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18S rDNA PCR based detection of hymenopteran parasitoids in the Indian Lac insect, *Kerria lacca* [Kerr]

Thamilarasi Kandasamy*, Tamoshree Roy, A Mohanasundaram, KK Sharma & R Ramani

Lac Production Division, ICAR-Indian Institute of Natural Resins and Gums, Ranchi-834 010, Jharkhand, India

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The Indian lac insect, *Kerria lacca* (Kerr) is an economically important insect yielding resin, dye and wax. *Aprostocetus purpureus* and *Tachardiaephagus tachardiae* are the two most important parasitoids of lac insect. Timely detection of the parasitoids infection is essential for their control. Host dissection and caging of adult lac insects are the existing methods for detection of parasitoids in lac culture. Host dissection is laborious, whereas caging helps to detect the presence of only adult but not immature stages of parasitoids. Hence in this study, a PCR based approach has been developed for detection of these two important parasitoids in lac insects. Primers were designed from the clustalW alignment of 18S rDNA sequences of parasitoids and lac insects. Subsequently, specificity of the primers was checked by PCR *i.e.*, *A. purpureus* and *T. tachardiae* specific primers amplified only the respective parasitoid DNA but not lac insect and another parasitoid DNA. The PCR methods thus developed were also found to work with field collected infected lac insect samples and were able to differentiate infected lac insects from the uninfected ones.

Keywords: *Aprostocetus purpureus*, Lac resin, Parasitoids, *Tachardiaephagus tachardiae*

Lac, a bioresource, unique in several respects, is nature's boon to humankind. It is secreted by a type of scale insect called lac insect. Out of different species secreting lac resin, the most common and widely cultivated species of lac insect in India is *Kerria lacca* (Kerr 1782) (Hemiptera: Tachardiidae). *K. lacca* yields three economically important materials *viz*., resin, dye and wax. As the products are natural, renewable, nontoxic and ecofriendly, they have wide range of applications[1]. Lac products are used in various industries including surface coating, electrical, food, textile, pharmaceutical and cosmetics. Lac insect is grown only on a specific group of plants called lac host plants mainly cultivated in tropical countries. India is the largest producer of lac in the world, accounting for about 55-60% of the total world lac production[2]. Lac production in India during 2016-17 was 16352 tons[3].

Lac productivity depends on various biotic and abiotic factors impinging upon lac insect ecosystem. The important biotic factors which affect lac productivity are density of lac insect settlement, sex ratio, host plant, pests and diseases. Parasitoids and predators are the major biotic players causing 50% of losses in the lac crop[4,5]. Fourteen species of parasitoids under 13 genera and 10 families were found associated with lac insects[6]. However, only few of them are economically important in lac production point of view. They lay eggs into the lac cell through the anal tubercles in or on the body of the lac insect. The grub that hatches feeds only on lac insect and not on lac resin. Of the regularly occurring parasitoids, *Aprostocetus purpureus* (Hymenoptera: Eulophidae) was the most abundant followed by *Tachardiaephagus tachardiae* (Hymenoptera: Encyrtidae)[5]. They occurred in large numbers in all season lac crops[7]. *A. purpureus* and *T. tachardiae* constituted 55.82 and 28.37%, respectively of the total population of parasitoids of *K. lacca*[6]; they constituted 82.7 and 13.2%, respectively in *Kerria yunnanenis* culture[8]. Parasitoids especially *A. purpureus* was the cause of large scale pre-summer mortality in lac insects[9]. In recent years, 538% increase in *A. purpureus* was observed over the previous four decades[10]. *T. tachardiae* posed major problem during rainy season crop[11]. Variations in emergence profile of different parasitoids and predators in different lac crop revealed that *T. tachardiae* and *A. purpureus* were abundant[12]. *A. purpureus* and *T. tachardiae* constitute majority of the parasitoid population of lac insects and cause economic damage to lac crop.

