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Intraspecific phylogenetic analysis of rice leaf folder, *Cnaphalocrocis medinalis* Guenee (Lepidoptera: Crambidae) populations across five agro-ecological units in Kerala

SEENA R SUBHAGAN[1]*, BERIN PATHROSE[1], MANI CHELLAPPAN[1], SMITA NAIR[1] and DHALIN D[2]

Kerala Agricultural University, Vellanikkara, Kerala 680 656, India

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ABSTRACT

The present study was carried out during 2023–2024 at College of Agriculture, Vellanikkara, Kerala to investigate the intraspecific molecular phylogeny by utilizing *mtCO1* gene sequences of the rice leaf folder, *Cnaphalocrocis medinalis* Guenee (Lepidoptera: Crambidae), across five major rice (*Oryza sativa*) growing agroecological units (AEUs), viz. AEU 3- Onattukara Sandy plains, AEU 4-Kuttanad, AEU 6-Kole lands Thrissur, AEU 20-Wayanad Central Plateau and AEU 23-Palakkad Eastern Plains in Kerala. While all populations clustered within the same clade, *C. medinalis* from Onattukara exhibited separate branching, albeit sharing similarities with Kuttanad populations. In contrast, populations from Thrissur and Wayanad displayed a closer resemblance, while those from Palakkad exhibited similarities with populations from China and Jharkhand in India. Moreover, the observed genetic structuring suggested regional differences in population dynamics, with populations from Southern Kerala (Onattukara and Kuttanad) exhibiting closer genetic grouping compared to those from Northern Kerala (Wayanad, Palakkad, and Thrissur). Analysis of percent identity matrices unveiled a high level of genetic similarity among *C. medinalis* populations across Kerala's AEUs, ranging from 98–99%. Notably, Kuttanad populations shared 99.85% of their identity with those from Onattukara and Wayanad, while Wayanad populations shared the same level of identity as Thrissur. Despite the regional differences, our study did not observe an apparent "barcode gap" typical of COI sequences. These findings shed light on the possibility of seasonal migration patterns, inbreeding events, and the subsequent distribution dynamics of the rice leaf folder across Kerala. Moreover, the potential impact of migration on disseminating resistant alleles among insect populations is a critical issue that warrants comprehensive investigation.

Keywords: Agro-ecological units (AEU), *Cnaphalocrocis medinalis*, Intraspecific phylogeny, Mitochondrial cytochrome oxidase subunit 1*(mtCO1*)

Rice (*Oryza sativa*) cultivation is a cornerstone of global agriculture, where rice is the primary sustenance for approximately half of the world's population. The recent decline of 10.34% in rice production in Kerala compared to 2020–2021, underscores the pressing issue of crop losses attributed to various stressors. Among these, insect pests emerge as formidable adversaries, causing about 25% losses and contributing significantly to an overall yield reduction, of approximately ₹240 billion (Babendreier *et al.* 2020). The increased reliance on nitrogenous fertilizers (Singh *et al.* 2019) and widespread use of insecticide sprays have fueled the outbreaks of rice leaf folder *Cnaphalocrocis medinalis*, inflicting substantial damage if left unchecked. In Kerala, the pest exhibits year-round activity, perpetuating from one crop to another (Samui *et al.* 2007). This persistent threat underscores the urgent need for intervention strategies to mitigate the detrimental effects of the rice leaf folder in Kerala.

During the larval stage, this pest exhibits voracious feeding behaviour, with a single larva capable of inflicting a detrimental impact on plant physiology and productivity (Alvi *et al.* 2003). Among the leaf folder complex in rice, *C. medinalis* emerges as the most dominant one (Rautaray *et al*. 2019) and the most destructive defoliator across rice ecosystems in Asia (Luo 2010). Confounding taxonomic intricacies further, *C. medinalis* has often been confused with *C. patnalis* (=*Marasmia patnalis*) in India due to their analogous behavioural patterns, shared host plant associations, and morphological similarities (Joshi *et al*. 1986).

