in 2010 and 2012, respectively.
[20] N, A and O indicated the sampling stages in nymph, pre-oviposition adult and post-oviposition adult, respectively.

Table 13. M-ratio test using the SMM (Ohta and Kimura, 1973) and the TPM (Di Rienzo *et al*, 1994) to detect a recent population. Significance tested using the Wilcoxon sum-rank test (α=0.05).

| Sample ID | WILCOXON Tests[21] | | Mode-Shift | M-ratio[22] |
|---|---|---|---|---|
| | TPM | SMM | | |
| 1 | **0.00781** | 0.65625 | Normal | 0.392 (0.164) |
| 2 | 0.07813 | 0.65625 | Normal | 0.571 (0.125) |
| 3 | 0.07813 | 0.5 | Normal | 0.442 (0.099) |
| 4 | 0.65625 | 0.98438 | Normal | 0.39 (0.128) |
| 5 | **0.01563** | 0.21875 | Normal | 0.513 (0.09) |
| 6 | 0.94531 | 0.99219 | Normal | 0.547 (0.09) |
| 7 | 0.65625 | 0.92188 | Normal | 0.542 (0.09) |
| 8 | 0.57813 | 0.78125 | Normal | 0.521 (0.09) |
| 9 | 0.94531 | 0.98438 | Normal | 0.559 (0.082) |
| 10 | 0.78125 | 0.9765 | Normal | 0.435 (0.053) |
| 11 | 0.21875 | 0.92188 | Normal | 0.418 (0.053) |
| 12 | **0.02344** | 0.57813 | Normal | 0.476 (0.073) |
| 13 | 0.5 | 0.78125 | Normal | 0.488 (0.126) |
| 14 | 0.57813 | 0.96094 | Normal | 0.492 (0.08) |
| 15 | 0.5 | 0.97656 | Normal | 0.547 (0.081) |
| 16 | 0.5 | 0.96094 | Normal | 0.494 (0.058) |
| 17 | 0.5 | 0.94531 | Normal | 0.554 (0.09) |
| 18 | 0.97656 | 1 | Normal | 0.541 (0.102) |
| 19 | 0.97656 | 0.98438 | Normal | 0.553 (0.07) |

[21]One-tail probability for an excess or deficit of observed heterozygosity relative to the expected equilibrium heterozygosity, computed from the observed number of alleles under mutation-drift equilibrium. SMM: stepwise mutation model, TPM: two-phase model of mutation

[22] M indicates the ratio of the number of alleles to the rage of allele size (Garza and Williamson, 2001). And variance was parentheses

**Genetic structure analysis for overall populations**

Global $F_{ST}$ was a low but significant 19 populations (uncorrected $F_{ST}$ = 0.0496, 95 % CI= 0.0289-0.0748, ENA corrected $F_{ST}$ = 0.0492, 95 % CI= 0.02795-0.07416). Estimates of pairwise $F_{ST}$ values, which were corrected from null allele using FreeNa, were ranged from -0.006 (DG_10 and CA1_12_N) to 0.15 (SW_12_N and CA4_12_N). Significance was determined with a Markov chain analysis (Table 14).

Generally, we analyzed the genetic structure for overall populations using Genetic Studio and STRUCTURE. The network that best fit the data contained 43 edges connecting the 19 nodes. Modularity optimization was divided four communities within genetic network (Table 15, Appendix 8). Otherwise, Bayesian analysis inferred the three genetic clusters among total populations; Ln (P) D revealed a maximum value of -9347.18, at $K$=3. Also, the maximum value among genotypes was 65.55, at $\Delta K$=2 (Evanno *et al*., 2005). Therefore, a total of three genetically substructured groups of populations were showed in Fig. 8. Between POPULATION GRAPH and STURUCTRE slightly showed different genetic composition between populations (Table 15). Number of assigned individuals, which immigrant probability at a threshold of $\alpha$=0.01, and average likelihood were calculated for each potential source population. Most populations,

excluding only three populations (sample ID: 10, 18 and 19), were assigned to its own population with low percentage assigned individuals from sampled in 2012 (Table 15).