*Correspondence:
E-mail: kthamilarasi@gmail.com

Timely detection of the parasitoid infection in lac cultivation is very much essential for their control. The conventional methods like caging and host dissection are followed regularly to detect parasitoid infection. Detection of parasitism is possible in caging only after the emergence of adult parasitoids. Caging of the field collected lac insect samples aids in detecting the presence of only live adult parasitoids but not immature stages or dead adult parasitoids inside the lac insect. Although host dissection is useful in detecting dead and immature stages of parasitoids, it is very laborious. Hence, an alternative method of detecting the parasitoids especially during early stage of parasitism is required.

Developments in molecular biology have provided a new direction in identification of organisms and to separate them from their closest relatives[13,14]. This has enormously helped in many useful studies; and some new species have also been discovered based on the differences in genome sequences. Few earlier studies took the advantage of sequence variations for detecting the parasitism in the host insect. Different genes such as COI[15-18], ITS1[19], ITS2[20-22] and 18S rRNA[23] have been used for this purpose. In the present study, we propose 18S rDNA PCR based method for specific detection of *Aprostocetus purpureus* and *Tachardiaephagus tachardiae* in the host lac insect.

Materials and Methods

Insect sample collection

Pure population of *K. lacca* was obtained from the National Lac Insect Germplasm Centre (NATLIGEC) at ICAR-Indian Institute of Natural Resins and Gums (IINRG) at Ranchi, India (Latitude $23^0$19'51.23"N, Longitude $85^0$22'06.86"E and altitude 634 MAMSL). The pure lac insect cultures were maintained on the lac host plant, *Flemingia macrophylla* grown in pots under protected conditions. The cultures were kept enclosed in synthetic mesh sleeves (60 mesh) to protect the insects from parasitoids and predators. The cultures were regularly sprayed with carbendazim (0.01%) to protect them from fungal infestation.

Lac insect parasitoids, *A. purpureus* and *T. tachardiae* were collected from their host (lac insect) cultivated at Institute Research Farm of IINRG, Ranchi, India (Latitude $23^0$19'51.23"N, Longitude $85^0$22'06.86"E and altitude 634 MAMSL). Samples of lac encrusted branches of host plants were collected two times per month. The lac encrusted branches weighing approximately 500 g were caged in wooden cages having holes to trap the emerging parasitoids. Transparent vials were fitted in the holes of the cages and placed in front of a light source for the parasitoids to emerge. The parasitoids emerged after 2-3 days of caging, and got collected in the transparent vials owing to their photo-tactic behavior. Different types of parasitoids emerged from the cages were collected and stored in absolute alcohol. These parasitoids were viewed under microscope and pure populations of *A. purpureus* and *T. tachardiae* were separated based on their morphological characteristics. Field collected samples of lac insects were obtained from places in and around Ranchi, India (GIS co-ordinates for the places of collection for samples 1 to 3 was Latitude $23^0$25'.746''N, Longitude $85^0$22'.478''E, altitude 397 MAMSL, for samples 4 to 6 was Latitude $23^0$19'51.23"N, Longitude $85^0$22'06.86"E and altitude 634 MAMSL and for samples 7 and 8 was Latitude $23^0$39'38.44"N, Longitude $85^0$57'10.49"E and altitude 1120 MAMSL).

Genomic DNA isolation

Mature female lac insects were kept in absolute ethanol for overnight at room temperature to remove the resinous covering and were serially washed with 70% ethanol to eliminate residual resin and other impurities. The cleaned insects were kept in absolute ethanol in 1.5 mL micro centrifuge tubes and stored at –20°C. Genomic DNA was isolated from 50 mg of lac insects and parasitoids adopting HiPurA™ insect DNA purification kit (HiMedia, MB529-50PR), with few minor modifications such as washing the column before DNA elution for 3-4 times to remove the pigments instead of 2 times and elution of DNA at 65°C instead of room temperature. The isolated DNA was eluted in 50-60 µL of elution buffer. The extracted DNA was individually quantified in Nanodrop 2000 spectrophotometer (Thermo Scientific) and was also checked on 1% agarose gel.

