Molecular Detection and Genetic Diversity of Casuarina Moth, *Lymantria xylina* (Lepidoptera: Erebidae)

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Abstract

The casuarina moth, *Lymantria xylina* Swinhoe (Lepidoptera: Erebidae), is an important pest in the Australian pine tree, *Casuarina equisetifolia*, forest in the coastal area of South China. At the same time, as a closely related species of *Lymantria dispar* L. (Lepidoptera: Erebidae), it is also a potential quarantine pest. In the present study, specific primers were designed for identification of *L. xylina* based on the COI barcoding sequence between *L. xylina* and four other common forest pests. A 569-bp fragment was successfully amplified from 40 *L. xylina* from five geographical populations in four Chinese provinces. In addition, even through the analysis came from five highly diverse populations of *L. xylina*, the genetic distances ranged from 0.001 to 0.031. The neighbor-joining tree showed that the species from Hubei and Chongqing were clustered within a distinct group.

Key words: *Lymantria dispar*, molecular detection, genetic diversity, haplotypes

The casuarina moth, *Lymantria xylina* Swinhoe (Lepidoptera: Erebidae), once belonged to the family Lymantriidae in superfAMILY Noctuoidae. But recent classifications have lowered Lymantriidae to the subfamily Lymantriinae under the family Noctuoidae, and then it was incorporated into Erebidae (Fibiger and Lafontaine 2005, Lafontaine and Fibiger 2006, Zahiri et al. 2011). In addition, Noctuoidae and Erebidae are both classified into the superfamily Noctuoidea. Therefore, it is now noted that *L. xylina* belongs to Lepidoptera–Noctuoidea–Erebidae–Lymantriinae and the genus *Lymantria*, in which many species are known as significant forestry pests (Pogue and Schaefer 2007). In particular, the gypsy moth, *L. dispar* L., has been reported to be a significant worldwide invasive moth species, which is an extremely serious forest pest in some parts of the United States and Eastern Canada (Pogue and Schaefer 2007). Based on the known biology and behavior, *L. xylina* is considered to be a potential invader to the United States, Canada, and Europe in the future (Pogue and Schaefer 2007, deWaard et al. 2010).

Similar to the gypsy moth, *L. xylina* is univoltine with a 9-mo egg period from July to April, in which a diapause is recorded with little detail. The process of the diapause is not quite clear (Hwang et al. 2007). Normally, it is reported that there are five to six instars, but it is more likely to have seven instars in Fujian Province (unpublished data). The pupal stage lasts about 2 wk. Adult males emerge first, followed several days later by the adult females. Soon after mating, adult females lay a single, light brown, hair-covered egg mass, consisting of about 100–1,500 eggs (Shen et al. 2006, Hwang et al. 2007). The egg mass are often attached to container boxes or vessels, leading to long-distance movements (Kang et al. 2015). After the eggs hatch, the larva act as an extremely polyphagous herbivore, and its recorded host plants include, and most likely to exceed, 65 species of broadleaf trees and shrubs (Shen et al. 2006).

So far, the defoliator *L. xylina* has been found in Japan, India, Korea, and China including Taiwan (Shen et al. 2006, Kang et al. 2015, Lee et al. 2015). Although *L. xylina* infested many plants, outbreaks of infestations has only been found in casuarina plantations in last decades, especially in the eastern coastal of China. However, serious defoliation on commercial fruit trees including Longyan (*Euphoria longana* Lam.) and Lychee (*Litchi chinensis* Sonn.) has recently occurred (Shen et al. 2006).

On the other hand, little is known about *L. xylina*, in spite of its potential serious effect on hardwood and fruit trees in China. It could be misidentified as gypsy moth, or other species in the genus, by using morphological characteristics, since similarity is occurred on the close related species (Kang et al. 2015), and the casuarina moth is highly likely to have a much wider distribution in China. Various infestations and the expanding outbreak areas indicated that there is a real threat posed by this moth.

