Indian Journal of Entomology Online published Ref. No. e24838 DoI. No.: 10.55446/IJE.2024.1838

# EVALUATION OF A 16S RRNA AMPLICON ILLUMINA SEQUENCING METHOD FOR EXAMINING THE GUT MICROBIOME OF *LIPAPHIS ERYSIMI* (KALT)

**Archana Shukla[1]*, Ashwani Kumar[2] and Pradeep Kumar Shukla[3]**

[1,3]Department of Biological Sciences, Faculty of Science, [2]Department of Entomology,
Naini Agricultural Institute, Sam Higginbottom University of Agriculture,
Technology and Sciences, Prayagraj 211007, Uttar Pradesh, India
*Email: archushukla001@gmail.com (corresponding author): ORCID ID 0000-0003-0288-0251

**ABSTRACT**

**Herbivore specialists adapt to feed on a specific group of host plants by evolving various mechanisms to respond to plant defenses. Gene enrichment in the metagenome, accompanied by functional identification, revealed an essential role of specific gut bacteria in the breakdown of plant cell walls, detoxification of plant phenolics, and synthesis of amino acids. The *Lipaphis erysimi* (Kalt) gut bacteriome is an essential aspect for herbivory adaptation and this study is aimed at the microbiome of the musatrd Aphid *L. erysimi* gut by targeting the V3-V4 hyper variable region of the 16S rRNA gene with the Illumina platform sequencing using adult *L. erysimi* was performed to understand the bacteriome variations. A total of 103883 reads were obtained revealing the gut of the *L. erysimi* was found to be dominated by the main symbiont Buchnera a Protobacteria.**

**Key words:** Mustard Aphid, V3-V4 metagenomic sequencing, operational taxonomical units (OTUs), endosymbionts, phloem-feeders, Illumina sequencing, 16S rRNA gene, gut microbiome, PE250 paired-end sequencing, NovaSeq sequencing

The connections insects formed with diverse microbes are largely responsible for their ability to adapt to various circumstances (Chu et al., 2013; Rosenberg et al., 2010; Takeshita et al., 2015; Moran et al., 2019). Aphids (Hemiptera: Aphididae), which feed on phloem sap, provide as an appropriate model for studying such insect-bacteria interactions (Buchner et al., 1965). They have developed relationships with facultative (or secondary) symbionts, which can be found in a variety of host tissues and can improve, while obligate (or primary) endosymbionts, which are housed in particular host cells called bacteriocytes and primarily provide nutritional roles. As an illustration, facultative symbionts have demonstrated their capacity to enhance host heat tolerance (Montllor et al., 2002; Burke et al., 2010), host resistance to natural enemies (Oliver, 2003; Schmitz et al., 2012; Łukasik et al., 2013; Costopoulos, 2014), host plant specialization (Leonardo et al., 2003; Tsuchida et al., 2004), and host survival (Koga et al., 2003) as well as hinder predation by causing aphid body-color alteration (Tsuchida et al., 2014). The eukaryote gut co-evolves with a microbiome that plays important roles in digestion, nutrition, development, and immune responses (Warnecke et al., 2007; Engel et al., 2012; Engel and Moran, 2013b). Additionally, aphids host a variety of facultative endosymbionts, are not necessary for life or reproduction. However, in some environmental circumstances, their presence can improve the fitness of their hosts (Oliver et al., 2010).

