**Determination of key predators of pear psylla using molecular bioassays for prey detection**

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**Abstract**

**Two molecular probes were designed to determine which generalist predators consume pear psylla under field conditions. The probe can be used to determine what fraction of a population of predators has eaten pear psylla within approximately the past 24 hours. One probe was shown to react to all species of psylla whereas a second probe was found to bind to only psyllas in the same genus as pear psylla. The probe did not react to a variety of other potential prey or other generalist predators. The probe was highly sensitive and capable of detecting minute levels of psylla DNA. The successful development of the molecular probe will now be used to determine which predators appear to be most important for regulating pear psylla. The data from the probe will need to be incorporated into larger datasets containing additional information such as predator densities, number of prey eaten per predator, and availability of alternative prey. The probe will be used as part of a multi-state, multi-institutional effort to enhance our ability to predict and augment the effective biological control of pear psylla.**

**Introduction**

Pear psylla continues to remain a key pest of pears despite a number of studies looking at a host of generalist predators as potential agents for regulating pear psylla populations. With pheromone mating disruption becoming increasingly more common in California pears, opportunities for biological control of pear psylla are more likely. However, one of the issues for predicting effective biological control of psylla has been the large of array of generalist predators that have been found in association with pear psylla populations.

Many species have been shown to feed on psylla in lab or field trials. However, what has not been established is the relative importance of the different predators and how their relative efficacy may change over the season. For example, do these predators shift to more preferred species at different points in the growing season or do the predators vary in their likelihood to feed on a psylla? The ultimate impact of these generalists will then depend on several variables: a) which species are feeding on pear psylla b) what fraction of these predators are feeding on psylla at any point in the season c) the size of the predator population d) how many psylla does an individual predator consume each day or during its lifetime and e) how does psylla densities influence the first 3 variables?

Identification of which predators are actually feeding on psylla and what proportion of the predator population is eating psylla at different times of the year potentially can be determined with the use of novel molecular tools. Three general techniques have now been tried to identify if the gut contents of a predator contain a specific prey: a) direct examination b) serological techniques and c) molecular approaches. We hope that these data help improve our ability to predict pear psylla

biological control such that growers will be able to exclude the more expensive psylla materials, e.g. abamectin, from their IPM programs as prophylactic treatments. While biological control of psylla is clearly possible in many years and in many orchards, it is also clear that risk varies by orchard region and by year.

The specific objectives for 2000 included the development of a molecular probe for a DNA sequence found in pear psylla, but not in potential generalist predators. Several steps were required as follows: a) sequencing of a specific region of the mitochondria of pear psylla, other prey species, and generalist predators b) determination of how long the probe could detect the psylla DNA after the initial feeding by the predator c) how sensitive was the probe at detecting low levels of pear psylla in the gut contents of its predators.

**Materials and Methods**
*Insects/ Mites, or Spiders*

The list of species used in the development of the pear psylla probe is shown in Table 1. The eggs, nymphs, and adults of pear psylla, *Cacopsylla pyricola* Foerster (Homoptera: Psyllidae), were obtained from laboratory colonies at the USDA-ARS in Wapato, Washington, as well as the predators *Deraeocoris brevis* (Uhler) (Heteroptera: Miridae), *Anthocoris tomensosus* Pericart and *Anthocoris antevolens* White (Heteroptera: Anthocoridae). Predators were reared on black bean aphid *Aphis fabae* Scopoli (Homoptera: Aphididae). This aphid together with the twospotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae), a predacious beetle, *Harmonia axyridis* (Coccinelidae), and 2 species of spiders, *Phidippus clarus* (Salticidae) and *Xysticus cunctator* (Thomisidae) also came from colonies reared in Wapato, WA. A second species of psylla found on pears, *Cacopsylla pyri* (L) (Homoptera: Psyllidae), was obtained from pear fields located in Lleida (Spain). Samples were collected and frozen before shipment to the US. A third species of psylla, *Acizzia uncatoides* (Ferris and Klyver) (Homoptera: Psyllidae), was collected in the field in Albany, California. Predator species tested in the study included the mullein bug, *Campylomma verbasci* (Meyer) (Heteroptera: Miridae), the minute pirate bug, *Anthocoris nemoralis* (Fabr.) (Heteroptera: Anthocoridae) and brown lacewing, *Hemerobius pacificus* Banks (Neuroptera: Hemerobiidae). The "willow psylla" (Homoptera: Psyllidae) came from French Canyon, WA. *Cydia pomonella (L)* (Lepidoptera: Tortricidae) and *Lygus hesperus* Knight (Heteroptera: Miridae) were obtained from colonies reared at the University of California in Berkeley,CA.

