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Larval species composition and genetic structures of *Carposina sasakii*, *Grapholita dimorpha*, and *Grapholita molesta* from Korea

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Abstract

Rapid determination of the larval species composition and understanding of their genetic structure is important to establish the appropriate management system for multiple species infesting in fruits. We established accurate and rapid diagnostic methods based on multiplex polymerase chain reaction (PCR) diagnostic techniques to discriminate the three major lepidopteran species in orchard, *Carposina sasakii*, *Grapholita dimorpha*, and *Grapholita molesta*. Each species was identified by amplifying species-specific PCR products (375 bp for *C. sasakii*, 125 and 234 bp for *G. dimorpha*, and 125 bp for *G. molesta*). Based on species composition analysis from six types of infested fruits, *G. dimorpha* constituted the highest proportion (47.8%), followed by 35.2 and 13.5% for *G. molesta* and *C. sasakii*, respectively. Interestingly, high prevalence was found in *G. dimorpha* and *G. molesta* for plum and peach, respectively. Based on genetic diversity analysis, the three insect species exhibited moderate or high haplotype diversity and low nucleotide diversity, ranging from 0.319 to 0.699 and 0.0006 to 0.0045, respectively. Demographic expansion was not detected according to either a neutrality test or mismatch distribution analysis. Moreover, no significant genetic structure corresponding to province, host plant, fruit type, or collection period was observed. These results suggest that the population of each species would have high dispersal ability following fruit-generating periods via intrinsic host adaptation ability regardless of the spatial and temporal conditions. Determination of larval composition on fruit is valuable for establishing appropriate management systems that take the species into consideration; additionally, population genetic approaches can be utilized to understand the effects of environmental factors (province, host fruit, fruit type, etc.) on population structures.

Keywords: species diagnosis, multiplex PCR, *Carposina sasakii*, *Grapholita dimorpha*, *Grapholita molesta*, genetic structure

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Introduction

Three lepidopteran species, including *Carposina sasakii* Matsumura (Tikhonov, 1962; Liu *et al*., 1997; Kim *et al*., 2000; Ishiguri & Shirai, 2004), *Grapholita dimorpha* Komai (Komai, 1979; Park & Kim, 1986; Oku *et al*., 1988; Yan *et al*., 1998; Komai, 1999; Choi *et al*., 2009), and *Grapholita molesta* Busck (Machida & Aoyama, 1931; Chaudhry, 1956; Pree *et al*., 1994;

Yang *et al.*, 2003; Kirk *et al.*, 2013), have been identified as major pests that damage various types of fruit (i.e., apples, apricots, peaches, pears, plums, quince, etc.) at the larval stage in East Asia. The distribution of *G. molesta* is worldwide (Kirk *et al.*, 2013) and *C. sasakii* is endemic to China, Japan, Korea, and the Russian Far East. *G. dimorpha* was first reported in Japan in the 1970s (Komai, 1979) and was later identified in China (Yan *et al.*, 1998), Korea (Choi *et al.*, 2009), and the Russian Far East (Beljaev & Ponomarenko, 2005). Considering their feeding habits, *G. molesta* can damage the shoot and fruit, whereas *G. dimorpha* and *C. sasakii* can only damage the fruit.

Investigation of the species composition at different larval stages from infested fruits has been hindered by the presence of multiple species and the high morphological similarities among different species. It is difficult to discriminate species when multiple species are observed on a single fruit. Several morphological characters have been described to help distinguish fruit-infesting larvae. In comparison between *G. dimorpha* and *G. molesta*, the number of anal proleg crochets can serve as a key character due to its distinctive difference (12–17 and 18–28 for *G. dimorpha* and *G. molesta*, respectively) (Lee *et al.*, 2015). In addition, the size of prothorax spiracles, arrangements of subventral setae, and the location of subdorsal setae are considered key morphological characters for species identification of *G. molesta* and *C. sasakii* (Lee *et al.*, 2013). However, these characters exhibit a range of variation between individuals and require a high-cost facility utilizing electron microscopy, which can result in the delay of identification. Moreover, it is difficult for a non-taxonomist to identify many specimens.

As an alternative, mitochondrial DNA sequences have been widely used for species identification of fruit-infesting larvae such as *C. sasakii* Matsumura, *Cydia pomonella* (L.), *G. dimorpha* Komai, *Grapholita funebrana* Treitschke, *G. lobarzewskii* Nowicki, *G. molesta* Busck, *Grapholita prunivora* Walsh, and *Grapholita packardi* Zeller (Barcenas *et al.*, 2005; Song *et al.*, 2007; Chen & Dorn, 2009; Hada & Sekine, 2011; Ahn *et al.*, 2013; Jung & Kim, 2013). The nucleotide sequences of the larvae were compared with the adult nucleotide sequences deposited into GenBank by BLAST analysis to confirm the species. Moreover, polymorphisms within the nucleotide sequences that were highly specific to a species were further utilized as genetic markers for the development of diagnostic methods. For example, polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) analyses (Song *et al.*, 2007; Chen & Dorn, 2009; Lee *et al.*, 2013, 2015; Choi *et al.*, 2016) and species-specific PCR (Barcenas *et al.*, 2005; Ahn *et al.*, 2013; Jung & Kim, 2013) were employed extensively using the sequence polymorphisms of several mitochondrial genes such as cytochrome *c* oxidase subunit I, NADH dehydrogenase 4, mitochondrial cytochrome c oxidase subunit I (COI), 16s rRNA, and cytochrome b. These methods are valuable for detecting the species without sequence analysis by reducing the cost. However, the accuracy of PCR–RFLP and gene-specific PCR is reduced by the existence of nucleotide polymorphisms at restriction enzyme sites or at priming sites, due to the existence of various haplotypes in each individual (Ahn *et al.*, 2013; Choi *et al.*, 2016). Hence, it is necessary to choose the restriction enzyme site and priming site taking into consideration the various types of haplotypes by species for PCR–RFLP and species-specific PCR, respectively.

C. sasakii, *G. dimorpha*, and *G. molesta* have high migratory ability and move to different host plants following the host-plant developmental stages. For example, 1st and 2nd generation *G. molesta* mainly affect stone fruits such as plum and peach; however, 3rd and 4th generation *G. molesta* move to pome fruits such as apple and pear (Phillips & Proctor, 1970; Makaji, 1987; Yang *et al.*, 2001). To understand the population's attributes such as migration and host preference, population genetic studies have been adopted to determine the genetic structure of *G. molesta* (Kirk *et al.*, 2013; Zheng *et al.*, 2013; Wei *et al.*, 2015) and *C. sasakii* (Wang *et al.*, 2015) populations. These studies have provided valuable information and helped us understand the world population's genetic structure and relationship with the host plant. In the case of *G. molesta*, significant positive correlations were detected between genetic differentiation and geographic distances in China (Zheng *et al.*, 2013). Moreover, the migration route was investigated by approximate Bayesian computation analysis (Wei *et al.*, 2015). The existence of two cryptic lineages has been observed and grouped by a molecular signature in *C. sasakii* (Wang *et al.*, 2015). In Korea, three larval species was collected on apple fruits and their genetic diversities were shown as low range of genetic distance among populations (Kwon *et al.*, 2017). These previous studies have been very informative for our understanding of the genetic attributes associated with environmental factors such as the host plant, collection site, collection period, etc., and have enabled us to understand the effects of intrinsic factors on behavior.

