Endosymbion microbials low abundance of Nilaparvata *lugens* (Stal) (Hemiptera: Delphacidae) from Indonesia rice production center based on metagenomic study using 16Sr RNA

Awaluddin[1*], Dadang[1], *Ruly* Anwar[1], and Giyanto[1]

[1]Department of Plant Protection, Faculty of Agriculture, IPB University, Indonesia

Abstract. Insects have a long history of association with endosymbiont microbes. There are several indications that endosymbiont microbes are associated with insects and participate in the degradation of organic and inorganic molecules. This research aims to identify the profile of abundance and diversity of endosymbiont microbes associated with *Nilaparvata lugens* in Konawe (Southeast Sulawesi), Pasuruan (East Java), Klaten (Central Java), and Karawang (West Java) using a metagenomic study approach. We analyzed 15 pairs samples of *N. lugens* obtained from several regions in Indonesia. Endosymbionts were identified using a full-length primer 27F and 1492R targeting the 16S rRNA gene. The results showed that the proteobacteria phylum dominated all samples. *Arsenophonus nasoniae* (Morganellaceae; Enterobacterales) contributed the highest abundance (50-62%) based on total NumRead nucleotide base sequences from each sample. The bacterial diversity in these four samples was classified as moderate. This research showed that there were similarities in the communities and profiles of endosymbiont microbial constituents in each region, and this study becomes the basis for further research regarding the role of endosymbiont microbes on their hosts.

1 Introduction

The brown rice planthopper *Nilaparvata lugens* Stal (Hemiptera: Delphacidae) is the primary pest of rice plants, especially in Indonesian rice production centers, such as Konawe (Southeast Sulawesi), Pasuruan (East Java), Klaten (Central Java), and Karawang (West Java) districts since 1970. *N. lugens* attacks cause significant losses and pose a severe challenge to achieving food self-sufficiency in all rice production centers. Pest control efforts still focus on the use of pesticides. To survive, these insects are able to fight toxic substances, including pesticides. Recent findings suggest that microbes living on insects as endosymbionts can protect their hosts from toxins. The symbiosis that is formed is a defense mechanism against pesticides and the host's natural enemies. Microbial communities

* Corresponding author: ipb2019awaluddin@apps.ipb.ac.id

associated with insects are dynamic and responsive to various stressors [1]. Microbiota, such as insects, can be subject to natural selection pressure caused by exposure to insecticides [2]. Pesticide-degrading bacteria are widespread in nature and have been identified in various insect orders, such as Hemiptera [3], Diptera [4] and Coleoptera [5].

Endosymbiont microbes are microbes that beneficial to the host in specific contexts [6]. Endosymbiont microbes have been discovered and contribute to nutrition, development, reproduction, speciation, and defence against natural enemies of insects host [7]. However, the definition of endosymbiont microbes differs when examining symbiosis on ecological versus evolutionary timescales. From an ecological point of view, endosymbiont microbes have a positive effect on fitness host by playing directly role in colonizing the host [8]. Some insect fitness traits are strongly influenced by host microorganisms [9]. The association of insects with the microbiota is significant for evolution, influences insects in food discovery and specific functions, such as protection from enemies and intra-species communication [10,11]. Endosymbiont microbes associated with the host also enable insects to feed on foods that are difficult to digest and poor in nutrients [12]. Itoh [13] stated that endosymbiont microbes play an essential role in detoxifying secondary metabolite compounds that are harmful to the host.

Metagenomics is an approach to obtaining genetic information from the total number of microbes in an environment without having to isolate and culture the cells or microbes [14]. This term comes from statistical meta-analysis and genomics, which are the latest advances in microbial genomics, PCR amplification, and gene cloning directly from the environment [15]. An important result of metagenomic studies is that researchers can estimate the abundance of a taxon or functional group based on the determination of the nucleic acid possessed by the organism. Therefore metagenomic studies constitute next-generation sequencing (NGS). Microbes that have the 16S DNA gene (a measure of DNA commonly found in bacteria) will be able to be read accurately and entirely with this NGS technology. The approach used for metagenomic DNA extraction is usually similar to the approach used for DNA extraction from pure cultures. The basic preparation process in DNA extraction that must be carried out includes destroying or grinding the sample, lysis cell, separating DNA cell, and DNA purification [16]. Next, the purity quality of sample was measured in each microbus using Nanodrop.

