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分子生物学和同工酶电泳法鉴定烟粉虱幼期寄生蜂浅黄恩蚜小蜂

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摘要：浅黄恩蚜小蜂 *Encarsia sophia*（Hymenoptera：Aphelinidae）**是一种有潜在应用价值的烟粉虱寄生蜂，在实验室条件下，浅黄恩蚜小蜂与丽蚜小蜂** *Encarsia formosa*（Hymenoptera：Aphelinidae）**共同存在时，表现出竞争优势。目前还很难准确判断早期被蚜小蜂寄生的烟粉虱，因此寻找一种有效快速鉴定烟粉虱的寄生蜂早期寄生的方法非常重要。本研究发现在检测烟粉虱寄生蜂早期寄生的每个阶段（卵、1 龄和 2 龄幼虫）时，基于线粒体 DNA 细胞色素氧化酶 COI 基因的 PCR 方法比同工酶电泳的更灵敏，同工酶电泳只能在寄生蜂发育至 3 龄及以上时才能检测出烟粉虱的寄生情况。PCR 方法同时也能够区分早期发育阶段的浅黄恩蚜小蜂和丽蚜小蜂。本研究所建立的 PCR 方法快速、灵敏、可靠，可代替同工酶电泳和解剖的方法，用于烟粉虱早期寄生的鉴定，从而实现实验室和田间烟粉虱寄生率的快速评估。**

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Molecular and iso-enzymatic identification of the nymphal parasitoid, *Encarsia sophia*（Hymenoptera：Aphelinidae）of the whitefly, *Bemisia tabaci* Genn.（Homoptera：Aleyrodidae）

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Abstract： *Encarsia sophia*（Hymenoptera：Aphelinidae）is a parasitoid of *Bemisia tabaci* which has potential application values. Compared to *Encarsia formosa*（Hymenoptera：Aphelinidae），it shows a competitive advantage when they co-exist in the condition of laboratory. It is not easy to judge the parasitic situation accurately by aphelinidae parasitoids of *B. tabaci* at the earlier stages. So it is necessary to find an effective and rapid method to identify early period parasitism of *B. tabaci* inside aphelimid. It has been observed in our findings that the PCR analysis system based on the cytochrome oxidase subunit I（COI）gene of mitochondrial DNA（Mt DNA）is more sensitive than isoenzyme method in the detection of *B. tabaci* parasitoids in every early stage（egg，1st and 2nd instar），while electrophoresis can only detect the presence of *B. tabaci* parasitoids when it developed to 3rd instar. PCR method also can distinguish early instars of *E. sophia* and *E. formosa*. The PCR analysis system described in our study is an alternative method of iso－enzyme analysis and dissection detection because

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PCR method was observed to be a rapid, sensitive and reliable test method for the early parasitism of *B. tabaci*. We think that it would be used to assess the parasitic rate of *B. tabaci* accurately both in the laboratory and fields.

Key words: *Bemisia tabaci*; *Encarsia sophia*; nymphal parasitoid; COI gene; electrophoresis isoenzyme

