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First record of Bondar's nesting whitefly, *Paraleyrodes bondari* Peracchi (Hemiptera: Aleyrodidae), occurrence and infestation in the cotton ecosystem of Tamil Nadu, India

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Abstract
The first report of Bondar's Nesting Whitefly, *Paraleyrodes bondari*, in the Cotton environment of Tamil Nadu, India, is of its emergence and infestation. Eggs (7.7±0.16 days), I nymphal instars (3.5±0.44 days), II and III nymphal instars (7.4±0.20 days), IV instars (4.5±0.21 days), and adults (16.7±0.38 days) were the life stages recorded from egg to adult. The entire life cycle ranges is retraced. To confirm the pest, morphological and molecular taxonomic characterizations were performed. An "X"-marking on the adult forewing, as well as a little powdery covering across the wings and body. The flower-petal shaped ovoid cells on abdomen compound pores confirmed the immature stage of taxonomic character. A 658 bp COI sequence amplified and deposited in the NCBI was used for molecular confirmation at Barcode Biosciences Pvt. Ltd., Bengaluru.

Keywords: Bondar's nesting whitefly, invasive pests, paraleyrodes bondari, DNA barcoding, cotton

1. Introduction
Cotton (*Gossypium* spp., Family: Malvaceae) is one of the most important cash crops in the world and is cultivated in more than 75 countries around the world [8]. It has been reported in countries such as Uganda [16], Florida [1], Comorus [19], Madeira [12], Mauritius, Reunion, Puerto Rico, Hawaii and Brazil [12]. It has also been reported in India [14], and the Andaman and Nicobar Islands [22]. India, the largest producer of cotton in the world, produced 18,550 thousand metric tons from an area of 16 million ha with an average yield of about 1.16 metric tons [2]. The crop is more prone to insect pests, plant pathogens, abiotic stresses and semi-tolerant to salinity stress [7]. Whiteflies (Aleyrodidae) are one of the most serious pests of cotton in the world. Both nymphs and adults of the whitefly suck the sap from the undersurface of the leaves and also transmit the viral diseases of cotton [4, 17] and the sweet potato whitefly, *Bemisia tabaci* Gennadius, which infest cotton plants on a regular basis. The Bondar's nesting whitefly (BNW), *Paraleyrodes bondari* is one of the recent invasive species that was introduced into India during 2018 and was recorded as an insect pest on coconut (*Cocos nucifera* L.) trees in [9]. From the time of its introduction, the BNW has been known to also infest other crops such as citrus (*Citrus limon*), guava (*Psidium guajava*), cassava (*Manihot esculenta*), custard apple (*Annona reticulata*), jackfruit (*Artocarpus heterophyllus*), bell pepper (*Capsicum annuum*), cinnamon tree (*Cinnamomum verum*), subabul (*Leucaena leucocephala*), Indian mulberry (*Morinda citrifolia*), mango (*Mangifera indica*), teak (*Tectona grandis*) and banana (*Musa paradisiaca*) that are being cultivated in India [22].
The BNW adults are smaller when compared with the spiraling whitefly, *Aleurodicus dispersus*. The eggs of BNW are laid and covered with waxy threads. Thus, it attains the appearance of eggs being mixed into larger amounts of woolly wax glassy threads and adult females usually lie in a circular nest. These features give the name Bondar's nesting whitefly [6]. Recently, the incidence of invasive whitefly, *Paraleyrodes bondari* has been reported in different parts of the world. The occurrence, diversity, and how this is impacted by host and variety variability, geographical dispersion, and environmental variability of whiteflies in India are unknown. Because this is a relatively new whitefly species in the country, this research will fill in the gaps in knowledge about the most vulnerable types, crop ages, cropping systems, and how widespread the whitefly is in the area, as well as the most impacted places in the country.