Female predators were starved for 24 h at 25ºC prior to testing. After starving, individual predators were placed in a small plastic box and allowed to consume *C. pyricola* nymphs for 90 min (ingestion period). After this period of time, only the predators that had eaten 1-5 *C. pyricola* nymphs were frozen at -20ºC until their assay. Additional predators were starved for 24 h and frozen for their use as negative controls.

*DNA extraction*

Total DNA was extracted from one individual of the prey or predator following the protocol for animal tissues of the DNeasy Tissue Kit, Qiagen. Total DNA obtained was re-suspended in 100 µl TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and stored at -20ºC.

*Sequencing*

DNA of pear psylla nymphs, three other species of psyllids (*C. pyri, A. uncatoides,* willow psylla), an aphid *A. fabae,* codling moth *C. pomonella*, and 5 predators for the pear psylla *(C. verbasci, D. brevis, A. nemoralis, A. tomensosus, A. antevolens)* was amplified. Two primers were used with genomic DNA template for amplification of mitochondrial segments with the polymerase chain reaction (PCR). These primers amplify a region of the gene COI. Each reaction (50 µl volume) contained: 4 µl of re-suspended DNA, 5µl of 10 x buffer (20 mM Tris-HCl (pH 8.0 at 25°C), 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol, 0.5% glycerol, 0.5% Tween$^®$20 and 0.5% Nonidet$^®$P40), 4 µl of 25mM $MgCl_2$, 0.2 µl of Taq DNA polymerase (Promega, Madison, WI), 1µl of 2.5mM dNTPs, and 2 µl of each primer 5 pmols/µl. Samples were amplified in a PTC-100, MJ Research, Inc. Version 7.0 thermal cycler for 35 cycles at: 94ºC, 30 s; 45ºC, 30 s; 72ºC, 45 s. A first cycle of denaturation was carried out at 94ºC for 2 min, and a last cycle of extension at 72ºC for 5 min. Double-stranded polymerase chain reaction (PCR) product was purified (QIAquick PCR Purification Kit; Qiagen) and sequenced (ABI PRISM$^{TM}$ Dye Terminator Cycle Sequencing Ready Reaction Kit; Perkin Elmer, Norwalk, CT, U.S.A.) in an ABI PRISM 310 Genetic Analyser according to the dideoxychain-termination method.

*Design of primers and analysis of COI markers*

Twelve oligonucleotides were designed to be used as specific primers from the *C. pyricola* fragment. Guidelines proposed for the design of efficient and specific primers by Innis & Gelfand (1990) and Saiki (1990) were followed. Primers contained around 20 internal bases, avoiding the sequence of the original primer. Two pairs of these primers were chosen for our study and synthesized. Amplification reactions using these primers (25 µl volume) contained: 6 µl of re-suspended DNA, 2.5µl of 10 x buffer, 3 µl of 25mM $MgCl_2$, 0.25 µl of Taq DNA polymerase (Promega, Madison, WI), 0.25µl of 5mM dNTPs, and 1 µl of each primer 10 pmols/µl. Samples were amplified in a PTC-100, MJ Research, Inc. Version 7.0 thermal cycler for 35 cycles at: 94ºC, 30 s; 58ºC, 30 s; 72ºC, 45 s. A first cycle of denaturation was carried out at 94ºC for 2 min, and a last cycle of extension at 72ºC for 2 min. Amplification products were all resolved eletrophoretically in 2% agarose gels.

