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Genetic diversity and variability in populations of the white wax insect *Ericerus pela*, assessed by AFLP analysis

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ABSTRACT. The white wax insect *Ericerus pela* Chavannes (Hemiptera: Coccoidea) is an economically valuable insect species that has been used for over a thousand years in China. The present study focuses on assessing the genetic variability in different populations of *E. pela* collected from seven Chinese provinces. The amplified fragment length polymorphism technique was used to generate DNA fingerprints of individuals from each population using nine primer combinations (*Eco*RI-*Mse*I). A total of 435 polymorphic loci were generated; fragment sizes ranged from 200 to 1000 bp. The percentage of polymorphic loci was 85.29%. Nei's genetic diversity and Shannon index indicated consistency in the results, which showed that the Sichuan population had the highest diversity, followed by Yunnan and Zhejiang populations. Dendrogram analysis showed the shortest genetic distance between the Sichuan and Yunnan populations, suggesting that they probably form sister groups. High genetic differentiation between

population values among all sampled populations indicated a low degree of genetic variability within each population (40.85%) and higher variation among populations (59.15%). Gene flow estimate values were low in all samples, suggesting low gene flow from events such as interbreeding and migration. Low gene flow values also suggested that populations among species of *E. pela* might become genetically heterogeneous, due to counteracting forces such as strong differential selection. Our data support the probability that *E. pela* will remain localized, and has a low potential to spread beyond current habitats.

Key words: White wax insect; Genetic diversity; Genetic variability; Gene flow

INTRODUCTION

The white wax insect *Ericerus pela* Chavannes (Hemiptera: Coccoidea: *Ericerus*), an important beneficial insect, has been cultured in China for over a thousand years (Chen, 2011). Male larvae secrete a white, waxy substance that is being increasingly utilized in many industries, such as print, medicine, food, chemical and machinery (Chen and Feng, 2009) (Figure 1).

Ericerus pela are widely distributed in Japan, Korean, Vietnam and China, from subtropical to temperate regions (Chen, 2011; Yang et al., 2012). The main ecological characteristics for suitable growth areas of *E. pela* are described as annual average temperatures of between 11° and 16°C, light 1900 to 2500 h/a (hours per year), rainfall 800 to 1200 mm, and annual relative humidity between 65 and 75% (Chen et al., 2007). It is reported that the volume of wax excretion of Kunming and Zhaotong populations of the white wax insects is higher than for other regional populations in China. Chinese privet (*Ligustrum lucidum*) and Chinese ash (*Fraxinus chinesis*) are excellent hosts for *E. pela* (Figure 1); the harvest of wax is higher from these than from other host plants (Chen et al., 1998; Chen, 2011).

Transcriptome analysis of *E. pela* during peak wax secretion has provided an overview of gene expression at the transcriptional level. Five genes related to white wax biosynthesis, including *far* and *ws,* were identified (Yang et al., 2012). Genetic diversity is of great importance to the sustainability of populations (Hamrick et al., 1991). For management of a species, knowledge of intraspecific genetic variations improves the ability to assess inbreeding and evolutionary potential in a changing world (Hedrick, 2001). To our knowledge, the genetic diversity and variability within and among populations of white wax insect has not been previously reported. Furthermore, due to a lack of data, it is unknown whether development of commercial culture of *E. pela* might bring ecological imbalance, a possibility that has caused considerable controversy thus far.

Amplified fragment length polymorphism (AFLP) is a useful DNA fingerprinting technique to study genetic diversity within a species, because it allows detection of genetic variation of organisms, based on DNA from any source and complexity, without prior knowledge of gene structure or sequences (Vos et al., 1995). This technique has been successfully used in several population genetic studies of insects (Cervera et al., 2000; Smith et al., 2003; Chandra et al., 2011; Taylor and Miller, 2014). In the present study, we used AFLP makers to study the genetic composition of seven populations of *E. pela*, collected from seven provinces in China (Table 1). The objectives of this research were to: 1) examine genetic diversity and variability in different *E. pela* populations; 2) explore genetic distance and phylogenetic relationships among *E. pela* populations in China; and 3), to assess the potential risk of diffusion of *E. pela* in its natural habitat.

Table 1. Locations of different populations of *Ericerus pela*.

