Bulletin of Entomological Research (2015) **105**, 736–742 doi:10.1017/S000748531500070X

Population genetic differentiation of the black locust gall midge *Obolodiplosis robiniae* (Haldeman) (Diptera: Cecidomyiidae): a North American pest invading Asia

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Abstract

Obolodiplosis robiniae is native to North America and is an important introduced insect pest that forms leaf margin roll galls on species of genus *Robinia* (Fabaceae) in China. It was first detected in China in 2004, but subsequently spread and provoked local outbreaks. An analysis of a 676-bp sequence of the mitochondrial DNA cytochrome oxidase subunit I was conducted in 560 individuals from 28 populations, in order to (1) assess population genetic structuring and (2) explore possible explanations for the rapid spread and invasion success of *O. robiniae*. Yet, only four haplotypes were identified and the nucleotide diversity was low ($\pi = 0.00005$) and among the 560 specimens studied, only ten showed haplotypic variation involving no more than three substitutions. The result showed a low degree of genetic diversity among populations of the successful invasive gall midge, which suggested that the pest experienced a severe genetic bottleneck and a loss of genetic diversity after its introduction. The successful establishment and spread of *O. robiniae* in China is attributed to the wide distribution of its host plant, thus allowing ample opportunities for gene flow in the pest species, and to the advantageous life history characteristics of *O. robiniae*.

Keywords: *Obolodiplosis robiniae*, cytochrome oxidase I, population genetic differentiation

(Accepted 18 August 2015; First published online 8 September 2015)

Introduction

The black locust gall midge, *Obolodiplosis robiniae* (Haldeman, 1847) (Diptera: Cecidomyiidae), is an important invasive insect pest that attacks the locust tree *Robinia* (Fabaceae) in China. It became one of the serious defoliators and provokes 100% infection rates in the provinces of Liaoning and Hebei (Yang *et al.*, 2006). *O. robiniae* is native to eastern North America (Haldeman, 1847), but the midge has spread to many countries and regions of Europe and some areas of Asia with international trade activities (Kodoi *et al.*, 2003; Woo *et al.*, 2003; Navone & Tavella, 2004; Duso *et al.*, 2005; Uechi *et al.*, 2005; Csoka, 2006; Hoffmann *et al.*, 2007; Wermelinger & Skuhravá, 2007; Roskam *et al.*, 2008; Skrzypczynska, 2008; Jorgensen, 2009; Pernek & Matosevic, 2009; Tóth *et al.*, 2009).

O. robiniae was first recorded in China in Qinhuangdao City, Hebei Province in 2004 by Yang *et al.* (2006). Since that time, it spread quickly, and by 2010, the pest had been found in a total of 27 cities in the Jilin, Liaoning, Shandong, and Hebei provinces and Beijing (Yang *et al.*, 2006; Lin *et al.*, 2007; Yan *et al.*, 2007; Zhang *et al.*, 2008; Mu *et al.*, 2010). However, as

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confirmed in this study, *O. robiniae* is even more widespread and exists in more areas in which its host is distributed in China. The midge damages its host's leaves causing them to roll, which makes the plant to decline and become more vulnerable to infestation by other pest insects, such as longhorn beetles and jewel beetles.

The locust tree is an important type of afforestation tree species in China because of its many important characteristics such as fast growth, strong adaptability, high ability to tolerate drought, salt, and poor soil, and especially its significant roles in water and soil conservation, protection against wind and sand, and street and road greening. There are two species and two varieties of the genus *Robinia* in China: *Robinia pseudoacacia* L., *Robinia hispida* L., *Robinia pseudoacacia* var. *pyramidalis* (Pepin) Schneid, and *Robinia pseudoacacia* var. *umbraculifera* DC. The locust tree was introduced from Europe in 20th century, and became economically and ecologically important. Therefore it was planted extensively in broadleaved deciduous forests in China, such that currently the planted area exceeds ten million hectares (Xu & Yang, 2006; Zhang *et al*., 2009).

According to the international standards of phytosanitary measures (ISPM No.2), a pest risk analysis of the gall midge was performed to predict its potential distribution areas in China. The results revealed that the potential distribution range of this species in China is 98.30°–132.03°E and 24.33°–47.41°N using CLMEX software (2nd edition) (Zhang *et al*., 2009). However, *O. robiniae* was not distributed over this whole area, possibly because its host tree, *R. pseudoacacia* L., is primarily distributed in northern China only.