The close relationship between *N. lugens* and endosymbiont microbes makes it interesting to study their profile and abundance. Currently, there is no information on the endosymbiont microbial community associated with *N. lugens* in rice production centers in Indonesia, so it is necessary to conduct a metagenomic study of endosymbiont microbes as an initial step in the management of this pest. This research aims to identify the community, abundance, and diversity of endosymbiont microbes using a metagenomic approach. Next, a functional analysis was carried out based on the sequence of the endosymbiont microbial community in *N. lugens*.

2 Methods

The research was carried out in Konawe (Southeast Sulawesi), Pasuruan (East Java), Klaten (Central Java), Karawang (West Java) Regencies and the Physiology Insect and Toxicology Laboratory, Plant Protection Department, Agriculture Faculty, IPB University. This research was carried out in June-August 2022

2.1 Sample preparation

N. lugens samples from each location sampling, were put into jars containing rice plants one-week-old. At each sampling location, location coordinates were recorded using a Global

Positioning System (GPS) tool. Preparation of analytical samples obtained from each research location was carried out by soaking 15 pairs of male and female *N. lugens* imago from each location in 5% NaOCl and rinsing with sterile distilled water five times to remove surface microbial contamination [17]. After the washing process, the sample was put into a 1.5 ml tube, and 96% alcohol was added. Next, the sample is sent to a nucleotide sequencing service company for the DNA extraction process and whole genome 16S rRNA sequencing.

2.2 DNA extraction and 16S rRNA gene sequencing of the *Nilaparvata lugens* endosymbiont microbe

The genomic DNA (gDNA) extraction process was carried out using a commercial DNA extraction kit, gSYNC DNA Extraction Kit (Gene aid, GS100). It was carried out according to the with modifications protocol listed. The extracted DNA concentrate was quantified and tested for purity using NanoDrop and a fluorometer (Qubit). Next, a library preparation process was carried out, which included amplification of the 16S rRNA region using full-length primers 27F and 1492R, to which special adapters were added (Table 1).

The amplification results were added with a special adapter, which acts as a sequencing ID using a kit from Oxford Nanopore Technology. After the library preparation process, the next stage is the Nanopore sing sequencing process operated with the MinKNOW 22.05.7 program. The base-calling process was carried out using the Guppy 6.1.5 program with a high-accuracy model [18]. The quality level of data in FASTQ format (Nanopore sequence results) was observed by visualizing it using the Nanoplot program, and then a quality filtering process was carried out using the Nanofit program [19,20].

Table 1. The primary used to amplify DNA of the endosymbiont microbe *Nilaparvata lugens* and their base sequences.

Primary name	Primary nucleotide sequence (5'-3')	Target genome
27F/1492R	AGAGTTTGATCCTGGCTCAG/ ACGGTTACCTTGTTAGGACTT	16S rRNA (full length)

2.3 Data analysis

The 16S rRNA gene sequence results with good quality were identified using the Centrifuge 1.0.4 program [21]. The bacterial and Archie index was constructed using the NCBI 16S Ref Seq database. The identification results were analyzed for the profile, diversity, and abundance of the endosymbiont microbial species *N. lugens* in each sample using the α diversity index such as Shannon-Wienner (H'), and the Simpson index (1/D), each of it can be calculated with the following formula:

$$= -\sum_{i=1}^{s} P_i \, (lnP_i) \qquad (1)$$

H' = Shannon-Wiener diversity index
Pi = proportion of first species in the community

The value of the Shannon-Wiener index ranges from 1.5 – 3.5 [22]. The higher H' index value, the higher species diversity and ecosystem stability at a location. The criteria used to interpret the Shannon-Wiener index value are:

$H' \geq 3$ = high species diversity
$1 < H' < 3$ = moderate species diversity
$H' \leq 1$ = low species diversity

$$D = 1/\sum_{i=1}^{s} P_i^2 \quad (2)$$

D = Simpson diversity index
s = number of morphospecies
Pi = proportion of first species in the community