The *Lipaphis erysimi*, and its obligatory bacterial endosymbiont, *Buchnera*, are well-known examples of nutritional symbioses. Aphids feed on phloem sap throughout their development, a diet deficient in vitamins and essential amino acids (EAAs) required for aphid growth and survival. *Buchnera* offers critical nutrients to its aphid host that the aphid cannot produce on its own, allowing the aphid to feed on this specialized plant niche (Shigenobu and Wilson 2011). *Buchnera* is mostly found in bacteriocytes. Bacteriocyte cell number and size increase throughout nymphal development (Simon et al., 2016; Colella et al., 2018). This fast expansion in bacteriocytes coincides with the aphid's high nutritional need throughout nymphal development as it expands, moults, and generates longer embryonic chains (Koga et al., 2012). *Buchnera* population growth rates can also be affected by development (Simon et al., 2016; Zhang et al., 2019), Metagenomics is a powerful tool for studying gut microbial diversity in order to better understand their physiology (Tringe et al., 2005). Complete taxonomic profiles of the holobiont can provide information into the co-evolution of this herbivore's gut microbiota. The aim of this study is to document the bacteriome of the *L.erysimi* using

16S rRNA amplicon-based Illumina sequencing and analytical procedure

## MATERIALS AND METHODS

A susceptible adult mustard aphid *L. erysimi* sample was collected in February 2023 from Indian mustard, *Brasssica Juncea* (L.) Czern. farm field SHUATS (25.4137°North and 81.8491° E), Naini, Prayagraj, U.P. India. The specimens were kept in 70% ethanol at 6°C immediately after collection. DNA samples enriched in bacteria were prepared by using Charles and Ishikawa (1999) protocol. 10-15 aphids were first rinsed three times in ultrapure water (Qiagen, Germany) and then crushed in 1 mL of buffer A (35 mM Tris –HCl, 25 mM KCl, 10 mM $MgCl_2$ and 25 mM sucrose, pH 7.5) with a Teflon pestle and a glass mortar. The resulting homogenate was then successively filtered and DNA were extracted from the residual pellets, each DNA sample was amplified in triplicate along with a control following Jousselin et al. (2015) with the DNeasy Blood and Tissue Kit (Qiagen,Germany). A Thermo Fisher Scientific Inc., Waltham, USA, NanoDrop spectrophotometer was used to measure the extracted DNA's concentration and quality. Anuvanshiki (OPC) Private limited, Delhi prepared and sequenced the high-throughput paired-end Illumina MiSeq libraries from the extracted aphid DNA samples. A limited cycle PCR reaction was carried out to produce a single amplicon of the V3 and V4 variable region (Rintala et al., 2017). All PCR products (250bp) were quantified and purified before sequencing then, sequencing libraries were generated using TruSeq. The libraray quality was assessed on the FastQC (version 0.11.5) (Andrews 2010) and MultiQC at last the library was sequenced on an Illumina PE250 platform at Novagen 6000 (Beijing, China), and 250 bp paired-end reads were generated. Total 132806 raw sequence was obtained for each sample.

QIIME 2 was followed in the analysis of 16s metagenomics sequencing data (Bolyen et al., 2019). The sequencing center provided fastq primer-trimmed MiSeq paired-end reads, which were processed using DADA2 (version 1.26.0) for denoising in order to obtain amplicon sequence variations (ASVs), (Salomon et al., 2023).We utilized modified versions of the universal primers (16S-V4F:GTGCCAGCMGCCGGTAA) and 16S-V4R: GGACTACHVGGGTWTCTAATCC). DADA2 (version 1.26.0) A naive Bayes taxonomy classifier was used to categorize each ASV against the SILVA 138.1 reference database in order to create the taxonomy table (Wasimuddin et al., 2020). Using R Studio (version 4.2), statistical analysis of the filtered sequences were carried out. Population diversity for the alpha diversity study with in sample was measured and calculated using the shannon entropy and the Chao1 index, a richness estimate index that calculates estimated ASV based on observed ASV from sequence data (Chiu et al., 2014).