*Gut content detection*: *Effect of digestion time*

*A. tomentosus* females that had eaten 1-5 *C. pyricola* nymphs were frozen immediately after feeding (t=0) or maintained for 2, 4, 6, 8, 16, 24 or 32 h at 22ºC until freezing. Four replicates of five individuals (n=20 females) were assayed for each pair of primers. Each replicate was tested three times in a different PCR reaction. The number of *A. tomentosus* in which amplification was shown was calculated for each replicate and the mean (± SE) was calculated for each period of time and expressed as a percentage. Starved females were added as negative controls.

*Sensitivity and species specificity*

The sensitivity was determined by assaying 10 samples containing DNA of 1 *C. pyricola* five-instar nymph in 100 μl of TE ($10^{-2}$) and serial dilutions of these extractions at a $10^{-3}$, $10^{-4}$, $10^{-5}$, $10^{-6}$, $10^{-7}$ and $10^{-8}$.

Species specificity was tested using DNA from *C. pyricola* eggs, nymphs and adults, three other species of psyllids (*C. pyri, A. uncatoides* and the "willow psylla", as well as other species of potentially important pests in the same crop, like the aphids *D. plantaginea* and *A. pomi*, the lepidopteran *C. pomonella*, the heteropteran *L. hesperus* and the acari *T. urticae*. The predators tested were the heteropteran *D. brevis*, *C. verbasci*, *A. tomensosus, A. antevolens* and *A. nemoralis*, the neuropteran *H. pacificus*, the coleopteran *H. axyridis*, and the spiders *P. clarus* and *X. cunctator*. The aphid *A. fabae* was also tested given that it was the prey consumed by the predators during rearing.

## Results

### COI markers of C. pyricola

Part of the COI gene (400 bp) was sequenced for *C. pyricola, C. pyri, A. uncatoides*, willow psylla, *C. verbasci, D. brevis, A. nemoralis, A. tomensosus, A. antevolens, A. fabae* and *C. pomonella*. The alignment of these fragments with the other prey and predator species allowed the design of twelve primers from the sequence of the pear psylla, which allow 25 possible combinations. In this study we used two primer combinations that allowed the amplification of 271 bp and 188 bp fragments, respectively.

### Gut content detection: Effect of digestion time

The minute pirate bug predator, *A. tomentosus* females that had eaten 1-5 *C. pyricola* five-instar nymphs and been frozen at t=0, 2, 4, 6, 8, 16, 24 and 32 h were assayed for the presence of *C. pyricola* DNA in their gut. Using primer pairs 1 and 2 (271 and 188 bp, respectively), prey DNA was detected in the gut of *A. tomentosus* females at all times tested (Fig. 1 and 2), but with different percentages. Detection of *C. pyricola* DNA was higher in all times using primer pair 2, indicating how the efficiency to detect prey in predator gut increased as the size of amplified fragment decreased (Fig. 3).

The mean (± SE) percentage of *A. tomentosus* scoring positive for *C. pyricola* DNA in their gut at t=0, 2, 4, 6 and 8 h was 100% using primer pair 1 and 2. For primer pair 1 the curve decreased up to 90% (±5.77) at t = 16h, 55% (±15) at t= 24h and 15% (±9.57) at t= 32h. With primer pair 2, the curve decreased up to 70% (±5.77) at t = 16h, 40% (±18.26) at t= 24h and 5% (±5) at t= 32h (Fig. 3).

### Sensitivity and species specificity

When primer pair 1 and 2 were tested to determine the sensitivity of detecting *C. pyricola* nymph extracts, the detection threshold was at a $10^{-5}$ dilution of a *C. pyricola* whole five-instar nymph for all replicates. Detection of *C. pyricola* DNA at a $10^{-6}$ dilution was found in 5 of the 10 extracts. $10^{-2}$, $10^{-3}$ and $10^{-4}$ dilutions yielded positive responses for all samples, while $10^{-7}$ and $10^{-8}$ dilutions were always negatives (Fig. 4).