Current research on rice leaf folder lacks data on its genetic variation and demographic patterns, which are crucial for improving the understanding and management of this pest (An *et al*. 2014). Population genetic studies using

[1]Kerala Agricultural University, Vellanikkara, Kerala; [2]Kelappaji College of Agricultural Engineering and Technology (Kerala Agricultural University, Vellanikkara, Kerala), Tavanur, Kerala. *Corresponding author email: seena.subhagan@kau.in

the mitochondrial cytochrome oxidase subunit I (*mtCOI*) gene are effective and reliable in revealing the adaptive strategies of leaf folder species. Previous attempts to analyze its molecular phylogeny using *mtCOI* sequences (Mashhoor *et al*. 2019) have not covered the different agroecological units (AEUs) in Kerala. This study attempted to fill this gap by characterizing *C. medinalis* across five AEUs in Kerala, using the *mtCOI* gene as a molecular marker.

MATERIALS AND METHODS

The present study was carried out during 2023–2024 at College of Agriculture, Vellanikkara, Kerala. During the seasonal cycles of Autumn/Virippu (June–September) and Winter/Mundakan (October–January), larval and adult specimens of the *C. medinalis* were collected from five distinct agroecological units (AEUs) in Kerala using sweep nets. These collection sites were strategically chosen to represent major rice-growing areas in the state, including AEU 4-Kuttanad (9.519773ºN, 76.359784ºE), AEU 23-Palakkad (10.86966ºN, 76.2959ºE), AEU 3-Onattukara (9.101141ºN, 76.554231ºE), AEU 6-Kole lands of Thrissur (10.567219º N, 76.131089º E) and AEU 20-Wayanad (11.849598ºN, 76.043735ºE). A sample of *C. patnalis (=M. patnalis*) was collected from AEU 4-Kuttanad (9.42798ºN, 76.426554ºE). The collected specimens from each sampling site were preserved separately and stored at -20°C until further analysis.

Total genomic DNA was extracted from single, third-instar larvae, with three larvae taken from each location. Each larva was thoroughly cleaned in distilled water. Total DNA was extracted using a DNeasy blood and tissue (Qiagen) kit as per the manufacturer's protocol. The DNA was analyzed on a 0.8% agarose gel stained with ethidium bromide (0.5 g/ml). The quantified DNA was subsequently employed for PCR analysis. The universal forward primer (LCO 1490 - 5′- GGTCAACAAATCATAAAGATATTGG -3′) and reverse primer (HCO 2198 - 5′- TAAACTTCAGGGTGACCAAAAAATCA -3′) (Folmer *et al.* 1994), were used to amplify a partial *mtCOI* gene fragment of 710 bp from all the samples.

The PCR amplification was performed in a 20 µL reaction mixture containing 10 µL of *Taq* DNA polymerase Master Mix (TaKaRa), 0.6 µL each of forward and reverse primer, 7.8 µL nuclease-free water and 1 µL template DNA. The PCR was programmed for an initial denaturation of 1 min at 95 °C followed by 35 cycles of denaturation for 15 sec at 95 °C, annealing for 15 sec at 56 °C, elongation for 30 sec at 72 °C, and a final extension at 72 °C for 7 min on Invitrogen Veriti PCR thermal cycler (Applied Biosystems). The PCR products were separated on 2% agarose gel using electrophoresis and good quality bands of expected size were purified from the gel and sequenced using the forward and reverse primers at GeneSpec Biosciences, Kakkanad, Kerala. The forward and reverse sequences were assembled using the CAP3 sequence assembly program (Huang and Madan 1999) and subjected to BLASTN search. Representative sequences were submitted to the GenBank database.