## RESULTS AND DISCUSSION

This study uncovered the variety and composition of Mustard Aphid's gut microbiome. 49 ASVs were found in a sample after the resultant merged count and taxonomy table was filtered, merged, chimera removed, and further curated.104788 sequences were retrieved, with a mean of 103883 reads per sample. Using a Naive Bayes classifier, all of these sequence reads were categorized into Operational Taxonomic Units (OTUs), which accounted for all of the sequences. 10 distinct bacterial genera belonging to 7 phyla, 10 classes, 10 orders, and 10 families and 10 species were produced by the classification using the classify-sklearn method database. The highly abundant fraction of *L. erysimi's* microbiome (>1% abundance) was dominated by 99% Gammaproteobacteria, while the Alphaproteobacteria and Proteobacteria accounted for 1% and 2% of the total (Fig. 1) Most prevalent genera was *Buchnera* accounted for 90% while the Aeromonas accounted for 10% (Fig. 2) The three most prevalent species being *Aeromonas caviae* and *Achromobacter piechaudii* both endosymbiont *caviae* and *piechaudii* accounted for 50% (Fig. 3) Alpha diversity index used to describe species richness within sample (Whittaker 1972) by using Shannon's entropy (H') which was 0.13 showed lesser uncertainty (Duan et al., 2020) (Fig. 4). The

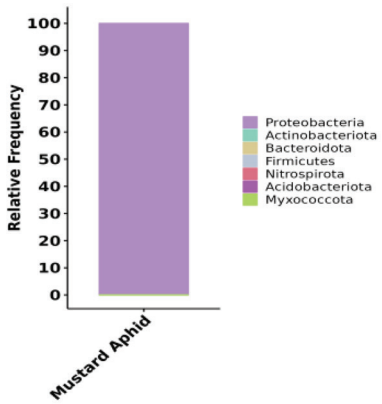


Fig. 1. The bacteriome classification at second level of taxonomy with Proteobacteria being the most dominant phyla reported in *L. erysimi*

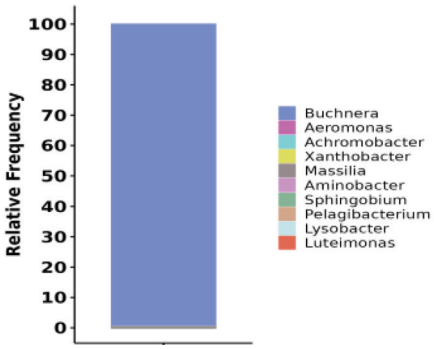


Fig. 2. The bacterial composition in the gut of *L. erysimi* at the genus level with highest abundance of *Buchnera*

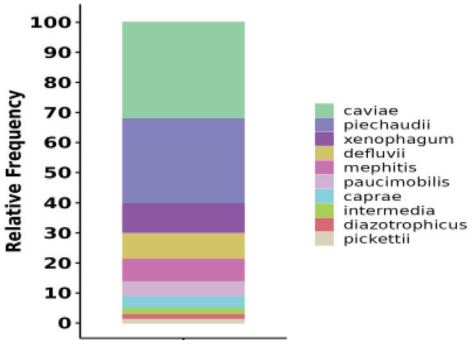


Fig. 3. The bacterial composition in the gut of *L.erysimi* at the species level with highest abundance of *caviae* followed by *piechudii* and *xenophagum*

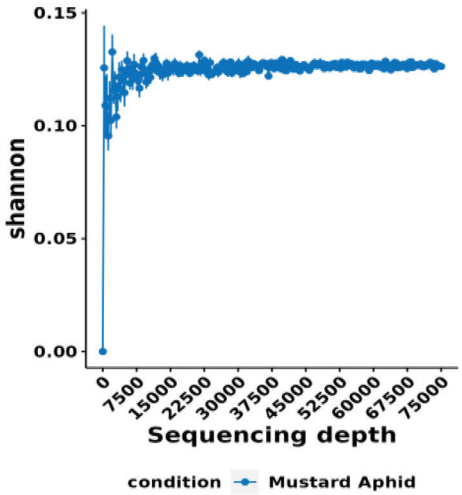


Fig. 4. Shannon Alfa refraction at 0.15 displaying good coverage of gut bacteriome of *L. erysimi*

sequencing depth index (Good's coverage) exceeded 99.9%, indicating that the sequencing depth is sufficient to demonstrate the microbial diversity of the samples and can reflect the actual situation of microorganisms carried by the samples. The species refraction curves also reflected the abundance of the *L. erysimi* microbial community. The refraction curves attained saturation (0.16) in every sample that was tested, indicating that the sequencing depth was sufficient to cover the majority of species.