When both pairs of primers were tested for species specificity against DNA from three other psyllas, three aphids, one lepidopteran, three arachnida and six other predator species, the 188 bp fragment was only obtained with species, which belong at the family Psyllidae. The 271 bp fragment seems to be a little bit more specific, detecting only genera *Cacopsylla*. Amplification patterns are shown in Fig. 5.

**Fig. 1.** Amplification pattern of COI mitochondrial DNA using the primers for 271 bp. Lane 1 molecular-size marker 100 bp, lane 2 *C. pyricola*; lane 3 starved *A. tomensosus*; lanes 4, 5, 6, 7, 8, 9, 10 and 11 *A. tomensosus* after eating 1-5 *C. pyricola* nymphs at t = 0, 2, 4, 6, 8, 16, 24 and 32 h. The specific COI markers are indicated by arrows.
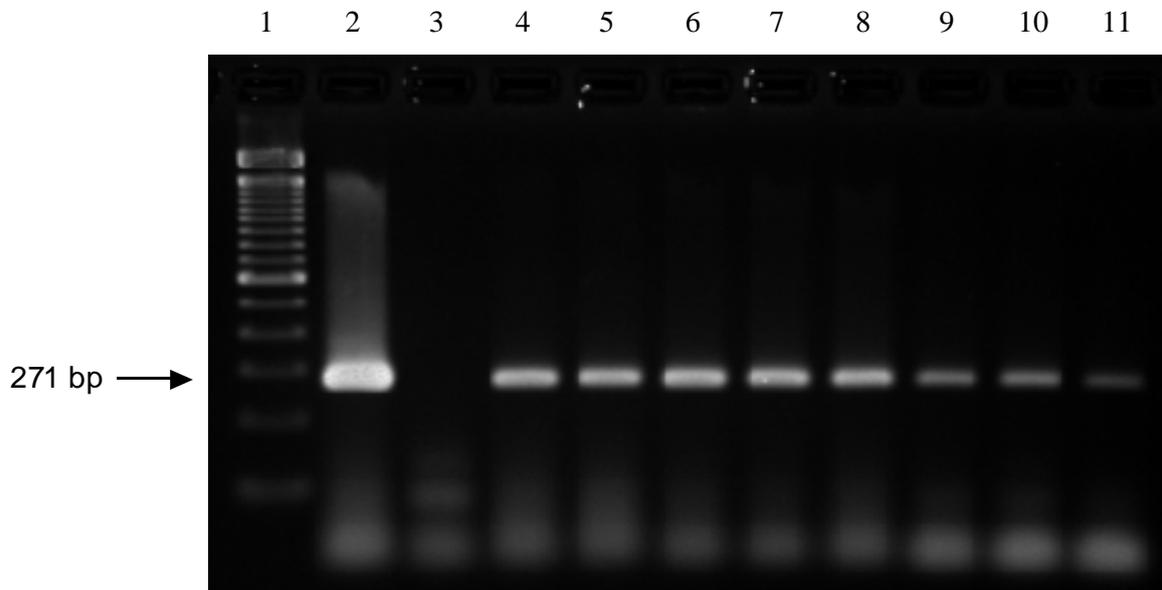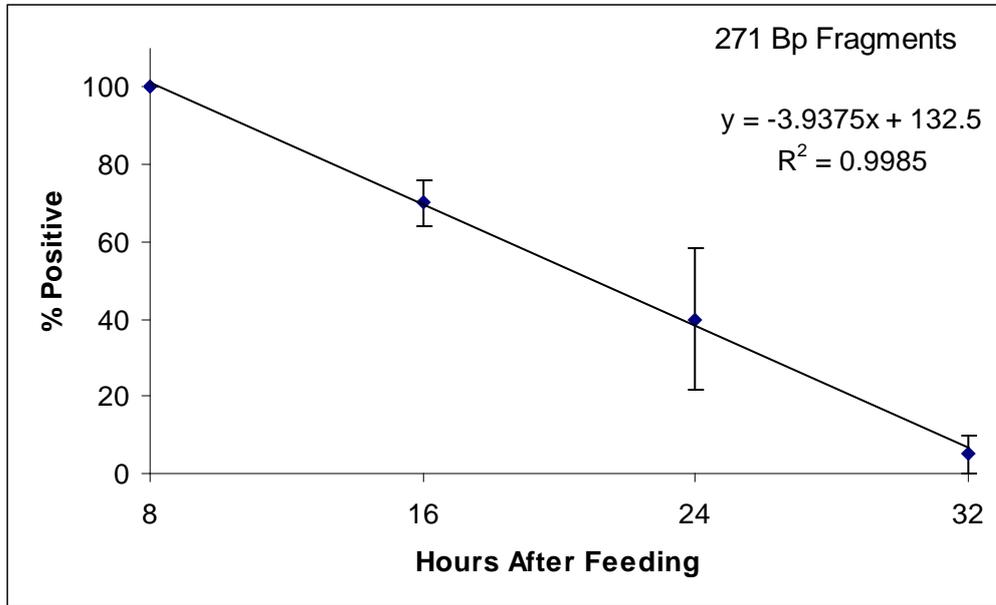


