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Diversity of the Gut Bacterial Communities Associated with Melon Fruit Flies, *Zeugodacus cucurbitae* (Coquillett) (Diptera : Tephritidae)

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Abstract

Insects are associated with greater diversity of symbiotic microorganisms and these organisms influence on the development and survival of the insects. With this contest, the present study was under taken to know the bacterial diversity in different stages of melon fly, *Zeugodacus cucurbitae viz*., egg, larva, pupa and adult. The results of the experiment were revealed based on molecular identification. There were 16 species of bacterial endosymbiont belonging to 11 genera isolated with Enterobacteriaceae being the dominant family in all the stages of melon fly. High bacterial diversity was noticed in pupal stage followed by larval stage, where it harboured proteobacteria, firmicutes, bacteroidetes and actinobacteria where in other stages were associated only with proteobacteria. The egg harboured 8 per cent of the isolated bacteria, larval stage with 28 per cent, pupa and adult were associated with 32 per cent each of the total endosymbionts isolated. The above results have thrown light on the diversity and recycling of endosymbionts in the gut of melon fly.

Keywords : *Zeugodacus cucurbitae*, Endosymbionts, Bacterial diversity

It is well known that arthropod gut harbours an array of microbes which sway their growth and fitness. These microorganisms have a long history of coevolution with the arthropods ranging from parasitism to obligate symbiosis (Dale and Moran, 2006). In the absence of symbiotic bacteria, insects have been reported to have a reduced growth rate and high mortality. Insects acquire endosymbionts by horizontal and or vertical transmission patterns (Lauzon *et al.,* 2009). Various endosymbionts have close interactions either intracellularly or extracellularly. The bacterial endosymbionts play an important role in host nutrition, development, fitness, survival, modulation of immune responses and communication. The presence of such an enormous bacterial diversity within insect digestive tracts is due to their different feeding habits, physicochemical properties and gut structures. (Hadapad *et al.*, 2015 and Andongma *et al.,* 2015).

Tephritid flies harbour different bacterial symbionts in their digestive system, which influence different fitness parameters (Pramanik *et al*., 2014). Gut microbes play an important role in insect morphogenesis, nutrition, antifungal toxin production, pheromone production, regulation of pH, synthesis of vitamins, temperature tolerance, development of resistance against parasitoids and detoxification of toxic compounds. (Genta *et al*., 2006).

The melon fly, *Z. cucurbitae*, being one of the devastating pests of cucurbitaceous vegetables (Subhash *et al*., 2018), has been recorded on more than 125 species of plants, including tomatoes (Weems *et al*., 2015). Cucurbits, the most economically nutritious crops, suffer a quiet high damage by fruit flies. The melon fruit fly, *Z. cucurbitae* is reported to causes 30.0 to 100.0 per cent losses in cucurbits depending on the host species and season (Dhillon *et al.*, 2005).

Several bacterial species belonging to family Enterobacteriaceae and Bacillaceae were isolated from the alimentary tracts of adult melon fly. Many species of bacteria were also identified in the alimentary tract, fly faeces, host fruit surfaces, oviposition sites and larvae infested fruit tissues (Gujjar *et al.,* 2017). These endosymbiont being very essential to the insects, they harbour them across all the stages. There is no much work on isolation in stage and gender

specific association of cultivable bacterial diversity in *Z. cucurbitae*. Therefore, the study was carried out to isolate and characterize the cultivable gut bacteria associated with the *Z. cucurbitae* using 16S rRNA gene sequence based on analysis.

Material and Methods

Fruit Fly Collection and Rearing

Gherkins infested with *Z. cucurbitae* were collected from the field in a cage and the emerged larvae were reared on the natural host in the box at room temperature. A layer of sand was provided filling in the rearing box for the maggots to go for pupation after development. Once it pupates, the pupae were carefully collected and kept in a rearing cage for adult emergence. The adult was provided with sugar, yeast and water in cotton swabs. Gravid females of *Z. cucurbitae* were exposed to gherkins for 24 hours for oviposition. The oviposited gherkins were replaced with fresh and oviposited gherkins kept in rearing box with a layer of sand for further development.