Genetic variation is regarded as an important factor in the colonization of invasive species (Sakai *et al*., 2001; Facon *et al*., 2006; Roman & Darling, 2007; Amouroux *et al*., 2013; Horst & Lau, 2015). However, invasive species are often expected to undergo reductions in genetic diversity during founding events because new habitats are usually colonized by only a few individuals, which carry only (small) part of the allelic diversity of the source population (Nei *et al*., 1975). Yet, sometimes genetic diversity in founder populations can be increased because the founding individuals come from different source populations (Davis, 2009). Hence, multiple introductions are predicted to increase genetic diversity in founding populations, and as such may contribute to the rapid population growth and expansion of a colonizing species. In this way, they underpin the success of invasions by facilitating local adaptation in new environments and by increasing new trait diversity (Facon *et al*., 2006).

Successful invasions imply a match between the exotic species and the new local environment (Facon *et al*., 2006). Prins & Gordon (2014) proposed 11 hypotheses to explain biological invasions. Yet, insects appear to be among the more difficult taxa to uncover the biological and ecological factors that explain how some species can colonize new habitats (Suarez *et al*., 2001; Boubou *et al*., 2011; Perdereau *et al*., 2011; Amouroux *et al*., 2013).

Molecular genetics is a useful tool in understanding population structure (Kirk *et al*., 2013). The mitochondrial cytochrome oxidase subunit I (COI) gene has been effectively used to analyze insect genetics, evolution, and phylogenetics because it possesses more phylogenetic signal than other mitochondrial genes (Folmer *et al*., 1994; Zhang & Hewitt, 1997; Knowlton & Weigt, 1998; Shirota *et al*., 1999; Hebert *et al*., 2003; Lin *et al*., 2010) and is commonly regarded as one of the most conserved protein-coding genes in the mitochondrial genomes of animals (Brown *et al*., 1999). In the present study, we therefore screen COI sequence variation in introduced populations of *O. robiniae* in China, in order to explore population genetic factors that may correlate with the invasion success of this gall midge.

Materials and methods

Collection and preservation of O. robiniae

Rolled leaves of the host containing mature larvae or pupae of the gall midge were collected from 28 cities in China (fig. 1). Table 1 shows the details of the collection sites. As many samples as possible were collected in each site. Samples were stored in plastic bags of 60 × 40 cm^2. Infested leaves were usually selected randomly from different trees, but occasionally when infestation was low, single trees were more thoroughly sampled. All samples were collected between May and June 2013–2014 except HD and YA in August 2013 (table 1).

Twenty mature larvae or pupae were peeled out of the galls, then put into 100% ethanol, and stored at −20°C. They were used as supplementary materials for DNA extraction if there was not enough adult material. All other larvae and pupae were placed in transparent glass jars (15 × 30 cm^2) covered with moistened gauze and kept at room temperature (approximately 25°C) to rear adults. After emergence, 20 adults per location were put into a 1.5 ml centrifuge tube, and were frozen (−20°C) for DNA extraction within a period of 1 month. The remaining adults were preserved in 100% ethanol at −20°C.

Because the larvae are usually parasitized by their natural enemy *Platygaster robiniae* Buhl and Duso (Hymenoptera: Platygasteridae), and high rates of parasitism were observed in our study, we selected adults for DNA extraction from each site except for Chengdu, Guiyang, Nanjing, Xian, and Yantai. From these localities, we had to use larvae because we could not rear sufficient numbers of adults. These larvae were collected from the first or second generation, even if the locust gall midge may produce up to six generations per year (Wang, 2009; Mu *et al*., 2010; Shao *et al*., 2010). This is because the parasitic load in the first two *O. robiniae* generations is lower than in later generations.