The traditional taxonomic keys are primarily based on the adult moths, requiring the proper preparation and preservation of the...
specimens and a trained taxonomist. Adult moths specimens with a fragile physical structure are often seriously damaged due to being transported a long distance from the fields they infest. In the case of a collected larva, the identification may be more difficult than with adults, and thus lead to a misidentification. Moreover, time-consuming preparation and identification makes it impractical for not only urgent laboratory experiment but also for monitoring in the field (Briski et al. 2011). In contrast, molecular identification is not hindered by the phenotypic polymorphism, sex, or developmental stage variation of the target species (Zhang et al. 2012). Thus, molecular detection has been used to distinguish very similar and closely related species in Lepidoptera with high reliability and resolution (de Waard et al. 2010, Nieukerken et al. 2012, Kang et al. 2015).

There is no doubt that the accurate species identification is a principal and fundamental component of a pest management, as well as confirming the pest’s distribution. In addition, population genetic studies are likely to reveal a microevolution and ecological adaption strategies of an insect pest in agro-ecosystems. This is significant for the design and optimization of sustainable pest management strategies (Bennett et al. 2011, Chen et al. 2016).

Therefore, in this study, 1) a species-specific primer, which was designed according to variations in COI barcodes, was used to identify L. xylina, to develop a polymerase chain reaction (PCR)-based assay for the rapid and accurate identification of the moth and 2) the genetic diversity of L. xylina in its putative range, and its genetic structure in a key casuarina growing area of China, was investigated.

Materials and Methods

Insect Collections

In total, 40 samples, collected from wild by sex-pheromone traps, were used for DNA barcoding (Table 1, Fig. 1). Each eight isolated samples from Fujian-Pingtan, Fujian-Jinjiang, Zhejiang, Chongqing, and Hubei were treated as a treatment and compared with each other. For a more accurate result, four other important forest moths in south of China, including the gypsy moth L. dispar asiatica (represented as L. dispar in the following text, Lab specimens from Hunan), and the other three species from Fujian, i.e., the pale tussock moth Calliteara pudibunda L., Dasychina sp. (Lepidoptera: Erebidae), and the nun moth Lymantria monacha L. (Lepidoptera: Erebidae) were analyzed for comparison and verification at the same time.

Genomic DNA extraction and PCR amplification

Genomic DNA of all samples was extracted by using Qiagen DNeasy Blood and Tissue Kit (Valencia, CA) following by the user’s manual. The universal primer sets used were LCO1490 (5′-GGTCAACAATCATAAAGATATGG-3′) and HCO2198 (5′-TAAACTCACGGTGACCGAAAAATCA-3′) reported by Folmer et al. (1994). Besides, the primers (F: GGTCAACAATCATAAAGATATGG and R: ATAATAATAGAAAAAGCTG) were used for molecular detection of L. xylina. And, the PCR amplification were processed under certain conditions as follows: Ex-Taq kit (TaKaRa, Dalian, China) with a 25-μl volume with 2.5-μl 1x Ex-Taq buffer, 2-μl dNTPs, 1 μl of each PCR primer, 0.25-μl Ex-Taq, and 1 μl of genomic DNA, respectively. PCR was performed using a Bio-Rad PCR System T100 (Bio-Rad, Hercules, CA) under the following conditions: 94°C for 3 min, 35 cycles of (94°C for 30 s, 50°C for 30 s, 72°C for 1 min), a final extension step was done at 72°C for 10 min. Amplified genomic fragments were purified using the E.Z.N.A. Gel Extraction Kit (Omega Bio-Tek Inc., Norcross, GA). The purified PCR fragments were cloned into a pMD19-T vector (TaKaRa) and then transformed into competent cells of Escherichia coli DH5α following the manufacturer’s instructions. Three DH5α clones were collected for a sample and the inserts of the expected size were bidirectionally sequenced using M13 primers. After that, the sequences were visualized on an ABI 3100 Genetic Analyzer (ABI, CA). Finally, the sequences were visualized on an ABI 3100 Genetic Analyzer in both directions (Invitrogen Co., Shanghai, China).

Specificity and Sensitivity of L. xylina-Specific Primers

To test the specificity of the L. xylina-specific primers, a PCR were conducted with 40 isolates of L. xylina, 3 isolates of L. dispar, 3 isolates of C. pudibunda, 3 isolates of Dasychina sp., and 1 isolate of L. monacha, which were widely distributed in China.