In this study, we obtained partial COI sequences by sequence analysis and accurately identified 440 fruit-infesting larvae collected from six different fruits using BLAST to determine the species composition at various larval stages. In addition, we developed multiplex PCR-based diagnostic methods to be able to use single reactions based on a total of 25 representative haplotypes as a standard template from three major pest species in Korea: *C. sasakii*, *G. dimorpha*, and *G. molesta*. Moreover, we evaluated the genetic structures from each species based on the haplotype frequency of COI nucleotide sequences considering spatial and temporal conditions using population genetic approaches.

Materials and methods

Specimen collection

Larvae-infested fruits were collected with the permission of farm owners or were obtained in public places. Fruits that had fallen to the ground due to larval infestation were collected.

A total of 1813 larvae were collected from an average of 89.2 fruits (10–304 fruits) that had a dark hole or digging lesions from internal feeders on the surface from six types of fruits (plum, apricot, pear, apple, peach, and quince) in 17 regions of South Korea from June to October, 2015–2016 (fig. 1 and online Supplementary table S1). Larvae were collected carefully using forceps after dissecting the infested region on the fruits and stored immediately at −20°C.

Genomic DNA extraction

The number of individual by each population for the genomic DNA extraction was roughly determined by considering the sample size. When the sample size of each population was over 60 individuals, 20–40% of individual from each population was randomly chosen. On the other hand, if there were fewer than 25 individuals in a population, more than 90% of

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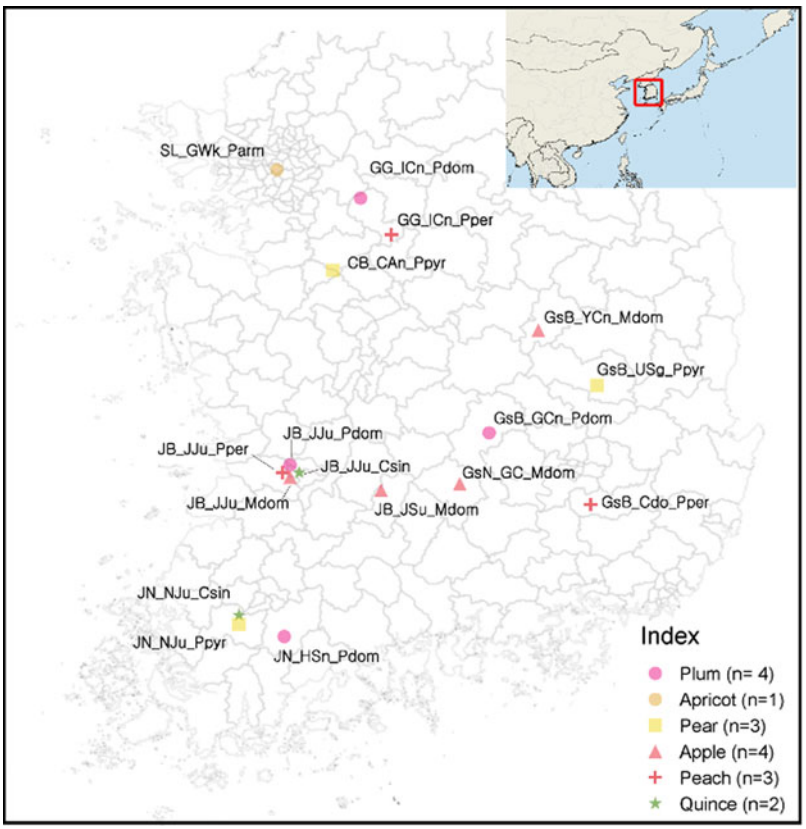

Fig. 1. Location of sample collection from six different host fruits.

individual were used for analysis. Based on this, the genomic DNAs were extracted individually from 440 larvae (24.3% of the total sample) using the DNeasy Blood and Tissue Kit (QIAGEN Korea Ltd., Seoul, Korea) following the manufacturer's instructions. Briefly, the whole body was homogenized in 200 µl of lysis buffer (190 µl AL buffer and 10 µl proteinase K) using a BioMasher II disposable grinder (BIOFACT Co., Ltd, Daejeon, Korea) and incubated at 56°C for 1 h. The homogenate was transferred to a DNA-binding column and centrifuged at 8000 *g* for 1 min to purify the genomic DNA. After washing with AW1 and AW2 buffers, genomic DNA was eluted with 100 µl of elution buffer and stored at −20°C before use.

PCR amplification of COI gene fragments and species identification

A fragment of the COI gene (~700 bp), which was a part of DNA barcode fragment was amplified and its nucleotide sequence was utilized for species identification. PCR amplification was conducted in 50-µl reaction mixtures containing 10 mM dNTP, 10 µM each forward and reverse primer (LCO1490 vs. HCO2198, online Supplementary table S2) (Folmer *et al*., 1994), 100–200 ng genomic DNA, and 5 U *Taq* DNA polymerase (BIONEER Corp., Daejeon, Korea) under the following conditions: 60 s at 94°C, 60 s at 50°C, and 30 s at 72°C for 35 cycles. The PCR products were further purified using the *AccuPrep* PCR Purification Kit (BIONEER Corp.) and used directly for sequence analysis on an ABI 3730 DNA analyzer (Thermo Fisher Scientific, Waltham, MA) in a BIONEER sequencing facility (BIONEER Corp.). The individual COI amplicon sequence (555 bp) was further used as a query for standard nucleotide BLAST searches (BlastN) at the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch).