Aphids feed on plants, however because these plants lack critical amino acids, the primary supply of nutrition is insufficient for the aphids' growth and reproduction (Nalam et al., 2019). A symbiotic bacterium called *Buchnera* synthesizes vitamins and other nutrients for the host insect, influencing its growth and development. (Manzano-Marı et al., 2020; Liu et al., 2023). According to Janda and Abbott (2010), *A. caviae* are a kind of facultative anaerobic bacteria that are Gram-negative and commonly found in insects, humans, and fish which contains antimicrobial resistance (AMR) genes (Roy et al., 2013; Dubey et al., 2022). According to Koga et al. (2003), mild rifampicin therapy preferentially removed the obligatory symbiont *Buchnera* from the host insect, whereas ampicillin treatment selectively eradicated the facultative symbiont. *A. piechaudii* which is linked to endophytes (Patel et al., 2023) but may reportedly enter insect guts with completelyunknown consequences. Aphids are more likely to pick up these bacteria from the plant surface or from the honeydew, where they are most likely expelled (Stavrinides et al., 2009), (Sabri et al., 2013). If these bacteria are allowed to move into the phloem, they may also be consumed from plant sap (Caspi-Fluger et al., 2012). To determine the significance of aphid gut companions, more research is necessary. Thus due to AMR genes present in facultative bacteria allowing *L. erysimi* to be an evolutionarily adaptable herbivore (Shigenobu and Wilson, 2011). In summary, these investigations, discovered that bacterial communities linked to mustard a *Buchnera* symbiont predominates in aphid hosts.

The current work raises a number of significant questions about how microbial communities affect the aphid hosts they inhabit. Notably, they include the nature and relative forces that influence the structure of microbial communities, with special emphasis to ecological considerations and transmission patterns, as well as the effective relevance and role of gut associates. NGS (Next generation sequencing) technology, together with more concentrated biological and functional investigations, will undoubtedly aid in the resolution of these important concerns.

## ACKNOWLEDGEMENTS

Authors thank the Sam Higginbottom University of Agriculture Technology and Sciences, Prayagraj, Uttar Pradesh, India for providing all the necessary facilities.

**FINANCIAL SUPPORT**

The study received no financial support.

**AUTHOR CONTRIBUTION STATEMENT**

AS wrote the paper and crated the experimental procedure. AK and PKS translated the manuscript.

**CONFLICT OF INTEREST**

No conflict of interest.

**REFERENCES**

Andrews Simon. 2010. FastQC: a quality control tool for high throughput sequence data. 1-1.

Bolyen Evan, Jai Ram Rideout, Matthew R Dillon, Nicholas A Bokulich, Christian C Abnet, Gabriel A. Al-Ghalith, Harriet Alexander et al. 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nature Biotechnology 37(8): 852-857.

Buchner Paul. Endosymbiosis of animals with plant microorganisms. *(No Title)* (1965).

Burke Gaelen, Oliver Fiehn, Nancy Moran. 2010. Effects of facultative symbionts and heat stress on the metabolome of pea aphids. The ISME Journal 4(2): 242-252.

Callahan Benjamin J, Joan Wong, Cheryl Heiner, Steve Oh, Casey M. Theriot, Ajay S Gulati, Sarah K. McGill, Michael K Dougherty. 2019. High-throughput amplicon sequencing of the full-length 16S rRNA gene with single-nucleotide resolution. Nucleic Acids Research 47(18): e103-e103.