To evaluate the sensitivity of the L. xylina-specific primer, a serial 10-fold dilution of genomic DNA ranging from 1,000 to 0.1 μg/μl were performed. Besides, the moths in different life stages (larval, pupal, and adult stages) were also tested. Double-distilled water was used as a negative control. The reaction mixture and thermal cycling conditions were the same as the description of the PCR above.

Analysis of COI Sequence

The sequences and the phylogenetic tree were constructed with MEGA 6.0 using the neighbor-joining method (Tamura et al. 2013). Robustness of the nodes of the phylogenetic tree was assessed from 1,000 bootstrap replicates. In addition, 13 more sequences of L. xylina obtained from GenBank of NCBI or BOLD (Table 2) were used to construct split-decomposition network by Splits Tree (4.13.1).

Results

Mitochondrial DNA COI Barcode Sequences

The specific primers LCO1490/HCO2198 were used to amplify 709-bp COI sequence from 50 isolates of the five species. Products from all amplifications were cloned and sequenced. The alignment of the COI sequence of the five common forest pests was shown in the Fig. 2. The DNNAM (Woffelman et al. 2004) with default setting

Table 1. Sampling of L. xylina from five areas in four provinces of China

| Region     | Sublocation | LC | Coordinates                  | D         | Host      | N  |
|------------|-------------|----|------------------------------|-----------|-----------|---|
| Fujian     | Pingtan     | PT | E119°48′04″ N25°30′28″        | 16 May    | Casuarina | 21 |
|            | Jinjiang    | JJ | E118°26′12″ N24°31′24″        | 22 May    | Casuarina | 33 |
| Zhejiang   | Taizhou     | ZJ | E121°24′64″ N28°05′21″        | 3 June    | Casuarina | 17 |
| Hubei      | Enshi       | HB | E109°04′59″ N29°56′56″        | 21 June   | NA        | 12 |
| Chongqing  | Chongqing   | CQ | E105°59′52″ N29°10′32″        | 25 June   | NA        | 9  |

Information including region, sublocation, location code (LC), latitude and longitude, collection date (D), and host and number of individuals (N).
was used to alignment. Here, we selected the individual from Pingtang to align with other species. As the Fig. 2 showed, those five species exhibited high similarity with each other (>93.51%) in the DNA barcoding region. Based on the variation in this area, we designed a specific primer set to recognize L. xylina. The location of primer was also shown in the Fig. 2.

### Specificity of the PCR Primers

The specificity of the PCR primers was assessed using the isolates of L. xylina, L. dispar, C. pudibunda, Dasyctena sp., and L. monacha form China listed in Materials and Methods section. It is showed that a fragment of 709 bp was amplified from all individuals (Fig. 3A), suggesting that the primer set was suitable for recovering the COI gene of the five species. In contrast, our specific PCR primer generated 569 bp for all 40 isolates of L. xylina, while no corresponding band was detected from the other species (Fig. 3B), indicating that the primer had extremely high specificity and reproducibility.

### Sensitivity of the PCR Primers

To confirm the utility and fidelity of our specific PCR, DNA from L. xylina was extracted at different life stages, including larvae, pupae, and adults. It showed that no matter the life stage of the L. xylina, the primer set was able to amplify the 569-bp specific band successfully (Fig. 4).

In addition, a serial dilution of cDNA ranging from 0.1 to 1,000 μg/μl was used to test the sensitivity of the L. xylina primers. After the PCR, a 569-bp band was detected as shown in Fig. 5, indicating the detection limit with the primer set was 0.1 μg/μl.

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**Table 2. Reference sequences for DNA barcoding of the L. xylina**