Multiplex PCR larval diagnosis

PCR-based larvae diagnostic methods were established to discriminate three fruit-infesting larvae: *C. sasakii*, *G. dimorpha* and *G. molesta*. The species-specific primers were designed from relatively highly variable regions based on the representative haplotype nucleotide sequences of COI with different amplicon sizes (online Supplementary table S2). The genomic DNA templates for haplotype sequences from each species were used as standard templates to evaluate the specificity of the species-specific primers. To determine the optimum PCR conditions, we tested each species-specific primer pair separately on all representative haplotypes (eight haplotypes for *C. sasakii*, 12 haplotypes for *G. dimorpha*, and five haplotypes for *G. molesta*) as templates. The PCR amplification was conducted in 25-µl reaction mixtures containing 10 mM dNTP, 2.5 µM three pairs of primer sets for each species (online Supplementary table S2), 10 ng genomic DNA, and 0.5 U *Taq* DNA polymerase (BIONEER Corp.) under the following conditions: a cycle of 2 min at 94°C and 30 cycles of 20 s at 94°C, 20 s at 57°C, and 30 s at 72°C. The specificity of species detection was confirmed by gel electrophoresis on a 1.2%

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agarose gel. The primer concentration and annealing temperature exhibiting the highest specificity were chosen by changing each condition empirically.

When optimizing the PCR conditions for the detection of the three species, we employed multiplex PCR, which is able to detect the three species in a single-tube reaction. The multiplex PCR was conducted with 10 mM dNTP, species-specific primer mixtures (2, 3, and 4 μM for each primer pair for *C. sasakii*, *G. dimorpha*, and *G. molesta*, respectively; online Supplementary table S2), 10 ng genomic DNA, and 0.5 U *Taq* DNA polymerase (BIONEER Corp.) in 25-μl reaction mixtures. Thermal cycler conditions were the same as above except 27 cycles were used. The PCR products were electrophoresed in 1.2% agarose gels to discriminate the larval species. Images were taken with a GDS-200C digital gel documentation system (Korea LabTech, Seongnam-si, Korea).

Larval distribution

Statistical analysis of mean comparisons of larval composition by fruit type was conducted using one-way analysis of variance (ANOVA) with *post hoc* Tukey's HSD test with IBM SPSS Statics version 23 software (IBM Korea Inc., Seoul, Korea).

Population genetic analysis

The COI nucleotide sequences were aligned by using MUSCLE (Edgar, 2004) and trimmed at 555 bp by using MEGA software (version 6.0) (Tamura *et al.*, 2013). The genetic diversity of each population was determined by obtaining the number of segregations (*S*), the number of haplotypes (*H*), haplotype diversity (*Hd*), and nucleotide diversity (π), which were estimated using DnaSP version 5.10 software (Librado and Rozas, 2009). To understand the demographic history, we conducted a neutrality test and mismatch distribution (spatial expansion) using ARLEQUIN ver. 3.5.1.2 (Excoffier and Lischer, 2010) with 1000 simulations. Genetic differentiation among the populations was calculated using pairwise F_{ST} values with ARLEQUIN. Analysis of molecular variance (AMOVA) was calculated using ARLEQUIN to determine the genetic structures and to compare the three fixation indices (F_{ST}, F_{SC}, and F_{CT}) by province, host plant, fruit type, and collection period. Populations with less than three individuals of each species were excluded from the analysis of demographic history, genetic differentiation, and AMOVA.

Results

Number of larvae from infested fruits

A total of 1813 larvae were collected from 1516 larvae-infested fruits obtained from six types of fruit (online Supplementary table S1). The average number of larvae per total fruit was 1.2 ± 0.2, which ranged from 0.2 to 3.3 (fig. 2). Quince had the highest capacity to attract egg laying females (up to 2.3 larvae per fruit; 1–6 larvae were observed on single quince fruits) (online Supplementary table S1). Generally, 1–3 larvae were found on a single fruit from each of peach, pear, and plum. In apricot and plum, the average number of larvae ranged from 0.2 to 0.7 larvae per fruit due to the presence of larvae-empty fruit.

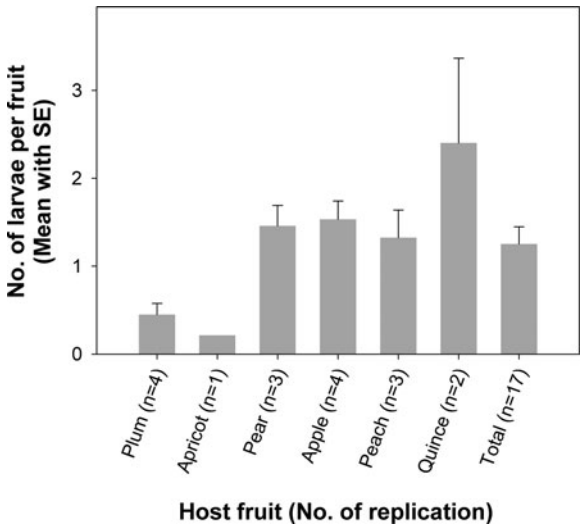

Fig. 2. Number of larvae per fruit in six different host fruits.

Species and haplotype composition determination via COI nucleotide sequence

Individual larvae (440; 24.3% of total specimens) were chosen randomly and their COI nucleotide sequences (555 bp) were used as queries for species identification using standard nucleotide BLAST searches at the NCBI website. In total, five species were identified, including *C. sasakii*, *Conogethes punctiferalis*, *Euzophera pyriella*, *G. dimorpha*, and *G. molesta*, with sequence identities as high as 98–100% (online Supplementary table S3). Two individual larvae collected from quince at Naju revealed the highest identity (94%) with *Euzophera bigella* (JF85985.1). An individual larva collected from peach at Jeonju revealed the highest identity with *Spilonota albicana* (96% identity; KF523835.1). However, neither species was considered an exact species due to their low identity (<98% identity) (online Supplementary table S3).

Sequence analysis revealed that 98% of the larval composition from 15 populations comprised *C. sasakii*, *G. dimorpha*, and *G. molesta* (online Supplementary fig. S1). Among them, *G. dimorpha* exhibited the highest proportion (46.4%). In the analysis of haplotype composition, eight haplotypes (Csas_Hap1–Csas_Hap8), 12 haplotypes (Gdim_Hap1–Gdim_Hap12), and five haplotypes (Gmol_Hap1–Gmol_Hap5) were found in *C. sasakii*, *G. dimorpha*, and *G. molesta*, respectively (online Supplementary tables S3 and S4). Csas_Hap2, Gdim_Hap1, and Gmol_Hap4 exhibited the highest proportion of each species at 42.1, 57.4, and 81.1%, respectively (online Supplementary table S4). *Conogethes punciferalis* and *E. pyriella* were observed as minor proportions (0.5 and 0.9%, respectively). Three (Epyr_Hap1–Epyr_Hap3) and two (Cpun_hap1 and Cpun_Hap2) haplotypes were found in *E. pyriella* and *C. punciferalis*, respectively (online Supplementary tables S3 and S4). The haplotype frequencies of *C. sasakii*, *G. dimorpha*, and *G. molesta* were further used as representative templates for the establishment of species-specific diagnostic PCR and population genetic analysis.