Table 14. Pairwise estimates of genetic differentiation ($F_{ST}$) (below the diagonal) between *L. delicatula* populations.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | - | | | | | | | | | | | | | | | | | | |
| 2 | $0.065^{NS}$ | - | | | | | | | | | | | | | | | | | |
| 3 | $0.066^{NS}$ | $0.036^{NS}$ | - | | | | | | | | | | | | | | | | |
| 4 | $0.083^{NS}$ | $0.065^{NS}$ | $0.058^{NS}$ | - | | | | | | | | | | | | | | | |
| 5 | $0.031^{NS}$ | $0.066^{NS}$ | $0.05^{NS}$ | $0.079^{NS}$ | - | | | | | | | | | | | | | | |
| 6 | $0.088^{*}$ | $0.072^{*}$ | $0.05^{*}$ | $0.091^{*}$ | $0.016^{NS}$ | - | | | | | | | | | | | | | |
| 7 | $0.101^{NS}$ | $0.075^{*}$ | $0.047^{*}$ | $0.11^{*}$ | $0.025^{*}$ | $0.002^{NS}$ | - | | | | | | | | | | | | |
| 8 | $0.152^{*}$ | $0.132^{*}$ | $0.098^{*}$ | $0.062^{*}$ | $0.076^{*}$ | $0.062^{*}$ | $0.062^{*}$ | - | | | | | | | | | | | |
| 9 | $0.12^{*}$ | $0.104^{*}$ | $0.072^{*}$ | $0.058^{*}$ | $0.05^{*}$ | $0.029^{*}$ | $0.027^{NS}$ | $0.007^{NS}$ | - | | | | | | | | | | |
| 10 | $0.119^{NS}$ | $0.12^{*}$ | $0.073^{*}$ | $0.09^{*}$ | $0.043^{NS}$ | $0.031^{NS}$ | $0.033^{NS}$ | $0.02^{NS}$ | $0.013^{NS}$ | - | | | | | | | | | |
| 11 | $0.081^{NS}$ | $0.067^{NS}$ | $0.03^{*}$ | $0.067^{*}$ | $0.019^{*}$ | $-0.007^{NS}$ | $0.008^{NS}$ | $0.045^{NS}$ | $0.015^{NS}$ | $0.021^{NS}$ | - | | | | | | | | |
| 12 | $0.05^{NS}$ | $0.064^{NS}$ | $0.054^{NS}$ | $0.092^{*}$ | $0.006^{*}$ | $0.03^{*}$ | $0.018^{NS}$ | $0.076^{NS}$ | $0.06^{NS}$ | $0.059^{NS}$ | $0.03^{NS}$ | - | | | | | | | |
| 13 | $0.122^{*}$ | $0.08^{*}$ | $0.08^{*}$ | $0.146^{NS}$ | $0.048^{*}$ | $0.017^{NS}$ | $0.028^{NS}$ | $0.116^{*}$ | $0.085^{*}$ | $0.093^{*}$ | $0.03^{*}$ | $0.047^{NS}$ | - | | | | | | |
| 14 | $0.084^{NS}$ | $0.057^{*}$ | $0.029^{NS}$ | $0.093^{NS}$ | $0.019^{NS}$ | $0.0002^{*}$ | $-0.006^{NS}$ | $0.068^{NS}$ | $0.04^{NS}$ | $0.04^{NS}$ | $0.009^{*}$ | $0.017^{NS}$ | $0.024^{NS}$ | - | | | | | |
| 15 | $0.11^{*}$ | $0.09^{*}$ | $0.07^{*}$ | $0.045^{*}$ | $0.046^{*}$ | $0.03^{*}$ | $0.042^{NS}$ | $0.005^{*}$ | $0.005^{*}$ | $0.02^{*}$ | $0.024^{*}$ | $0.051^{*}$ | $0.075^{*}$ | $0.036^{*}$ | - | | | | |
| 16 | $0.109^{*}$ | $0.108^{*}$ | $0.083^{*}$ | $0.056^{*}$ | $0.042^{*}$ | $0.03^{*}$ | $0.041^{*}$ | $0.017^{*}$ | $0.01^{*}$ | $0.014^{*}$ | $0.026^{*}$ | $0.057^{*}$ | $0.086^{*}$ | $0.039^{*}$ | $0.002^{*}$ | - | | | |
| 17 | $0.139^{NS}$ | $0.128^{*}$ | $0.09^{*}$ | $0.049^{*}$ | $0.087^{*}$ | $0.073^{*}$ | $0.08^{*}$ | $0.017^{*}$ | $0.024^{*}$ | $0.04^{*}$ | $0.057^{NS}$ | $0.086^{*}$ | $0.134^{*}$ | $0.075^{*}$ | $0.01^{*}$ | $0.012^{*}$ | - | | |
| 18 | $0.087^{NS}$ | $0.079^{*}$ | $0.049^{*}$ | $0.037^{*}$ | $0.037^{*}$ | $0.026^{*}$ | $0.043^{*}$ | $0.026^{*}$ | $0.017^{*}$ | $0.028^{*}$ | $0.009^{*}$ | $0.047^{*}$ | $0.066^{*}$ | $0.039^{*}$ | $0.006^{*}$ | $0.011^{*}$ | $0.016^{*}$ | - | |
| 19 | $0.091^{*}$ | $0.076^{*}$ | $0.059^{*}$ | $0.048^{*}$ | $0.032^{*}$ | $0.023^{*}$ | $0.019^{*}$ | $0.02^{*}$ | $0.011^{*}$ | $0.025^{*}$ | $0.021^{*}$ | $0.027^{*}$ | $0.067^{*}$ | $0.02^{*}$ | $0.002^{*}$ | $-0.002^{*}$ | $0.018^{*}$ | $0.012^{*}$ | - |

[1]Probability of being different from zero following correction for multiple comparisons.$^{*}P < 0.05$; NS: not significant. The adjusted nominal level (5%) for multiple comparisons was 0.000292.

Table 15. Genetic clustering (STRUCTURE and POPULATION GRAPH) and assignment (GeneClass 2.0) of *L. delicatula* specimens collected from 4 regions and 19 populations in South Korea.

| Sample ID | STRUCTURE (K=3)[23] | | | POPULATION GRAPH[24] | | % of assigned individuals in first rank (assigned sample ID)[25] |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | Network Community | Eigenvalues | |
| 1 | 0.041 | **0.887** | 0.072 | 3 | 0.2321 | 84.21 (1) |
| 2 | 0.111 | **0.695** | 0.194 | 2 | -0.3911 | 52.38 (2) |
| 3 | 0.174 | 0.405 | **0.421** | 2 | 0.17 | 45.45 (3) |
| 4 | 0.339 | **0.521** | 0.141 | 3 | -0.09761 | 63.33 (4) |
| 5 | 0.097 | **0.692** | 0.211 | 1 | -0.2331 | 37.93 (5) |
| 6 | 0.233 | 0.201 | **0.565** | 3 | -0.2174 | 19.23 (6) |
| 7 | 0.214 | 0.181 | **0.606** | 2 | 0.1474 | **16 (7,14)** |
| 8 | **0.659** | 0.13 | 0.21 | 3 | -0.06689 | 26.67 (8) |
| 9 | **0.58** | 0.162 | 0.258 | 1 | -0.01442 | 25.93 (9) |
| 10 | **0.528** | 0.162 | 0.311 | 2 | -0.004084 | 17.86 (8) |
| 11 | 0.288 | 0.224 | **0.488** | 2 | 0.05486 | 25 (11) |
| 12 | 0.087 | **0.616** | 0.298 | 1 | -0.2427 | 50 (12) |
| 13 | 0.132 | 0.188 | **0.68** | 4 | 0.6128 | 60 (13) |
| 14 | 0.133 | 0.162 | **0.706** | 3 | -0.2885 | 28.13 (14) |
| 15 | **0.568** | 0.138 | 0.295 | 3 | 0.1766 | 25 (15) |
| 16 | **0.592** | 0.127 | 0.281 | 3 | 0.08428 | 21.88 (16) |
| 17 | **0.676** | 0.094 | 0.231 | 3 | -0.2593 | 26.09 (17) |
| 18 | **0.498** | 0.194 | 0.308 | 2 | 0.02079 | 21.88 (8) |
| 19 | 0.342 | 0.240 | **0.419** | 1 | 0.01017 | 15.53 (16) |

[23] Average coefficient of ancestry obtained from a structure analysis with $K$ = 3 for Ln (P(D)) = -9347.18. The maximum value among genotypes was 65.55 at $\Delta K$=2 (Evanno *et al*., 2005).

