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Mitochondrial COI Gene Sequences Based Molecular Variation in Rugose Spiraling Whitefly [*Aleurodicus rugioperculatus* Martin](Hemiptera: Aleyrodidae) Species from Different Geographic Locations of India

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Abstract

Rugose spiraling whitefly (RSW), Aleurodicus rugioperculatus has been recognized as an economically significant pest, causing serious concerns in various regions of the country. Its polyphagous nature is enabling it to feed on a wide range of host plants which contributes to its invasive potential and the resultant economic impact. To establish a comprehensive molecular database of RSW and validate the genetic variation within the species, sampling was conducted across different geographic regions of the country. Genomic DNA was extracted from the collected RSW samples using the Cetyl Trimethyl Ammonium Bromide (CTAB) method. The molecular identity of the RSW specimens was confirmed through a NCBI BLAST search and all the analyzed nucleotide sequences have been deposited in the International GenBank (NCBI), USA, under accession numbers OP024187 to OP024192, OP024194, and OP024195. Phylogenetic analysis of the collected samples, comprising 15 populations and 7 host species, revealed existing genetic distance within the RSW populations. Specifically, the Tamil Nadu population obtained from a coconut plantation exhibited the maximum genetic distance compared to the other populations. This finding highlights the distinct genetic characteristics of this particular RSW population, suggesting variations in its adaptation.

Keywords: Rugose Spiraling Whitefly; DNA Barcoding; Molecular Phylogenetics; Host Plants; Species Identification.

Introduction

Whiteflies (Hemiptera: Aleyrodidae) have become a serious menace in crop production. They constitute a major part of the insect invasion to numerous crops in India namely, spiralling whitefly, *Aleurodicus disperses* in 1995 (David & Regu, 1995) and *Solanum whitefly, Aleurothrixus trachoides* in 2014 (Dubey & Sundararaj, 2015). In 2016, infestation of Rugose Spiralling Whitefly (RSW), *Aleurodicus rugioperculatus* Martin was reported for the first time from Pollachi, Tamil Nadu of India which drew the attention of farmers as well as scientists nationwide. It was first reported from coconut, *Coccus nucifera* L. in 2004 in Belize (Martin, 2004) and it is believed to have originated in Central America, the pest threatened coconut palms in Florida in 2009 (Stocks & Hodges, 2012). India is the only country in the oriental region where this whitefly has been introduced. The exact mode of entry of RSW into India remains unknown, but it is speculated that the pest might have entered into the country through trade, possibly accompanying ornamental plants. The pest attained significance within a short period of time and it spreads to adjacent states on several crops viz. coconut, *C. nucifera* L., oil palm, *Elaeis guineensis* Jacq. guava, *Psidium guajava* L. banana, Musa spp. L. cocoa, *Theobroma cacao* L. etc (Shanas et al., 2016; Pradhan et al., 2020; Sankarganesh et al., 2023).

Insect pests, particularly invasive species are a major production constraint and concern for farmers across the World. In the current global agricultural scenario, invasive insect pests hold great socio-economic and ecological importance as they have risen significantly worldwide over the past two centuries due to high intercontinental human-mediated interactions (Pradahan et al., 2021). The clear discrimination between morphological characters of sucking insect pests is quite challenging in the

immature stages. However, molecular diagnostic tools could be used to precisely identify those pest species. It provides valuable support for the rapid and accurate identification of morphologically indistinct species supported by taxonomic data (Mainali, 2008). This understanding is pivotal in devising effective strategies for managing the pest including RSW. The use of molecular and genomic tools facilitates understanding genetic makeup of individuals.

DNA-based identification has gained importance, as a transformative technology where the species of an individual organism is identified by using small regions of the mitochondrial genome (cytochrome 'c' oxidase subunit or COI gene) to discriminate species (Hebert et al., 2003). This approaches have been employed to characterize cryptic and taxonomically challenging insect species. The cytochrome 'c' oxidase subunit 1 (COI) is a general marker utilized to diagnose species across the animal kingdom. A DNA barcode database 'Barcode of Life Data Systems' (BOLD) was set up as a DNA barcode reference library facilitating information sharing. Apart from species identification, DNA barcoding complements both molecular phylogenetics and population genetics (Hebert et al., 2004).