The regular survey conducted to monitor the insect pest incidence on the cotton crop during 2019-2021 has revealed the incidence of such whiteflies in a complex manner on cotton fields of Tamil Nadu, India. In particular, the nested presence of whiteflies on the undersurface of cotton leaves was noticed. Such infestations were taken up for taxonomical identification and their damage potential to cotton.

2. Materials and Methods

2.1 Survey

The extensive field survey was conducted in different cotton growing ecosystems of Tamil Nadu, at Coimbatore (11.020127° N; 76.928993° E), Salem (11.823917° N; 77.662261° E), Erode (11.1592° N; 77.07269° E), Perambalur (11.1623° N; 78.91571° E) and Tiruchirappalli districts (11.7524° N; 78.73683° E) during the months of December 2020 to April 2021. The cotton crops that were aged between 45 and 95 days were subjected to scrutiny for the presence of insect infestation. An average of 10 random observations on 3 leaves representing the top, middle and bottom of the canopy in an area were taken. The whiteflies in a nested fashion were observed and were carefully collected, packed and stored for transport to the laboratory for further analysis. Simultaneously, the presence of *P. bondari* on different host plants that are associated with and nearest to the cotton ecosystem was also observed during this survey.

2.2 Morphological analysis

Morphological identification was done by mounted slides and was prepared as per the procedure of Hodges and Evans (2005) for *P. bondari* using a pseudo-pupal stage and examined under a Stereozoom Microscope (M205 C) fitted with a camera Leica DMC 2900 and a Phase Contrast Microscope (LEICA DM750) fitted with a camera LEICA DFC295. The microscopic images were compared with the standard key characteristics given by Martin (2004).

2.3 Molecular analysis

DNA was isolated from the BNW adult populations collected from cotton at different locations in Tamil Nadu, which was preserved in 70% ethanol. From those preserved or fresh adult samples, DNA extraction was done using the Hot SHOT DNA extraction protocol [15]. Briefly, the adult BNW specimens were subjected to the prime step of washing with MilliQ water and then dried on tissue paper. After that, they were transferred into a 0.2 ml Eppendorf® tube, which had 20 µl of alkaline lysis buffer. The specimens were crushed opposed to the side of the Eppendorf® tube and these crushed samples were incubated for 30 minutes under 95 ° C in water and kept at 4 ° C for 3-4 minutes. After that, 20 µl of neutralizing buffer was added to the lysate in the Eppendorf®tube. Prior to *PCR*, these samples were vortexed briefly and spun down and finally kept at 4ºC or otherwise, samples were kept at-20ºC for long term storage. The absorbance at 260 nm in a Nanodrop Spectrophotometer (ND-1000) was measured for the DNA extracted and, if DNA dilutions were required, they were made using 1X TE buffer to a final DNA concentration of 50 ng/µl for use in Polymerase Chain Reaction (PCR) mixures.

A fragment (658bp) of the mitochondrial cytochrome c oxidase subunit I (*mtCOI*) gene was amplified using the universal primers amplified using (forward primer LCO 1490 - 5′ GGTCAACAAATCATAAAGATATTGG 3′ and reverse primer HCO 2198 - 5′ TAAACTTCAGGGTGACCAAAAAATCA 3′) [3]. The PCR was performed in 25µl volume in a thermocycler (Bio- Rad DNA Engine) and the PCR conditions for amplification were: initial denaturation at 95º C for 4 minutes, followed by 35 cycles each consisting of denaturation for 1 minute at 92º C, annealing for 1 minute at 54º C with extension for 1.5 minute at 72°C followed by final extension for 10 minutes at 72°C. The amplified products were separated in a gel electrophoresis unit in TBE buffer and visualized under a UV transilluminator. The size of individual DNA fragments was compared with a co-migrating 100 bp DNA ladder. The 20 µl of unpurified amplified PCR product was sent for sequencing at Barcode Biosciences Pvt. Ltd, Bengaluru, India and sequenced through single pass DNA analysis in both forward and reverse directions with forward and reverse primers (5µl for each sample).