Polymerase chain reaction (PCR)

Total genomic DNA isolated from all the samples were amplified using the insect specific 18S rDNA primers, 18SFrontF (5' CTGGTTGATCCTGCCAG TAGT 3') and 18SBackR (5' TCCTTCCGCAGGTTC ACC 3')[20]. The PCR reactions were performed in a final volume of 50 µL using 0.5 µL of *Taq* DNA polymerase enzyme (Fermentas Inc, # EP0402), 5 µL of dNTP mixture (1.0 mM), 5 µL of 10X *Taq* Buffer (with $(NH_4)_2SO_4$ of Fermentas Inc), 4 µL of $MgCl_2$ (25 mM), 20 picomoles of forward and reverse

primers of 18S rRNA gene and 50 ng of genomic DNA. The thermal cycling reactions were carried out on a Gene-amplification PCR system (Sensoquest Lab Cycler) and the PCR profile was as follows: Initial denaturation was carried out at 94°C for 2 min for 1 cycle, followed by 35 cycles of 30 s at 94°C (denaturation), 30 s at 56°C (primer annealing) and 2 min at 72°C (extension); and final extension step of 10 min at 72°C. The obtained PCR products were sequenced using 18S FF and 18S BR primers at Chromous Biotech Pvt. Ltd., Bengaluru, India. The consensus sequences obtained were deposited in GenBank.

Specific primer designing for *A. purpureus* and *T. tachardiae*

The 18S rDNA sequences of lac insect and parasitoids were aligned using ClustalW alignment. *A. purpureus* and *T. tachardiae* specific primers were designed in such a way that the primer annealing positions were present only in the respective parasitoid and either absent or mutated in the host and another parasitoid. The specific primers for *A. purpureus* are A.p_18S rRNA_F_2 (5' CAATCG GTGGCGGGCTTGCT 3') and A.p_18S rRNA_R_2 (5' TGGACCGCCCCGAAAGGCTA 3'). The specific primers for *T. tachardiae* are T.t_18S rRNA_F_1 (5' ATCGGTGGCGGACTCCTC 3') and T.t_18S rRNA_R_1 (5' GCGAACGCACCGCGCG 3'). PCR reaction was performed in 25 µL sample volume. It contained 25 ng DNA template, 1X PCR buffer, 2 mM $MgCl_2$, 0.1 mM dNTPs, 1.25 U *Taq* DNA polymerase, 10 picomoles of each primer and distilled water. The PCR programme started with an initial denaturation at 94°C for 3 min, 35 amplification cycles of 94°C for 30 s, 61°C (*A. purpureus* specific primers) and 63°C (for *T. tachardiae* specific primers) for 30 s and 72°C for 30 s and final elongation step at 72°C for 10 min. Five microlitres of each PCR product was run on an 1% agarose gel containing ethidium bromide (0.5 µg/mL) for 45 min at 80 volts. Infected lac insect genomic DNA was also amplified with the same primers. PCR was carried out with the DNA isolated from lac insect, *A. purpureus* and *T. tachardiae* and field collected lac insect samples. For filed collected lac insect samples a control PCR with 28S rRNA primers of lac insects was also conducted. Primers for this control PCRs were 3HK 28SqF1 (5' TTGCGTAAC AAAACGGTTCA 3') and 3HK 28SqR1 (5' TCGGC AGATTCCTTCATCAT 3'). The PCR conditions followed was same as that for parasitoid PCRs except for the annealing temperature (60°C). For host dissection method, around 0.5 metre of lac insect encrustations of the field collected lac insect samples were analyzed under microscope to find out the parasitoid infection.

Sensitivity of PCR assays

To check the sensitivity of PCR assays, parasitoid DNA of 500 ng/µL was serially diluted in host DNA up to 10^4 times i.e., up to 0.05 ng/µL. The serially diluted DNA was used for PCR with parasitoid specific primers.