The utilization of molecular data, particularly the sequencing of a specific gene region i.e. mitochondrial cytochrome oxidase subunit-1 (COX I), offers a powerful tool. This genetic region serves as a distinct genetic signature that aids in the validation of intraspecific diversity, estimation of species richness, identification of intraspecific variation and delimitation of biologically and taxonomically distinct entities through phylogenetic studies. The COI gene has been successfully used by previous researchers in the determination of host associated differences in insect species. (Ghosh et al., 2017; Firake et al., 2017; Firake et al., 2021; Kranthi et al., 2021). It is widely applied method, based on mitochondrial gene and tends to be highly conserved among related species.

The molecular studies are largely focused to resolve the genetic relationship of insect species (Murthy et al., 2009). Despite its economic importance, there is a paucity of information about the genetic variability in populations of RSW. Whitefly populations have been studied previously in India with regard to their colonization on host plants and geographic regions. (Prasanna et al., 2015; Boopathi et al., 2019; Prabhulinga et al., 2021). Also, the phylogenetic of invasive whitefly, *Paraleyrodes bondari* from the southern region of the country were revealed. (Josephrajkumar et al., 2019). However, efforts to apply mtCOI sequences on spiraling whitefly are scanty which is considered important for developing sustainable approaches for managing the pest population. Furthermore, a thorough assessment of the genetic variation within the RSW species is essential for implementing comprehensive management strategies at the national or area-wide level. With this backdrop, the present study aimed to identify RSW using COI gene sequences and meticulously quantified the extent of genetic variation. The study also sought to establish evolutionary relationships among RSW populations originating from diverse habitats and locations. It may provide valuable insights that can help in mitigating the impact of RSW on agriculture and ecosystems.

Materials and Methods

Sampling and Preservation

Multiple insect samples of RSW, *A. rugioperculatus* were collected from various regions of southern, eastern and north-eastern India, which have experienced invasion of whiteflies (Fig.1). The collected specimens were preserved in 10ml screw cap vials containing 70% ethanol and were suitably labeled. Subsequently, they were stored at -20°C until DNA extraction. Voucher specimens were maintained for each geographical location.

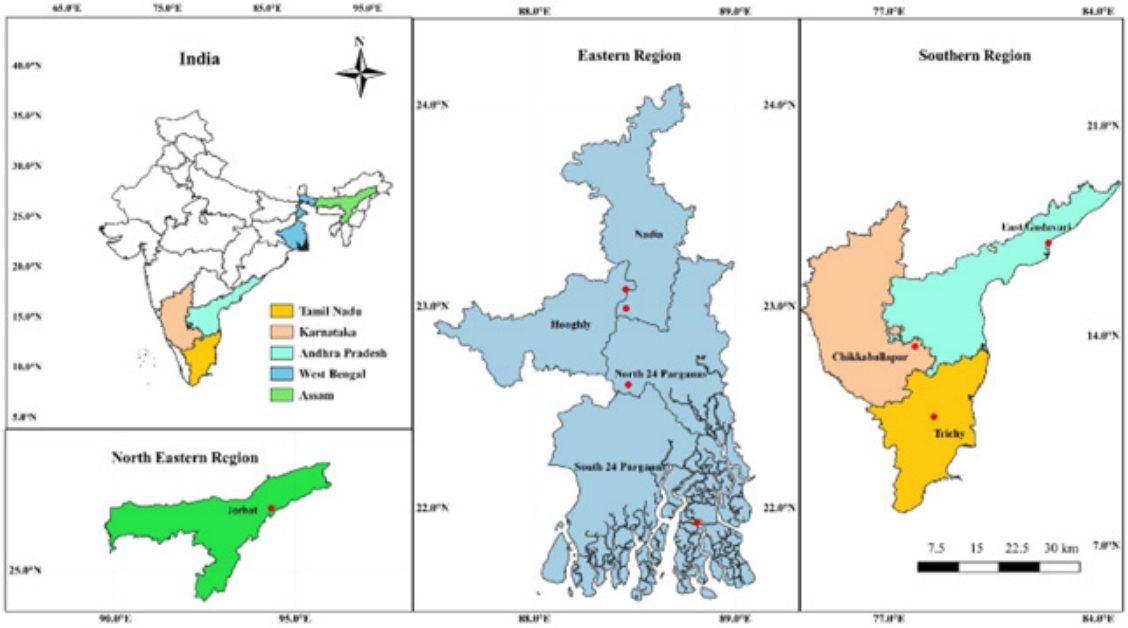

Figure 1: Map showing the RSW, *A. rugioperculatus* sampled states and locations in India