3 Results and discussion

3.1 The endosymbiont microbes abundance

The results of endosymbiont microbes 16S rRNA gene sequencing analysis of samples obtained from each region were grouped at the operational taxonomic unit (OTU) based level on the number sequence of nucleotide bases (NumRead) determined top 10 taxa from phylum to species as presented (Fig. 1). OTUs at the phylum level from all sampling areas were found to be 99% Proteobacteria, and Karawang had the highest phylum diversity of 9 phylum, although the population was below 1%. Of the 34 thousand bacterial groups of endosymbiont microbial populations based on NumRead on *N. lugens* samples from Konawe, 99% of the phylum Proteobacteria has 33.8 thousand NumRead of the dominant species, that was *Arsenophonus nasoniae* (Morganellaceae; Enterobacterales) with a population of 24.1 thousand and the smallest species population is Pantoea brenneri (Erwiniaceae; Enterobacterales), that was 264 (Fig. 1A) while samples from the other three regions tend to show relatively the same quantity of node width (Fig. 1B,C and D). The relatively similar distribution pattern of endosymbiont microbes is thought to be caused by relatively similar sampling topography, namely below ≤ 200 meters below sea level (MDPL) and a uniform plant cultivation system.

The genus Arsenophonus was first described because of its ability to exert a male-killing effect on the parasitic wasp Nasonia vitripennis [23,24]. Previous research has stated the relationship of the genus Arsenophonus with different ranging hosts from parasitism to mutualism [25], including male killing [23], and mandatory nutritional supplementation [26], genome analysis suggests a potential role for *A. nasoniae* in synthesizing vitamin B for the host [27].

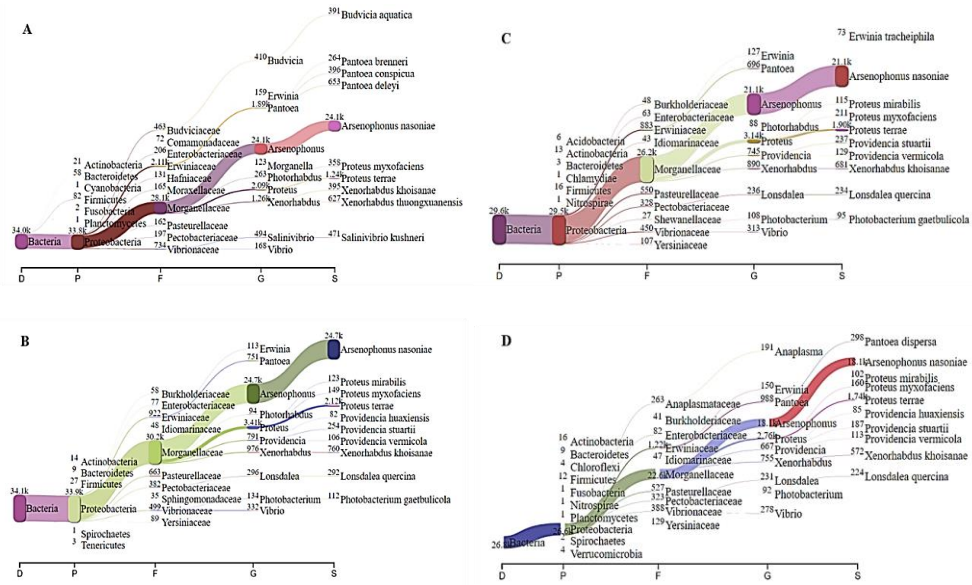

Fig. 1. Visualization of the top 10 endosymbiont microbes of *Nilaparvata lugens*. (A) in Konawe, (B) Pasuruan, (C) Klaten, and (D) Karawang. Node width is proportional to quantity to describe hierarchical changes between taxonomic nodes over time (phylum, famili, genus and species).