Introduction

Bemisia tabaci Genn. (Homoptera: Aleyrodidae: Aleyrodinae) is an important pest of cotton, vegetables, and ornamental plants all over the world (Servin – Villegas *et al.*, 2001; De Barro *et al.*, 2011). It was recorded in China for the first time in 1949 (Zhou, 1949). The cotton whitefly was regarded as a secondary pest until 1980s (Byrne *et al.*, 1990) and in the last two decades it was regarded as a primary pest (Schuster, 2003) because of its wide host – range, higher fecundity and the development of resistance to insecticides (Palumbo *et al.*, 2001, 2003; Ahmad *et al.*, 2002; Nauen and Denholm, 2005; Jones, 2003). The cotton whitefly causes damage directly by feeding and indirectly by the transmission of pathogens on plants (Byrne *et al.*, 2000). There is need of alternative or supplementary strategies to control *B. tabaci* as it has developed resistance to insecticides (Palumbo *et al.*, 2001, 2003; Ahmad *et al.*, 2002; Nauen and Denholm, 2005). Many parasitoid species in the genera of Encarsia (Hymenoptera: Aphelinidae) are important natural enemies of the two whitefly species (Gerling, 1998). Over 170 species in the genus Encarsia have been described worldwide (Hayat, 1989). *Encarsia sophia* Girault & Dodd [= *E. transvena* (Timberlake)] (Hymenoptera: Aphelinidae)] is one of the most important parasitoid species parasitizing many whitefly species, including *B. tabaci* (Kapadia and Puri, 1990; Gerling *et al.*, 1998; Hunter & Kelly, 1998; Antony *et al.*, 2003; Zang & Liu, 2008, 2009; Shi *et al.*, 2009). Female eggs are laid internally in whitefly nymphs and develop as primary parasitoids. There are many species of *B. tabaci* nymphal parasitoids (Wang *et al.*, 2011) but it is only well documented in literature. Compared to *E. Sophia*, reproductive technology of *E. formosa* had achieved remarkable importance as nymphal parasitoid in case of whitefly (Cheng *et al.*, 1986). But under laboratory conditions it has been evaluated that *E. sophia* have a higher competitiveness than *E. formosa*. *E. sophia* is a potentially useful nymphal parasitoid of *B. tabaci* at present (Li *et al.*, 2008; Anyony *et al.*, 2003). So it is important to work on the identification of this parasitoid at nymphal stages of *B. tabaci*. There are some traditional methods for the identification of *B. tabaci* nymphal parasitoids which are based on dissection or rearing of the parasitoids until they emerged. These both methods not only consumed much time and needed more skilled labor but also have low rate of success. Electrophoresis iso – enzyme method had been successively used to identify another nymphal parasitoid, *E. bimaculata* of *B. tabaci* (Antony, 2004) but not studied against *E. sophia*. By looking at the biology of this parasitoid it is important to find out a rapid and accurate method to detect the *E. sophia* parasitization at earlier stages of *B. tabaci* nymphs.

Keeping this importance in view, it has been decided to use molecular (PCR) and iso – enzymeatic method for the identification of nymphal parasitoid, *E. sophia* in *B. tabaci* nymphs.

Materials and methods

1. Host plants and Insects culture

Host plants: Bt – cotton (*Gossypium hirsutum*) seed of jiyou – 768 variety was provided by Institute of Vegetable Research, Beijing Academy of Agriculture and Forestry Sciences (BAAFS). The cotton seedlings were grown in 12 cm diameter plastic flower pots in greenhouse inside glass cages (60 × 40 cm) under 25 ~ 30℃, RH 60% ~ 70% and 16L : 8D photoperiod.

***B. tabaci*:** whiteflies used in all experiments were collected from the Institute of Plant and

Environment Protection (BAAFS) where it was reared on cotton plants.

E. Sophia: individuals used in the trials were provided by the Department of Entomology, Agricultural Experiment Station, Texas A & M University, USA where it was cultured on *B. tabaci* nymphs as host.

Experimental Arena: Cotton leaves with *E. sophia* pupae were detached and shifted into experimental arena as shown in Fig. 1. Each petri dish (9 cm diameter) has a hole (4 cm dm) on upper lid and gauzed by muslin cloth to provide ventilation while at the bottom of each petri dish, two pieces of 9cm diameter filter paper were placed to maintain the moisture for detached leaves. A 5 ×2 cm soaked cotton wool strip was used as paste on the stems of detached leaves to keep moisture constant. After adult insect emergence, both male and female *E. sophia* were isolated and shifted separately and then they were fed with honey – water solution (10%) for 24 hours. Mated females were released into the petri dish with non – parasitized third nymphal instar of *B. tabaci*. Parasitic *B. tabaci* nymphs inside *E. Sophia* were used as experimental material (Zhou *et al.*, 2010; Szabo *et al.*, 1993), single detection and each sample repeated 10 times. Adult parasitoids of *E. formosa* and *E. sophia* were reared and collected under the same environmental conditions as mentioned above. Collection methods of experimental insects are given in table 1.

Table 1 The situation of test insect

Code	Required stage of larval parasitoid	Collection method
B0	non – parasitized *Bemisia tabaci*	Remove the leaves with third or fourth *B. tabaci* nymph
EB0 (EF0)	Eggs of *Encarsia sophia* (*Encarsi formosa*) parasitized *B. tabaci*	When female oviposition behavior finished, singled out the egg of *E. sophia* (*E. formosa*) parasitized *B. tabaci*
EB1 (EF1)	First instar of *E. sophia* (*E. formosa*) parasitized *B. tabaci*	When introduced the female for 2 days, singled out the first instar of *E. sophia* (*E. formosa*) parasitized *B. tabaci*.
EB2 (EF2)	Second instar of *E. sophia* (*E. formosa*) parasitized *B. tabaci*	When introduced the female for 3 to 4 days, singled out the second instar of *E. sophia* (*E. formosa*) parasitized *B. tabaci*.
EB3 (EF3)	Third instar of *E. sophia* (*E. formosa*) parasitized *B. tabaci*	When introduced the female for 4 to 6 days, singled out the third instar of *E. sophia* (*E. formosa*) parasitized *B. tabaci*.
EB4 (EF4)	Pupa of *E. sophia* (*E. formosa*) parasitized *B. tabaci*	When introduced the female for 7 days, singled out the pupa of *E. Sophia* parasitized *B. tabaci*.
EF	Adult of *Encarsia formosa*.	Adult of *Encarsia formosa*.