DNA extraction, gene amplification, and sequencing

For each individual, total DNA was extracted from the entire body using the methods described by Zhou *et al*. (2007). A region of the COI gene was polymerase chain reaction (PCR)-amplified (Saiki *et al*., 1988) with the following pair of primers: LCO1490 5′-GGTCAACAAATCATAAAGATATTG G-3′ (forward) (Folmer *et al*., 1994), and COIA 5′-CCCGGTAAAATTAAAATATAAACT TC-3′ (reverse) (Funk *et al*., 1995). The COI fragment amplified by LCO1490 and COIA is approximately 676 bp long (Uechi *et al*., 2011) corresponding with positions 1752–2190 of the mitochondrial genome of *Drosophila yakuba* Burla (Diptera: Drosophilidae) (Clary & Wolstenholme, 1985). This COI region covers a part of the COI region that is used for DNA barcoding (658 bp from the 5′end) (Hebert *et al*., 2003) and a region that has been adopted for molecular phylogenetic analyses of Cecidomyiidae (439 bp from the 3′-end) (Kodoi *et al*., 2003; Uechi *et al*., 2011). The PCR reactions were performed in 0.5 µl of each primer (10 µmol), 12.5 µl 2× Taq PCR Master Mix (TIANGEN), 10.5 µl ddH_2O and 1 µl DNA template in a final volume of 25 µl. The amplifications were performed in

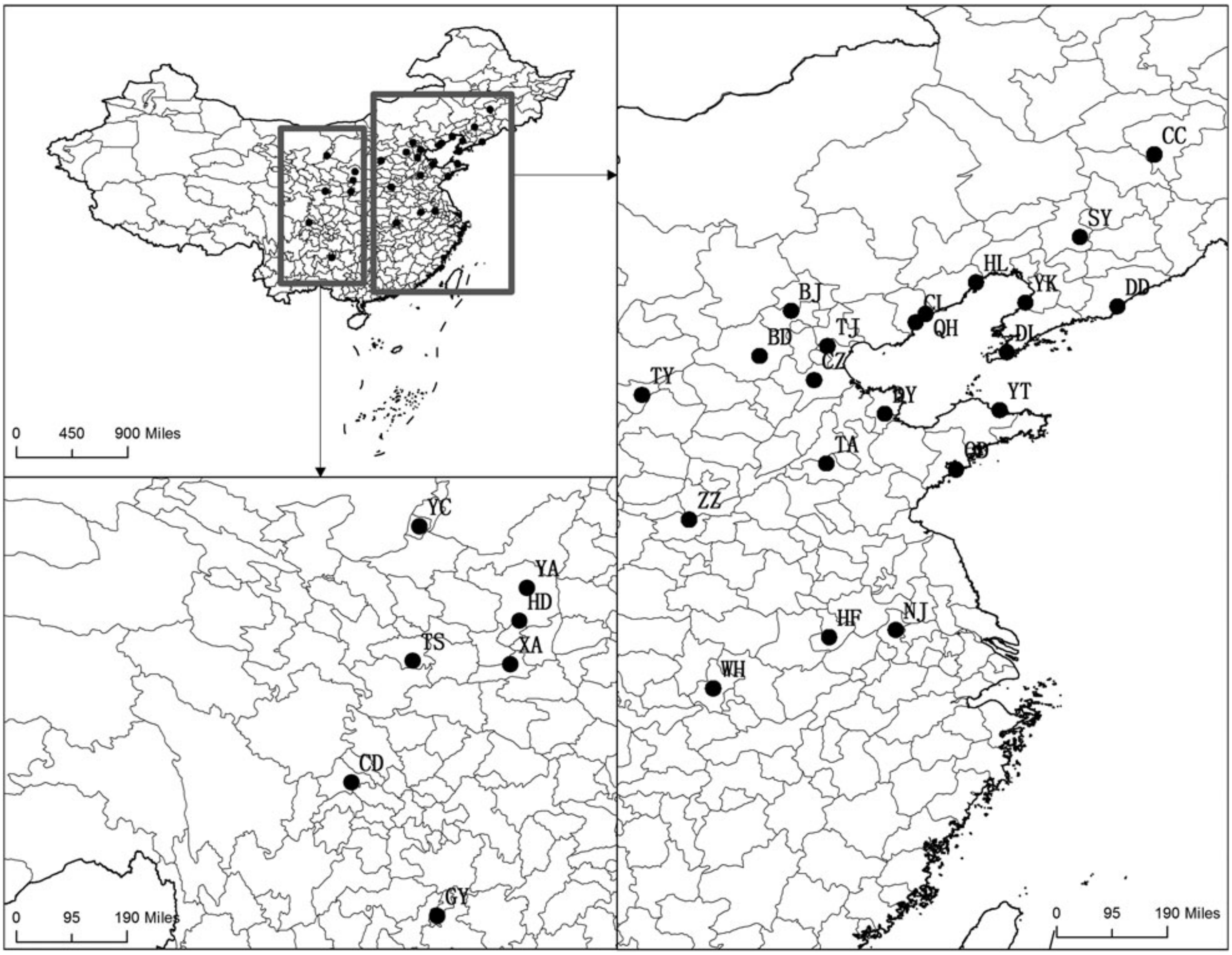

Fig. 1. *Obolodiplosis robiniae* collection locations in China.