Caspi-Fluger, Ayelet, Moshe Inbar, Netta Mozes-Daube, Nurit Katzir, Vitaly Portnoy, Eduard Belausov, Martha S Hunter, Einat Zchori-Fein. 2012. Horizontal transmission of the insect symbiont Rickettsia is plant-mediated. Proceedings of the Royal Society B: Biological Sciences 279, no. 1734 (2012): 1791-1796.

Charles Hubert, Hajime Ishikawa. 1999. Physical and genetic map of the genome of *Buchnera*, the primary endosymbiont of the pea aphid Acyrthosiphon pisum." Journal of Molecular Evolution 48: 142-150.

Chiu Chun-Huo, Yi-Ting Wang, Bruno A Walther, Anne Chao. 2014. An improved nonparametric lower bound of species richness via a modified good–turing frequency formula. Biometrics 70(3): 671-682.

Chu Chia-Ching, Joseph L Spencer, Matías J Curzi, Jorge A Zavala, Manfredo J Seufferheld. 2013. Gut bacteria facilitate adaptation to crop rotation in the western corn rootworm. Proceedings of the National Academy of Sciences 110(29): 11917-11922.

Colella Stefano, Nicolas Parisot, Pierre Simonet, Karen Gaget, Gabrielle Duport, Patrice Baa-Puyoulet, Yvan Rahbé et al. 2018. Bacteriocyte reprogramming to cope with nutritional stress in a phloem sap feeding hemipteran, the pea aphid *Acyrthosiphon pisum*. Frontiers in Physiology 9: 1498.

Costopoulos Kelly, Jennifer L Kovacs, Alexandra Kamins, Nicole M Gerardo. 2014. Aphid facultative symbionts reduce survival of the predatory lady beetle *Hippodamia convergens*. BMC Ecology 14: 1-7.

Duan M, Wu X, Teame T, Hao Q, Ding Q, Liu H, Ran C, Zhang Z. 2020. Use of a paraprobiotic and postbiotic feed supplement (HWF™) improves the growth performance, composition and function of gut microbiota in hybrid sturgeon (*Acipenser baerii x Acipenser schrenckii*). Fish & Shellfish Immunology 104: 36-45.

Dubey Saurabh, Eirill Ager-Wick, Jitendra Kumar, Indrani Karunasagar, Iddya Karunasagar, Bo Peng, Øystein Evensen, Henning Sørum, Hetron M. Munang'andu. 2022. *Aeromonas* species isolated from aquatic organisms, insects, chicken, and humans in India show similar antimicrobial resistance profiles. Frontiers in Microbiology 13: 1008870.

Engel Philipp, Nancy A Moran. 2013. The gut microbiota of insects–diversity in structure and function. FEMS Microbiology Reviews 37(5): 699-735.

Engel Philipp, Vincent G. Martinson, Nancy A Moran. 2012. Functional diversity within the simple gut microbiota of the honey bee. Proceedings of the National Academy of Sciences 109(27): 11002-11007.

Janda J Michael, Sharon L Abbott. 2010. The genus *Aeromonas*: taxonomy, pathogenicity, and infection. Clinical Microbiology Reviews 23(1): 35-73.

Jousselin Emmanuelle, Astrid Cruaud, Gwenaelle Genson, François Chevenet, Robert G. Foottit, Armelle Cœur d'acier. 2013. Is ecological speciation a major trend in aphids? Insights from a molecular phylogeny of the conifer-feeding genus *Cinara*. Frontiers in Zoology 10: 1-18.

Koga Ryuichi, Xian-Ying Meng, Tsutomu Tsuchida, Takema Fukatsu. 2012. Cellular mechanism for selective vertical transmission of an obligate insect symbiont at the bacteriocyte–embryo interface. Proceedings of the National Academy of Sciences 109(20): E1230-E1237.

Koga Ryuichi, Tsutomu Tsuchida, Takema Fukatsu. 2003. Changing partners in an obligate symbiosis: a facultative endosymbiont can compensate for loss of the essential endosymbiont *Buchnera* in an aphid." Proceedings of the Royal Society of London. Series B: Biological Sciences 270 1533: 2543-2550.