2 PCR detection

2.1 Genomic DNA isolation

Dissection of EB0, EB1, EB2, EB3 and EB4 stages (see detail in table 1) was directly under the Motic – microscope and if parasitoids identified at the corresponding stages, they were provoked in the centrifuge tube (1.5 mL) respectively by the anatomy needle and stored at –20℃ until they were used in experiments. The total DNA was extracted by TIANamp Genomic DNA Kit (TAKATA).

2.2 PCR amplification

PCR products were conservative sequences of cytochrome oxidase I gene. LA Taq polymerase and dNTP bought from Takara. Specific primers of *E. sophia* that we designed based on the inter – comparison of *E. sophia* and *B. tabaci* COI gene sequence. We used universal primer (Forward primer sequence c1 – j – 2183F: 5′ – CAACATTTATTTTGATTTTTTGG – 3′ and Reverse primer sequence L2 – N – 3014R: 5′ – TCCAATGCACTAATCTGCCATATTA

－3′) to attain their COI genes sequence of *E. sophia* and *B. tabaci*, then sequencing (Sanboyuanzhi Biotechnology Co., Ltd in Beijing). CLUSTALW2 was used to compare the two sequences, at last we got the specific primers of *E. sophia*: Forward primer AE42F: 5′－TGGGGTTATAGGAATG－3′ and Reverse primer AE669R: 5′－AACTAATCCCTCC CAG－3′. Show it in Figure 1.

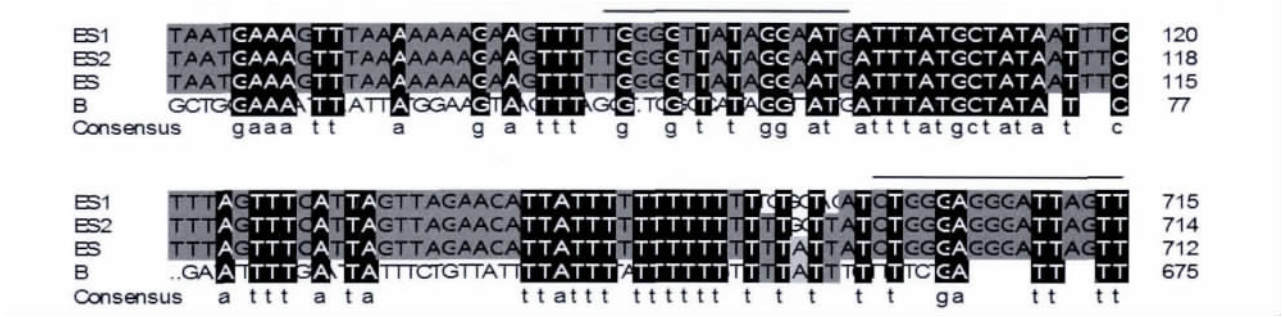

Fig. 1　Partial alignments of sequence COI gene, *Encarsia sophia* sequence identity compared with *Bemisia tabaci*. Point design primers are shown under the black line. ES1, Second instar of *E. sophia*; ES2, Third instar of *E. sophia*; ES, *E. sophia*; B, *B. tabaci*.

The reaction mixture (25 μL volume) contained LaTaq polymerase 0.5 μL (5 u/μL) 17 μL ddH_2O, 2 μL dNTP (2.5 mM 400 μL), 2.5 μL 10 × buffer Ⅱ (Mg^{2+} plus), 1 μL of each primer and 1 μL of template DNA. The PCR cycling involved pre－denaturation (94℃ for 5 min), initial denaturation at 94℃ for 45 s, annealing at 50℃ for 45 s, with elongation at 72℃ for 1min, followed by 35 cycles of step 2 to the end step, and then stored at 4℃. The PCR product was analyzed by gel electrophoresis using 1% agarose and PowerPac Universal electrophoresis (American Bio－Rad. Gel. DOC™ XR+), 1 × TAE buffer, voltage 95V, after 30 min it was analysed by gel imaging system (American Bio－Rad). 5 kb DNA Ladder (trans 2 K plus) and DL 2 kb DNA Ladder were used as marker to analyse the size of the PCR products.