**Fig. 2** Amplification pattern of COI mitochondrial DNA using the primers for188 bp fragments. Lane 1 molecular-size marker 100 bp, lane 2 *C. pyricola*; lane 3 starved *A. tomensosus*; lanes 4, 5, 6, 7, 8, 9, 10 and 11 *A. tomensosus* after eating 1-5 *C. pyricola* nymphs at t = 0, 2, 4, 6, 8, 16, 24 and 32 h. The specific COI markers are indicated by arrows.
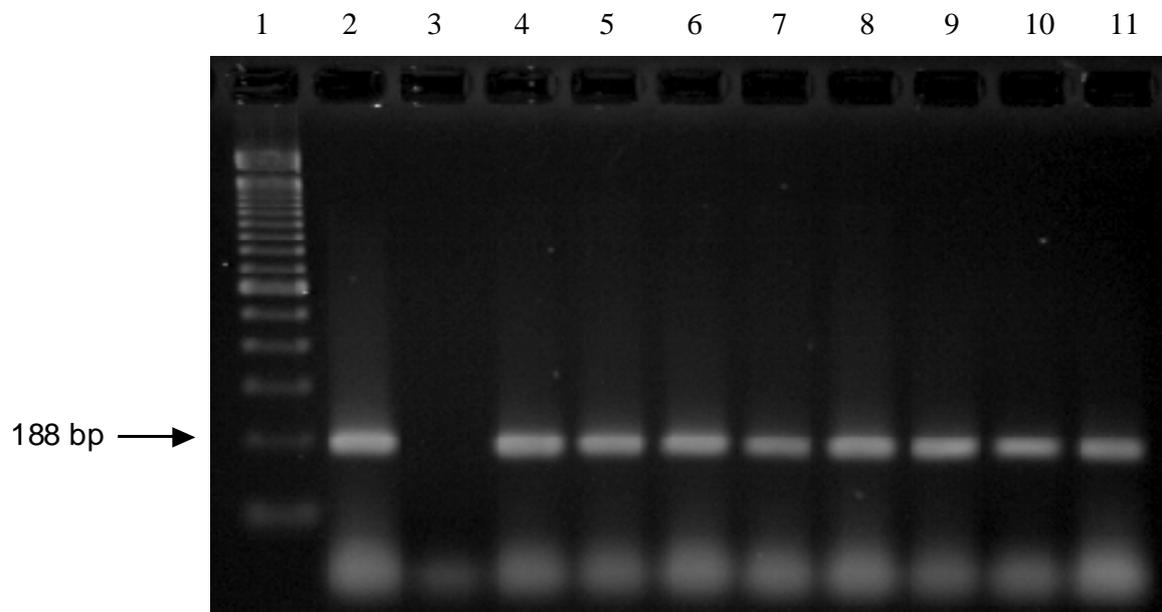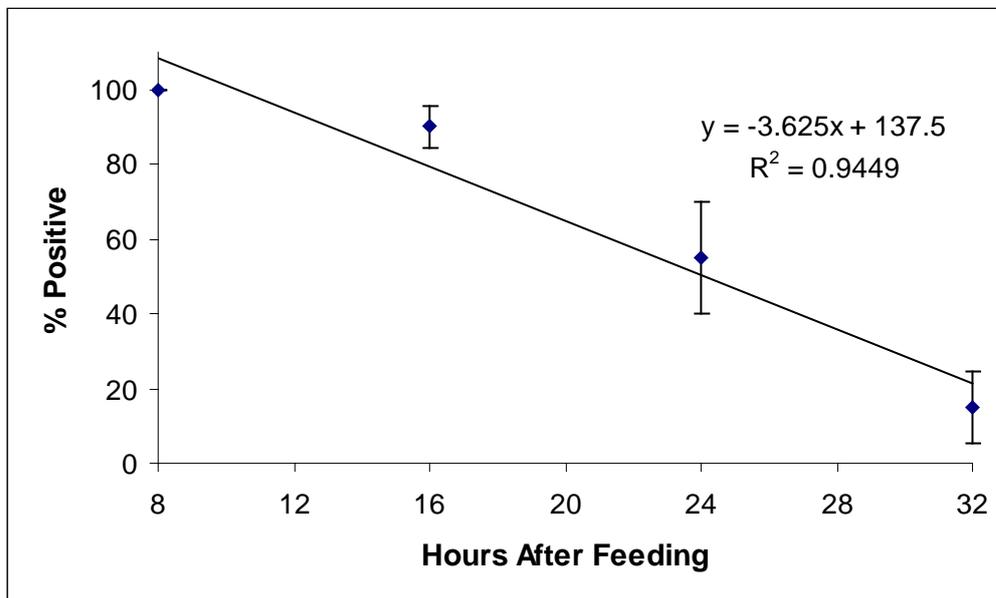
**Fig. 3** Means (±SE) (n = 20 in all cases) for detection percentages of *A. tomentosus* females scoring positive after eating 1-5 *C. pyricola* nymphs: (A) using primers for 271 bp fragments; (B) using primers for 188 bp. The best fitting model (higher R2 value) was linear in each case.