Extraction of Genomic DNA from Whitefly Samples

The genomic DNA (gDNA) was extracted from the adult RSW using the modified Cetyl Trimethyl Ammonium Bromide (CTAB) method (Gawel & Jarrett, 1991; Boopathi et al. 2014). Air-dried specimens were placed in 1.5 ml Eppendorf tubes containing 50 µl of pre-heated CTAB extraction buffer. The samples were then crushed using a micro pestle. The tubes were incubated in a water bath at 65°C for 45 minutes with gentle inversion at regular intervals to allow lysis in extraction buffer. After incubation, the tubes were removed from the water bath and allowed to cool at room temperature for 5 minutes. To facilitate DNA extraction, a 1:1 proportion of chloroform: isoamyl alcohol (24:1) was added to the tubes, which were then mixed by inversion for 15 minutes. Then, the tubes were centrifuged at 12,000 rpm for 15 minutes at 4°C using a micro centrifuge (Eppendorf, Model No. 5430). The clear aqueous phase containing the DNA was carefully transferred into a new sterile tube. To precipitate the DNA, one-third volume of ice-cold isopropanol was added, and the tubes were gently inverted for a few seconds. The DNA was allowed to precipitate overnight at 20°C. After the overnight incubation, the tubes were centrifuged at 12,000 rpm for 20 minutes at 4°C to pellet the DNA, and the supernatant was discarded. The DNA pellet was then washed with 70% ethanol and centrifuged again at 5,000 rpm for 5 minutes at 4°C. After washing, the supernatant was decanted, and the DNA pellets were air-dried at room temperature. Finally, the dried pellets were re-suspended or dissolved in sterile water or 1X TE buffer (pH 8.0) based on the size of the pellet and stored at -20°C until further use.

Qualitative and Quantitative Estimation of DNA

The integrity of the genomic DNA was assessed by visualizing it on a 0.8 percent agarose gel. For qualitative and quantitative estimation, 2µl of DNA from each sample was analyzed using a nanodrop spectrophotometer (Thermo Fisher Scientific). The DNA was measured at a wavelength of 260/280 nanometers to determine its quality. Simultaneously, a blank control (1X TE) was maintained for comparison during the estimation process. Based on the measured concentration, stock solutions of the DNA samples were prepared and stored at -20°C for PCR amplification.

PCR Amplification

For amplification of the standard barcoding region of the cytochrome oxidase I (COI) gene of mitochondrial DNA (mtDNA), universal primers: LCO1490 (forward) 5'-GGTCAACAAATCATAAAGATATTGG-3' and HCO2198 (reverse) 5'-TAAACTTCAGGGTGACCAAAAAATCA3' were utilized (Folmer et al., 1994). PCR amplifications were performed in a PCR machine (Thermo Fisher Scientific), with a final volume of 50 µl. The reaction mixture included 4 µl of genomic DNA, 4 µl each of forward and reverse primers, 5 µl of taq buffer, 2 µl of dNTPs, 0.8 µl of taq polymerase, and 30.2 µl of molecular biology-grade water. The PCR profile consisted of an initial denaturation step at 95°C for 2.06 minutes, followed by 35 cycles of denaturation at 95.0°C for 45 seconds, annealing at 52.0°C for 1 minute, and extension at 72.0°C for 25 seconds. A final extension step was performed at 72.0°C for 7 minutes. After completion of all the cycles, the samples were held at 4°C in the PCR machine and then stored at -20°C for further use. The amplified PCR products were subjected to electrophoresis on a 2.5 percent agarose gel (Sigma) using 2 µl of a 100 bp DNA ladder (BR Biochem) as a molecular weight standard. The gel was visualized under UV trans-illuminator and gel was documented in gel documentation system (Zenith). Once the desired band size was detected, the amplicons were processed commercially for Sanger dideoxy sequencing for all the samples. A total of 45 µl of post-PCR product, maintained in a frozen condition, was submitted for sequencing.