The BNW mtCOI sequences received were annotated and manually edited using the DNA MAN programme and then submitted to GenBank (Id: MZ604316). The resultant sequences were compared with the reference sequence submitted to the GENBANK, National Centre for Biotechnological Information (NCBI), Bethesda, USA to determine the similarity between the sequences. The Phylogenetic analysis included all available mtCOI sequences from GenBank and sequences from this study. Out-groups were included in each analysis. Finally, the sequences were edited using Bioedit Sequence Alignment Editor Version 7.0.5.3, followed by Clustal W Multiple alignment [21] using MEGA-X and the dendrogram was constructed using the Neighbour-joining method with the bootstrap analysis of 1000 replicates in MEGA-X software.

2.4 Target sequence retrieval

The protein sequence of cytochrome c oxidase subunit I of *P. bondari* was retrieved from NCBI with accession number QXT61001. This protein sequence was taken as a target for modeling using Homology modelling approach.

2.5 Modelling and validation

The retrieved protein sequence was subjected to homology modelling using SWISS-MODEL [23], an automated protein structure Homology modelling server. The predicted model for the protein target was validated using SAVES v6.0-Structure validation server (https://saves.mbi.ucla.edu/). The Ramachandran plot for the backbone conformation of each residue in the protein model was generated using PROCHECK [11]. The stereo-chemical quality of the modeled protein was verified from the Ramachandran plot.

3. Results and Discussion

The survey results showed that the population of nesting whiteflies, *Paraleyrodes bondari* was recorded from 5.3 to 6.9 numbers per three leaves. The *Bemisa tabaci* population was recorded from 7.1 to 8.9 numbers per three leaves (Table 1).

Table 1: Occurrence of *P. bondari* and *B. tabaci* in different cotton ecosystem of Tamil Nadu

S. No	Location	Geo coordinates		Whiteflies (No. of adults / 3 leaves)	
		Latitude	Longitude	*P. bondari*	*B. tabaci*
1	Lakkampatti, Salem	11.8239° N	77.66226° E	6.4	7.6
2	Alamarathupatti, Salem	11.8496° N	77.70936° E	6.9	7.3
3	Melakudikkadu, Perambalur	11.1623° N	78.91571° E	6.8	8.5
4	Palaiyur, Trichirappalli	11.7524° N	78.73683° E	5.8	7.1
5	Vazhaiyur, Trichirappalli	11.0610° N	78.75897° E	5.3	7.4
6	PN,Pudur, Coimbatore	11.2433°N	76.92673° E	6.9	8.9
7	TNAU, Coimbatore	11.0201° N	76.92899° E	6.7	8.7
8	Shenbagapudur, Erode	11.4411° N	77.20809° E	6.1	7.9
9	Periyakodiveri, Erode	11.5040° N	77.29821° E	6.5	7.5
10	Kittampatti, Erode	11.7097° N	77.68514° E	6.8	7.8
11	Illippili, Erode	11.6651° N	77.65139° E	6.4	8.8
12	Kurumpalayam, Erode	11.1592° N	77.07269° E	6.2	8.7

3.1 Taxonomical identification

Paraleyrodes bondari puparia were used to make the slide mounts. When compared to other Aleyrodidae, the puparia have around 5-6 compound pores, with one or two pairs in the anterior region being smaller than the remaining four abdominal pairs and the cephalic pair, the thorax has about 5-6 compound pores (Fig. 1a-b). On segments III to VIII, there are six pairs of abdominal compound pores. The two pairs in front are smaller than the four pairs in back. The microscopic characteristics of nymphal instars identified were flower-petal shaped ovoid cells on abdominal compound pores (Fig. 1c-d).