**Fig. 3A**



271 Bp Fragments

$y = -3.9375x + 132.5$
$R^2 = 0.9985$

% Positive

Hours After Feeding

**Fig 3B.**



$y = -3.625x + 137.5$
$R^2 = 0.9449$
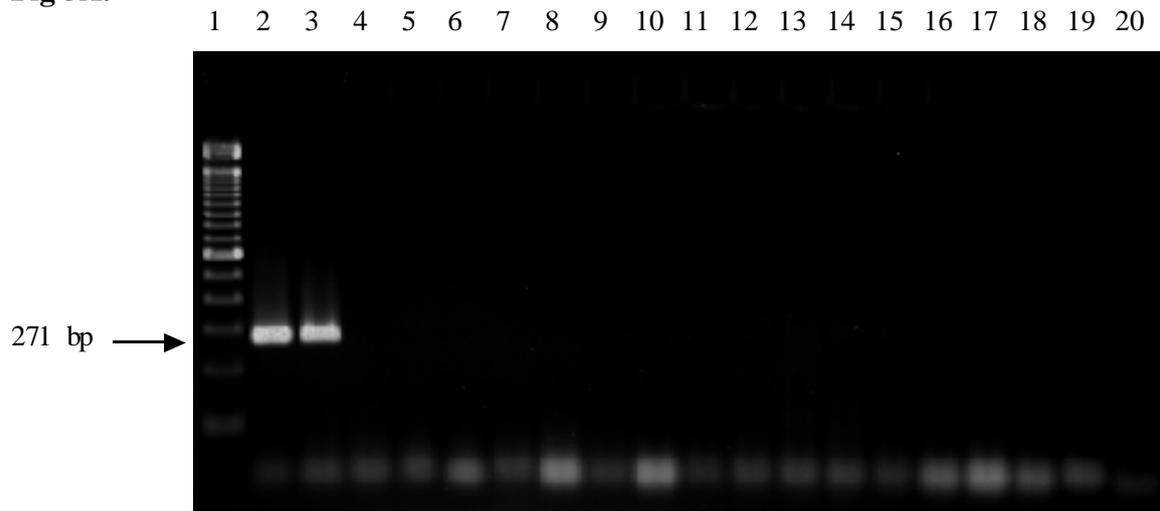
% Positive