Development of a multiplex PCR diagnosis

For rapid and accurate detection focusing on the three target species (*C. sasakii*, *G. dimorpha*, and *G. molesta*), we

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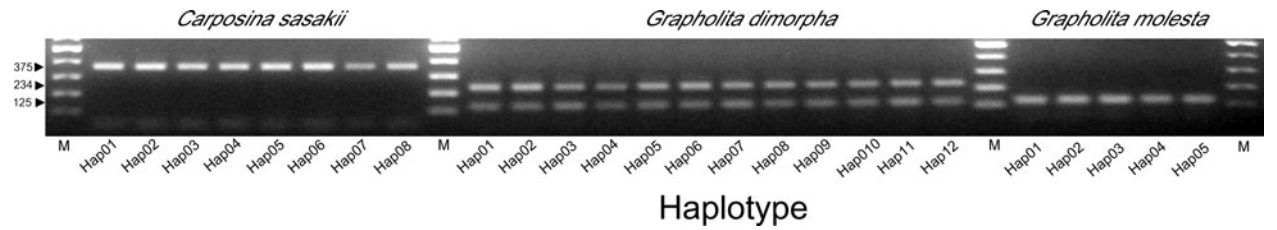

Fig. 3. Results of multiplex PCR using species-specific primers on 1.2% agarose gels using representative haplotypes from *C. sasakii*, *G. dimorpha*, and *G. molesta*. M represents the 100-bp molecular marker.

developed diagnostic PCR methods based on multiplex PCR. First, species-specific primers were designed considering the low diversity from various haplotypes in a species and the high diversity among the three species by using representative haplotype which could not be overlapped after genotyping from the three species (8, 12, and 5 haplotypes for *C. sasakii*, *G. dimorpha* and *G. molesta*, respectively) (online Supplementary table S3). The species-specific primers successfully amplified the expected target products (*C. sasakii*, 375 bp; *G. dimorpha*, 234 bp; and *C. molesta*, 125 bp) from the representative haplotype from each species with high specificity (online Supplementary fig. S2). Next, multiplex PCR was conducted by combining the three sets of species-specific primers in a single reaction mixture using same representative haplotype templates from each species (fig. 3). Interestingly, single PCR products of the expected sizes of 375 and 125 bp were produced from *C. sasakii* and *G. molesta*, respectively (fig. 3). On the other hand, two PCR products (125 and 234 bp) were generated from the *G. dimorpha* template, indicating the reduced specificity of *G. molesta*-specific primers in multiplex PCR mixtures. However, these primers were useable for species identification because *G. dimorpha* primers were still able to amplify the *G. dimorpha* genomic DNA. As a result, PCR products for the target species with sizes of 375 bp for *C. sasakii*, 125 and 234 bp for *G. dimorpha*, and 125 bp for *G. molesta* were amplified specifically (fig. 3).

When using multiplex PCR to identify the haplotypes of *C. punctiferalis* and *E. pyriella*, no PCR amplification was observed (data not shown), indicating that the diagnostic methods was highly specific for the identification of the three species.

Species composition in infested fruits

The remaining 1373 larvae were identified by diagnostic multiplex PCR methods, and species composition was determined from a total of 1813 larva. 95.4% of larvae (1730 larvae) consisted mainly of *C. sasakii* (24.4%), *G. dimorpha* (42.3%), and *G. molesta* (28.7%) (fig. 4a). The remaining 4.6% (83 larvae) did not yield amplification products, suggesting the existence of either other species with low frequency on the fruit such as *C. punctiferalis* or *E. pyriella* or unscored haplotypes of the three target species that could not produce PCR amplification.

G. dimorpha was observed in the fruit with the highest proportion (45.0 ± 10.4%) among three species from all fruits. In the ANOVA analysis by fruit types, a significant mean difference was observed (99.1 ± 0.8% and 50.9 ± 16.8%) in plum

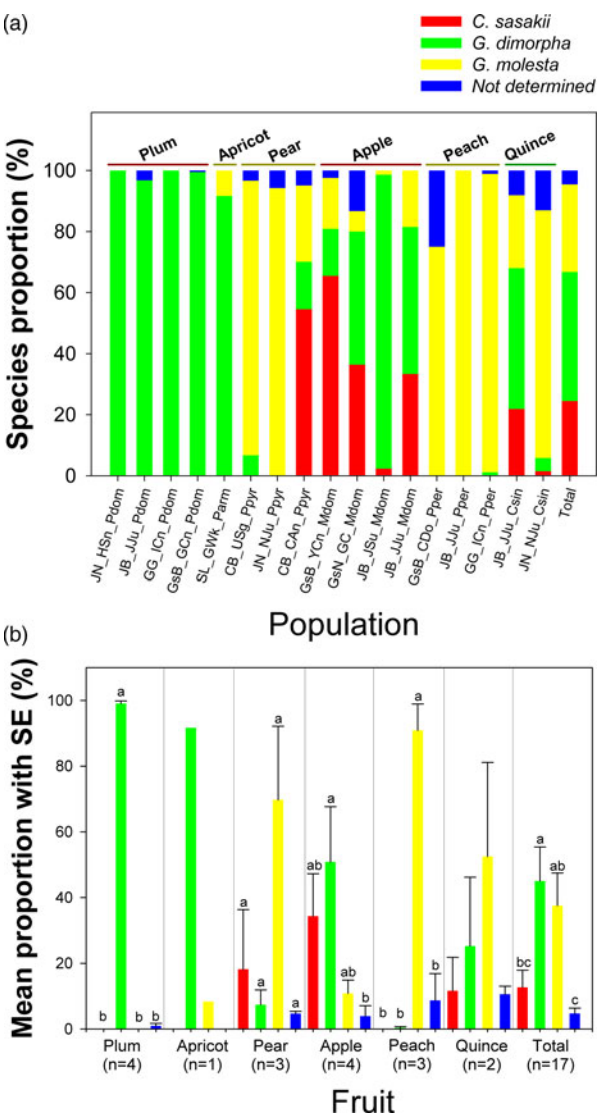

Fig. 4. Larval-species composition of three species obtained by multiplex PCR from 1813 larvae from six different host fruits. (a) Species proportion by each population. (b) Mean proportion of the three species by fruit. The small characters represent the statistical difference determined by Tukey's HSD and ANOVA.

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Table 1. The summarized genetic diversity indices from three species, *C. sasakii*, *G. dimorpha*, and *G. molesta*.