[24] Eigenvector based modularity maximization for genetic network. Numbers under the heading Network Community represent node clustering based on modularity maximization.

[25]Percentage (%) of assigned individuals and most likely assigned population ID was shown in parentheses. Bold indicates the equal values of assignment both populations.

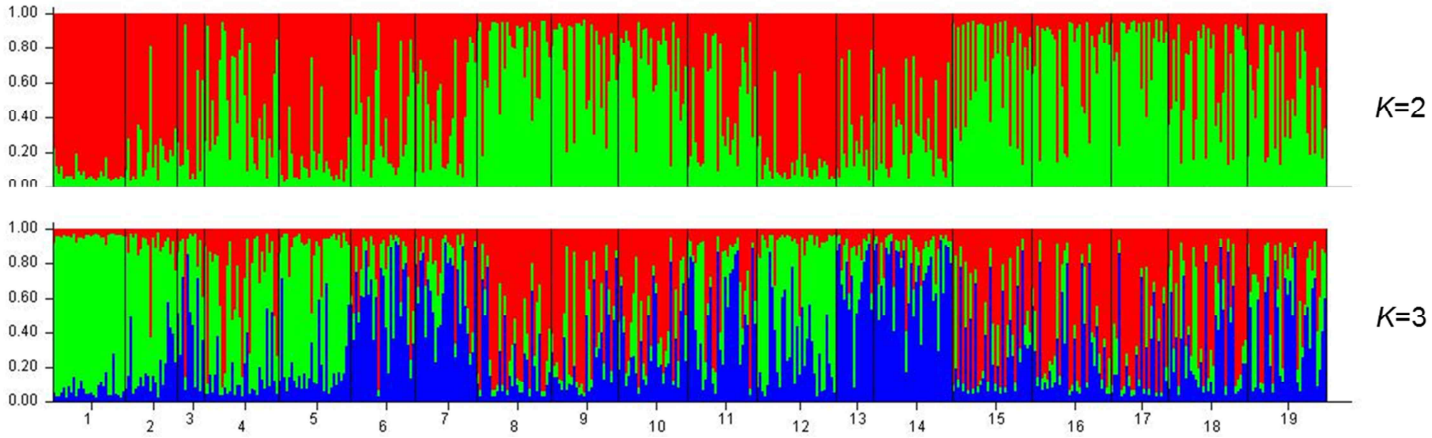



Fig. 8. Individual membership of 514 *L. delicatula* specimens collected from 19 populations computed by STRUCTURE. (a) genetic structure was inferred at *K*=2 with maximum value among genotypes was 63.55 at Δ*K* = 2, using Δ*K*= m|*L*"(*K*)|/s[*L*(*K*)] (Evanno *et al.,* 2005). (b) with maximum value of Ln (P) D was -9456.52, at *K*=3.

**Effective population size and migration rate**

Estimated maximum likelihood (ML) of $N_e$ and $m$ between two years of *L. delicatula* was shown Table 16. $N_e$ and $m$ of CA were relatively larger (102.75 -119.91) and low (0.043 - 0.069) than other regions. This result implied that CA was source population, while other regions (DG, GJ and SW) may act as sinks in Korea (Fig. 9).

Table 16. The maximum-likelihood both effective population size ($N_e$) and immigration rate ($m$) of four locations between two generations in Korea calculated using the ML$N_e$ (Wang and Whitlock, 2003).

| Pop. | | Source population | | | |
|---|---|---|---|---|---|
| | | CA | DG | GJ | SW |
| CA | *N*e | - | 104.77 (155.82-78.58)[26] | 111.91 (171.1-82.56) | 102.75 (150.89-77.79) |
| | *M* | - | 0.043 (0.068-0.025) | 0.058 (0.09-0.033) | 0.069 (0.11-0.04) |
| DG | *N*e | 21.75 (33.77-15.44) | - | 21.77 (34.76-15.77) | 20.82 (32.09-14.87) |
| | *M* | 0.11 (0.18-0.06) | - | 0.13 (0.22-0.07) | 0.15 (0.26-0.08) |
| GJ | *N*e | 45.93 (134.28-27.042) | 43.28 (131.5-25.24) | - | 40.79 (102.23-25.52) |
| | *M* | 0.12 (0.25-0.039) | 0.11 (0.24-0.033) | - | 0.22 (0.53-0.075) |
| SW | *N*e | 24.75 (37.79-18.13) | 22.8 (33.93-16.67) | 35.31 (76.48-22.89) | - |
| | *M* | 0.26 (0.41-0.15) | 0.27 (0.45-0.155) | 0.18 (0.35-0.077) | - |

[26] Likelihood and ± 95% CI was shown in parentheses.