No.	Location	Code	No.	Location	Code
1	Sichuan, Yilong	SCYL	12	Yunnan, Kunming	YNKMB
2	Sichuan, Guangyuan	SCGY	13	Yunnan, Zhaotong	YNZT
3	Sichuan, Leshan	SCLSL	14	Yunnan, Jianshui	YNJS
4	Sichuan, Emei	SCEM	15	Guizhou, Jinsha	GAJS
5	Sichuan, Xide	SCXD	16	Shanxi, Xi'an	SXXA
6	Sichuan, Jinkouhe	SCLSG	17	Shanxi, Ningqiang	SXNQ
7	Yunnan, Guangji	YNJNG	18	Guangxi, Xing'an	GXXQ
8	Yunnan, Jinlin	YNJNS	19	Guangxi, Rongjiang	GXRJ
9	Yunnan, Chenggong	YNCG	20	Hunan, Suining	HNSN
10	Yunnan, Anning	YNAN	21	Hunan, Jianghua	HNJH
11	Yunnan, Dashiba	YNKMD	22	Zhejiang, Hangzhou	ZJHZ

MATERIAL AND METHODS

Insects

Seven populations of ***Ericerus pela*** were collected from *Ligustrum lucidum* (Chinese Privet) and *Fraxinus chinesis* (Chinese Ash) in twenty-two different sites in China. Fresh samples of mature adult females were preserved in 75% ethanol at -20°C for genetic analysis. Dry specimens were stored at ambient temperature in the museum of Research Institute of Resource Insects, Kunming, China. No specific permits were required for the described field studies, as all insects were collected from natural distribution sites and used only in laboratory-contained experiments.

DNA isolation

Twenty individuals were chosen randomly from each population for isolation of total genomic DNA using a standard proteinase K, phenol/chloroform extraction technique (Marchant, 1988; Chen et al., 2013). In brief, insects were individually homogenized in TNES extraction buffer (40 mM Tris-HCl, pH 7.5; 80 mM NaCl; 80 mM EDTA, pH 8.0; 0.5% sodium dodecyl sulfate) and the homogenate was incubated with 200 µg/mL proteinase K at 65°C for 1 h. Proteins were precipitated by addition of buffer saturated phenol and mixture (chloroform:isoamyl alcohol; 24:1) with subsequent centrifugation. DNA was obtained by ethanol precipitation and diluted with 50 µL TE buffer (QIAGEN, Maryland, USA), then all the samples were stored at -20°C until use.

AFLP pre- and selective amplification

Master mix (20 µL) for pre-amplification contained 2 µL 10X Taq buffer; 1 µL Taq polymerase (1 U/µL); 1.6 µL $MgCl_2$ (25 mM); 1.6 µL dNTP mix (2.5 mM); 1 µL *Eco*RI-C (10 µM); 1 µL *Mse*I-A (10 µM); 13.8 µL ultrapure sterile water; and 3 µL DNA temple. Each reaction tube was then placed in a thermal cycler and amplified using 20 PCR cycles of 94°C for 2 min, 94°C for 20 s, 56°C for 30 s and 72°C for 2 min, followed by 60°C for 30 min.

Selective amplification reactions were performed in 20 µL final volume master mix containing 2 µL 10X Taq buffer; 1 µL Taq polymerase (1 U/µL); 1.6 µL $MgCl_2$ (25 mM), 1.6 µL dNTP mix (2.5 mM); 1 µL *Eco*RI-C (10 µM); 1 µL *Mse*I-A (10 µM); 13.8 µL ultrapure sterile water; and 3 µL DNA temple. Each reaction tube was then placed in a thermal cycler and amplified using 20 PCR cycles of 94°C for 2 min, 94°C for 20 s, 56°C for 30 s (lowering the annealing temperature by 1°C

per cycle), and 72°C for 2 min; then 20 cycles of 94°C for 20 s, 56°C for 30 s and 72°C for 2 min, followed by 60°C for 30 min before holding at 4°C.

Statistical analysis

AFLP markers were scored as 1 when bands were present, and 0 when bands were absent. Data were scored using the Cross Checker software (version 2.91; Buntjier, 1999). All bins with bands were converted to 1 and all empty bins were converted to 0. Resulting data were analyzed using the POPGENE package software (version 1.32; Yeh and Boyle, 1997) to assess percent polymorphism; gene diversity estimates of total populations (H_T) (Nei, 1973); genetic differentiation between populations (G_{ST}); and gene flow estimates (N_m) (McDermott and McDonald, 1993). A molecular dendrogram was constructed, based on Nei's genetic distance, using MEGA3 and the UPGMA method (Kumar et al., 2004).

RESULTS

AFLP amplification

For each population, twenty individuals that produced banding patterns using all nine primer pairs were used in AFLP analysis. Nine primer combinations for analysis of 140 individuals from the seven ***E. pela*** populations generated a total of 435 polymorphic loci, with fragment sizes ranging from 200 to 1000 bp, and a high average polymorphic percentage (85.29%; Table 2). Proportions of polymorphisms observed within each population ranged from 2.55%, for the Zhejiang population, to 73.77%, for the Sichuan population (Table 3).