Species	Population	*N*	*S*	*H*	*Hd*	π	Tajima's D	Fu's FS	SSD	Raggedness index	Remarks
Carposina sasakii	CB_CAn_Ppyr	20	8	4	0.6737	0.00364	−0.35 (0.418)	1.82 (0.851)	0.041 (0.416)	0.185 (0.504)	
,,	GsB_YCn_Mdom	19	3	3	0.6784	0.00215	1.06 (0.858)	1.6 (0.823)	0.005 (0.524)	0.047 (0.834)	
,,	GsN_GC_Mdom	17	9	5	0.7353	0.00368	−0.84 (0.206)	0.48 (0.639)	0.018 (0.485)	0.053 (0.734)	
,,	JB_JJu_Csin	18	3	3	0.6209	0.00166	0.16 (0.609)	0.93 (0.684)	0.009 (0.278)	0.097 (0.517)	
,,	JB_JJu_Mdom	17	9	4	0.6691	0.00334	−1.1 (0.129)	1.31 (0.799)	0.023 (0.555)	0.092 (0.659)	
,,	JB_JSu_Mdom	3	1	2	0.6667	0.0012	0 (0.991)	0.2 (0.41)	0.093 (0)	0.556 (1)	
,,	JN_NJu_Csin	1	na	1	na	na	na	na	na	na	Excluded
,,	Total	94	11	8	0.6994	0.00294	−0.63 (0.288)	0.41 (0.63)	0.003 (0.616)	0.031 (0.816)	
Grapholita dimorpha	CB_CAn_Ppyr	19	11	4	0.4561	0.00272	−1.87 (0.021)	0.93 (0.724)	0.018 (0.3)	0.151 (0.776)	
,,	GG_ICn_Pdom	7	3	3	0.7143	0.00257	0.75 (0.8)	0.67 (0.631)	0.047 (0.352)	0.204 (0.589)	
,,	GG_ICn_Pper	1	na	1	na	na	na	na	na	na	Excluded
,,	GsB_GCn_Pdom	20	12	4	0.6158	0.01022	2.43 (0.996)	6.34 (0.989)	0.135 (0.052)	0.379 (0.413)	
,,	GsB_YCn_Mdom	18	12	5	0.719	0.00521	−0.64 (0.285)	1.57 (0.788)	0.04 (0.107)	0.109 (0.316)	
,,	GsN_GC_Mdom	29	15	5	0.6256	0.00551	−0.68 (0.26)	2.82 (0.892)	0.045 (0.335)	0.117 (0.763)	
,,	JB_JJu_Csin	18	2	3	0.2157	0.0004	−1.51 (0.034)	−1.74 (0.009)	0.001 (0.13)	0.372 (0.858)	
,,	JB_JJu_Mdom	25	4	3	0.4767	0.00119	−1.02 (0.186)	0.5 (0.565)	0.018 (0.056)	0.178 (0.437)	
,,	JB_JJu_Pdom	14	12	5	0.7802	0.0082	0.82 (0.819)	2.39 (0.864)	0.076 (0.163)	0.14 (0.614)	
,,	JB_JSu_Mdom	20	11	4	0.6053	0.00294	−1.68 (0.022)	1.21 (0.762)	0.034 (0.167)	0.156 (0.51)	
,,	JN_HSn_Pdom	20	3	3	0.6263	0.00193	0.7 (0.784)	1.38 (0.775)	0.001 (0.848)	0.037 (0.954)	
,,	JN_NJu_Csin	2	0	1	0	0	0 (1)	0 (na)	na (na)	na (na)	Excluded[1]
,,	SL_GWk_Parm	11	2	3	0.6364	0.00131	0.2 (0.647)	−0.02 (0.393)	0.041 (0.028)	0.248 (0.233)	
,,	Total	203	24	12	0.6305	0.00449	−1.06 (0.135)	−0.03 (0.572)	0.028 (0.512)	0.081 (0.786)	
Grapholita molesta	CB_CAn_Ppyr	17	1	2	0.4412	0.00079	0.95 (0.883)	1.07 (0.592)	0.012 (0.001)	0.208 (0.491)	
,,	GG_ICn_Pper	11	2	3	0.4727	0.00092	−0.78 (0.233)	−0.66 (0.11)	0.017 (0.177)	0.17 (0.632)	
,,	GsB_YCn_Mdom	13	1	2	0.1539	0.00028	−1.15 (0.182)	−0.54 (0.103)	0 (0.167)	0.503 (0.974)	
,,	GsN_GC_Mdom	6	1	2	0.6	0.00108	1.45 (0.978)	0.8 (0.552)	0.06 (0)	0.4 (0.546)	
,,	JB_JJu_Csin	20	2	3	0.5105	0.00098	−0.09 (0.416)	−0.06 (0.366)	0.025 (0.003)	0.201 (0.253)	
,,	JB_JJu_Mdom	8	0	1	0	0	0 (1)	0 (na)	0 (0)	0 (0)	
,,	JB_JJu_Pper	10	1	2	0.4667	0.00084	0.82 (0.87)	0.82 (0.541)	0.016 (0.006)	0.222 (0.603)	
,,	JB_JSu_Mdom	2	1	2	1	0.0018	0 (1)	0 (0.235)	na (na)	na (na)	Excluded
,,	JN_NJu_Csin	29	1	2	0.133	0.00024	−0.75 (0.19)	−0.41 (0.154)	0 (0.106)	0.556 (0.924)	
,,	JN_NJu_Ppyr	15	0	1	0	0	0 (1)	0 (na)	0 (0)	0 (0)	
,,	SL_GWk_Parm	1	na	1	na	na	na	na	na	na	Excluded
,,	Total	131	4	5	0.3193	0.00059	−0.73 (0.26)	−1.34 (0.205)	0.004 (0.002)	0.237 (0.585)	

[1]Those populations were excluded by low number of individual.

($df_{3,12} = 8282.1$, $P < 0.001$) and apple ($df_{3,12} = 3.9$, $P < 0.05$), respectively (fig. 4b). The proportion of *G. molesta* was 37.6 ± 9.9%; it was found mainly in peach, pear, quince, apple, and apricot (fig. 4). Interestingly, 90.9 ± 8.0% were found in peach, representing a significant difference ($df_{3,8} = 59.7$, $P < 0.01$) (fig. 4b). *C. sasakii* was observed at the lowest proportion (12.7 ± 5.2%), observed only in pear, apple, and quince (fig. 4).

Genetic diversity and demographic history of the target species

Genetic diversity indices (*S*, *H*, *Hd*, and π) of the three species are summarized in table 1. The total *Hd* values were 0.699 (0.621–0.735), 0.631 (0.216–0.719), and 0.319 (0–0.6) in *C. sasakii*, *G. dimorpha*, and *G. molesta*, respectively. The total π values were 0.0029 (0.0012–0.0037), 0.0045 (0.0004–0.010), and 0.0006 (0.0002–0.0018) in *C. sasakii*, *G. dimorpha*, and *G. molesta*, respectively. The diversity of *C. sasakii* and *G. dimorpha* was higher than that of *G. molesta*.