| Number | GenBank or BOLD | The geographic origin of the samples | Location code | References                  |
|--------|-----------------|-------------------------------------|--------------|------------------------------|
| 1      | BOGDA069-08     | Taiwan, Taipei                      | TW-1         | NA                           |
| 2      | AF075276        | Taiwan, Taipei                      | TW-2         | Bogdanowicz et al. (2000)    |
| 3      | DQ116158        | Taiwan, Kau                         | TW-3         | Armstrong and Ball (2005)     |
| 4      | DQ116170        | Taiwan, Kau                         | TW-4         |                              |
| 5      | DQ116171        | Taiwan, Kau                         | TW-5         |                              |
| 6      | KX436295        | Taiwan, Yilan                       | TW-6         | Stewart et al. (2016)         |
| 7      | KX436399        | Japan, Kimitsu                      | JP-1         |                              |
| 8      | KX436387        | Japan, Okinawa                      | JP-2         |                              |
| 9      | KX436388        | Japan, Okinawa                      | JP-3         |                              |
| 10     | KF746258        | United States, MA (Lab colony)      | USA-1        | Kang et al. (2015)           |
| 11     | KF746259        | United States, MA (Lab colony)      | USA-2        |                              |
| 12     | KF746256        | Korea, Yeosu harbor (quarantine inspection) | KR-1    |                              |
| 13     | KF746257        | Korea, Yeosu harbor (quarantine inspection) | KR-2    |                              |
Fig. 2. The alignment of the COI sequence for the five common forest pests. The specific primer was marked as the red on the top of sequence location.

Fig. 3. Specificity test of *L. xylina* specific primer. A) Lanes 1–8: adult males of *L. xylina* from Pingtan; Lanes 9–16: adult males of *L. xylina* from Jinjiang; Lanes 17–24: adult males of *L. xylina* from Zhejiang; Lanes 25–32: adult males of *L. xylina* from Chongqing; Lanes 33–40: adult males of *L. xylina* from Hubei; lanes 41–43: adult males of *L. dispar*; lanes 44–46: larvae of *C. pudibunda*; lanes 47–49: larvae of *Dasychira* sp.; lane 50: larva of *L. monacha*; lane 51: negative control (dd H2O). And, the lanes in the part B were the same as that of part A.
Mitochondrial DNA COI Barcode Sequences Analysis

We used a PCR method with the specific primers LCO1490/HCO2198 to amplify COI sequence from the genomic DNA of 40 individual *L. xylina*. Products from all amplifications were cloned and then sequenced. The results revealed that there were 673 conserved sites, 36 variable sites, 27 parsimony-informative sites, and 9 singleton sites. The nucleotide composition was AT rich, which is consistent with the characteristic of nucleotide composition in insect mitochondria.

Forty sequences were obtained with variable haplotypes from all species. Sixteen haplotypes were identified (Hap1–Hap16, Table 3), and the proportion of haplotypes characterized from different species ranged from 25.0 to 62.5%. The average number of haplotypes of COI over all populations was 4.5, indicating a high degree of diversity. The largest numbers (5) of haplotypes were observed in

![Fig. 4. Sensitivity test of *L. xylina* specific primer of different developing stages. Lane 1: larva of *L. xylina*; lane 2: pupae of *L. xylina*; lane 3: adult of *L. xylina*; Lane 4: negative control (ddH₂O).](image)

![Fig. 5. The sensitivity test with a serial DNA concentration of *L. xylina* specific primer. Lane 1: 1,000 μg/μl, lane 2: 100 μg/μl, lane 3: 10 μg/μl, lane 4: 1 μg/μl, lane 5: 0.1 μg/μl.](image)

| Species | H | Hap1 | Hap2 | Hap3 | Hap4 | Hap5 | Hap6 | Hap7 | Hap8 | Hap9 | Hap10 | Hap11 | Hap12 | Hap13 | Hap14 | Hap15 | Hap16 | N | H | Hd | π |
|---------|---|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|-----|-----|-----|-----|
| JJ      | 8 | 3    | 5    | 0    | 0    | 0    | 0    | 0    | 0    | 0    | 0    | 0    | 0    | 0    | 0    | 0    | 0    | 0   | 16  | 8   | 0.607 | 0.00096 |
| PT      | 8 | 2    | 6    | 0    | 0    | 0    | 0    | 0    | 0    | 0    | 0    | 0    | 0    | 0    | 0    | 0    | 0    | 0   | 16  | 8   | 0.250 | 0.00071 |
| ZJ      | 8 | 3    | 6    | 0    | 0    | 0    | 0    | 0    | 0    | 0    | 0    | 1    | 1    | 0    | 0    | 0    | 0    | 0   | 16  | 8   | 0.464 | 0.00107 |
| HB      | 8 | 5    | 0    | 0    | 0    | 2    | 2    | 1    | 2    | 1    | 1    | 0    | 0    | 0    | 0    | 0    | 0    | 0   | 16  | 8   | 0.892 | 0.00504 |
| CQ      | 8 | 5    | 0    | 0    | 0    | 0    | 0    | 0    | 0    | 0    | 0    | 0    | 1    | 4    | 4    | 1    | 1    | 0   | 16  | 8   | 0.786 | 0.00178 |
| Total   | 40| 16   | 17   | 2    | 1    | 2    | 1    | 2    | 1    | 2    | 1    | 1    | 1    | 4    | 4    | 1    | 1    | —   | —   | —   | —   | —   | —   | —   |