The *mtCOI* sequences of *C. medinalis* from all five AEUs, *C. patnalis* from AEU 4, and the sequences of *C. medinalis, C. patnalis* and *C. bilinealis* retrieved from the GenBank (Table 1) were trimmed and subjected to multiple sequence alignment using Clustal W (Thompson *et al*. 1994). To analyze the phylogenetic relationship between *C. medinalis* from different AEUs, a phylogenetic tree was structured using the neighbor-joining method (Saitou and Nei 1987) with MEGA software version 11 (Tamura *et al*. 2021). A typical rice pest worldwide, rice stem borer, *Chilo suppressalis* (Lepidoptera: Crambidae) (GenBank Accession No. NC_015612), was selected as an outgroup. The bootstrap analysis (Felsenstein 1985) indicated the percentage of replicate trees where the related taxa clustered together, based on 1000 replicates. Evolutionary distances were calculated using the Maximum Composite Likelihood method (Tamura *et al*. 2004), expressed as the number of base substitutions per site. The study included 18 partial sequences of *mtCOI*, encompassing 631 positions in the final dataset, with gaps and missing data positions excluded from the analysis.

The evolutionary divergence between *mtCO1* sequences of *C. medinalis* from Kerala was estimated using the Kimura 2-parameter model (Kimura 1980) with MEGA11.0 (Tamura *et al*. 2021). The percentage identity matrix among the nucleotide sequences of *C. medinalis* from five different agroecological units of Kerala was prepared using Clustal W (Thompson *et al*. 1994).

RESULTS AND DISCUSSION

In this study, good quality genomic DNA with absorbance 260/280 nm ratios between 1.8 and 2, and yield ranging from 290 to 376 µg was obtained from third instar larvae from the five populations studied. In the PCR amplification of COI loci, expected sized bands of 710 bp were obtained for all the samples (Fig. 1).

In the BLASTN analysis, all the sequences showed more than 99% identity and 99% query coverage with the

Table 1 Details of sequences retrieved from GenBank for phylogenetic analysis

Name of insect	Accession number	Country
Cnaphalocrocis medinalis	MK854566	Jharkhand, India
	JQ647917	Korea
	JQ305693	China
	MK301227	Pakistan
	GU681924	Canada
	MK566596	Australia
	NC_015985	USA
	HM906228	Papua New Guinea
Cnaphalocrocis patnalis	NC_060868	USA
	MK566597	Australia
Cnaphalocrocis bilinealis	JF840498	Canada

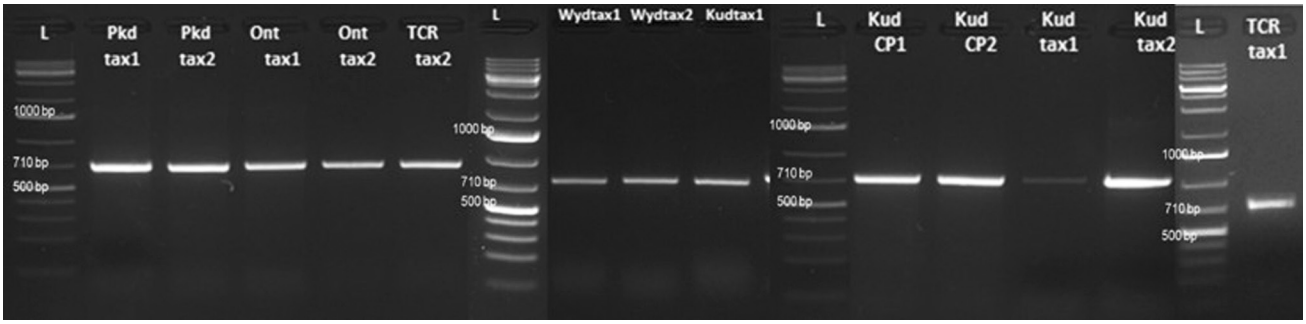

Fig. 1 Gel images with expected band size of 710 bp for CO1 loci in *C. medinalis.*
(L: 100kb, Pkdtax 1,2 – AEU 23, Onttax 1, 2 – AEU 3, TCRtax 1, 2 – AEU 6, Wydtax 1, 2 – AEU 20, Kudtax 1, 2 – AEU 4, KudCP 1, 2 – *C. patnalis* AEU 4).