Demographic expansion was tested through neutrality tests (Tajima's *D* and Fu's F_S), where a significant negative value could be considered an indicator of population expansion. No significant negative value was observed from the three species using the entire populations (table 1). In the mismatch distribution from the spatial expansion model, bimodal distribution was shown by *C. sasakii* and *G. dimorpha* (fig. 5a, b), suggesting demographic equilibrium. In the case of *G. molesta*, unimodal distribution was observed; however, this result was not consistent with that from the neutrality test (fig. 5c). Moreover, a moderately large SSD and a non-significant *P*-value were observed, supporting the failure of the null hypothesis of demographic expansion (table 1).

In summary, moderate *Hd* and low π were observed from all three species. The demographic history of the three species is likely to be in demographic equilibrium according to the non-significant negative value in the neutrality test and the bimodal pattern of mismatch distribution for *C. sasakii* and *G. molesta*. However, there were inconsistent values between the neutrality test and mismatch distribution in *G. molesta*.

Genetic differentiation and structure

Genetic differentiation was calculated by pairwise comparisons of the F_{ST} test and ranged between −0.159 and 0.145, −0.047 and 0.512, and −0.086 and 0.595 for *C. sasakii*, *G. dimorpha*, and *G. molesta*, respectively, by using 428 individual based on the sequence variations of COI genes (tables 2–4, respectively).

The total genetic variation in *C. sasakii*, *G. dimorpha*, and *G. molesta* was 0.037 ($P = 0.077$), 0.141 ($P < 0.001$), and 0.081 ($P < 0.05$), respectively, suggesting that all three species have low genetic variation among the populations. In addition, no population genetic structure for *C. sasakii* was observed, while little but significant population genetic structure was observed for both *Grapholita* species (table 5).

We investigated the genetic structures of the three species according to province, host plant, fruit type, and collection period using AMOVA (table 5). The fixation indices of F_{CT} value represented the non-significant low values as −0.067 to 0.071, −0.018 to 0.049, and −0.096 to −0.013 in *C. sasakii*, *G. dimorpha*, and *G. molesta*, respectively, suggesting that genetic structure would not be occurred by the classification to province, host plant, fruit type, and collection period.

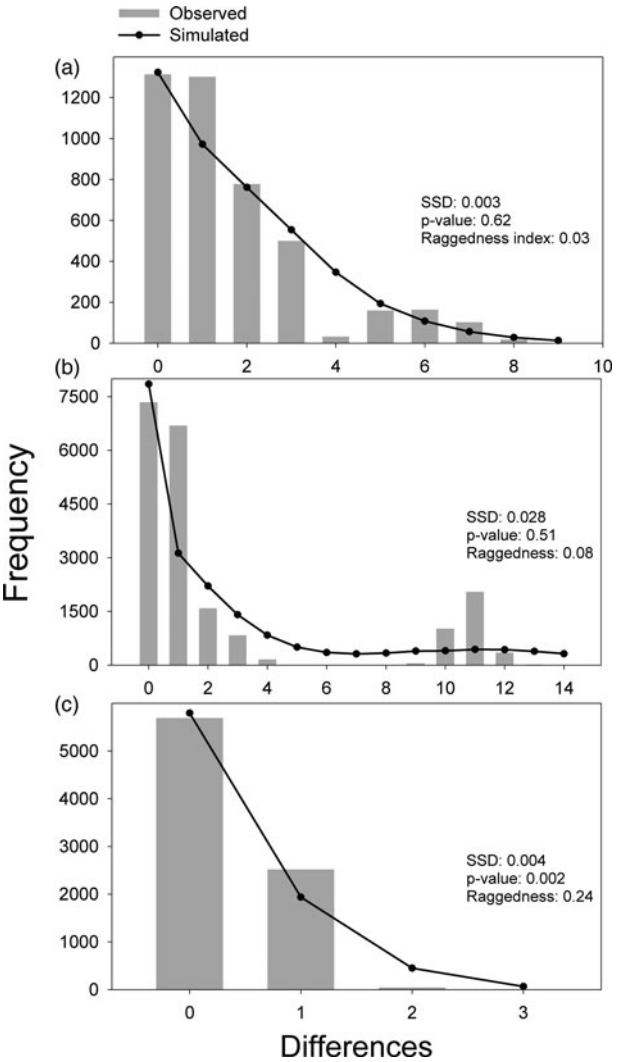

Fig. 5. Mismatch distribution by spatial expansion using the entire populations from *C. sasakii* (a), *G. dimorpha* (b), and *G. molesta* (c).

Discussion

We determined the larval composition of six different fruits by employing sequence analysis and a molecular diagnostic technique based on multiplex PCR methods. The species identification of larvae is an obstacle to accurately estimating the population size of fruit-infesting larvae. PCR-based diagnostic techniques have been widely employed to detect two species (*G. molesta* vs. *G. dimorpha* or *G. molesta* vs. *C. sasakii*); however, there are no methods currently available to detect three species in a single PCR. In this study, we identified species from among 440 larvae. Species-specific primers were designed from 8, 12, and 5 haplotypes from *C. sasakii*, *G. dimorpha*, and *G. molesta*, respectively. The primers displayed high specificity by generating species-specific PCR amplicons (online Supplementary table S2). However, this method requires three PCRs for diagnostic purposes. To decrease the cost of PCR, we employed multiplex PCR by preparing primer mixtures. The annealing temperature, thermal cycler conditions,

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Table 2. Genetic differentiation by pairwise F_{ST} from *C. sasakii*. Lower diagonal and upper diagonal represent the F_{ST} and significant differences (+, $P < 0.05$).

Population	CB_CAn_Ppyr	GsB_YCn_Mdom	GsN_GC_Mdom	JB_JJu_Csin	JB_JJu_Mdom	JB_JSu_Mdom
CB_CAn_Ppyr	0	+	−	+	+	+
GsB_YCn_Mdom	0.074	0	−	−	−	−
GsN_GC_Mdom	0.071	0.025	0	−	−	−
JB_JJu_Csin	0.119	−0.036	0.004	0	−	−
JB_JJu_Mdom	0.105	−0.024	−0.011	−0.022	0	−
JB_JSu_Mdom	0.145	−0.039	−0.107	−0.088	−0.159	0

and primer concentrations were the main factors that affected the specificity. *G. dimorpha* was identified by two bands at 125 and 234 bp due to the loss of specificity of the *G. molesta* primers caused by increasing the forward primer concentration (90% identity at the forward region of the oligonucleotide between *G. dimorpha* and *G. molesta*), resulting in the amplification of two bands from *G. dimorpha*. However, the primer mixture used for multiplex PCR was able to discriminate *G. molesta* by generating a single band (125 bp). The diagnostic methods have an advantage to detect larval species without the use of restriction enzymes and can easily be adopted at local diagnostic centers.