3. Isozyme electrophoretic detection

Esterase isozyme electrophoretic was used in vertical slab polyacrylamide gel electrophoresis tests. Single insect was shifted in each centrifuge tube (1.5 cm), samples were homogenized with homogenate buffer (pH 6.8), and then 5 μL bromophenol blue indicator solution was added and mixed to get the suspensions. The condition of electrophoretic: stacking gel (5%), superposed on 8% resolving gel, electrophoresis buffer (pH 8.2), current of 25 mA. Esterases were visualized by the staining of 0.005% 1－naphthyl acetate, 0.2 M phosphate buffer (pH 6.6) and fast blue RR salt (100 mg/100mL). Gels were photographed and analyzed by gel imaging system (American Bio－Rad. Gel. DOC™ XR+).

Results

1. The result of PCR detection

PCR was able to accurately identify the *E. sophia* parasitized *B. tabaci* nymph as early as 1 hour after spawning. Figure 2 shows that there was a band at 627 bp when *E. sophia* parasitize *B. tabaci* nymphs. After sequencing and comparing by the sequence of NCBI BLAST website, it was exactly similar to *E. sophia* M95107 cytochrome oxidase subunit I (COI) gene (GenBank: AY264338), similar rate reached to 99%. There are three different bases of ES1 and ES2 sequence (Figure 1), maybe they are different individuals or at different instars. Figure2 shows that non－parasitized *B. tabaci* nymphs did not show bands. We also used the same specific primer of *E. Sophia* to identify *E. fromosa*, the result showed that there is no band at 627 bp, so this pair of primers could distinguish early instars of *E. Sophia* and *E. fromosa* by PCR detection.

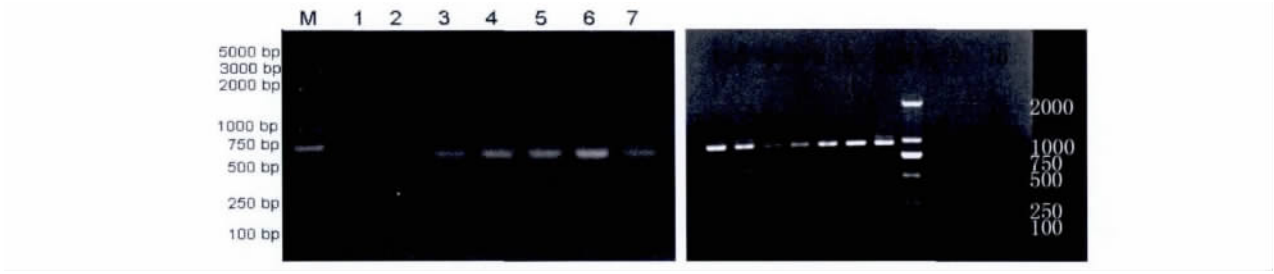

Fig. 2 PCR detection of *Encarsia sophia* (A) and *Encarsia formosa* (B) parasitized and non – parasitized *Bemisia tabaci* nymphs
A: (M: maker; 1: B0; 2: B0; 3: EB0; 4: EB1; 5: EB2; 6: EB3; 7: EB4). B: (M: maker; 1: EF; 2: EF; 3: EF0; 4: EF1; 5: EF2; 6: EF3; 7: EF4; 8: B0; 9: B0; 10: B0)

2. The result of Isozyme electrophoretic detection

Fig. 3 shows the esterase – banding pattern of non – parasitized and *E. sophia* parasitized *B. tabaci* nymphs. Non – parasitized *B. tabaci* and EB2, EB1, EB0 strain had three bands while EB4, EB3 (mR: 0.021 and 0.064) had two esterase bands. The esterase electrophoretic could only detect the later larvae of *E. sophia* parasitized *B. tabaci* nymphs. Only when *E. sophia* develops to the third instar or later, this method of detection *E. Sophia* parasitized and non – parasitized *B. tabaci* nymphs can work. Map shows the relative mobility rate (mR) where mR is the distance from protein samples to the sample (cm) divided by the distance from bromophenol blue to the sample (cm).