complete mitochondrial genome of *C. medinalis* reported from Korea in 2013 (accession number JQ647917) and specimen isolated in China in 2014 (accession number JQ305693). The CO1 sequences of *C. medinalis* and *C. patnalis* were deposited in the NCBI nucleotide database, and the accession numbers were obtained (Table 2). A segment of the *mtCOI* gene has been widely employed as a DNA barcode in insects, facilitating differentiation even among closely related species (Hebert *et al*. 2003a). The species richness of *C. medinalis* across various paddy growth stages in Malaysia was investigated, and their DNA barcode was documented for the first time by Yaakop *et al*. (2020), who reported 98–99% similarity with existing GenBank data. Similarly, Jindal (2019) reported that population of *C. medinalis* from Hoshiarpur-2, Moga, and Sangrur-2 in Punjab, India, exhibited 99% identity in their *mtCOI* gene sequences with those in the GenBank database.

The phylogenetic analysis of the 18 taxa examined ensued in an unrooted tree (Fig. 2) with a sum of branch length (SBL) of 0.1285. The outgroup, rice stem borer, *C. suppressalis* (NC015612), with a branch length of 0.0418, was seen to form a distinct clade divergent from the *Cnaphalocrocis* genus. The clusters, including the *Cnaphalocrocis* genus, diverge from a single node into 17 branches and are in the same clade. It further branches into a small cluster, which includes the species *C. patnalis*, and a bigger cluster, which includes the rest of the members in the clade. Even though *C. patnalis* from Kuttanad, Kerala (OR856006) arise from the same node as that from Australia (MK566597) and the USA (NC060868), it forms a separate branch. *C. bilinealis,* separates from *C. medinalis* and forms a single branch in the second major subcluster.

Among the 13 nodes of the *C. medinalis* taxa, the populations from the different agroecological units of Kerala showed more similarity to the *C. medinalis* population from other parts of the world. The first molecular phylogeny of insects, using a reference set of DNA sequences from identified species, was conducted over 35 years ago (Wheeler 1989). The study population from Palakkad (AEU 23) was grouped with *C. medinalis* from China and Jharkhand, India. The populations from the Kole lands of Thrissur (AEU 6) and Wayanad (AEU 20) separated from the same node, but the Wayanad population was more related to that from Canada (GU681924) and the USA (NC015985). The study