The results of the 16S rRNA gene sequencing of Konawe samples at the species level based on NumRead found 38.953 species accounts 62% from the phylum Proteobacteria, namely the species *A. nasoniae* with a NumRead of 24.071 accounts. In contrast, the other species were ≤ 3%. The population abundance of the species *A. nasoniae* accounts for 63% of the Gammaproteobacteria class, 68% of the Enterobacterales order, and 79% of the Morganellaceae family (Fig. 2A). In the Pasuruan sample, 45.098 species accounts were found, 55% from the phylum Proteobacteria, 24.697 accounts of the *A. nasoniae* species, and ≤ 5% for other species. The population abundance of the species *A. nasoniae* accounts for 55% of the Gammaproteobacteria class, 61% of the Enterobacterales order, and 70% of the Morganellaceae family (Fig. 2B).

The Klaten sample has 39.464 species accounts consisting of 54% of the phylum Proteobacteria, the *A. nasoniae* species has a NumRead count of 21.066 accounts, while the other species are ≤ 6%. The population abundance of the *A. nasoniae* species accounts for 54% of the Gammaproteobacteria class, 59% of the Enterobacterales order, and 69% of the Morganellaceae family (Fig. 2C). The species level based on NumRead in the Karawang sample found 36.274 species accounts, 50 % from phylum Proteobacteria, the species *A. nasoniae* with a NumRead of 18.121 accounts, while the other species were ≤ 5 %. The population abundance of the species *A. nasoniae* accounts for 52% of the Gammaproteobacteria class, 57% of the Enterobacterales order, and 69% of the Morganellaceae family (Fig. 2D).

In general, the endosymbiont microbial population of the species *A. nasoniae* has a relatively high population abundance with a presentation of ≥ 50%. These results indicate that this microbial species is an endosymbiont associated with *N. lugens.* On the other hand, it strengthens the notion that endosymbiont microbes play an important role in the metabolism and sustainability of their host populations in each region. However, we cannot yet confirm whether endosymbiont microbial populations with relatively small abundances

have the same function [28], stated that certain endosymbiont microbial populations of the genus *Arsenophonus* strains were associated with insecticidal susceptibility to *N. lugens* hosts through changes in UGT and P450 gene expression, transcriptome and metabolome analysis showed downregulation of xenobiotic metabolism, and increased accumulation of amino acids in hosts infected with *Arsenophonus* type S. A wide variety of functions mediated by endosymbiont microbes associated with insects contribute to the overall fitness of their insects host. However, the main contribution of endosymbiont microbes is related to their ability to provide nutrients. Secondary bacterial symbionts enhance host immunological responses to entomophagy [29] and entomopathogens [30].