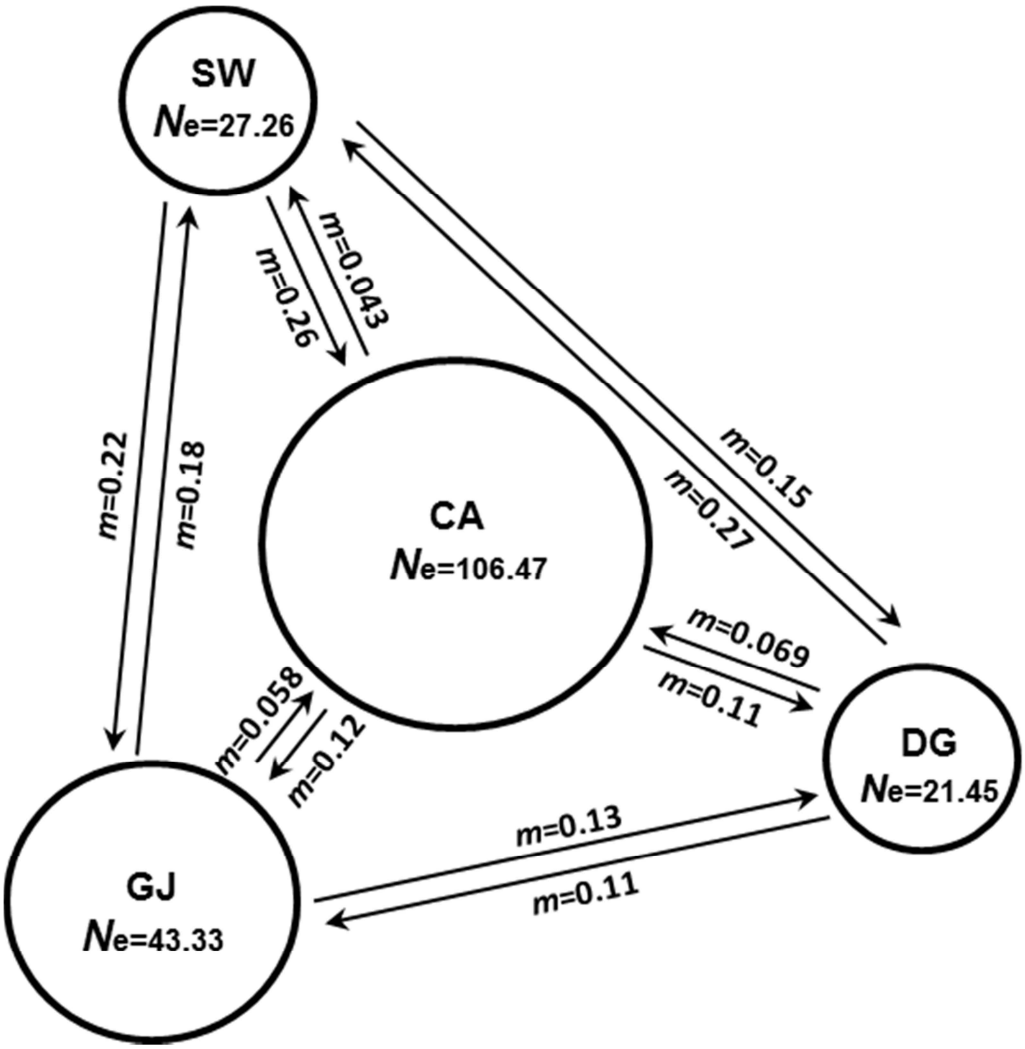



Fig. 9. Schematic summary of dynamics between mean effective populatiion size (*N*e) and esimated migration (*m*) between each population pair in Korea.

**Genetic variation among regions and life stages**

Global $F_{ST}$ was a low but significant by collected from 2012 (uncorrected $F_{ST}$ = 0.033, 95 % CI= 0.0252-0.0425, ENA corrected $F_{ST}$ = 0.0323, 95 % CI= 0.0243-0.0416). *L. delicatula* showed low but significant overall $F_{ST}$ and $F_{IS}$ between nymph ($F_{ST}$=0.0253, $p$ <0.01; $F_{IS}$=0.147, $p$<0.01) and pre-oviposition adult ($F_{ST}$=0.0452, $p$<0.01; $F_{IS}$=0.11, $p$<0.01) from different four sites. Also, nymph ($F_{ST}$=0.02, P<0.01; $F_{IS}$=0.147, $p$<0.01) and adults of the post-oviposition ($F_{ST}$= 0.009, P<0.01; $F_{IS}$=0.061, $p$<0.01) were shown within CA. But, these fixation indices were indicated not significantly different among groups in the one sided $p$-values after 1000 permutations.

Full graph has 29 edges with edge probability of 0.1429 from collected 15 populations in 2012 (Fig. 10). The Mantel test between *c*GD and geographical distance revealed a positive correlation ($r^2$=0.237, $p$=0.01; Fig. 11), indicating the IBgD. Dispersal ability of *L. delicatula* was restricted in geographical scale across the life-stage in Korea (Fig. 11). The probabilities of subgraph within network were determined using the binomial test from Genetic Studio program. There are non-significant deficiency of edges between ‘pre-oviposition’ and ‘nymph and post-oviposition’ was found [$p(X \leq K_{btw})$ = 0.2695]. But, a significant deficiency of

edges were detected in ‘nymph’ and ‘pre- and post-oviposition’ [$p(X \leq K_{btw})$ = 0.03205] and ‘post-oviposition’ and ‘nymph and pre-ovipsition’ [$p(X \leq K_{btw})$ = 0.001161], respectively (Table 17). This suggests that oviposition has acted as active dispersal of *L. delicatula* resulting in two subgraphs of nymph and post-oviposition within the network.