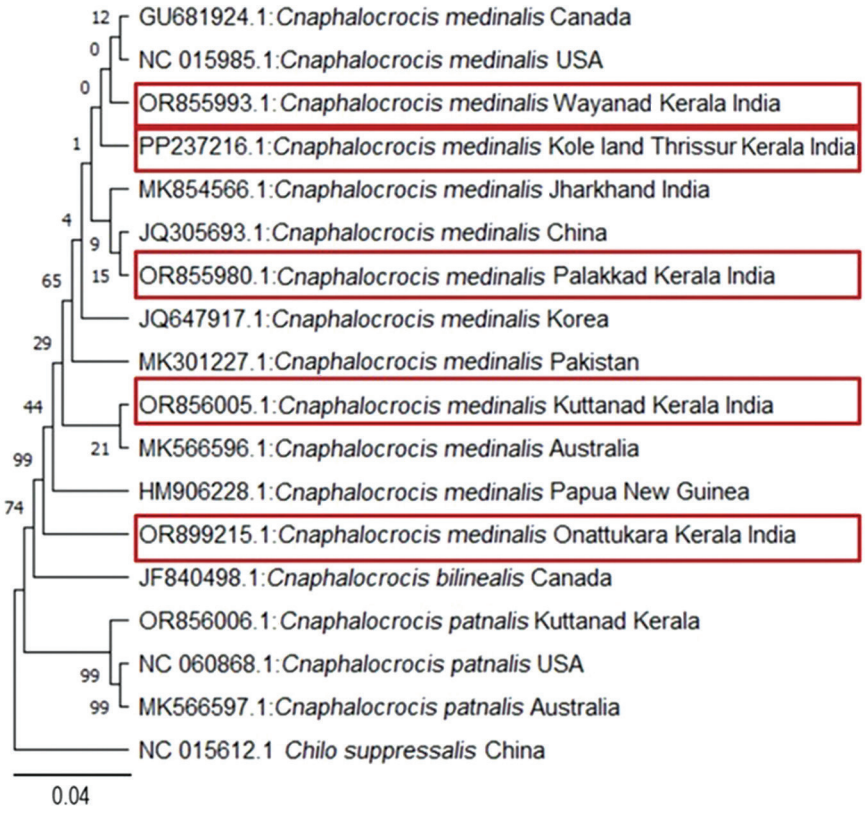

Fig. 2 Phylogenetic tree constructed by the neighbour-joining method with MEGA software based on partial COI gene sequences showing phylogenetic relationships between *Cnaphalocrocis medinalis* sampled from different AEU of Kerala and other sequences retrieved from the database with *Chilo suppressalis* as the outgroup. Bootstrap values are expressed as percentages of 1000 replications.

Table 2 Details on agroecological units (AEU) and GenBank accession numbers of leaf folder populations from Kerala

Species	Agroecological units (AEU)	Location	GPS coordinates		GenBank accession numbers
			Latitude N	Longitude E	
Cnaphalocrocis medinalis	4 Kuttanad	Kuttanad	9.519773	76.359784	OR856005
	4 Kuttanad	Kuttanad	9.519773	76.359784	PP724649
	4 Kuttanad	Kuttanad	9.519773	76.359784	PP724720
	23 Palakkad Eastern Plains	Palakkad	10.86966	76.2959	OR855980
	23 Palakkad Eastern Plains	Palakkad	10.86966	76.2959	PP724379
	23 Palakkad Eastern Plains	Palakkad	10.86966	76.2959	PP724381
	3 Onattukara Sandy Plain	Onattukara	9.101141	76.554231	OR899215
	3 Onattukara Sandy Plain	Onattukara	9.101141	76.554231	PP724749
	3 Onattukara Sandy Plain	Onattukara	9.101141	76.554231	PP724750
	6 Kole Lands	Thrissur	10.567219	76.131089	PP237216
	6 Kole Lands	Thrissur	10.567219	76.131089	PP725470
	6 Kole Lands	Thrissur	10.567219	76.131089	PP725475
	20 Wayanad Central Plateau	Wayanad	11.849598	76.043735	OR855993
	20 Wayanad Central Plateau	Wayanad	11.849598	76.043735	PP725290
	20 Wayanad Central Plateau	Wayanad	11.849598	76.043735	PP725421
Cnaphalocrocis patnalis	4 Kuttanad	Kuttanad	9.42798	76.426554	OR856006
	4 Kuttanad	Kuttanad	9.42798	76.426554	PP724741
	4 Kuttanad	Kuttanad	9.42798	76.426554	PP724742

population from Onattukara (AEU 3), Kerala (OR899215) branches out separately from the rest of the cluster. The population from Kuttanad (AEU 4), Kerala (OR856005) was more similar to that from Australia (MK566596) and Papua New Guinea (HM906228). In a previous report by Mashhoor *et al*. (2019), *C. medinalis* from Papua New Guinea was found to be the nearest relative of *C. medinalis* isolated from Kerala (GenBank Accession: KF671954). Similarly, in an analysis of *mtCOI* sequences of *C. medinalis* from China, Papua New Guinea, Australia, Pakistan, Korea, and India by Jindal (2019), the population from Papua New Guinea formed an independent cluster from the rest of the populations.