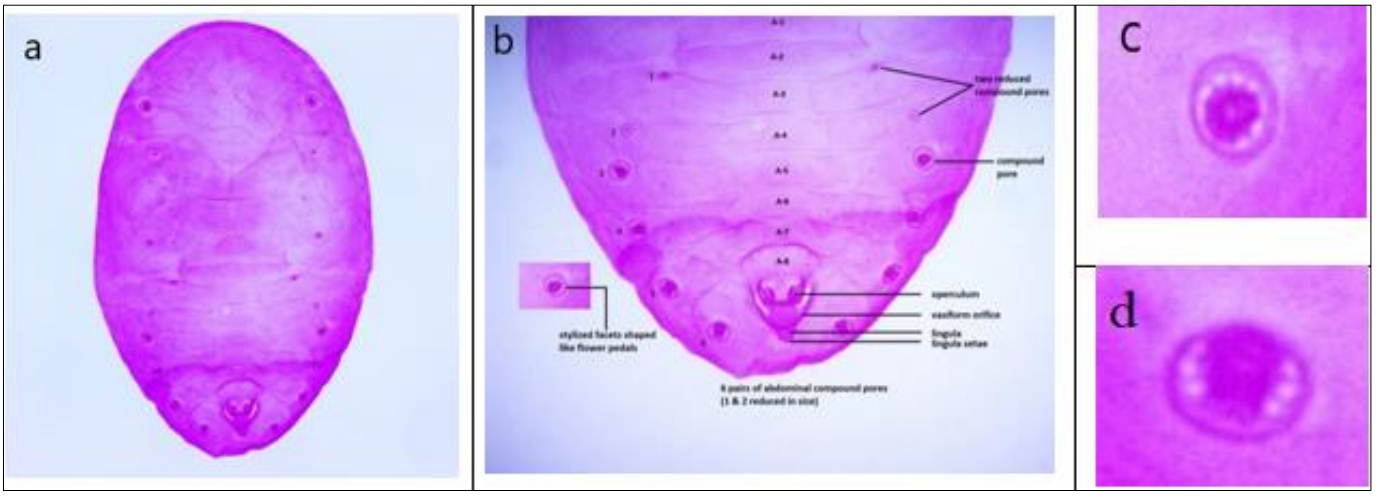

Fig 1: a-d. a: general features of the puparium b: features of posterior part of puparium c-d: a large compound pores with flower- petal shaped ovoid facets.

3.2 Molecular confirmation

The nucleotide sequences received from Barcode Biosciences Pvt. Ltd, Bengaluru, Karnataka with a 658 bp size were used for homology search using the BLAST algorithm against the NCBI nucleotide sequence data library. The investigated barcode sequences indicated a 99% match to the previously deposited *P. bondari* specific *COI* gene sequence. It was also found that the DNA barcode developed in this work had 100% identity with a DNA barcode established for the same species taken from coconut that was reported for the first time in India (NCBI Accession No. MK343480) and with the DNA barcode developed for a population sampled in Florida, USA (NCBI Accession No. KP032215). Thus, it has been unequivocally identified that the pest found during the survey was BNW (*P. bondari*) and the DNA barcode sequence obtained from this study was submitted to GenBank **(**NCBI Accession No. MZ604316).

3.3 Molecular Phylogenetic analysis by Maximum Likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei 2004 model. The tree with the highest log likelihood (-1737.69) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 15 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 548 positions in the final dataset (Fig. 2). Evolutionary analyses were conducted in MEGA7 [10].