Table 2. Primers used to characterize AFLP band patterns in *Ericerus pela* populations.

No.	Primer	Total loci	Polymorphic loci	Polymorphic percentage (%)
1	E-AAC/M-CAG	72	48	66.67
2	E-AAC/M-CTT	54	39	72.22
3	E-ACA/M-CAT	47	36	76.60
4	E-ACG/M-CAG	63	58	92.06
5	E-ACG/M-CTG	56	49	87.50
6	E-ACT/M-CAC	69	63	91.30
7	E-AGC/M-CAA	61	56	91.80
8	E-AGG/M-CAG	40	31	77.50
9	E-AGG/M-CTG	48	44	91.67
	Total	510	435	85.29

Table 3. Nei's genetic diversity and Shannon diversity index of *Ericerus pela* populations.

Population ID	Polymorphic loci	Polymorphic proportion (%)	Nei's genetic diversity index (means ± SD)	Shannon diversity index (means ± SD)
Sichuan	373	73.33	0.30 ± 0.20	0.44 ± 0.28
Guizhou	80	15.69	0.07 ± 0.15	0.09 ± 0.22
Yunnan	282	55.29	0.22 ± 0.21	0.32 ± 0.30
Hunan	90	17.63	0.07 ± 0.16	0.11 ± 0.23
Zhejiang	13	2.55	0.01 ± 0.06	0.01 ± 0.09
Shanxi	77	15.10	0.06 ± 0.15	0.09 ± 0.22
Guangxi	67	13.14	0.05 ± 0.14	0.08 ± 0.20
All	435	85.29	0.27 ± 0.17	0.42 ± 0.24

Genetic diversity

Through analysis of the seven *E. pela* populations using Nei's genetic diversity and Shannon index, consistency of results was demonstrated by two indices (Table 3). Nei's genetic diversity index was 0.01-0.30; the Sichuan population was the highest, followed by the Yunnan population then the Zhejiang population, which scored the lowest. Shannon's diversity index was 0.01-0.44, in which the Sichuan population was highest, followed by the Yunnan and then the Zhejiang populations.

Genetic variation

Population heterozygosity (H_T), a measure of total genetic variance, revealed a high genetic diversity value (0.2473) among populations. Genetic variation within and among populations was measured as G_{ST}. Higher G_{ST} values (>0.5) indicate a majority of genetic variability resides between populations. In the present study, G_{ST} among all sampled populations was high (0.5915), indicating a low degree of genetic variability within populations (40.85%) and high variation among populations (59.15%). Gene flow is commonly measured as N_m. Where N_m values are >1, a high level of gene flow is indicated and the effects of gene flow on population differentiation are likely to be greater than the effect of random genetic drift (Bossart and Pashley Prowell, 1998). In the present study, N_m values were low in all samples (Table 4) suggesting low gene flow from events such as interbreeding and migration. Low gene flow values also suggest that local populations will become genetically heterogeneous, considering they are subjected to differential selection and other counteracting forces.

Table 4. Genetic differentiation coefficient and gene flow analysis among *Ericerus pela* populations.

No. of populations	H_T	H_S	G_{ST}	N_m
7	0.2473	0.1010	0.5915	0.3454

H_T, total genetic variance; H_S, genetic variance within population; G_{ST}, genetic differentiation coefficient; N_m, gene flow.

Genetic distance and dendrogram

Results of Nei's analysis revealed that a genetic distance among populations of 0.0423-0.367 (Table 5). The lowest value, observed between Sichuan and Yunnan populations, was 0.0423, while the highest value, between the Guizhou and Zhejiang populations, was 0.3679.

The dendrogram shows the genetic distance and phylogenetic relationships among different geographic populations of *E. pela*. The genetic distance of the Sichuan population and Yunnan populations was the shortest and these populations formed a sister group, consistent with the idea that the Sichuan and Yunnan populations have a close genetic relationship. The Guangxi and Shanxi populations also formed a sister group, again consistent with high genetic similarity between two populations. Genetic separation between the Zhejiang and Hunan populations was pronounced and each formed a distinct branch. Due to its genetic isolation, the Guizhou population showed the most distant phylogenetic relationship from other populations.

Table 5. Genetic identity and distance in *Ericerus pela* populations.