0.2 ml tubes in an Eppendorf B Mastercycler (Eppendorf, Germany) with the following profile: the mixtures were incubated for 5 min at 94°C (initial denaturation step), followed by 30 cycles consisting of 94°C for 45 s, 54°C for 45 s and 72°C for 1 min, then 5 min at 72°C, and finally held at 16°C. Successful amplification was verified with 1% agarose gels using a slab gel apparatus with 3 µl of the amplification products. The sequencing reaction was performed using an ABI BigDye Terminator version 3.1 cycle sequencing kit on an ABI 3730XL (SinoGenoMax, China).

Data analysis

The sequences were edited and aligned with the Staden Package (Staden *et al.*, 1999) and Clustal X version 1.81 (Thompson *et al.*, 1997). The aligned DNA sequences were imported into MEGA version 5.0 (Tamura *et al.*, 2011) to calculate the nucleotide and haplotype diversities for each population. The genetic diversity indices, including the haplotype diversity, number of polymorphic (segregating) sites (S), nucleotide diversity (π, the mean number of differences between all pairs of haplotypes) and the average number of nucleotide differences (k) were calculated using DnaSP version 5.0 (Librado & Rozas, 2009). The significances of pairwise Fst values among populations were calculated using same software and ARLEQUIN 3.11 (Excoffier *et al.*, 2005).

The nucleotide sequence data reported in this paper have been deposited in the GenBank nucleotide sequence databases with the following accession numbers KM984772–KM984799 and KP184500–KP184502.

Results

Sequence composition

A total of 560 individuals from 28 populations were sequenced, and a final COI alignment of 676 bp in length was obtained by using the primers for LCO1490 and COIA. No insertions or deletions were found. The average compositions of the nucleotides A, G, C, and T were 28.8, 13.8, 15.4 and 42.0%, respectively. As expected, the sequences were extremely rich in A + T, which accounted for an average of 70.8% of the nucleotides.

Sequence variation

The 676-bp gene sequences revealed 673 conserved sites and only three variable sites two of which were parsimony informative. Of these three substitutions, one (125) occurred at

Table 1. *Obolodiplosis robiniae* collection data.