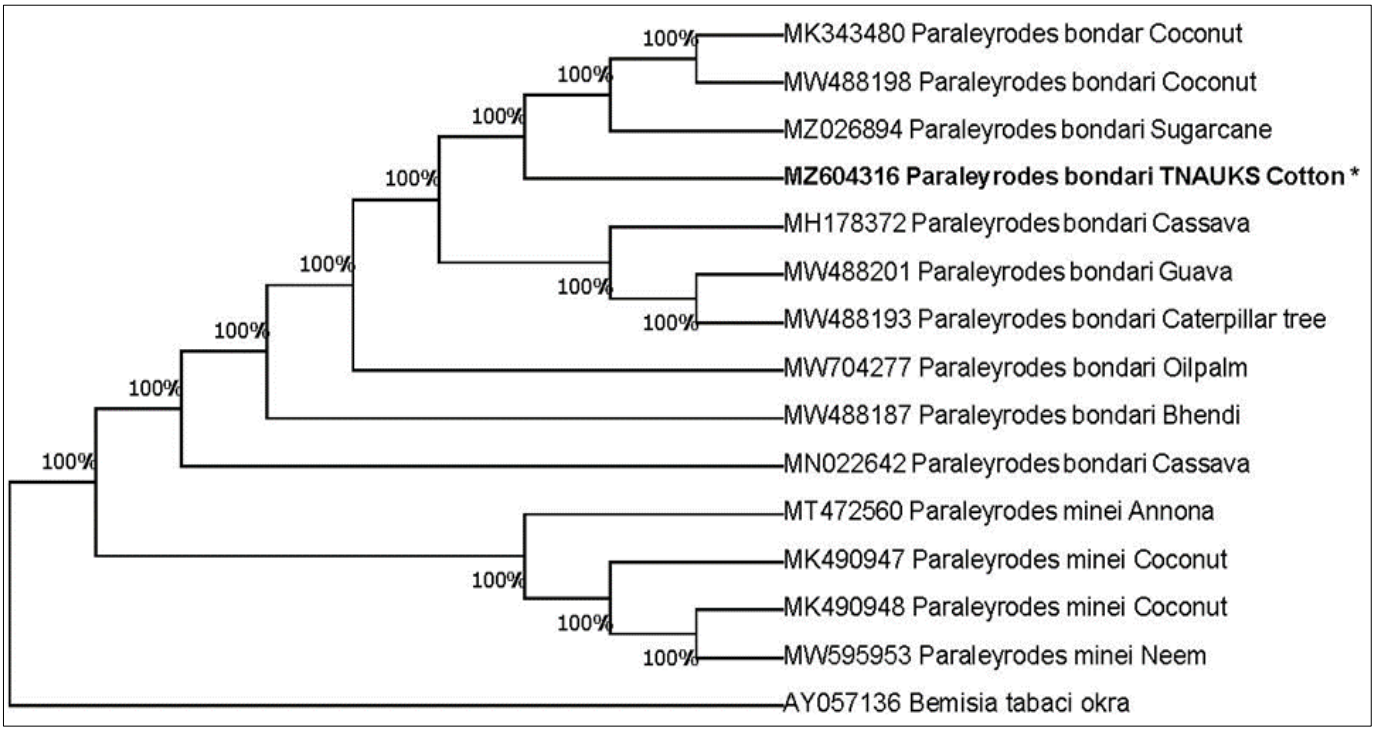

Fig 2: Molecular Phylogenetic analysis by Maximum Likelihood method

3.4 Homology modelling and structure validation

The three-dimensional structure of the target protein of *P. bondari* was developed using SWISS-MODEL server. The Ramachandran plot for the predicted protein model was analysed and given in (Fig. 3a-b). The report of Ramachandran plot showed that 91.3% of residues present in the allowed region of the plot (Table 2), which indicates high quality of geometry of the modelled structure.

Table 2: Statistics report of Ramachandran plot

S. No.	Protein target	Ramachandran Plot Statistics			
		Residues in most favoured regions (%)	Residues in additional allowed regions (%)	Residues in generously allowed regions (%)	Residues in disallowed regions (%)
1.	*P. bondari*	91.3	8.2	0.5	0.0