Kozich James J, Sarah L Westcott, Nielson T Baxter, Sarah K Highlander, Patrick D Schloss. 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Applied and Environmental Microbiology 79(17): 5112-5120.

Leonardo Teresa E, Gladys T Muiru. 2003. Facultative symbionts are associated with host plant specialization in pea aphid populations. Proceedings of the Royal Society of London. Series B: Biological Sciences 270, no. suppl_2: S209-S212.

Liu Hui-ping, Qiao-yan Yang, Jing-xing Liu, Inzamam Ul Haq, Yan Li, Qiang-yan Zhang, Kotb A. Attia, Asmaa M Abushady, Chang-zhong Liu, Ning Lv. 2023. Host plant-mediated effects on *Buchnera* symbiont: implications for biological characteristics and nutritional metabolism of pea aphids (*Acyrthosiphon pisum*). Frontiers in Plant Science 14: 1288997.

Łukasik P, H Guo, M Van Asch, J Ferrari, H C J Godfray. 2013. Protection against a fungal pathogen conferred by the aphid facultative endosymbionts Rickettsia and Spiroplasma is expressed in multiple host genotypes and species and is not influenced by co-infection with another symbiont. Journal of Evolutionary Biology 26(12): 2654-2661.

Manzano-Marín, Alejandro, Armelle Coeur d'acier, Anne-Laure Clamens, Céline Orvain, Corinne Cruaud, Valérie Barbe, Emmanuelle Jousselin. 2020. Serial horizontal transfer of vitamin-biosynthetic genes enables the establishment of new nutritional symbionts in aphids' di-symbiotic systems. The ISME Journal 14(1): 259-273.

Montllor Clytia B, Amy Maxmen, Alexander H Purcell. 2002. Facultative bacterial endosymbionts benefit pea aphids *Acyrthosiphon pisum* under heat stress. Ecological Entomology 27(2): 189-195.

Moran Nancy A, Howard Ochman, Tobin J Hammer. 2019. Evolutionary and ecological consequences of gut microbial communities. Annual Review of Ecology, Evolution, and Systematics 50: 451-475.

Nalam Vamsi, Joe Louis, Jyoti Shah. 2019. Plant defense against aphids, the pest extraordinaire. Plant Science 279: 96-107.

Oliver Kerry M, Patrick H Degnan, Gaelen R Burke, Nancy A Moran. 2010. Facultative symbionts in aphids and the horizontal transfer of ecologically important traits. Annual Review of Entomology 55: 247-266.

Patel Hiren K, Riddhi A Makampara, Rishee K Kalaria, Maitrik P Joshi. 2023. Endophytes: A novel tool for sustainable agriculture." In Endophytic Association: *What, Why and How*, pp. 37-55. Academic Press.

Quast Christian, Elmar Pruesse, Pelin Yilmaz, Jan Gerken, Timmy Schweer, Pablo Yarza, Jörg Peplies, Frank Oliver Glöckner. 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools." Nucleic acids research 41(D1): D590-D596.

Rintala Anniina, Sami Pietilä, Eveliina Munukka, Erkki Eerola, Juha-Pekka Pursiheimo, Asta Laiho, Satu Pekkala, Pentti Huovinen. 2017. Gut microbiota analysis results are highly dependent on the 16S rRNA gene target region, whereas the impact of DNA extraction is minor. Journal of biomolecular techniques: JBT 28(1): 19.

Rosenberg Eugene, Gil Sharon, Ilil Atad, Ilana Zilber-Rosenberg. 2010. The evolution of animals and plants via symbiosis with microorganisms. Environmental Microbiology Reports 2(4): 500-506.

Roy Rudra Prasad, Min Bahadur, Sudip Barat. 2013. Isolation, identification and Antibiotic resistance of *Aeromonas* spp. and Salmonella spp. from the fresh water loach, *Lepidocephalichthys guntea* and water of Terai River Lotchka, West Bengal, India. Zoologica Poloniae 58(1-2): 5.