Sequence Analysis

The trace DNA sequences obtained were analyzed using the MEGA 11 molecular biology software (Tamura et al., 2021). To determine the identity and homology of the analyzed sequences, a BLAST search was performed. The sequences were deposited in the gene bank of the National Center for Biotechnology Information (NCBI) and accession numbers were obtained for all the submitted sequences (Table 2).

Establishment of Evolutionary Relationships

The evolutionary relationship within the species was determined based on the nucleotide sequences of the partial COI gene. Sequence alignment was performed using the MUSCLE algorithm, which was implemented in Seaview (Thompson et al., 1994). A phylogenetic tree was constructed using the Neighbor-Joining (N-J) algorithmic method (Tamura et al., 2021). To assess the robustness of the tree, the bootstrap test was employed (Felsenstein, 1985). The test involved 1,000 replicates to estimate the evolutionary distances between all sequences. The constructed tree incorporated the obtained sequences from the study, as well as relevant sequences retrieved from the NCBI gene bank. The similarity among the sequences was estimated within the tree. All the evolutionary analyses were performed using MEGA-11 software.

Results and Discussion

The genomic DNA was extracted from freshly collected female specimens (identified based on pincers at the abdominal end) of RSW, *A. rugioperculatus* obtained from different regions (Table 1). The A260/280 ratio was ranged from 1.31 to 1.83 across the samples. The targeted region of the PCR fragment was successfully amplified in all the specimens, with no variation in band size. Following sequencing, the quality of the partial COI gene sequences varied in length, ranging from 684 bp to 691 bp across the species. Ambiguous regions at the 5' and 3' ends of the sequences were trimmed, resulting in obtaining final high-quality sequences. The chromatographs from each sequence displayed a high level of confidence, indicating the good quality of each chromatograph within its respective sequence. Additionally, the complete open reading frames were inspected for all sequences, and no stop codons were detected.

The identity and homology of all the analyzed sequences were evaluated. A BLAST search was performed for each sequenced isolate to compare its similarity with known sequences in the database (Table 1). The BLAST search of the sequenced isolate collected from banana, *Musa* spp. (RSWAP) showed 100% similarity with *A. rugioperculatus* having the accession no. MT542036; the sequence of the isolate collected from coconut, C. *nucifera* (RSWAJ) having the accession no. MK159741 showed 97% similarity with *A. rugioperculatus*. The sequence of the isolate collected from *C. nucifera* (RSWKT) showed 99% similarity with the *A. rugioperculatus* having the accession no. ON739017. Moreover, the sequence of the isolate collected from *C. nucifera* (RSWTN) showed 97% similarity with *A. rugioperculatus* having the accession no. MT542036. Further, the sequence of the isolate collected from *Musa* spp. (RSWHG) showed 98% similarity with *A. rugioperculatus* having the accession no. MK159741. Nucleotide sequence (accession no. MT542036) study of the whitefly isolate collected from *C. nucifera* (RSWKY) showed 99% similarity with *A. rugioperculatus*. The nucleotide sequence of the arecanut, *Areca catechu* isolate (RSWNP) exhibited 99% similarity with *A. rugioperculatus* having the accession no. ON739017 while the nucleotide sequence of the *C. nucifera* isolate (RSWSB) bearing accession no. MK159733 revealed 100% similarity with *A. rugioperculatus*. These findings provide ample evidence of the identity of the analyzed sequences and their close similarity to *A. rugioperculatus*.