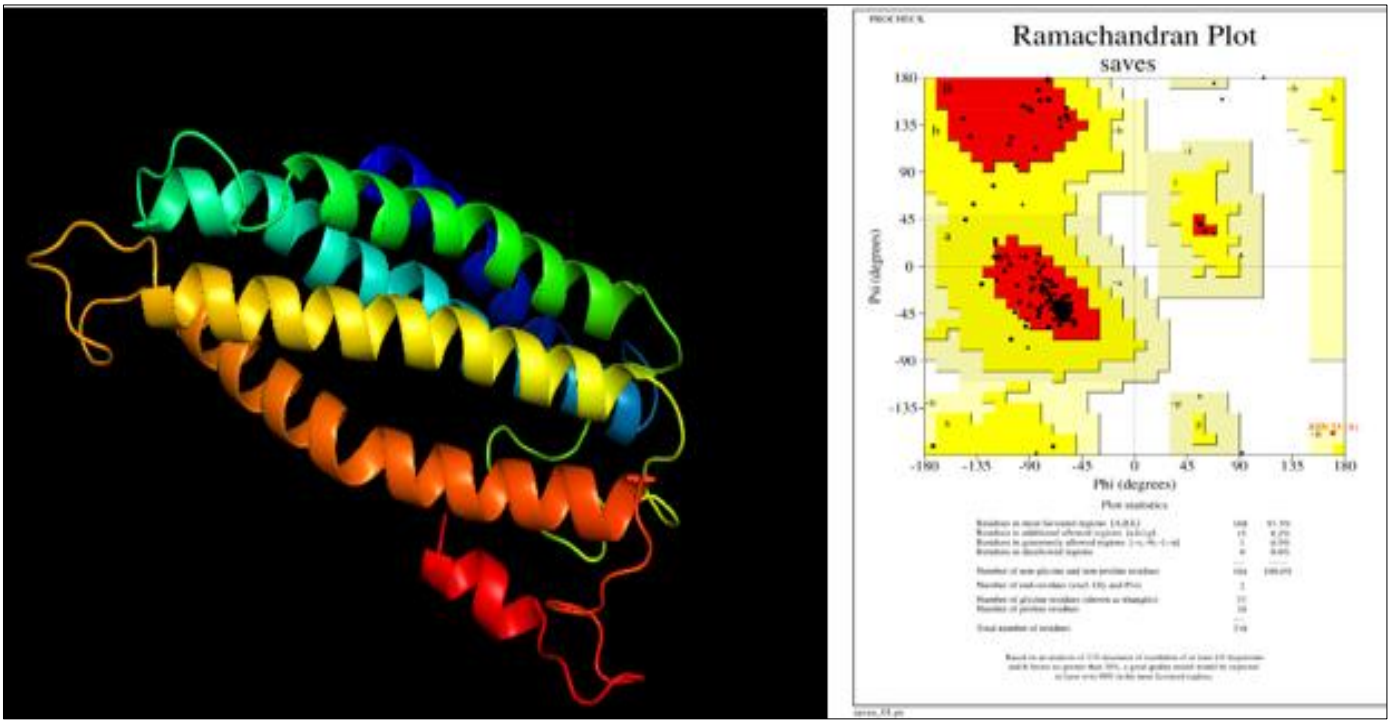

Fig 3: a-b. a: three-dimensional structure of the target protein of *P. bondari* b: protein model