N, number of sampling individuals; H, number of unique haplotypes; Hd, haplotypes diversity; Hap, haplotype; π, nucleotide diversity.
Hubei and Chongqing populations. In addition, the nucleotide diversity was also calculated showing that nucleotide diversity of COI ranging from 0.071 to 0.504% (Table 3).

Analysis of Phylogenetic Tree

The neighbor-joining tree was inferred from a 709-bp fragment of COI from 40 isolates of casuarina moth collected in China. The tree showed that these sequences were separated into two main groups (Fig. 6). The specimens from Fujian (Jinjiang and Pingtan) and Zhejiang province were grouped together and well separated from the others, whereas the COI sequence from gypsy moth, L. dispar, was chosen as out-group (Fig. 6).

Additionally, the sequence of COI haplotypes from worldwide L. xylina populations (Table 2) was also used to construct split-decomposition network. The results showed the populations from outside of China were all clustered with the species from Fujian and Zhejiang. The other populations from Hubei and Chongqing also comprised with a distinct group (Fig. 7).

Intra- and Interspecies Genetic Distances

The genetically divergent cluster of casuarina moth was supported by the COI data using the Kimura2-parameter model. Pairwise genetic distances of 40 COI sequences for different casuarina moth species ranged from 0.001 to 0.031 (Table 4). The smallest genetic distance (0.001) was found between Pingtan and Jinjiang. In contrast, the largest genetic distance (0.031) was observed between Jinjiang and Hubei, as well as Jinjiang and Chongqing.

Discussion

In this study, L. xylina was discovered for the first time in Chongqing and Hubei by sex-pheromone traps, where L. dispar was being treated as a main forest pest (Chen et al. 2016). The L. xylina presence was confirmed by our specific primers. The casuarina moths probably were misidentified as less was known about this pest in the past. Normally, it is an important pest on Casuarina equisetifolia forests in the coastal area of South China. For instance, in Fujian province, the larvae of this moth damaged important ecological and economic forests, including casuarina, longan, and lychee, and more than 60 other species of host plants (Shen et al. 2006). However, the host plant of the moth in its new reported habitat in Chongqing and Hubei is still unknown, since sex-pheromone trap were used in their collections. However, finding it in a new area makes it more likely that its host range will be expanded. The expanding distribution of the moth required an integrated pest management program to control it.

Normally, casuarina moth and gypsy moth are distinguished by the female wing pattern: L. xylina has a straight and yellowish brown postemedial line; L. dispar has a blackish zigzagging fasciate...
postemedial line (Pogue and Schaefer 2007). Although the wing patterns of the two species are not too much alike, they still confuse forest ranger and other employees. In addition, the two species share common in male adult morphological characteristics, living habits, and larva features. Also, the external morphological features affected by environment and the development stage, so they show a great morphological plasticity in different regions or microhabitats (Dujardin et al. 1999, Liebherr 2003, Schlick-Steiner et al. 2010). Consequently, as an alternative, rapid and precise method, molecular identification for \( L. xylina \) is quite helpful for pest control. In this study, specific primers were designed for identification of \( L. xylina \) based on COI barcoding sequence. The specific primers are based on the COI barcode sequences from the five common forest pest species, which share a high sequence similarity (>93.51%) with \( L. xylina \). We also took geographical variation and life stages into consideration. In addition, the detection is limited with our primer with 0.1 ng/µl, which exceeded the concentration of the DNA we extracted (15.8–1367.9 ng/µl). This study illustrated that the molecular diagnosis was sufficient and effective to distinguish \( L. xylina \) between the five common forest pests and that shows potential for pest quarantine programmers.