	Samples	Sites	Location	Date	Altitude	Number
1.	CC	Changchun, Jilin	N:43°53.851′E:125°16.329′	05 Jun 2013	218	20
2.	SY	Shenyang, Liaoning	N:41°50.438′E:123°25.690′	04 Jun 2013	51	20
3.	DD	Dandong, Liaoning	N:40°06.906′E:124°21.536′	03 Jun 2013	33	20
4.	HL	Huludao, Liaoning	N:40°42.829′E:120°50.706′	31 May 2013	17	20
5.	YK	Yingkou, Liaoning	N:40°12.432′E:122°04.413′	01 Jun 2013	15	20
6.	DL	Dalian, Liaoning	N:38°58.531′E:121°36.800′	02 Jun 2013	67	20
7.	BJ	Beijing	N:40°00.184′E:116°14.363′	20 May 2013	76	20
8.	QH	Qinhuangdao, Hebei	N:39°56.161′E:119°35.411′	27 May 2013	17	20
9.	CL	Changli, Hebei	N:39°43.252′E:119°20.328′	30 May 2013	8	20
10.	BD	Baoding, Hebei	N:38°52.782′E:115°27.546′	21 May 2013	35	20
11.	TJ	Tianjin	N:39°07.935′E:117°08.818′	22 May 2013	5	20
12.	CZ	Cangzhou, Hebei	N:38°16.961′E:116°48.495′	23 May 2013	23	20
13.	YC	Yinchuan, Ningxia	N:38°28.933′E:106°11.983	28 May 2014	1115	20
14.	TY	Taiyuan, Shanxi	N:37°54.592′E:112°31.811′	26 May 2014	798	20
15.	DY	Dongying, Shandong	N:37°26.366′E:118°34.448′	24 May 2013	17	20
16.	YT	Yantai, Shandong	N:37°32.024′E:121°25.657′	15 Jun 2013	9	20
17.	QD	Qingdao, Shandong	N:36°03.367′E:120°20.934′	14 Jun 2013	24	20
18.	TA	Taian, Shandong	N:36°12.225′E:117°07.104′	20 Jun 2013	208	20
19.	YA	Yanan, Shaanxi	N:36°35.633′E:109°29.535′	06 Aug 2013	1121	20
20.	HD	Huangdiling, Shaanxi	N:35°35.165′E:109°15.965′	08 Aug 2013	883	20
21.	XA	Xian, Shaanxi	N:34°15.474′E:108°58.938′	04 Jun 2014	428	20
22.	ZZ	Zhengzhou, Henan	N:34°48.509′E:113°42.266′	07 Jun 2014	95	20
23.	TS	Tianshui, Gansu	N:34°21.405′E:106°00.034′	03 Jun 2014	1460	20
24.	NJ	Nanjing, Jiangsu	N:32°03.426′E:118°50.820′	18 Jun 2014	90	20
25.	HF	Hefei, Anhui	N:31°52.824′E:117°11.639′	20 Jun 2014	39	20
26.	CD	Chengdu, Sichuan	N:30°38.245′E:104°07.334′	30 Jun 2014	510	20
27.	WH	Wuhan, Hubei	N:30°36.733′E:114°17.772′	21 Jun 2014	40	20
28.	GY	Guiyang, Guizhou	N:26°33.531′E:106°45.003′	29 Jun 2014	1090	20

the second codon position, one (312) at the third codon position, and the other (571) at the first codon position. Two substitutions caused changes in the amino acids. The first (571) caused an H to Y change, and the second (125) caused an R to H change.

Only four haplotypes were detected (tables 2 and 3), confirming the low degree of polymorphism. As table 2 shows, haplotype I was characterized by G (125), C(312), and T(571) at the three variable sites. The three other haplotypes are named as follows: haplotype II with C(571), haplotype III with T(312), and haplotype IV with A(125). Haplotype I occurred in 550 of the 560 individuals (98.2%) and was found in all 28 populations. Haplotype II was observed in population DD (3 individuals), haplotype III in population TA (1 individual) and haplotype IV in population YK (6 individuals) (table 1). Each of these three haplotypes (i.e., II, III, and IV) can be derived from haplotype I via single substitutions, involving transitions. Therefore, it is likely that haplotype I is the ancestral haplotype, more so as it is by far the most frequent haplotype. The low genetic diversity of the 28 populations is also evident from the extremely low values of the average number of nucleotide differences, the haplotype (gene) diversity, and the nucleotide diversity, which were 0.035, 0.035, and 0.00005, respectively.

Genetic differentiation among populations

The pairwise F_{st} values among 28 populations varied between 0.0000 and 0.2632. Several significant F_{st} values were observed: the highest is 0.2632 between YK and other populations except DD and TA, followed by 0.2256 between YK and TA, then 0.2106 between YK and DD, followed by 0.1053 between DD and other populations, and 0.0792 between DD and

Table 2. Alignment of the variable sites of the partial COI haplotypes. The numbers indicate the positions of the variable sites in the alignment.

	Location sites		
Types	125	312	571
Haplotype I	G	C	T
Haplotype II	G	C	C
Haplotype III	G	T	T
Haplotype IV	A	C	T

Table 3. Haplotypes from 28 locations.

Samples	Haplotype	Number of samples	Samples	Haplotype	Number of samples
BJ	I	20	QH	I	20
BD	I	20	SY	I	20
CC	I	20	TA	I	19
CD	I	20		III	1
CL	I	20	TJ	I	20
CZ	I	20	TS	I	20
DD	I	17	TY	I	20
	II	3	WH	I	20
DL	I	20	XA	I	20
DY	I	20	YA	I	20
GY	I	20	YC	I	20
HD	I	20	YK	I	14
HF	I	20		IV	6
HL	I	20	YT	I	20
NJ	I	20	ZZ	I	20
QD	I	20			

TA. However, 324 of the 378 F_{st} values were ≤0, which suggests that, although there was a high level of genetic differentiation between YK, DD, and TA populations, the total genetic differentiation was very low.