The intraspecific molecular phylogeny analysis of two mitochondrial genes, COII and COIII, in *Reticulitermes speratus* (Isoptera: Rhinotermitidae) populations across the Japanese Archipelago and the Korean Peninsula revealed the formation of two major clades. These clades consisted of distinct groupings, notably encompassing populations from the Korean/Southern Japanese regions as one group and those from the Northern Japanese territories as another (Park *et al.* 2006). Likewise, in this analysis, while the *C. medinalis* populations are positioned within the same clade, it is noteworthy that populations from Southern Kerala (Onattukara and Kuttanad) exhibit a closer grouping relative to those from Northern Kerala (Wayanad, Palakkad, and Thrissur).

As presented in Table 3, the estimation of evolutionary divergence revealed close genetic relatedness between *C. medinalis* populations from Wayanad and Kole lands of Thrissur, with a Kimura two-parameter (K2P) distance of merely 0.155%. A similar pattern was observed between

Table 3 Estimates of evolutionary differences between sequences, presented as percentages using the Kimura-2 parameter model

	1	2	3	4	5
OR855993.1: *C. medinalis* Wayanad	-				
PP237216.1: *C. medinalis* Thrissur	0.155				
OR856005.1: *C.medinalis* Kuttanad	0.255	0.310			
OR899215.1: *C.medinalis* Onattukara	0.311	0.466	0.155		
OR855980.1: *C. medinalis* Palakkad	0.778	0.935	0.935	1.093	-

Table 4 Percentage identity matrix among the nucleotide sequences of *Cnaphalocrocis medinalis* from five different agroecological units of Kerala estimated using Clustal W

OR855980.1: *C. medinalis* Palakkad	100.00				
OR899215.1: *C. medinalis* Onattukara	98.92	100.00			
OR856005.1: *C.medinalis* Kuttanad	99.08	99.85	100.00		
OR855993.1: *C. medinalis* Wayanad	99.23	99.69	99.85	100.00	
PP237216.1: *C. medinalis* Thrissur	99.08	99.54	99.69	99.85	100.00
	1	2	3	4	5

populations from Kuttanad and Onattukara, showing an evolutionary distance of 0.155%. Interestingly, among the selected populations, the highest divergence of 1.093% was noted between *C. medinalis* populations from Palakkad and Onattukara. These findings align well with the phylogenetic analysis, suggesting consistency in estimating evolutionary distance across the studied populations.

However, the intraspecific genetic distance among the *C. medinalis* populations from different AEUs was found to be less than 3% (K2P distance) as against the 3% CO1 threshold employed to delineate subspecies across various geographical regions and taxonomic groups for lepidopterans (Hebert *et al*. 2003b). Consequently, the genetic variations within the species did not display clear ranges, and there was no noticeable "barcode gap" typically observed in COI sequences among the studied populations of *C. medinalis*. Jiang *et al*. (2013) documented similar findings regarding interspecific genetic distance in their molecular phylogenetic investigation of the butterfly genus *Polytremis* (Lepidoptera: Hesperiidae) in China.

The percent identity matrix (Table 4) demonstrated a high level of genetic similarity among rice leaf folder populations across five different AEUs, with identities ranging from 98–99%. Notably, the populations from Kuttanad and Onattukara had 99.85% genetic identity, as did the populations from Wayanad and the Kole lands of Thrissur. Such genetic similarity among leaf folder populations may be attributed to seasonal migration patterns of the pest. A similar possibility was discussed by Jindal (2019), who reported that rice leaf folder larvae from seven locations in Punjab, including 2 locations of Sangrur and Hoshiarpur, Moga, Ludhiana and Jalandhar, shared 99-100% genetic similarity. Notably, long-distance migration of *C. medinalis* has been documented from regions such as China, Japan, and Taiwan (Pan 1985), with studies indicating multi-stop migration capabilities and the ability to cover distances of up to 300 km per night for consecutive nights to reach suitable habitats (Wang *et al*. 2017). Furthermore, the fluctuations in population sizes and migration patterns of *C. medinalis* are known to be responsive to changes in rice planting systems, as observed by Qi (2008). A recent study by Hsieh *et al*. (2023) highlighted a lack of genetic diversity in populations of *C. medinalis*, indicating unrestricted gene flow between six geographic sites in Taiwan. Moreover, the absence of genetic divergence between Taiwanese populations and those in neighboring regions implied a shared origin. The researchers proposed the possibility of population expansion following a recent 'founder effect' for *C. medinalis* in East Asia, implying a single origin for these populations.