Sabri Ahmed, Sophie Vandermoten, Pascal D Leroy, Eric Haubruge, Thierry Hance, Philippe Thonart, Edwin De Pauw, Frédéric Francis. 2013. Proteomic investigation of aphid honeydew reveals an unexpected diversity of proteins. PloS one 8(9): e74656.

Salomon Jeffrey D, Haowen Qiu, Dan Feng, Jacob Owens, Ludmila Khailova, Suzanne Osorio Lujan, John Iguidbashian et al. 2023. Piglet cardiopulmonary bypass induces intestinal dysbiosis and barrier dysfunction associated with systemic inflammation. Disease models and Mechanisms 16(5): dmm049742.

Schmitz Antonin, Caroline Anselme, Marc Ravallec, Christian Rebuf, Jean-Christophe Simon, Jean-Luc Gatti, Marylène Poirié. 2012. The cellular immune response of the pea aphid to foreign intrusion and symbiotic challenge. e42114.

Shigenobu Shuji, Alex CC Wilson. 2011. Genomic revelations of a mutualism: the pea aphid and its obligate bacterial symbiont. Cellular and Molecular Life Sciences 68: 1297-1309.

Simonet Pierre, Gabrielle Duport, Karen Gaget, Michèle Weiss-Gayet, Stefano Colella, Gérard Febvay, Hubert Charles, José Viñuelas, Abdelaziz Heddi, Federica Calevro. 2016. Direct flow cytometry measurements reveal a fine-tuning of symbiotic cell dynamics according to the host developmental needs in aphid symbiosis. Scientific Reports 6(1): 19967.

Stavrinides John, Jodi K McCloskey, Howard Ochman. 2009. Pea aphid as both host and vector for the phytopathogenic bacterium *Pseudomonas syringae*. Applied and Environmental Microbiology 75(7): 2230-2235.

Takeshita Kazutaka, Yu Matsuura, Hideomi Itoh, Ronald Navarro, Tomoyuki Hori, Teruo Sone, Yoichi Kamagata, Peter Mergaert, Yoshitomo Kikuchi. 2015. *Burkholderia* of plant-beneficial group are symbiotically associated with bordered plant bugs (Heteroptera: Pyrrhocoroidea: Largidae). Microbes and Environments 30(4): 321-329.

Tringe Susannah Green, Christian Von Mering, Arthur Kobayashi, Asaf A Salamov, Kevin Chen, Hwai W Chang, Mircea Podar et al. 2005. Comparative metagenomics of microbial communities. Science 308, 5721: 554-557.

Tsuchida Tsutomu, Ryuichi Koga, Akiko Fujiwara, Takema Fukatsu. 2014. Phenotypic effect of Candidatus *Rickettsiella viridis*, a facultative symbiont of the pea aphid (*Acyrthosiphon pisum*), and its interaction with a coexisting symbiont. Applied and Environmental Microbiology 80(2): 525-533.

Tsuchida Tsutomu, Ryuichi Koga, Takema Fukatsu. "Host plant specialization governed by facultative symbiont. Science 303, 5666: 1989-1989.

Wasimuddin Klaus Schlaeppi, Francesca Ronchi, Stephen L. Leib, Matthias Erb, Alban Ramette. 2020. Evaluation of primer pairs for microbiome profiling from soils to humans within the One Health framework. Molecular ecology resources 20(6): 1558-1571.

Zhang Bo, Sean P Leonard, Yiyuan Li, Nancy A Moran. 2019. Obligate bacterial endosymbionts limit thermal tolerance of insect host species. Proceedings of the National Academy of Sciences 116(49): 24712-24718.

(Manuscript Received: December, 2023; Revised: June, 2024;
Accepted: July, 2024; Online Published: August, 2024)
Online First in www.entosocindia.org and indianentomology.org Ref. No. e24838