Discussion

Our study explores population genetic diversity in a new invasive pest, *O. robiniae*, which has been widely spread in Europe and Asia in recent years and provides insight into its invasion success. Our results revealed a low level of nucleotide diversity ($\pi = 0.00005$) in the partial COI sequences among populations from sites separated by distances of up to 1600 km from Yantai (Shandong Province) to Tianshui (Gansu Province) in the east and west, respectively, and approximately 1900 km from Changchun (Jilin Province) to Guiyang (Guizhou province) in the north and south, respectively.

This extremely limited genetic diversity probably reflects a founder effect, eventually combined with a bottleneck, causing a severe loss of haplotypes (Nei *et al.*, 1975). *O. robiniae* indeed arrived probably only very recently in China, since there are no records of this gall midge before 2006 (Yang *et al.*, 2006). Yet, its host *R. pseudoacacia* was introduced into China approximately 130 years ago (Pan & You, 1994) and is currently widely planted in vast areas. Such a low genetic diversity has also been reported in mango blossom gall midge, *Procontarinia mangiferae* (Felt) (Amouroux *et al.*, 2013).

Despite this low genetic diversity, *O. robiniae* spread successfully in China, probably through human activity and as a result of its own biological and ecological characteristics.

First, the host plant of *O. robiniae*, *R. pseudoacacia*, is widespread in China. Second, *O. robiniae* lays eggs on mature plants in spring when they rapidly develop. The gall midges then start attacking the root suckers from mid-summer onward when mature host trees grow slow and decline. Duso *et al.* (2011) reported two strategies by which the gall midges cope with adverse environmental conditions: (1) larvae diapause in summer and (2) pioneering adults are produced in late summer. Although we have no precise data on diapause of *O. robiniae* in China, we did observe larvae on the leaves of mature host trees in early summer, and adult midges on root suckers in late summer. Due to a lack of comparative data from *O. robiniae* in its native range, we do not know whether the two strategies mentioned above are new. Yet, a plastic diapause period in new habitats has been recorded in the gall midge *Procontarinia mangiferae* (Felt) (Amouroux *et al.*, 2013)

All in all, *O. robiniae* seems to have r-selected traits since (1) it requires around 15 days to develop from egg to adult in summer (Yang *et al.*, 2006), (2) it produces four to six generations a year in China, and (3) it may have up to 192 eggs in the ovary (Park *et al.*, 2009). Life-history strategies with more r-selected traits including short generation time, high fecundity, and high growth rate are commonly (Prins & Gordon, 2014), but not always (Sakai *et al.*, 2001), observed during the establishment of successful invasive species.

The lack of strong competitors could be another factor explaining the success of *O. robiniae* in China. Although several defoliators, such as the geometrid moths *Meichihuo cihuai* Yang, *Apocheima cinerarius* Erschoff, and *Zamacra excavata* Dyar (Qiang *et al.*, 2002), feed on the black locust tree in China, they all have only one generation per year and the period that their larvae attack their host plant involves only about 20 days from mid-April to mid-May (Qiang *et al.*, 2002), after which leaves of infested plants can still re-develop gradually. Furthermore, these defoliators are relatively rare as they were hardly found during our sampling. This suggests that *O. robiniae* probably does not suffer much from interspecific competition in China.

Acknowledgements

The authors would like to thank Ruixing Zhao (Forest Protect Station of Liaoning Province), Yanping Yu (Forest Protect Station of Jilin Province), Kaipeng Zhang (Jingyuetan National Forest Park), Chao Li (Forestry Survey and Planning Institute of Guizhou Province) and Xiangchen Cheng (General Station of Forest Pest Management, State Forestry Administration) for their great support and help with the sample collection in the fields. The authors also thank Professor Zhongqi Yang (the Biological control lab of the Chinese Academy of Forestry) for identifying the species. They also thank Dr, Yong Li for discussions about the data, and they sincerely thank the other members of the Plant quarantine lab and Plant Pathology Lab of the Chinese Academy of Forestry for helpful comments. This work was funded by Central Nonprofit Research Institutes Fundamental Research Fund Project CAFRIFEEP201102-3.

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