Despite the presence of numerous resistance genes, Corsican mosquito populations exhibited only a 14-fold increase in resistance to organophosphates, compared to a 117-fold increase in Californian populations (Georghiou *et al*. 1975). Georghiou *et al*. (1980) and Pasteur *et al*. (1981) further observed that this significant rise in organophosphate resistance in Californian field populations was attributable to a single resistance gene. These findings indicated that while local mutations play a role in developing resistance, the spread of resistant genes between populations can greatly enhance this effect. Raymond and Marquine (1994) have explained the possibilities of migration of resistance genes in Corsican *Culex pipiens* mosquito populations from California. The observed patterns of cross-border knockdown resistance (*kdr*) gene flow between *Aedes aegypti* (Diptera: Culicidae) populations, between the state of Amapa (Brazil) and French Guiana underscored the necessity for international collaboration to monitor and prevent the transnational movement of vectors and pathogens (Salgueiro *et al*. 2019). The significance of this research has been instrumental in enhancing vector control strategies for *A. aegypti*.

The rice leaf folder poses a persistent threat to rice cultivation, with infestations beginning from the nursery stage and peaking during the reproductive and ripening phases (Litsinger *et al*. 2006). In India, *C. medinalis* has become a significant and recurring pest of rice (Padmavathi *et al*. 2006). While damage during the vegetative stage

is generally manageable if the crop remains unstressed (Mohapatra 2008, Tanwar *et al.* 2019), the pest's rapid life cycle, completing two to three generations within a single crop, worsens the problem (Barrion *et al*. 1991). Regardless of extensive research on rice leaf folder migration in other countries (Wada 1979, Chang *et al*. 1980, Tu 1983), there is a notable dearth of studies and reports from India. This highlights a critical gap in our understanding of the dynamics in the migration of *C. medinalis* in the Indian context, underscoring the need for further investigation in this area. The increasing reports of crop failures, outbreaks (Balasubramani *et al*. 2000), and insecticide resistance (Sun *et al*. 2023) in rice leaf folders are a growing concern. Understanding the critical role of migration in the spread of insecticide-resistant genes is essential for developing effective strategies to manage and mitigate resistance in pest populations worldwide. This insight is vital for informing global pest management practices and ensuring sustainable agricultural productivity.

In conclusion, our research provides a comprehensive phylogenetic analysis of *C. medinalis* populations illuminating distinct genetic affinities among populations from different regions of Kerala. Specifically, those from southern locales such as Onattukara (AEU 3) and Kuttanad (AEU 4) exhibited closer genetic relatedness to species from Australia and Papua New Guinea, while those from northern regions like Palakkad (AEU 23) demonstrated a closer association with counterparts from China and Jharkhand, India. Moreover, Thrissur Kole (AEU 6) and Wayanad (AEU 20) populations exhibited genetic similarities with species from the USA and Canada. Evolutionary divergence was only 0.155% between *C. medinalis* populations from Wayanad and Kole lands of Thrissur and those from Kuttanad and Onattukara. Notably, *C. medinalis* populations across different AEUs in Kerala share a high degree of genetic identity, indicative of potential seasonal migration and inbreeding events. Our study lacks data to assess and track the migratory trajectory of *C. medinalis*. Importantly, our study confirms the monophyletic nature of *C. medinalis* populations in Kerala, underscoring the need to investigate the possible migration of this pest in India, the strategies pursued, and the associated fitness costs. This can provide valuable insights for evidence-based decision-making and interdisciplinary collaborations, ultimately contributing to developing effective integrated pest management approaches that safeguard agricultural productivity and environmental sustainability. Moreover, the possible role of migration in transferring insecticide-resistant alleles among insect populations is a significant concern and requires thorough investigation.

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