The results showed that PCR could be more effective than electrophoretic for the detection of *E. sophia* parasitized and non – parasitized *B. tabaci* nymphs, because it could distinguish the early parasitoid while Esterase electrophoretic could only detect *B. tabaci* nymphs inside *E. sophia* developing to third instar, prepupa and pupa. The reasons for different mR of the two B0 fastest band in Fig. 3 was might be the two B0 at different developmental stages. The two methods were compared in the table 2.

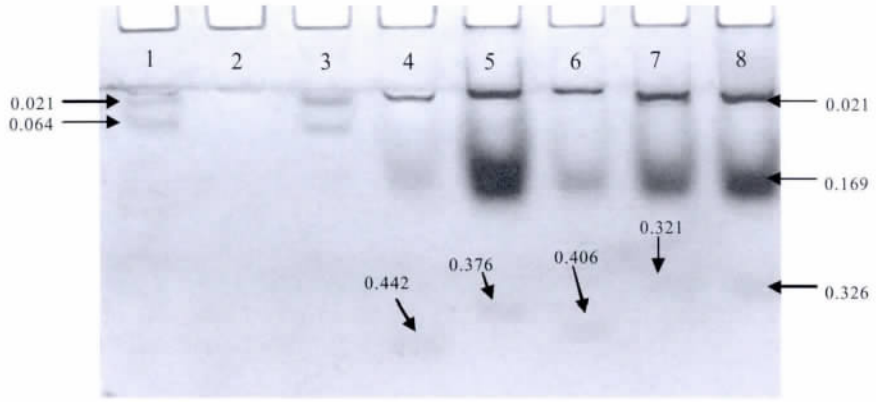

Fig. 3 Esterase – banding pattern of unparasitized and *Encarsia sophia* parasitized *Bemisia tabaci* nymphs.
1: EB4; 3: EB3; 4: EB2; 5: EB1; 6: EB0; 7: B0; 8: B0, The figure shows the migration rate of protein.

Table 2 Comparison of PCR and isoenzyme electrophoresis detection of *E. sophia* parasitized and non – parasitized *B. tabaci* nymphs

Method	The different stages of *E. sophia*					
	egg	First instar	Second instar	Third instar	prepupa	pupa
PCR	+	+	+	+	+	+
Isoenzyme electrophoresis	–	–	–	+	+	+

"+" shows that this method could detect *E. sophia* parasitized and non – parasitized *B. tabaci* nymphs; "–" shows that this method could not detect *E. sophia* parasitized and non – parasitized *B. tabaci* nymphs.

Discussion

In recent years, molecular biology developed rapidly, DNA barcoding technique could indentify species at different development stages (Ahrens *et al.*, 2007). All the molecular identification of parasitoids are based on adult detection (Qiu, 2005; Babcock and Heraty, 2000; De León *et al.*, 2010). It is the first report in our knowledge to use molecular identification to detect *E. sophia* parasitized and non-parasitized *B. tabaci* nymphs.

Antony *et al.* used electrophoresis to detect *E. bimaculata* parasitized and non-parasitized *B. tabaci* nymphs and considered that electrophoresis might make it possible to detect *E. bimaculata* at earlier stage (2004). However, our results showed that electrophoresis could only detect the *B. tabaci* parasitoids in the late larval instar (third instar and pupa). Lisha *et al.* (2003) reported that there were two fast moving and a slow band in cassava strain for esterase in a non-parasitized *B. tabaci*. Our results are consistent with the results reported in his paper. Compared with isoenzyme electrophoresis methods, PCR methods have more advantages such as rapid, accurate, efficient and so on. We used COI gene as an identified fragment gene to compare with other fragment genes, COI gene has some advantages such as relatively conservative, enough variation and the length of sequence is moderate (Hebert *et al.*, 2003). It could detect the early larvae of *E. sophia* parasitized *B. tabaci* nymphs. PCR could detect *E. sophia* parasitized *B. tabaci* nymphs rapidly, accurately and it is better than isozymes electrophoresis, dissection and rearing to parasitoid emergence. This method could detect the parasitoid rate in laboratory. In natural environment it would be used to assess the parasitic effect even the parasitoids were at the egg stage and it also would save both a lot of time and cost of labor. The PCR analysis system could provide a potential method for other parasitoids species identification and parasitic effect evaluation.