Isolation of Gut Bacteria

A total of one day old 50 eggs, five 3rd instar maggots, five pupa and 15 days old laboratory reared male and gravid female flies were separated, starved for 3 hrs and cold anesthetized at -20°C for 5 min, surface sterilization was done with 70 per cent alcohol for 60 seconds, followed by another round of sterilization for 60 seconds using 0.5 per cent sodium hypochlorite. The surface sterilized fruit flies were washed thoroughly with sterile distilled water twice. Individual surface-sterilized flies were dissected aseptically under laminar air flow. Individual dissected mid guts were washed thoroughly with sterile distilled water and transferred to a sterile microfuge tube containing 1 mL of phosphate buffered saline (PBS) and macerated using a micro pestle. The immature stages *viz.,* egg, maggot and pupa were directly crushed as whole in PBS. The homogenized samples were centrifuged at 2000 rpm for 10 minutes. Serial dilutions of samples were made up to 10^{-6} dilutions. The aliquot of 100 µL of all the dilutions were plated on Nutrient Agar (NA) for isolating the symbiotic bacteria. Aliquot was spread using sterilized spreader. The plating was done by spread plate technique. Each plate was incubated at 28°C for 48 hours. After every 24 hours, plates will be observed for microbial growth (Thaochan, *et al.*, 2009).

Colonies that differed in appearance will be typically different bacterial strains, species or genera. Based on size, shape, pigmentation, elevation, consistency and margins of the isolates colonies differentiated and pure culture was obtained by sub culturing on the same media.

Molecular Identification of Gut Bacterial Isolates

Total DNA was extracted from bacterial colonies by inoculating the single colony of bacterial culture in nutrient broth and incubated at 37°C for 24 hrs. Transfered 1.5 mL culture to micro centrifuge tube and centrifuged at 10000 rpm for 3 minutes and collected pellets. Resuspended the pellets on 400 µL sucrose buffer and vertex. Added 32 µL lysozyme, incubated for 10 minutes at 60°C. Then added 45 µL 10 per cent SDS and 5µL proteinase, mix well and incubate again in water bath for 10mins at 60°C. Added 240 µL NaCl and 140 µL freshly prepared 10 per cent CTAB and kept in water bath for 10 minutes. Added 500 µL Chloroform:Isoamylalcohol (24:1), mixed well and centrifuged at 12000 rpm for 10 minutes. Transfered the upper aqueous phase into new tube and added 50 µL 3M sodium acetate and 300 µL of isopropanol, mixed gently and incubated overnight at -20°C. Spun at 12000 rpm for 15 min to pellet down the DNA. Added 1ml 70 per cent ethanol and spun at 12000 rpm for 10 min (twice). Discarded the supernatant and allowed drying. Resuspended the DNA in 40 µL TE Buffer, added 2 µL RNAse and incubated at 37°C for 30 min (William *et al.,* 2012 and Swathi *et al.*, 2015).

The 16SrRNA gene was amplified from bacterial colonies by PCR, using universal eubacterial primer pairs eu27.F (5'-AGAGTTTGATCCTGGCTCAG-3') and eu1495.R (5'-ACGGCTACCTTGTTACGACTT-3'). PCRs were carried out in 30 µL reactions with each reaction tube containing 1.5 mM of each primer,

~ 15ng of template DNA, 3 µL Taq buffer, 1.5 µL Taq Polymerase. The following condition was used for the PCR reactions: 98°C for 1 min, 59°C for 30 seconds and 72°C for 1 min for 30 cycles and a final extension of 72°C for 10 min. PCR products were subsequently subjected to Agarose gel electrophoresis. Aliquots (2 µL) of each PCR product were resolved electrophoretically on 1 per cent agarose gel using 10X TAE buffer. The PCR products visualized with an UV transilluminator and photographed with a gel documentation system (Gel Doc 200, BIO-RAD, USA) after staining the gel with ethidium bromide (0.5µg mL–1) (Promega), the DNA molecular weight marker, a 1-kbp DNA ladder (Promega) (Plate 1) was used to determine the size of the amplified fragments (Lorenz, 2012 and Swathi *et al.,* 2015).