The predominant larval composition of three species would be affected mainly by the host preference, egg-laying habit, and number of generations per year. The presence of *G. dimorpha* larvae in the highest proportion on the fruit may be explained by the number of generations (four generations per year) (Choi *et al.*, 2009) and egg-laying habit, which can infest only the fruit. In addition, the proportion of *G. dimorpha* was observed in almost all fruit types in this study (fig. 4). In the case of *G. molesta*, its contribution to the larval composition was less than that of *G. dimorpha*, even though a similar occurrence pattern was revealed along with the highest proportion of the adult population density in the field. In the monitoring of population density by pheromone traps in plum and apple orchards during 2011–2012, *G. molesta* was shown to be the dominant species (Choi *et al.*, 2009; Jeong *et al.*, 2012); however, its larval composition was less dominant than that of *G. dimorpha*. This could be explained by its egg-laying habit; it can lay eggs both on fruit and on the shoot. By expanding the adaption range for egg-laying to include the shoot, the larval composition would be less than that of *G. dimorpha* regardless of the population density of the adult. The population density of *C. sasakii* was the lowest among the three species; this is typically caused by the relatively low number of generations (two generations on apple and plum per year in Korea) (Kim *et al.*, 2001; Jeong *et al.*, 2012). Interestingly, the high proportion of prevalence of fruit *G. dimorpha* and *G. molesta* was found for plum and pear, respectively, in this study (fig. 4b). This result is not consistent with the adult population density; rather, it may be related to the egg-laying habit of adults on the fruit of each host plant. Further analysis is necessary to understand the intrinsic habit of adult species on host plants by comparing the number of adults and the larval population composition in the field.

Focusing on the host preference on plum, we conducted AMOVA to confirm the genetic structures. However, there was no significant difference with regard to host plant or provinces among these three species. Therefore, this result might be caused by the high dispersal rate following the wide host preference. A population movement was proposed in several studies of *G. molesta*, in which the movement occurred from plum to apple fruit of different stages of species (Choi *et al.*, 2008). The movement of *G. molesta* in apple orchards was investigated using random amplified polymorphic DNA (RAPD) markers (Kim *et al.*, 2009). The allelic frequency increased during early April after its first occurrence; however, it decreased as time progressed. Moreover, the clustering of the local population fluctuated in April, July, August, and September. These results strongly support the active movement of the *G. molesta* population. In this study, we observed high/moderate haplotype diversity and low nucleotide diversity, which have been observed in highly dispersal species, such as that seen in *Laodelphax striatella* (Sun *et al.*, 2015). This also suggests that the dispersal ability of the three species would be an active movement. Almost all species exhibited demographic equilibrium with insignificant Tajima's *D* and Fu's F_S values (table 1). Moreover, the bimodal pattern of mismatch distribution was observed in *C. sasakii* and *G. molesta*. Though a skewed-unimodal pattern was observed in *G. moelsta*, there was no obvious basis for demographic expansion from a bottleneck effect (fig. 5 and table 1). As a result, the three species would reach demographic equilibrium and it would be mediated by their high migration abilities. The movement following the fruit would also disrupt the population structure by the host plant and collection date. More genetic markers are also necessary to understand the genetic structure in detail as a further study.

The existence of *E. pyriella* was first observed in Korea. The biology and damage of *E. pyriella* has not been well-reported in Korea. However, its importance in pear orchards has been reported in Xinjiang, China (Song *et al.*, 1994; Ma *et al.*, 2014). Its hosts are also known: *Malus pumila* Mill, *Ziziphus jujube* Mill, *Prunus armeniaca* Linn, *Amygdalus persica* Linn, and *Ficus carica* Linn (Song *et al.*, 1994). In this study, only pears and quince were found with other *Euzophera* spp. It is necessary to survey its distribution and damage in Korea as a preliminary survey for proper management.

In conclusion, molecular diagnostic techniques based on multiplex PCR should be considered as a practical technique to detect three species in a single PCR while reducing both the cost and time required for analysis. The existence of three major pests, *C. sasakii*, *G. dimorpha*, and *G. molesta*, was confirmed, and information on larval composition will be utilized as preliminary data to establish the appropriate pest management systems considering the ecology of each species. The population genetic approach is a powerful technique used to understand intrinsic genetic attributes and is valuable for tracing the genetic structure by province, host plant, and collection period. Although no genetic structure was found in this study, new polymorphic markers such as microsatellites and

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Table 3. Genetic differentiation by pairwise F_{ST} from *G. dimorpha*.

Population	CB_CAn_Ppyr	GG_ICn_Pdom	GsB_GCn_Pdom	GsB_YCn_Mdom	GsN_GC_Mdom	JB_JJu_Csin	JB_JJu_Mdom	JB_JJu_Pdom	JB_JSu_Mdom	JN_HSn_Pdom	SL_GWk_Parm
CB_CAn_Ppyr	0	+	+	−	−	−	−	−	−	+	−
GG_ICn_Pdom	0.221	0	+	−	−	+	+	+	+	+	−
GsB_GCn_Pdom	0.223	0.253	0	−	+	+	+	−	+	+	+
GsB_YCn_Mdom	−0.018	0.121	0.114	0	−	+	+	−	−	+	−
GsN_GC_Mdom	−0.012	0.114	0.126	−0.041	0	−	+	−	−	+	−
JB_JJu_Csin	−0.002	0.512	0.334	0.090	0.066	0	−	+	−	+	+
JB_JJu_Mdom	0.009	0.439	0.339	0.075	0.072	0.061	0	+	−	+	+
JB_JJu_Pdom	0.066	0.150	0.004	−0.025	−0.009	0.202	0.181	0	−	+	−
JB_JSu_Mdom	−0.047	0.209	0.215	−0.029	−0.015	0.030	0.003	0.050	0	+	+
JN_HSn_Pdom	0.244	0.231	0.332	0.151	0.139	0.442	0.428	0.220	0.229	0	−
SL_GWk_Parm	0.089	0.121	0.260	0.042	0.038	0.351	0.307	0.129	0.083	0.062	0

Lower diagonal and upper diagonal represent the F_{ST} and significant differences (+, $P < 0.05$).

Table 4. Genetic differentiation by pairwise F_{ST} from *G. molesta*.