Table 1: Details of RSW, *A. rugioperculatus* samples with Accession number

State	Location (Village/district)	GPS Position	Host Plant	Sample Id	Accession number
West Bengal	Serpur, Hooghly	23.02042°N 88.41956°E	Banana, *Musa* spp. L.	RSWHG	MK159741
West Bengal	New Town, North 24 Parganas	22.60892°N 88.46615°E	Arecanut, *A. catechu* L.	RSWNP	ON739017
Tamil Nadu	Vairichettipalayam, Trichy	11.28195°N 78.49548°E	Coconut, *C. nucifera* L.	RSWTN	MT542036
Andhra Pradesh	Amaravalli, East Godavari	17.08328°N 82.33094°E	Banana, *Musa* spp. L.	RSWAP	MT542036
Karnataka	Kammanahalli, Chikkaballapura	13.6245°N 77.866°E	Coconut, *C. nucifera* L.	RSWKT	ON739017
West Bengal	Kalyani, Nadia	22.98766°N 88.45528°E	Coconut, *C. nucifera*, L.	RSWKY	MT542036
Assam	Barbheta, Jorhat	26.722°N 94.342°E	Coconut, *C. nucifera* L.	RSWAJ	MK159741
West Bengal	Dayapur, South24 Parganas	21.92503°N 88.80814°E	Coconut, *C. nucifera*, L.	RSWSB	MK159733

To establish the evolutionary relationships, a phylogenetic tree was constructed based on neighbor-joining method following the multiple sequence analysis using MUSCLE and inferred based on the mtCOI nucleotide sequence of RSW collected from various locations in India (8 sequences) and additional samples retrieved from the NCBI database (7 sequences) (Table 2). The resultant tree depicted two distinct groups (Fig.2). The isolates with the following accession numbers: OP024191 (Kammanahalli; Coconut, *C. nucifera*), OP024190 (Amaravalli; Banana, *Musa* spp.), OP024187 (Serpur; Banana- Musa spp.), OP024194 (Barbheta; Coconut, *C. nucifera*), OP024192 (Kalyani; Coconut, *C. nucifera*), OP024188 (New Town; Arecanut, *A. catechu*), OP024195 (Dayapur; Coconut, *C. nucifera*) clustered together in one group. However, the isolate with accession number OP024189 (Thuraiyur; Coconut, *C. nucifera*) branched off separately from this cluster. The phylogenetic tree exhibited two main branches and the retrieved sequences were mixed with the collected isolates within the first main branch. These sequences corresponded to the following accession numbers: KP032219 (Florida; Wild banana- *Strelitzia nicolai*), MW750563 (Pune; Banana- *Musa* spp.), MW750575 (Pune; Coconut- *C. nucifera*), MK598812 (Karnataka; Mango- *Mangifera indica*), MK926750 (Kolar; Jamun- *Syzygium cumini)*, MF445090 (Mangalore; Banana- *Musa* spp.), and MH321182 (Kannur; Sugarcane- *Saccharum officinarum*).

The first main branch, which contains both the isolated and retrieved species, diverged from the second branch with a distance of 0.06, indicating minor substitutions in the nucleotide sequence. Within the first branch, there are 14 sequences that form a single cluster, regardless of the host species from which they were collected and identified. However, the isolates with accession number OP024189 (Thuraiyur; Coconut, *C. nucifera*) collected from Tamil Nadu diverged from the first branch with a distance of 0.07, indicating a significant separation and suggesting its independent evolution (Fig. 2.). Interestingly, the sequences of *A. rugioperculatus* from different locations showed similarities with most of the isolates, indicating a lack of substantial diversity among the isolates. The subtle variations noticed in the sequences may find their roots in the diverse ecological niches where these whiteflies colonize and flourish. The act of feeding on different host plants might

induce certain epigenetic effects, potentially contributing to genetic variations. It is possible that mutations have occurred in specific loci, leading to dissimilarities in the sequences. This species may possess genetic plasticity in its alleles, affording it the ability to adapt and survive across a spectrum of host plants. This genetic adaptability likely plays a crucial role in the resilience and proliferation of the species in various environments. Further exploration of these genetic intricacies is warranted to comprehensively grasp the adaptive mechanisms and potential implications for effective pest management strategies.