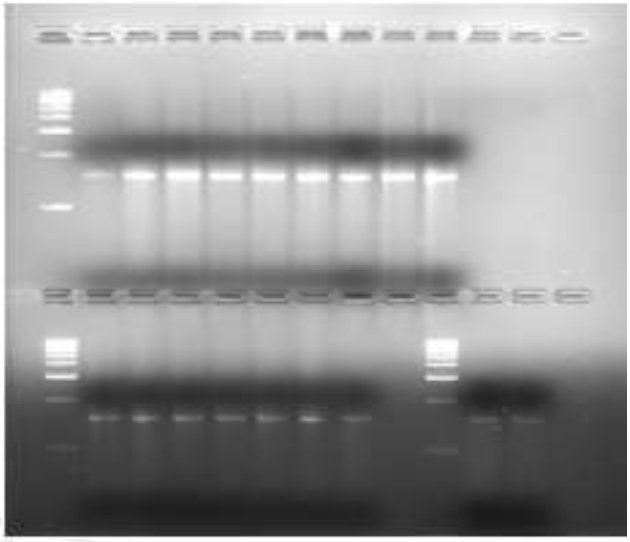


Plate 1: Gel electrophoresis after DNA amplification

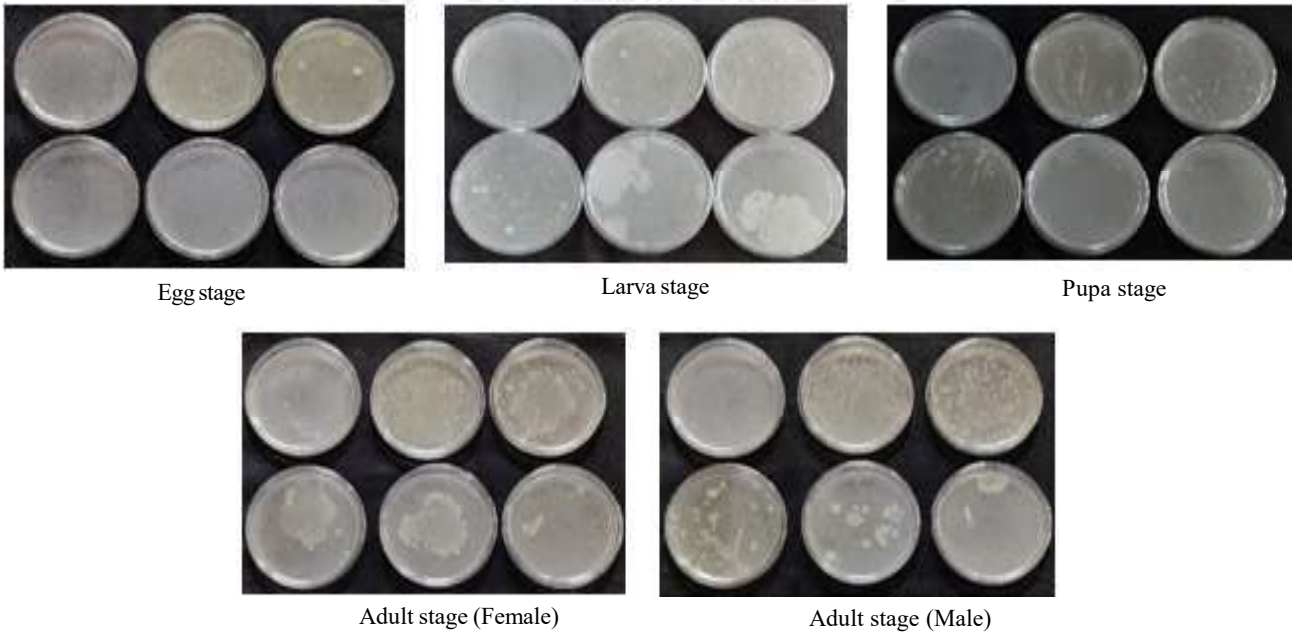
16S rRNA sequencing analysis

The purified PCR products were sent for sequencing. The nucleotide sequencing of the PCR fragments was performed. The DNA sequences corresponding to 16SrRNA gene, obtained from individual bacteria was reverse complemented using software Bioedit. The obtained sequences were analysed along with the sequences retrieved from the NCBI (National Centre for Biotechnology Information) GenBank using bioinformatics software and endosymbionts were identified.