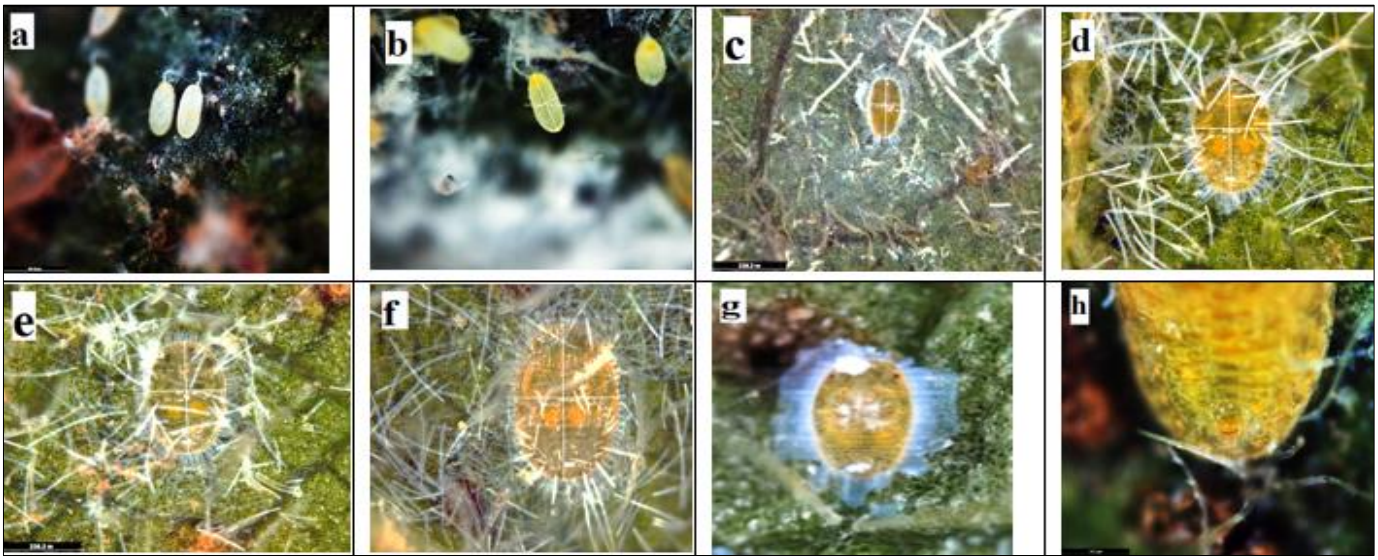

Fig 4: a-h. Developmental stages of P.bondari: a: eggs are small yellowish grape shaped with short pedicel b: length and breadth of egg c-f: various stages of nymphal instars (I- IV) g: nymphs possess a "skirt" surrounded by puparium h: well developed lingula and vasiform orifice.

3.5 Life stages

The BNW egg was a small yellowish grape shape with a short pedicel on the ventral surfaces of the leaves (Fig. 4a-b). Generally, four nymphal instars are associated with whitefly. The measurements like body length and breadth were obtained for various nymphal stages, eggs and adults (Fig. 4c – f and Table 3). Nymphs are translucent pale yellow in color and also secrete short wax filamentous threads from well-developed compound pores of the puparium, as described by Martin (1996). The lateral view of the nymphs possesses a "skirt" surrounded by a puparium with an interrupted row of short wax (Fig. 3g). The most peculiar character of the BNW puparium was its long, shiny wax threads and well-developed lingula and vasiform orifice (Fig. 3h). BNW is distinguished by an "X"-shaped marking on the adult forewing, as well as a light powdery coating across the wings and body regions (Fig. 5a-b). It has also been observed that BNW constructs irregular waxy colonies on the ventral surface of the leaves and constructs a nest which resembles a bird's nest (Fig. 5c). *P. bondari* constructed waxy colonies on the under surface of the leaves (Fig. 5d).

Fig 5: a-d. a: "X"- marking on adult forewing b: newly emerged bondar's adult without "X" – marking on forewing c: construct nest which resembles bird's nest d: colonies on the under surface of the leaves with male and female adult

Table 3: Different life stages of *P. bondari* with body measurements (Mean±SD)

Life stages	Body measurements	
	Length (µm)	Breadth (µm)
Eggs	263.8±10.3	114.4±0.7
1st nymphal instar/Crawler	344.5±9.8	167.0±4.5
2nd nymphal instar	683.9±9.6	440.2±5.9
3rd nymphal instar	708.8±1.0	457.4±9.1
4th nymphal instar/ Puparium	938.0±6.1	634.8±5.2
Adult	1104.8±3.4	388.5±7.7

3.6 Biology

Eggs are laid in woolly wax "nests" that are circular in shape. Freshly deposited eggs are oblong and pale yellowish, which turn into dark yellowish or orange with a lengthy pedicel before hatching (7.7± 0.16 days). Four nymphal instars consisting of crawlers, I nymphal instars (3.5 ± 0.44 development days), II and III nymphal instars (7.4± 0.20 development days), and IV instars were observed with a total nymphal development period of 4.5± 0.21 days. The puparium is oval in shape, yellowish to orange in color and encircled by filamentous wax supports that extend from the dorsum. Adults are pale yellow with a mild powdered wax coating on the body and flattened wings dorsally over the body (16.7±0.38). The total life cycle ranges from 35-40 days (Table 4).