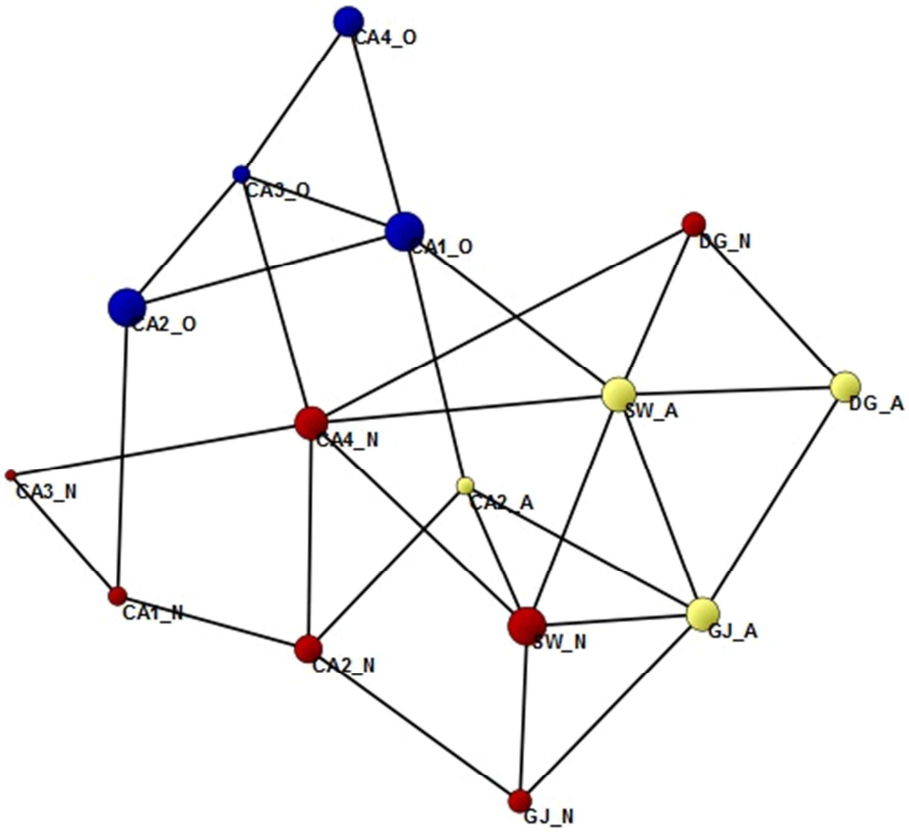



Fig. 10. A two-dimensional projection representing the genetic relationship among *L. delicatula* sampled in South Korea in 2012. Node size is proportional to increasing connectivity and edge length is proportional to the genetic distance between populations. Node color is Network Community see in the Table 12.

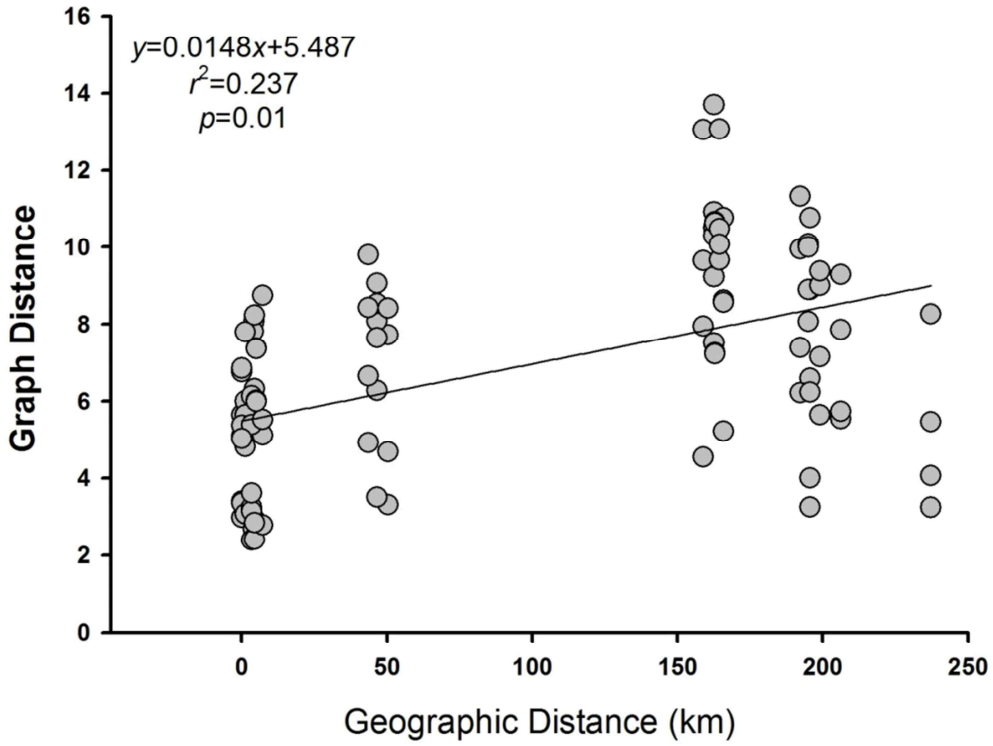



Fig. 11. The relationship between graph distance (shortest distance between pairs of nodes) and geographic distance among 19 nodes demonstrating the detection of Isolation By Graph Distance (IBgD) within the graph structure.

Table 17. The probability of the subgraph in the network determined among life stages conducted with the module Graph of the software Genetic Studio. Full graph has 29 edges with edge probability of 0.1429 across six microsatellite markers.

| Network | Bridge probability between life stage | Separating Edge Set between stage | $p(X<=\|E_{btw}\|)$[27] |
|---|---|---|---|
| Nymph | 0.533 | 10 | 0.032 |
| pre-oviposition | 0.419 | 10 | 0.2696 |
| post-oviposition | 0.419 | 4 | 0.001161 |

[27] Probability of sub-graph was determined by binominal test between two groups of life stages (i.e. in column of nymph indicates $p(X<=\left| E_{\text{nymph vs. pre- and post-oviposition}} \right|)$ in the network. Analyses were performed with the module Graph of the software Genetic Studio.

AMOVA analysis was not significant different among nymph and pre-oviposition adult populations in same site within four different regions in Korea. In the analysis, ~1 % of the overall molecular variation was explained by nymph and post-oviposition adults. But, a significant genetic differentiation existed among different life stages within CA ($p$=0.01). Although the proportion of explained variance was low (~3 %), a significant was shown among three groups along the life stages in CA 2 (Table 18).

In the PcoA, the mean factor scores were plotted along the first two principal component axes, which together accounted for 73.77 % of the total variance (55.89 % for axis 1 and 14.88 % for axis 2; Fig. 12a). The axis 1 revealed the genetic difference of geographical area along the CA except for 11 (CA4_N) and other populations (DG, SW and GJ). As result of the genetic difference in fine scale, nymph and post-oviposition were divided for 55.89 % for axis 1 and 14.88 % for axis 2 within CA (Fig. 12 b).