Results

Designing of parasitoid specific primers and PCR

18S rDNA of *K. lacca* and their parasitoids, *A. purpureus* and *T. tachardiae* were amplified using insect specific 18S rDNA primers, 18SFrontF and 18SbackR. The sequences were validated as 18S rDNA sequences by performing BLAST analysis. The length of 18S rDNA of *A. purpureus* and *T. tachardiae* was around 2 Kb and that of *K. lacca* was around 2.5 Kb [Suppl. Figs 1 & 2. Available only

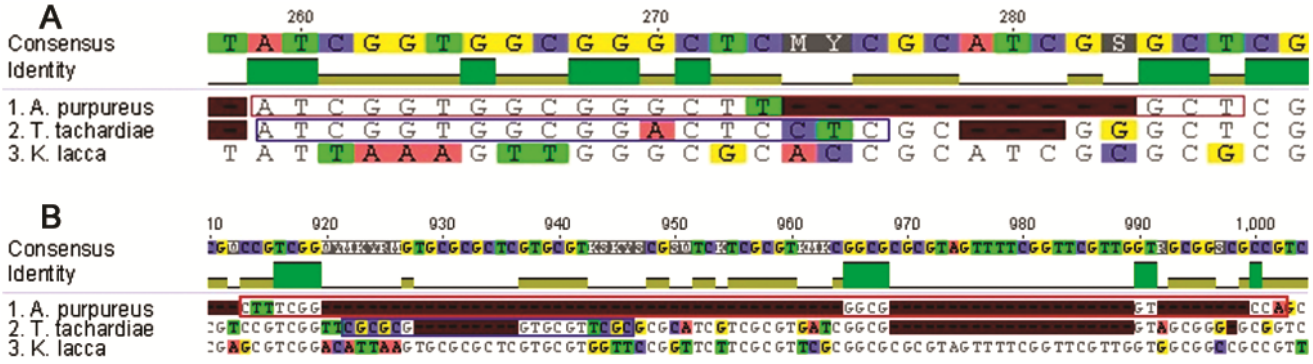

Fig. 1 — Clustal W alignment showing host and parasitoid 18S rDNA sequences of both (A) forward; and (B) reverse primers. Sequence in red colour box shows *A. purpureus* specific forward (A)/reverse (B) primer; and sequence in blue colour box shows *T. tachardiae* specific forward (A)/reverse (B) primer

online at repository (http://nopr.niscair.res.in) along with respective paper]. The sequences were submitted to GenBank and the accession numbers are JQ359003, JQ359004 and KC577447 for *A. purpureus*, *T. tachardiae* and *K. lacca* 18S rRNA sequences, respectively.

Host as well as parasitoid 18S rDNA sequences were aligned using ClustalW and the parasitoid specific regions were obtained. *A. purpureus* and *T. tachardiae* specific primers were designed from those parasitoid specific regions, respectively (Fig. 1 A & B). One of the primer pairs specific for *A. purpureus*, A.p_18S rRNA_F_2X A.p_18S rRNA_R_2 gave a PCR product of 528 bp with *A. purpureus* DNA only (Fig. 2A). The primers did not cross react with host *K. lacca* DNA and also with another related parasitoid *T. tachardiae.* On the other hand, *T. tachardiae* specific primer pair T.t_18S rRNA_F_1XT.t_18S rRNA_R_1 amplified only *T. tachardiae* DNA of 548 bp length but not *K. lacca* and *A. purpureus* DNA (Fig. 2B).