Among all samples collected, 40% individuals shared a singled haplotypes, which is found in samples collected from the coastal areas. Unique haplotypes were found for individuals from Hubei and Chongqing, indicating great genetic variability existence. In addition, we found that the number and diversity of haplotypes from the inner land of China is higher than that in the coastal region (Table 3). Therefore, we suspected the species originated in the inner land of China and expanded to the coastal region. A similar situation was reported in thrips \( Dendrothrips minowai \) Priesner, where they found the thrips was likely to have expanded from the eastern region to the west of Guizhou province due to less haplotypes diversity was in the western area (Lyu et al. 2016).

In this study, we investigated the genetic diversity and genetic structure of five populations using mitochondrial DNA markers. Among the five populations examined, the genetic distance within the population ranged from 0.1 to 0.5%, while the range of the interpopulation variability was between 0.1 and 3.1% (Table 4). Hebert et al. (2003) proposed that the upper boundary for intraspecific variability is 2%; here, the highest genetic distance (3.1%) was observed between Fujian/Zhejiang and Hubei/Chongqing. A geographical barrier is one of the most important issues to explain the genetic divergence for most insect populations (Yagi et al. 2001, Shoda-Kagaya 2007). Considering the geographical factors between those populations, there were more than 17 million kilometers, and thousands of nameless mountains and rivers, distributed between the costal (Fujian/Zhejiang) and mid-west of China (Chongqing/Hubei). In addition, the climate is another important factor to influence intraspecific genetic diversity and population genetic structure (Pauls et al. 2013). Microevolution of the adaptation to local environmental conditions would cause genetic variants in space and time (Hoffmann and Sgrò 2011). In the case of the small brown planthopper (\( Laodelphax striatellus \) Fallén), the mtDNA diversity and the distribution of mtDNA variations were shown to be associated with climate adaptation (Sun et al. 2015). Therefore, it seems that the geographical and climate factors are sufficient to explain the genetic divergence that we found.

In addition to the genetic distance, the NJ dendrogram clearly showed that the species from Fujian/Zhejiang was well separated from the one from Hubei and Chongqing (Fig. 6), while the
individuals from the same place were clustered in the same group. The same situation was also demonstrated in Fig. 7; the L. xylina from different locations were also separated as two groups. Hence, a clear difference in DNA barcoding region revealed the L. xylina would be divided into two subspecies, one from Hubei and Chongqing, and the others would be in a separate group, suggesting great genetic variability of L. xylina. However, we cannot date back to the real source of L. xylina outside China; therefore, this situation need to be clarified further. The same situation was illustrated in the gypsy moth L. dispar. It was found that the population in Guizhou, southwest of China, shared a distinct cluster in consensus tree of barcodes (Chen et al. 2016). Therefore, in addition to the geographical and climate factors, we considered the long space and time separation could generate a natural barrier to prevent gene flow between casuaria moth in Hubei and Chongqing and others from the coastal area; we even inferred the present morph-species might be comprised of two cryptic species.

As a potential quarantine pest, in the presence of our study, we provided a specific primer to detect the casuaria moth L. xylina and analyzed the genetic variance from different location. Given the high genetic variability, some ecological and nuclear evidence may be needed to explain the great variance of the two species between cos- tal and inner land of China. Furthermore, for quarantine purpose, we need to find out the whole distribution of the casuaria moth in China, and to work out a comprehensive map of the haplotypes of casuaria moth by using additional samples.

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Table 4. Mean intra- and interspecific genetic distances of L. xylina located in different area of China with standard deviations.

|     | JJ   | PT   | ZJ   | HB   | CQ   |
|-----|------|------|------|------|------|
| JJ  | 0.001 ± 0.001 | 0.001 ± 0.000 | 0.001 ± 0.000 | 0.001 ± 0.001 | 0.005 ± 0.002 |
| PT  | 0.001 ± 0.000 | 0.030 ± 0.006 | 0.031 ± 0.006 | 0.010 ± 0.003 | 0.002 ± 0.001 |
| ZJ  | 0.001 ± 0.000 | 0.030 ± 0.006 | 0.031 ± 0.006 | 0.010 ± 0.003 | 0.002 ± 0.001 |
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