Table 4: Biology of *P. bondari* in open field conditions

Life stages	Field conditions (days)
Eggs	7.7±0.16
1st nymphal instar	3.5±0.44
2nd &3rd nymphal instar	7.4±0.20
4th nymphal instar	4.5±0.21
Total nymphal instars	15.4±0.85
Adult	16.7±0.38
Total life cycle	39.8±1.39

3.7 Host range of *Paraleyrodes bondari*

Currently, *Paraleyrodes bondari* has been noticed in different cotton growing tracts of Tamil Nadu and also hosts a range of Indian crops, including banana (*Musa paradisiaca*), sugarcane (*Saccharum officinarum*), guava (*Psidium guajava*), coconut (*Cocos nucifera*), and jamun (*Syzygium cumini*) (Fig. 6a-e). Till now, there was no previous record of Paraleyrodes species invasion in the cotton ecosystem documented. *Paraleyrodes bondari* as a polyphagous pest causes economic damage to the majority of horticultural crops. Its recent invasion of the cotton ecosystem may lead to economic loss in the future.

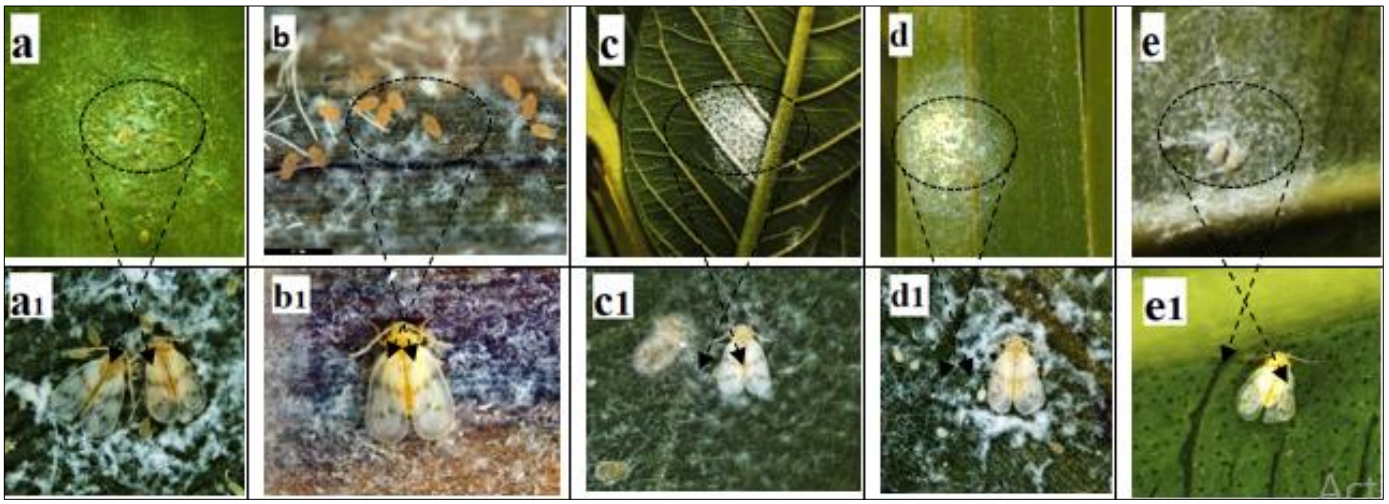

Fig 6: a-e. Host plants: a. banana b. sugarcane c. guava d. coconut e. jamun

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