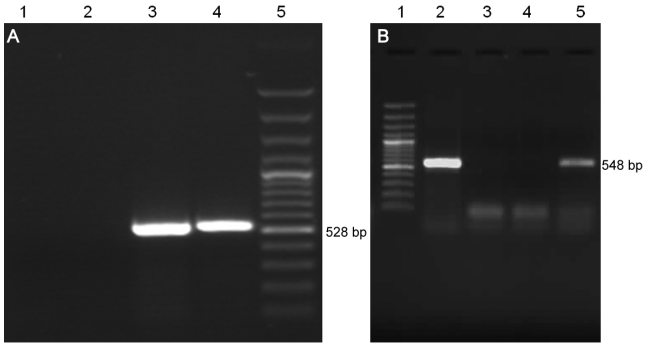

Fig. 2 — PCR with (A) *A. purpureus*; and (B) *T. tachardiae* specific primers. Panel (A): Lane 1, PCR with *T. tachardiae* DNA; lane 2, PCR with *K. lacca* DNA; lane 3, PCR with *A. purpureus* DNA; lane 4, PCR with field collected infected sample; and lane 5, 100 bp ladder; and Panel (B): Lane 1, 100 bp ladder; lane 2, PCR with *T. tachardiae* DNA; lane 3, PCR with *K. lacca* DNA; lane 4, PCR with *A. purpureus* DNA; and lane 5, PCR with field collected infected sample.

Sensitivity of PCR assays

To study the sensitivity of PCR assays parasitoid genomic DNA was serially diluted in *K. lacca* DNA and subject to PCR. *A. purpureus* specific primers could amplify *A. purpureus* DNA up to 1: 10000 dilutions i.e., from 500 ng/µL DNA up to 0.05 ng/µL DNA. Similar experiment with *T. tachardiae* specific primers yielded PCR product up to 1: 1000 dilutions i.e., from 500 ng/µL DNA up to 0.5 ng/µL DNA (Fig. 3).

Analysis of field collected lac insect samples

Subsequently genomic DNA of eight field collected lac insect samples were tested with parasitoid specific primers. Out of them, two samples were found to be infected with *A. purpureus* and one was infected with *T. tachardiae* (Fig. 4 B & C). Previously the same DNA samples were amplified with primers of a lac insect housekeeping gene, 28S rRNA to ensure that absence of amplification with the parasitoid specific primers was due to the absence of parasitoids and not because of failure of PCR reaction (Fig. 4A). Infection of the parasitoids in those samples was also confirmed by host dissection method by observing them under microscope.

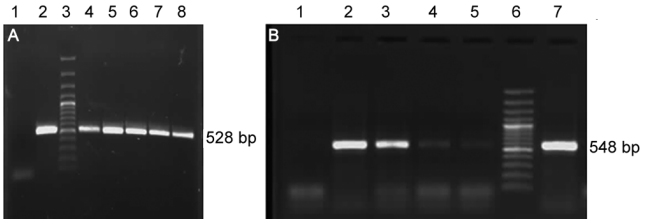

Fig. 3 — Sensitivity of (A) *A. purpureus*; and (B) *T. tachardiae* specific PCR. Panel (A): Lane 1, PCR with *K. lacca* DNA; lane 2, PCR with *A. purpureus* DNA; lane 3, 100 bp ladder; and lanes 4-8, *A. purpureus* DNA serially diluted in *K. lacca* DNA (1:1, 1:10, 1:100, 1:1,000 and 1:10,000); and Panel (B): Lane 1, PCR with *K. lacca* DNA; lanes 2-5, *T. tachardiae* DNA serially diluted in *K. lacca* DNA from 1,1 to 1,1,000; lane 6, 100 bp ladder; and lane 7, PCR with *T. tachardiae* DNA