Results and Discussion

Isolation and Identification of Gut Bacteria

Based on size, shape, pigmentation, elevation, consistency and margins 16 isolates were obtained from different stages of melon fruit fly (Plate 2). Majority of the colonies were small, round with smooth margin, white to yellow pigmented mucoid colonies,



Egg stage | Larva stage | Pupa stage

Adult stage (Female) | Adult stage (Male)

Plate 2 : Culture plate of different stages

TABLE 1
Colony characterisation of bacterial isolates obtained from all the stages of melon fruit fly

Organism	Size	Colour	Opacity	Margin	Shape	Elevation	Gram stain
Klebsiella michiganensis	Small	Creamy White	Translucent	Smooth	Round	Flat	Negative
Citrobacter farmeri	Medium	Off White	Translucent	Smooth	Round	Flat	Negative
Kluyvera cryocrescens	Small	Off White	Translucent	Smooth	Round	Flat	Negative
Klebsiella aerogenes	Small	Creamy White	Translucent	Smooth	Round	Flat	Negative
Providencia rettgeri	Small	Creamy White	Opaque	Irregular	Round	Raised	Negative
Leucobacter chromiireducens	Medium	White	Opaque	Irregular	Round	Raised	Positive
Myroides odoratus	Small	Yellow	Opaque	Smooth	Round	Raised	Negative
Providencia vermicola	Small	Orangish yellow	Opaque	Irregular	Round	Flat	Negative
Bacillus subtiles	Big	Off White	Opaque	Irregular	Irregular	Flat	Positive
Bacillus licheniformis	Big	Off White	Opaque	Irregular	Irregular	Flat	Positive
Paenochrobactrum gallinarii	Small	Creamy White	Opaque	Irregular	Round	Raised	Negative
Pectobacterium aroidearum	Small	Off White	Translucent	Smooth	Round	Flat	Negative
Pseudomonas putida	Medium	Creamy White	Translucent	Irregular	Irregular	Flat	Negative
Pectobacterium carotovorum	Small	Creamy White	Translucent	Smooth	Round	Flat	Negative
Morganella morganii	Small	Off White	Translucent	Smooth	Round	Flat	Negative

where few colonies were translucent and few were opaque with raised or flat elevation. Gram negative bacteria had an upper hand where only three out of 16 isolates were gram positive *viz., Leucobacter chromiireducens, Bacillus subtilis* and *B. licheniformis* (Table 1).

16S rRNA Gene Sequence Analysis

Based on the *16S rRNA* gene sequence analysis, 16 species belonging to 11 genera were identified from all the four stages of *Z. cucurbitae* reared on Gherkins, according to percentage similarity of the NCBI database (Table 2). Among the 16 bacterial isolates, 68.75 per cent of them belong to the phylum Proteobacteria followed by Firmicutes (12.5%), Bacteroides and Actinobacteria of 6.25 per cent each. Four *Klebsiella* sp. (18.75%), two *Providencia* sp. (12.5%), two *Bacillus* sp. (12.5%), two *Pectobacterium* sp. (12.5%) and one (6.25%) each of *Citrobacter, Kluyvera, Leucobacter, Myroides, Pseudomonas, Morganella* and *Paenochrobactrum* were identified. Most of the isolates belonging to the family Enterobacteriaceae, which comprised 43.75 per cent followed by Morganellaceae with 18.75 per cent and Bacillaceae with 12.5 per cent of all the isolates (Fig. 1). This is in agreement with the findings of Hadapad *et al.* (2019) who isolated bacterial endosymbionts from wild *Z. cucurbitae* and found diverse bacterial composition with Proteobacteria (87.72%) being the dominant phyla followed by Bacteroidetes, Firmicutes and Actinobacteria. Likewise, Mishra *et al.* (2018) identified Enterobacteriaceae as the most prevalent bacterial family in melon flies followed by Staphylococcaceae, Enterococcaceae, Bacillaceae and Brucellaceae. In