Table 18. Analysis of molecular variance (AMOVA) for three models of *L. delicatula* samples in South Korea.

| Model | Source of variation | d.f. | Sum of squares | Variance components | % of[28] variation | *P* -value[29] |
|---|---|---|---|---|---|---|
| Nymph and pre-oviposition populations from different regions throughout Korea | Among stages | 1 | 9.671 | 9.671 | 0 | n.s. |
| | Among Pop | 6 | 80.061 | 13.343 | 5 | 0.001 |
| | Within Pop | 210 | 1129.966 | 5.381 | 95 | 0.001 |
| Nymph and post-oviposition populations within Cheonan | Among stages | 1 | 14.42 | 14.416 | 1 | 0.001 |
| | Among Pop | 6 | 48.04 | 8.007 | 2 | 0.001 |
| | Within Pop | 224 | 1049.34 | 4.685 | 97 | 0.001 |
| Nymph, pre- and Post-oviposition Populations in CA2 | Among Pops | 2 | 11.76 | 5.88 | 3 | 0.001 |
| | Within Pops | 79 | 192.91 | 2.442 | 18 | 0.001 |
| | Within indiv. | 82 | 138.5 | 1.689 | 79 | 0.001 |

[28] The percentage of total variance was contributed by each component.
[29] The probability test *P*-value was calculated by 999 permutations.

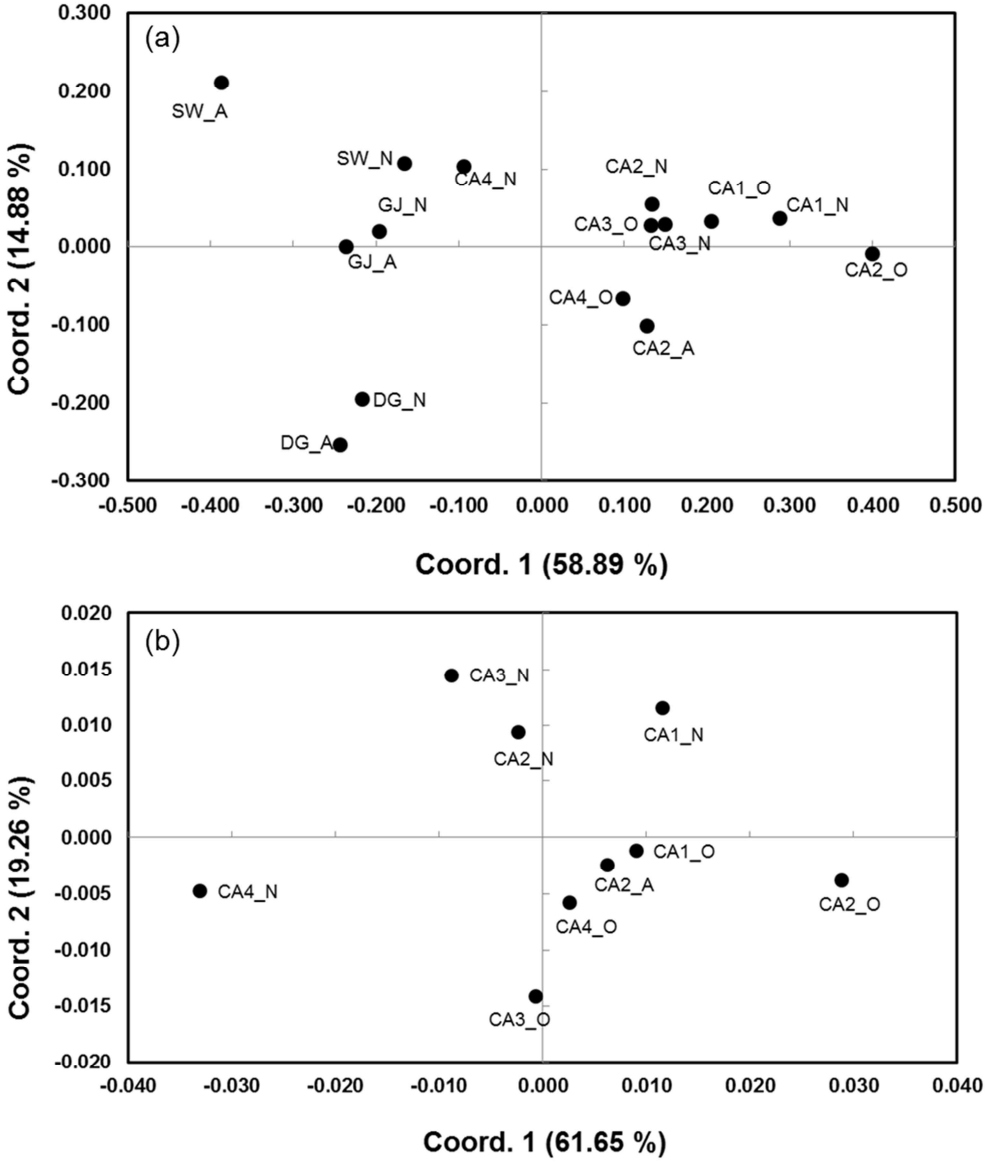



Fig. 12. Principal coordinates Analysis (PCoA) (a) four different regions in Korea (b) within Cheonan collected in 2012. The percentage of total variation attributed to each coordination. See table 12 for location abbreviations.

## 3-4. Discussions

We investigated the genetic variation and structures at six microsatellite loci in *L. delicatula* populations in Korea. Microsatellite provided not only modified genotypic resolution and parameter values, but also the power of tests assessing within and between populations (Delmotte *et al*., 2002).

In this study, we estimated $N_e$ between temporal variance of allele frequencies passed by two generations of *L. delicatula.* $N_{e,}$ extremely low in all collected sites, was not explained the real demographic of observation (*N*) from the field census. $N_e$ has been controversial to low predictability, especially insect (Watts *et al*., 2007). Extremely low $N_e$ in introduced range was probably resulted from the bottleneck from original sources, in particular *L. delicatula* populations as recently invaded in Korea. Also, population size of overwintering *L. delicatula* affected by winter temperature in Korea. In 2011, egg hatching rate was very low (ca. 33 %) from field observed in Cheonan, by severe winter temperature (see details in Chapter 2-1), vice versa naturally selected only 30% in Korea. Therefore, invisible hand was acts allele frequency following generation.

Relatively different values of estimated $N_{e,}$ that CA was larger than other locations, may have a persuasive to interpret of demographic events.