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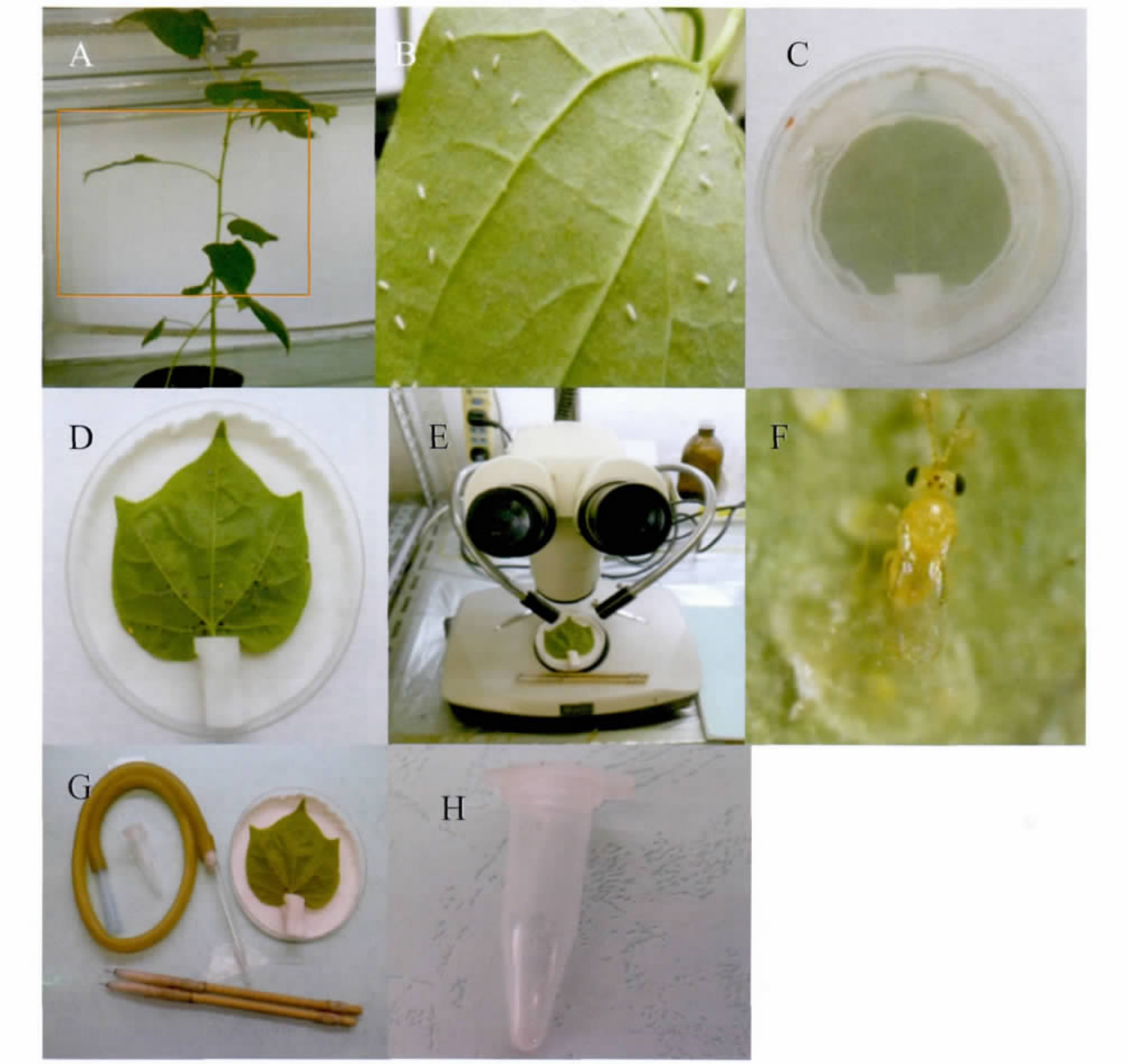

Fig. 4　Experimental design for getting the material to molecular experiments

A，Cotton seedling；B，Selection of whitefly infested leaf；C，Upper lid of petridish with hole；D，Parasitized *Bemisia tabaci* nymphs with cotton leaf，filter paper at the bottom，soaked cotton wool strip；E，Confirmation of the *B. tabaci* nymph parasitization by SMZ－168；F，*Encarsia Sophia* is parasiting *B. tabaci* nymph；G，Parasitized *B. tabaci* nymph dissection kit，anatomy needle；H，1. 5 mL centrifuge tube.