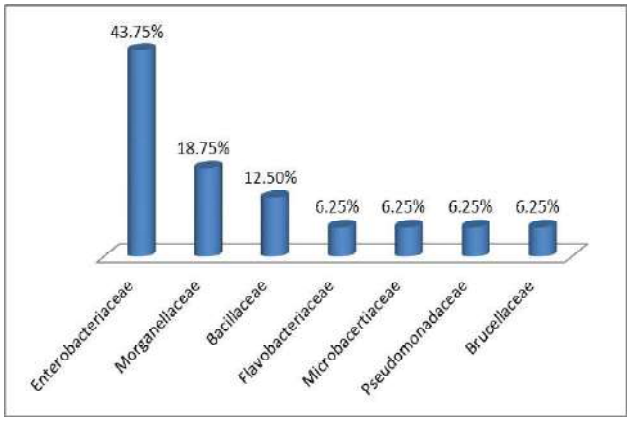


Fig. 1 : Relative abundance of bacterial families in the different developmental stages of the Melon fly

TABLE 2
Identity of bacterial isolates based on 16S rRNA gene identity

Phylum	Class	Order	Family	Organism	per cent identity
Egg Stage					
Proteobacteria	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	*Klebsiella oxytoca*	96.42
				Klebsiella michiganensis	97.73
Larval Stage					
Proteobacteria	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	*Klebsiella oxytoca*	95.79
				klebsiella michiganensis	91.97
			Morganellaceae	*Providencia vermicola*	96.48
Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus subtilis*	96.63
				Bacillus licheniformis	96.28
Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	*Myroides odoratus*	96.17
Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae	*Leucobacter chromiireducens*	94.24
Pupal Stage					
Proteobacteria	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	*Klebsiella oxytoca*	97.73
				klebsiella michiganensis	96.35
				Pectobacterium aroidearum	97.19
				Pectobacterium carotovorum	96.93
			Morganellaceae	*Providencia vermicola*	96.55
				Morganella morganii	95.39
		Pseudomonadales	Pseudomonadaceae	*Pseudomonas putida*	93.64
	Alpha Proteobacteria	Hyphomicrobiales	Brucellaceae	*Paenochrobactrum gallinarii*	97.21
Adult Stage					
Proteobacteria	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	*Klebsiella oxytoca*	95.14
				Klebsiella michiganensis	95.38
				Citrobacter farmer	*96.62*
Male					
Proteobacteria	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	*Klebsiella oxytoca*	95.51
				Klebsiella michiganensis	96.11
				Klebsiella aerogenes	96.00
				Kluyvera cryocrescens	96.49
			Morganellaceae	*Providencia rettgeri*	95.28

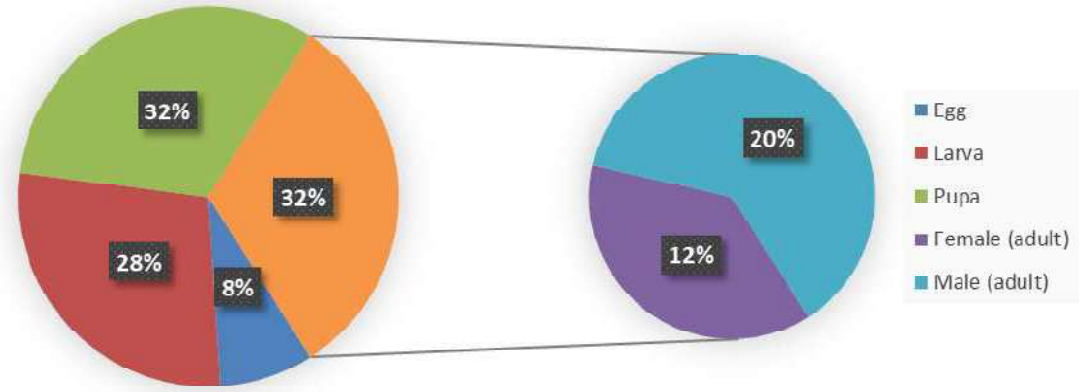


Fig. 2 : Diversity of endosymbionts in developmental stages of Melon fly

2015 Hadapad *et al.,* isolated 26 bacterial isolates from melon fly belonging to the families Enterobacteriaceae, Bacillaceae, Micrococcaceae and Staphylococcaceae with *Enterobacter* (34.6%), *Klebsiella* (19.2%), *Citrobacter* (7.7%), *Bacillus* (15.4%) and *Providencia* (7.7%) being the dominant genera. Many of the studies showed that Enterobacteriaceae is the most predominant family associated with Tephritidae