Collected samples from DG, SW and GJ were less favorable habitats for adult stage, which mainly composed by *A. altissima* in hilllock and no existed grape vine in nearby. Therefore, highly reduced adult population may correspond to reduced reproductive variance. Ratio of *Ne*/*N* was used to explain the discrepancy between $N_e$ and *N* in level of taxonomic or between natural and artificial environments (Banks and Thomson's, 1985; Brakefield *et al*., 2001). *Ne*/*N* ratio was not suggested in level of Order (Hemiptera) and Family (Fulgoridae) until now. In this study, *Ne*/*N* ratio was estimated from samples 0.43, 0.17, 0.27 and 0.06 in CA, DG, GJ and SW, respectively. Even if population size is still large, low *Ne*/*N* ratio may estimate a risk of population persistence by lacking the genetic variation (Saarinen *et al*., 2009). Also, relative effective population size and immigration rate may predict the influence of selection and genetic drift in the past, moreover, estimated the population size in near future. Therefore, this information can provide the management strategy by local area in asymmetric spreading phase with lag times in recipient regions.

A bottleneck was continuous observed in DG populations in 2010 and 2012 across nymph and adult under TPM (Table 13), also previously bottleneck test of DG population collected from 2011 (Park *et al*., 2013). A genetic bottleneck was expected the rapid and drastic decreases in allele

frequency, which negative effect on their fitness from general genetic concept (Reed and Frankham, 2003). After exploring bottleneck, selected population was adapted for their environment, so called by rapid adaptive evolution, in particular invasive species (Hänfling and Kollmann, 2002).

IBgD showed significance ($p$=0.01, $r^2$=0.237) across the geographical distance in Mantel test, indicating the restricted gene flow by geographical barrier (Fig. 11). Traditional IBD test also significant with low probability ($p$=0.03, $r^2$=0.0795) by the genetic distance using $F_{ST}$ / (1-$F_{ST}$) (data not shown in result). Previously, IBD test using the pairwise $F_{ST}$ of *L. delicatula* were not significant throughout Korea in 2012 (Park *et al*., 2013). Conditional genetic distance (*cGD*) is more sensitive than $F_{ST}$ and *D*, traditional measures of pairwise genetic distance, by calculating the length of the shortest path connecting pairs of nodes (Dyer, 2009). Also, study range affect the strength of gene flow in this study, because sample were collected in mainly western part, which is probably simultaneous establishment regions by structured same genetic cluster, except for DG populations (Park *et al*., 2013).

Seasonal Occurrence of *L. delicatula* was shown the different peak timing among host plants. Some plants, mainly nymph preferred, may acts the temporary stepping stones for insect as alternative resource by liking

the adults breeding. Age structure among host plants in field and the longevity in semi-field conditions were discussed (Appendix 3). This phenological event was speculated oviposition was crucial for active dispersal of *L. delicatula* in Korea. Passive dispersal of *L. delicatula* was detected mediate by human activity and ground transportation, relatively long distance which surpass to its movement ability (Park *et al*., 2013). Therefore, genetic variance among life stage was analyzed. AMOVA results are showed significant different genetic variation among nymph and post-oviposition populations of *L. delicatula* (Table 18). Our study documents not significant genetic variation among populations from nymph and pre-oviposition adults in the same site. This implied the *L. delicatula* was sedentary to their natal habitat, before oviposition. Also, molecular variation was different between nymph and post-oviposition populations. The probability of subgraph able to corroborate evidence the AMOVA among life stages. Therefore, all of these finding were indicated that oviposition was presumed as a trigger for active dispersal of *L. delicatula* in Korea.

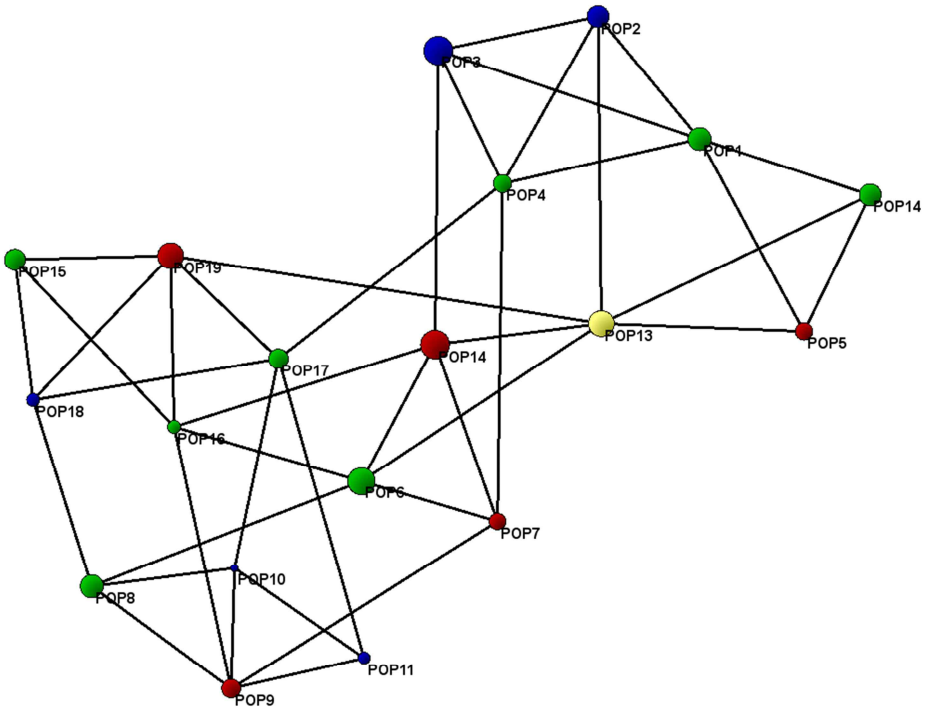
The effect of survival and reproductive choice among host plants was not revealed in this study. But, habitat structure and host plant quality were partially contributed to dispersive behavior of *L. delicatula* in Korea.

Although, adult of *L. delicatula* strongly preferred a few host plants for laying eggs, they have a low ability to discriminate among host plants (Kim *et al*., 2011). Recently, host specificity was evaluated by molecular characterization using the diagnostic gene with high reliability and relatively easy methodology. Laboratory simulations have a limitation to recreate the vegetation structure and microclimate situation in the environment and could misleading the host preference and recognition (Symondson, 2002). Advanced molecular technology like a DNA barcoding (Hebert *et al*., 2003) and gut content analysis (Juen and Traugott, 2005) would enable easily identification for trophic relationships.