Population	CB_CAn_Ppyr	GG_ICn_Pper	GsB_YCn_Mdom	GsN_GC_Mdom	JB_JJu_Csin	JB_JJu_Mdom	JB_JJu_Pper	JN_NJu_Csin	JN_NJu_Ppyr
CB_CAn_Ppyr	0	−	−	−	−	−	−	−	+
GG_ICn_Pper	−0.031	0	−	−	−	−	−	−	−
GsB_YCn_Mdom	0.074	−0.023	0	−	−	−	−	−	−
GsN_GC_Mdom	−0.025	0.069	0.336	0	−	−	−	+	+
JB_JJu_Csin	−0.052	−0.024	0.065	−0.033	0	−	−	+	+
JB_JJu_Mdom	0.160	0.029	−0.042	0.461	0.127	0	−	−	−
JB_JJu_Pper	−0.086	−0.053	0.078	−0.059	−0.075	0.187	0	−	−
JN_NJu_Csin	0.138	0.027	−0.058	0.448	0.127	−0.036	0.154	0	−
JN_NJu_Ppyr	0.234	0.104	0.011	0.595	0.192	0	0.291	0.003	0

Lower diagonal and upper diagonal represent the F_{ST} and significant differences (+, $P < 0.05$).

Table 5. The genetic structure by AMOVA from three species by province, host plant, fruit type, and collection period.

Species	Division	Source of variation	Df	S.S	Variance of component	% Variation	Fixation indices (*P*-value)
C. sasakii	Total	Among populations	5	6.3	0.031	3.7	F_{ST} = 0.037 (0.077)
„	„	Within populations	88	69.6	0.791	96.3	
„	„	Total	93	75.9	0.822		
„	Province (three provinces)	Among divisions	2	4.5	0.056	6.7	F_{CT} = 0.067 (0.163)
„	„	Among populations within divisions	3	1.8	−0.014	−1.6	F_{SC} = −0.017 (0.536)
„	„	Within populations	88	69.6	0.791	95	F_{ST} = 0.05 (0.069)
„	„	Total	93	75.9	0.840		
„	Host plant (three host plants)	Among divisions	2	4.3	0.060	7.1	F_{CT} = 0.071 (0.33)
„	„	Among populations within divisions	3	2.0	−0.011	−1.3	F_{SC} = −0.014 (0.601)
„	„	Within populations	88	69.6	0.791	94.2	F_{ST} = 0.058 (0.079)
„	„	Total	93	75.9	0.840		
„	Collection period (by month)	Among divisions	2	2.9	0.008	0.97	F_{CT} = 0.01 (0.35)
„	„	Among populations within divisions	3	3.4	0.025	3.02	F_{SC} = 0.031 (0.217)
„	„	Within populations	88	69.6	0.791	96.01	F_{ST} = 0.04 (0.066)
„	„	Total	93.0	75.9	0.824		
G. dimorpha	Total	Among populations	10	43.5	0.180	14.1	F_{ST} = 0.141 (P < 0.001)
„	„	Within populations	190	207.8	1.094	85.9	
„	„	Total	200	251.3	1.274		
„	Province (five provinces)	Among divisions	4	17.1	0.021	1.7	F_{CT} = 0.017 (0.344)
„	„	Among populations within divisions	6	26.4	0.165	12.9	F_{SC} = 0.131 (P < 0.001)
„	„	Within populations	190	207.8	1.094	85.5	F_{ST} = 0.145 (P < 0.001)
„	„	Total	200	251.3	1.280		
„	Host plant (five host plants)	Among divisions	4	14.8	−0.023	−1.8	F_{CT} = −0.018 (0.498)
„	„	Among populations within divisions	6	28.7	0.197	15.6	F_{SC} = 0.153 (P < 0.001)
„	„	Within populations	190	207.8	1.094	86.3	F_{ST} = 0.138 (P < 0.001)
„	„	Total	200	251.3	1.268		
„	Fruit type 1 (stone and pome fruit)	Among divisions	1	9.7	0.063	4.9	F_{CT} = 0.049 (0.055)
„	„	Among populations within divisions	9	33.8	0.148	11.3	F_{SC} = 0.119 (P < 0.001)
„	„	Within populations	190	207.8	1.094	83.8	F_{ST} = 0.162 (P < 0.001)
„	„	Total	200	251.3	1.305		
„	Fruit type 2 (apple and plum)	Among divisions	1	8.0	0.041	2.65	F_{CT} = 0.027 (0.204)
„	„	Among populations within divisions	6	28.7	0.186	12.18	F_{SC} = 0.125 (0.003)
„	„	Within populations	145	188.7	1.301	85.16	F_{ST} = 0.148 (0.001)
„	„	Total	152	225.3	1.528		
„	Collection period (by month)	Among divisions	3	11.5	−0.025	−1.94	F_{CT} = −0.019 (0.564)
„	„	Among populations within divisions	7	32.0	0.199	15.7	F_{SC} = 0.154 (P < 0.001)
„	„	Within populations	190	207.8	1.094	86.24	F_{ST} = 0.138 (P < 0.001)
„	„	Total	200	251.3	1.268		
G. molesta	Total	Among populations	8	2.6	0.013	8.1	F_{ST} = 0.081 (0.023)
„	„	Within populations	120	17.6	0.147	91.9	
„	„	Total	128	20.2	0.160		
„	Province (four provinces)	Among divisions	3	0.5	−0.011	−7	F_{CT} = −0.07 (0.854)
„	„	Among populations within divisions	5	2.1	0.020	12.8	F_{SC} = 0.12 (0.005)
„	„	Within populations	120	17.6	0.147	94.2	F_{ST} = 0.058 (0.019)
„	„	Total	128	20.2	0.156		
„	Host plant (four host plants)	Among divisions	3	0.2	−0.015	−9.6	F_{CT} = −0.096 (0.909)
„	„	Among populations within divisions	5	2.5	0.026	16.3	F_{SC} = 0.148 (0.005)
„	„	Within populations	120	17.6	0.147	93.3	F_{ST} = 0.067 (0.023)

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Table 5. (*Cont.*)

Species	Division	Source of variation	Df	S.S	Variance of component	% Variation	Fixation indices (*P*-value)
„	„	Total	128	20.2	0.157		
„	Fruit type (stone and pome fruit)	Among divisions	1	0.2	−0.005	−2.9	F_{CT} = −0.029 (0.501)
„	„	Among populations within divisions	7	2.5	0.014	9.2	F_{SC} = 0.09 (0.018)
„	„	Within populations	120	17.6	0.147	93.7	F_{ST} = 0.063 (0.017)
„	„	Total	128	20.2	0.157		
„	Collection period (by month)	Among divisions	2	0.6	−0.002	−1.31	F_{CT} = −0.013 (0.512)
„	„	Among populations within divisions	6	2.0	0.015	9.15	F_{SC} = 0.09 (0.03)
„	„	Within populations	120	17.6	0.147	92.15	F_{ST} = 0.078 (0.02)
„	„	Total	128	20.2	0.159		

SNP markers would aid in our understanding of the environmental effects on population diversity in the field.

Supplementary material

The supplementary material for this article can be found at https://doi.org/10.1017/S0007485317000694.

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