Table 2: Isolates of RSW, *A. rugioperculatus* collected from different host plants across the different geographical regions and NCBI retrieved sequences with their gene bank accession number

Gene bank Accession No.	Location	Host Plant
OP024187	Serpur, Hooghly	Banana, *Musa* spp. L.
OP024188	New Town, North 24 Parganas	Arecanut, *A. catechu* L.
OP024189	Vairichettipalayam, Trichy	Coconut, C. *nucifera* L.
OP024190	Amaravalli, East Godavari	Banana, *Musa* spp. L.
OP024191	Kammanahalli, Chikkaballapura	Coconut, *C. nucifera* L.
OP024192	Kalyani, Nadia	Coconut, *C. nucifera* L.
OP024194	Barbheta, Jorhat	Coconut, *C. nucifera* L.
OP024195	Dayapur, South 24 Parganas	Coconut, *C. nucifera* L.
KP032219	Florida, USA	Wild banana, *Strelitzia nicolai* Regel and Korn
MF445090	Mangalore, Karnataka	Banana, *Musa* spp. L.
MH321182	Kannur, Kerala	Sugarcane, *S. officinarum* L.
MK598812	Bengaluru, Karnataka	Mango, *M. indica* L.
MK926750	Kolar, Karnataka	Jamun, *S. cumini* (L.) Skeels.
MW750563	Pune, Maharashtra	Banana, *Musa* spp. L.
MW750575	Pune, Maharashtra	Coconut, *C. nucifera* L.

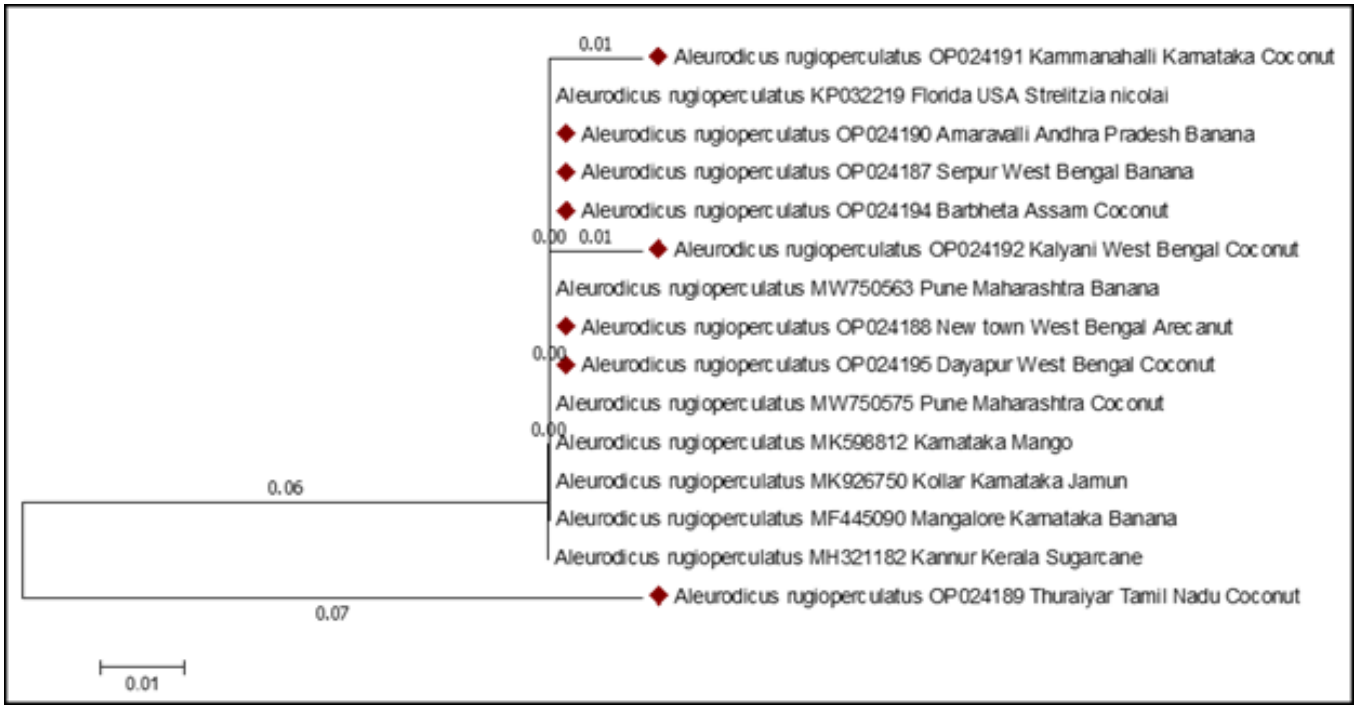

Figure 2: Neighbour-joining phylogenetic dendrogram of nucleotide sequences of RSW, *A. rugioperculatus* from different regions.