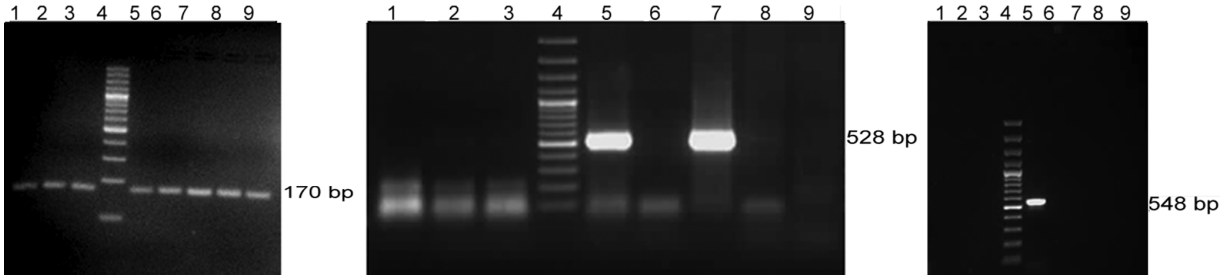

Fig. 4 — PCR of field collected lac insect samples with lac insect and parasitoid specific primers. (A): PCR with lac insect 28S rRNA primers; (B): PCR with *A. purpureus* specific primers; and (C): PCR with *T. tachardiae* specific primers. [Lanes 1-3: field collected samples no. 1-3; lane 4: 100 bp ladder and lanes 5-9: field collected samples no. 4-8]

Discussion

A PCR based robust molecular method has been successfully developed for detecting key parasitoids of lac insect for the first time. Conventionally detection and identification of the parasitization was done by host rearing and host dissection. Host rearing is a time consuming method[13,15] and there are chances of non-emergence of adult parasitoids from parasitized hosts[17]. Host dissection is a tedious method and cannot be performed in the early instar host larva owing to its smaller size. Identification of parasitoid larvae is highly difficult or impossible in host dissection[13]. Hence molecular method using either protein or DNA could be a better rescue[22]. Isozyme electrophoresis is one of the protein based methods for identifying parasitization. However, the method often lacks sensitivity[23] and cannot discriminate among related parasitoids[24]. On the other hand, DNA based methods are rapid, sensitive and specific. Hence a relatively easier and time saving DNA based molecular method has been attempted in this study.

We selected a house keeping gene, 18S rRNA which is present in high copy numbers in the insects. 18S rRNA is one of the highly preferred genes to be used for this purpose. It is used as a molecular marker in phylogenetic studies of higher groups of insects[25]. 18S rRNA is highly conserved rRNA gene and the probability of getting intraspecific and intra individual variation is less compared to other insect rRNA genes. Early detection of parasitization and detection of immature stages of parasitoids are made possible by this method. Weathersbee *et al*.[21] used species specific 18S rDNA PCR to precisely detect and differentiate hymenopteran parasitoids of brown citrus aphid even at earlier point of parasitization.

The present study is found to be very specific and sensitive to identify the two important key parasitoids of lac insect. The sensitivity test or dilution experiment is necessary to implicate how early parasitoids could be detected after the parasitoid has deposited the egg into the host insect[26]. To mimic the condition of the parasitized host, serial dilutions of parasitoid DNA was done with host DNA and PCR was performed to find out the quantity of parasitoid DNA required to generate a visible band on agarose gel. *A. purpureus* and *T. tachardiae* specific primers were able to detect the parasitoids up to 1:10000 and 1:1000 dilutions, respectively. The sensitivity is ten-fold less in *T. tachardiae* detection compared to *A. purpureus* detection. The sensitivity was only 10^{-3} adult wasp equivalent[18]. However, the ITS2 based PCR technique developed by Zhu *et al*.[27] (2004) was very sensitive and could detect $7.5X10^{-7}$ wasp DNA equivalents in *Lygus* bugs. Traugott *et al*.[26] (2006) reported that their COI based detection assays were able to detect *Cotesia glomerata* and *Cotesia rubecula* parasitoid up to the level of 0.05 and 0.03 pg/μL PCR mixture, respectively.

18S rDNA PCR is a simple foolproof technique to precisely detect key parasitoids of lac insect. It does not require knowledge on morphology of the parasitoids and the microscopic skill for host dissection. The time taken for parasitoid specific PCR may be slightly longer than the dissection method whereas time consumption is less than caging (equivalent to host rearing) method. Future scope of this study includes developing a PCR based diagnostic kit for the detection of key parasitoids in lac insect culture.

Conflict of Interest

The authors declare that there is no conflict of interests.

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