Diversity of Bacterial Endosymbionts in different Stages

While compared with the individual stages, egg stage harboured two species (8%) *Klebsiella oxytoca* and *K. michiganensis* belonging to Enterobacteriaceae while the later stage of larvae had acquired (28%) of seven species of bacteria belonging to the families Enterobacteriaceae, Bacillaceae, Morganellaceae, Flavobacteriaceae and Microbacteriaceae. The pupal stage was associated with eight species (32%) of the bacterial endosymbionts belonging to the families Enterobacteriaceae, Morganellaceae Pseudomona daceae and Brucellaceae. In adult, male harboured higher diversity of bacteria (20%) than that of the female (12%). (Table 2, Fig 2). Proteobacteria was the only phylum found in all the stages except for the larval stage which also hosted Firmicutes, Bacteroidetes and Actinobacteria. Similarly, Noman *et al*. (2021) isolated 14 species of 11 genera and eight families belonging to six bacterial phyla from larvae, pupae and male and female adult of *Zeugodacus tau.* Proteobacteria was the most represented phylum in all the stages except larvae. Among the classes of bacteria, Gammaproteobacteria was more abundant in pupae, male and female adult flies, whereas Bacilli were found more dominant in the larval stage.

Among the 16 bacteria isolated from different stages of the fruit fly, *Klebsiella oxytoca* and *K. michiganensis* were observed to be associated with all the four stages of the melon flies. Whereas, *Providencia vermicola* was found in larval as well as in pupal stage (Fig. 3). The rest of the bacteria were seen associated with the particular stage of the fruit fly. The endosymbiont load in insects are greatly modulated according to their need and it also depends on environment and habitat of that stage and their nutritional requirement, they host the symbionts in their body and eliminate them with somatic tissue once the purpose is served (Vigneron *et al.,* 2015).

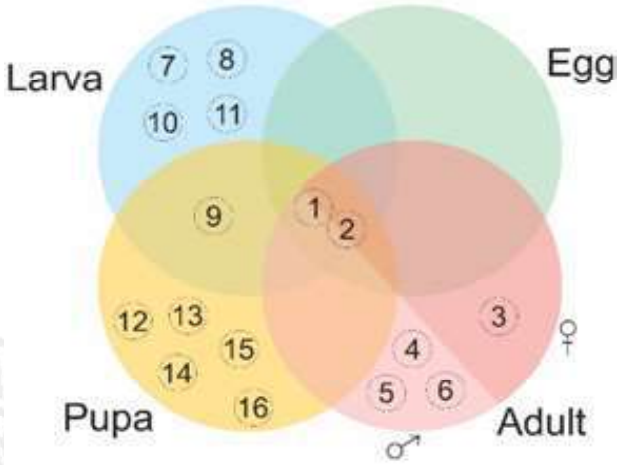


Fig. 3: *1. Klebsiella oxytoca, 2. Klebsiella michiganensis, 3. Citrobacter farmeri, 4. Kluyvera cryocrescens, 5. Klebsiella aerogenes, 6. Providencia rettgeri, 7. Leucobacter chromiireducens, 8. Myroides odoratus, 9. Providencia vermicola,10. Bacillus subtiles, 11.Bacillus licheniformis, 12. Paenochrobactrum gallinarii 13.Pectobacterium aroidearum 14. Pseudomonas putida, 15. Pectobacterium carotovorum, 16. Morganella morganii*

This study enables with deep understanding of the bacterial endosymbionts associated with the different life stages of *Z. cucurbitae*. This experiment revealed that the larval and pupal stage harboured more diversity of endosymbionts and male flies having more symbiotic association with the gut bacteria rather than that of the female flies in their mid-gut. The change in bacterial diversity in different stages have made a channel to study about how the endosymbionts are obtained at different stages and eliminated from their body. Understanding the specific functions of each endosymbionts and the transmission patterns will be a fertile area for future research.

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