Leveraging molecular and genomic tools in understanding the genetic makeup of invasive insect pests is vital for planning and developing efficient and sustainable control methods (Prasanna, 2020). It enhances our ability to manage pests and mitigate their adverse effects on agriculture and ecosystems. The findings of the present analysis have uncovered a close genetic relationship among RSW isolates, suggesting a shared ancestry between the *A. rugioperculatus* populations in India and Florida. These results challenge the notion that genetic variations among RSW populations are directly correlated with their geographical distribution. This insight implies that other factors, potentially ecological or biological in nature, may be exerting a more significant influence on the observed genetic diversity among RSW populations. In the present study, the evolutionary divergence among the sequences of *A. rugioperculatus* ranged from 0.06 to 0.07. The dendrogram generated from this analysis provided a visual representation of the genetic relationships among the RSW populations. It highlighted that the RSW population from Tamil Nadu was distantly related to the other isolates. This suggests that the genetic makeup of the Tamil Nadu population of *A. rugioperculatus* differs significantly from the populations in other regions.

Mitochondrial DNA (mtDNA) is widely utilized in evolutionary studies and phylogenetic analysis due to its characteristics, including its large size and high rate of nucleotide substitution (Hebert et al., 2003). It helps in flagging genetically distinct lineages and identifying cryptic species. Researchers have observed that variations in mtDNA can influence the speciation process (Wilson et al., 2010; Nieukerken et al., 2012; Behere et al., 2014; Shashank et al., 2015). In the present study, the aim was to investigate the genetic variation of *A. rugioperculatus* among the few Indian populations where this species is distributed. By analyzing the mtCOI sequences of *A. rugioperculatus*, the study pointed out the existing variations among the Indian population of this species. In a related study, a comprehensive exploration of phylogenetic relationships among whitefly species in Florida was undertaken (Dickey et al., 2015). The findings from this study demonstrated that several genera, including *Aleurodicus, Paraleyrodes, Aleurocanthus, Dialeurodes,* and *Trialeurodes*, clustered together, forming a cohesive group. Conversely, the genus *Bemisia* formed a distinct and separate cluster. Likewise, the ecological characteristics of *A. rugioperculatus* observed in this study align with the results of the phylogenetic analysis. These findings are consistent with the research conducted by Boopathi et al. (2019) who discovered that spiralling whiteflies on *Acalypha* and *Calotropis* exhibited greater genetic distinctiveness compared to whiteflies found on other host plants.

Genomic tools enable the development of early detection and monitoring systems for invasive pests. This is crucial for rapid response and containment, preventing the establishment and spread of invasive species in new regions. Timely and accurate identification of invasive pests can significantly contribute to minimizing their adverse effects on agriculture, ecosystems, and biodiversity. Genetic divergence can be influenced by various factors, including population size, population structure, natural selection, mutation rates, gene flow between populations and introgression resulting from hybridization. It is crucial to investigate patterns of genetic diversity as they provide valuable insights. For instance, if geographically distant populations exhibit high genetic diversity, it suggests that they possess greater evolutionary potential to adapt the increasing impacts they face. However, the comprehensive approach is important to provide a more complete understanding of the genetic dynamics and population structure of *A. rugioperculatus* across its range.

Conclusion

Resolving the species composition and diversity within the whitefly species complex is of utmost importance in order to plan and develop sustainable approaches for management. To arrive at a more conclusive understanding, further studies are necessary, involving the inclusion of additional Indian populations as well as locations of global significance. Additionally, it is essential to investigate the *A. rugioperculatus* population in areas experiencing severe infestations, with the aim of identifying the development of any biotypes. This information is crucial for formulating specific pest management strategies tailored to the unique characteristics of this pest population.

Conflict of Interest

The authors declare that there is no conflict of interest.

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