Hours After Feeding

**Fig. 4** Amplification pattern of *C. pyricola* COI mitochondrial DNA using the pairs of primers for 188 bp and 271 bp. Lanes 1 and 9, molecular-size marker 100 bp; lanes 2 and 10, $10^{-2}$ dilution of a nymph; lanes 3 and 11, $10^{-3}$ dilution of a nymph; lanes 4 and 12, $10^{-4}$ dilution of a nymph; lanes 5 and 13, $10^{-5}$ dilution of a nymph; lanes 6 and 14, $10^{-6}$ dilution of a nymph; lanes 7 and 15, $10^{-7}$ dilution of a nymph; lanes 8 and 16, $10^{-8}$ dilution of a nymph. The specific COI markers are indicated by arrows.
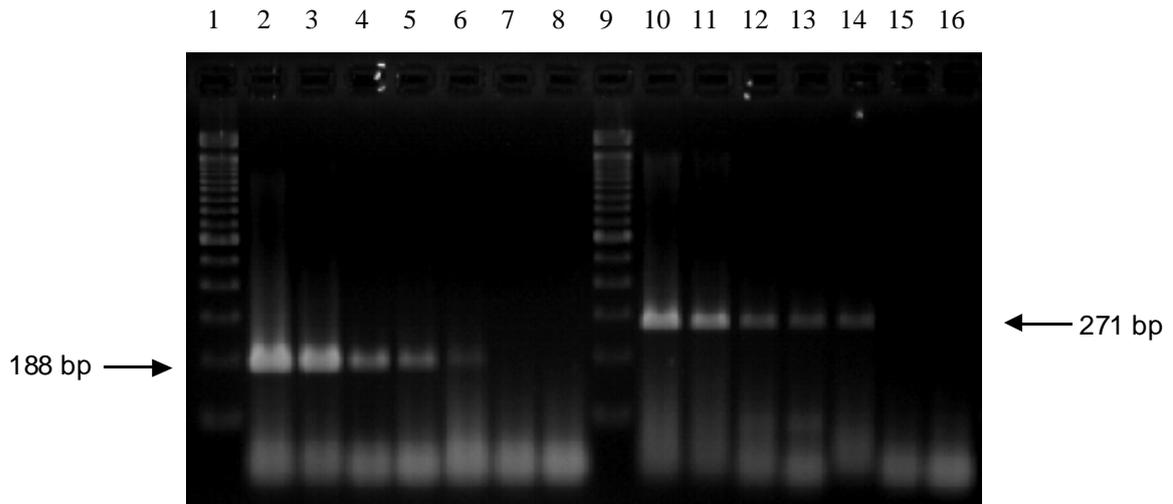
**Fig. 5** Agarose-gel eletrophoresis (2%) of PCR amplified DNA: A) using the COI specific primers for 271 bp; B) using the COI specific primers for 188 bp. Lane 1 molecular-size marker 100 bp, lane 2 *Cacopsylla pyricola*, lane 3 *Cacopsylla pyri*, lane 4 *Acizzia uncatoides*, lane 5 (willow psylla), lane 6 *Aphis fabae* (Black bean aphid), lane 7 *Dysaphis plantaginea* (Rosy apple aphid), lane 8 *Aphis pomi*, lane 9 *Campylomma verbasci*, lane 10 *Deraeocoris brevis*, lane 11 *Anthocoris nemoralis*, lane 12 *A. tomensosus*, lane 13 *A. antevolens*, lane 14 *Cydia pomonella*, lane 15 *Lygus hesperus*, lane 16 *Harmonia axyridis* (Coccinelidae), lane 17 *Phidippus clarus* (Salticidae), lane 18 *Xysticus cunctator* (Thomisidae), lane 19 *Tetranychus urticae*, lane 20 *Hemerobius pacificus*. The specific SCAR marker is indicated by an arrow.

**Fig 5A.**



**Fig 5B**