Reproductive strategy was critical for survival future generation. Oviposition site was determined by female avoiding the hybrid and providing the sufficient resource for their progeny (Fox and Mousseau, 1995), as well as reducing the impact of intra-specific competition (Craig *et al*., 2000). But, egg masses of *L. delicatula* were detected in wide ranges including non-host plants in field. Shortly, reproduction strategy of *L. delicatula* was probably developed toward to discordance between oviposition preference and offspring fitness in Korea. This phenomenon has a possibility as temporary event for rapidly adaptation of invaders in new environment in early invasion stage of *L. delicatula*.

The common driving force behind all displacement strategies is that ultimately the insect concerned are ‘slaves of the environment’ (Loxdale and Lushai, 1999). Population biology integrated with population ecology and genetics open the window the new insight against the invisible hand. Also, these finding may provide the practical solution from integrated ecology, genetics and evolutionary perspectives on invasion population of *L. delicatula* in Korea (Sakai *et al*., 2001).

Appendix 8. A two-dimensional projection representing the genetic relationship among *L. delicatula* sampled in South Korea in 2010 and 2012. Node size is proportional to increasing connectivity and edge length is proportional to the genetic distance between populations. Node color is Network Community see in the Table 15.



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## List of appendix

# 꽃매미(*Lycorma delicatula* (매미목: 꽃매미과)의 월동 생태와 개체군 유전학적 연구

박마라나

## 초록

꽃매미는 포도에 피해를 주는 농업해충으로 2004 년 국내에 처음 보고된 외래해충이다. 겨울철 알 단계로 월동하는 꽃매미의 월동 생존율은 다음 세대의 개체군 크기에 영향을 미친다. 월별 채집된 알을 저온처리(-5, -10, -15, -20 ℃)한 결과, 부화율은 채집시기에 따라 다르게 나타났다. 봄철 야외 부화율은 2010 년부터 2013 년까지 각각 72.10%, 33.12%, 84.33%, 52.30%로 연도별로 다르게 나타났다. 야외 부화율은 1 월의 일 평균온도와 일 최저온도로 가장 잘 예측되었고, 이를 이용하여 부화율과 온도와의 관계식을 제안하였다. 본 모델을 사용하여 예측 부화율과 실제 야외 부화율의 차이를 비교한 결과 좁은 편차범위를 나타냈다.

봄철 발생시기를 예측하기 위해 꽃매미 알의 온도에 따른 휴면 발달을 조사하였다. 꽃매미 알의 부화기간은 채집 시기가 봄철에 가까울수록 짧아졌다. 또한 휴면 후 발육단계의 발육영점온도는 11.13℃이고, 유효적산온도는 293.26 Degree days 였다. 온도에 따른 비선형 발육률은 Brière 2 모델을 사용하였다. 모델의 검증을 위해 4 월 1 일부터 일별 야외온도에 따른 발육률을 누적하고 이를 발육완료 분포모델에 적용하여 1 령의 발생시기를 비교하였다. 그 결과, 본 모델은 야외에서 꽃매미 1 령의 발생시기를 잘 예측하였다

꽃매미가 가해하는 포도나무, 가죽나무, 뽕나무에서 이들의 계절적 발생을 조사하였다. 꽃매미 약충은 가죽나무, 뽕나무에서 많이 발생하고, 성충 시기에 가죽나무와 뽕나무에서 밀도가 낮아지는 반면 포도나무에서 높아졌다. 또한 가죽나무에서 채집된 꽃매미 암컷성충은 35~45 %의 비율로 나타났다. 가죽나무에서 꽃매미의 발육단계 별 밀도를 logistic 모델에 적용하여 발생 최성기의 Degree Days 를 알아보았다. 그 결과, 1 령 271, 2 령 492, 3 령 620, 4 령은 908 Degree days 로 나타났다. 하지만 성충은 가죽나무에서 분산으로 인하여 발생밀도가 낮아 모델의 예측력이 낮았다.

꽃매미의 개체군 유전학 연구를 위해 hybridization-biotin enrichment 방법을 이용하여 총 8 개의 초위성체(Microsatellite) 마커를 개발하였다. 이를 이용하여 국내 꽃매미의 침입동태와 확산경로를 위한 개체군 유전학 연구를 하였다. 그 결과, 국내의 개체군은 낮은 유전적 분화도를 보였다. Isolation By Diatance(IBD)의 Mantel test 결과, 지리적

거리와 유전적 거리의 상관관계는 유의하지 않았다. PcoA 와 STRUCRUE 분석 결과, 유전적 클러스터는 총 3 개로 천안지역과 삼척지역의 유전구조가 다른 지역과 뚜렷하게 구별되었다. Assignment test 를 통해 각 조사지점 별 개체간의 이동여부를 조사한 결과, 복잡한 이동 경로를 나타냈다. 이를 통해 인위적 요인에 인한 국내 꽃매미 개체군의 빠른 확산 가능성을 보였다.

유효집단의 크기($N_e$)와 이주율($m$)을 천안, 수원, 광주, 대구에서 조사하였다. 그 결과, 천안은 상대적으로 다른 조사지역에 비해 유효집단이 크고 이주율이 낮았다. 발육단계 별(약충, 산란 전 성충, 산란 후 성충) AMOVA 와 Population graph 결과, 동일지역에서 약충과 산란 전 성충간의 유전적 변이를 보이지 않았고, 약충과 산란 후 성충간의 차이는 유의하게 나타났다. 이를 통해 꽃매미의 분산은 산란행동에 의해 영향을 받는 것으로 확인하였다.

통합적인 개체군 생태와 유전학 연구를 통해 꽃매미의 방제를 위한 월동 및 발생과 국내 침입동태 및 분산요인의 확인하였다. 따라서 본 연구를 통해 꽃매미 개체군의 효율적인 관리방안의 제시에 그 의의가 있다.

주요어: 꽃매미, 월동생태, 부화, 개체군 유전학, 생물학적 침입

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