	1	2	3	4	5	6	7
1	***	0.7966	0.9586	0.8434	0.8387	0.8843	0.8729
2	0.2274	***	0.7973	0.7987	0.6922	0.7063	0.6983
3	0.0423	0.2265	***	0.8650	0.8741	0.9360	0.9122
4	0.1703	0.2247	0.1451	***	0.8204	0.7873	0.7525
5	0.1759	0.3679	0.1345	0.1979	***	0.8613	0.8237
6	0.1229	0.3477	0.0662	0.2391	0.1493	***	0.9553
7	0.1359	0.3591	0.0919	0.2844	0.1939	0.0457	***

Above diagonal: Nei's genetic identity; below diagnonal: genetic distance. 1. Sichuan; 2. Guizhou; 3. Yunnan; 4. Hunan; 5. Zhejiang; 6. Shanxi; 7. Guangxi.

DISCUSSION

In this comprehensive analysis of genetic diversity of various populations of the white wax insect *E. pela* in China, the mean value of Nei's genetic diversity index was 0.27 and Shannon diversity index was 0.42. There was a high genetic diversity among all tested populations, which is likely linked to the widespread distribution of *E. pela*. Because of its different host plant species and habitat requirements, there are many differences in ecological adaptability, duration of wax secretion and degree of wax production between different geographical populations of the insect (Chen, 2011). In this study, we showed that there are large differences in the tested polymorphic loci among populations of *E. pela*. The polymorphic percentages of the Sichuan and Yunnan populations were 73.33 and 55.29% respectively, and were much higher than those of other populations. The Sichuan population had the highest Nei's genetic diversity index, followed by the Yunnan population, while the Zhejiang population had the lowest. Shannon's diversity index was in the range of 0.01-0.44, with the Sichuan and Yunnan populations ranking the highest and the Zhejiang population the lowest. Based on these data, it is conceivable that Yunnan and Sichuan represent the geographical center from which the white wax insect originated. As is often the case with primitive groups, there was much higher genetic diversity in Sichuan and Yunnan *E. pela* populations than in others.

Genetic variation was measured as G_{ST} within and among populations. Values >0.5 indicate that a majority of genetic variability resides between populations, while lower values (<0.5) indicate that a majority of the genetic variability resides within a given population (Chandra et al., 2011). In the present study, G_{ST} values among all sampled populations were high (0.5915) indicating a low degree of genetic variability within a given population (40.85%) and high variation between populations (59.15%; Table 4). These results indicate that there is considerable genetic differentiation among the sampled populations. Such abundance of genetic differentiation is generally considered the result of insufficient gene flow coupled with selective pressures, as well as genetic drift. Reduced dispersal between populations can lead to genetic subdivisions within populations and may facilitate local adaptation (Slatkin, 1987; Hülber et al., 2015). Environmental or physical barriers may also promote isolation of populations. These include geographic distance and barriers such as mountains, rivers, and stretches of unsuitable habitat (Seyahooei et al., 2011). In the current study, samples were collected from seven provinces in China with different environments and there was a correlation between geographical isolation and genetic differentiation. Thus, geographical isolation is likely to be the main reason for the observed high degree of genetic differentiation among white wax insect populations tested.

Gene flow is commonly measured as N_m, where N is the number of individuals in a

population, and m is the proportion of those individuals present as a result of immigration (Chandra et al., 2011). N_m values >1 indicate a high level of gene flow, and that the effects of gene flow on population differentiation are greater than the effect of random genetic drift (Bossart and Pashley Prowell, 1998; Avramidou et al., 2015). In the present study, N_m values were as low as 0.3454 in the tested regions, suggesting low gene flow from factors such as a lack of interbreeding and migration. Low gene flow values also suggest that species subpopulations will become genetically heterogeneous due to a range of counteracting forces such as strong differential selection. Low gene flow is possibly closely related to poor diffusion ability of *E. pela*. For instance, larvae in the first stage only move within ten meters, and larval survival rate was only 5% if larvae covered a distance of more than 2.5 meters (Chen, 2011). In consideration of the results on genetic diversity and variation presented here, we expect that *E. pela* will remain localized, and that there is a low risk that any subpopulations will spread beyond their current habitat. This is also the likely reason that to this day, *E. pela* has never been reported to cause damage to forests or crops, despite the fact that this insect has been cultured in China as a natural bio-resource for hundreds of decades. Concerns have been raised over the suggestion that increased usage of the white wax scale insect may generate invasive behavior, thus creating the potential for ecological harm to other ecosystems. We show here that there is minimal risk of this, due to low gene flow coupled with low mobility of this species.

Conflicts of interest

The authors declare no conflict of interest.

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