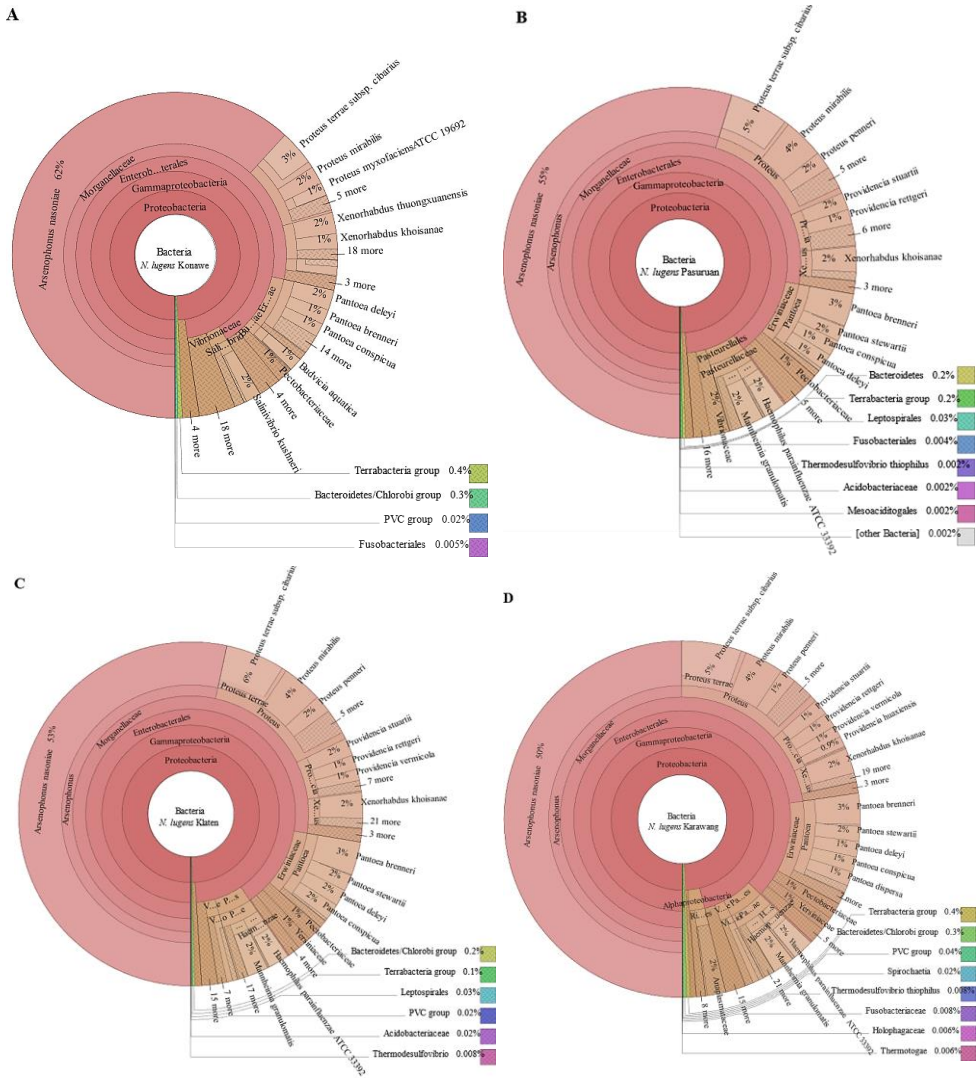

Fig 2. Visualization of the relative abundance of the endosymbiont microbial species *Nilaparvata lugens*. (A) in Konawe, (B) Pasuruan, (C) Klaten, and (D) Karawang.

The ability of microorganisms to utilize pesticides as a carbon source, depends on encoding the biochemical systems required to deal with those substrates [31]. Temperature and pH, nutrient availability, chemical concentrations, and bacterial population sizes all

influence pesticide metabolism [32,33]. The chemical composition and complexity of a pesticide play a role in how quickly and effectively bacteria use it as a food source [34].

3.2 Shannon-Wiener and Simpson diversity index in metagenome studies of the microbial endosymbiont *Nilaparvata lugens*

Based on the ACE and Chao l nonparametric estimator index, estimates of the diversity of endosymbiont microbial species show a high level of agreement between the observed and expected numbers based on NumReads of nucleotide sequences. This result was proven by the incidence (Chao1) and Abundance-based coverage estimator (ACE) values of more than 96% (Table 2).

Table 2. Shannon and Simpson's diversity index in a metagenomic study of the endosymbiont microbe *Nilaparvata lugens.*

Sample N. lugens	Observed	Chao1	se.Chao1	ACE	se.ACE	Shannon	Simpson
Konawe	645	1133	78.11	1095.73	19.250	1.79	0.49
Pasuruan	400	671.5	55.83	659.627	14.230	1.49	0.46
Klaten	419	701.31	55.65	702.14	15.10	1.57	0.48
Karawang	480	772.89	53,60	814.999	17.077	1.81	0.52

The results showed that the diversity and complexity index for endosymbiont microbial species was highest in the Karawang sample with values of 1.81 and 0.52 while the lowest was in the sample from Pasuruan with values of 1.49 and 0.46. The description of the *N. lugens* endosymbiont microbial species community in each sampling area explains that both Simpson and Shannon indices used to measure alpha diversity concepts are similar or less sensitive to differences in species richness based on NumReads of nucleotide sequences. The lower the Shannon index value, the lower the diversity, and the higher the Simpson index value (range: 0-1), the more complex the community and species diversity will be [22].

Endosymbiont microbial populations in the digestive tract of insects belonging to the phylum Firmicutes, Proteobacteria, Actinobacteria [35] and Bacterioidetes can influence host biology [33]. Several research results have found that the microbial community consists mainly of Firmicutes [36], especially Enterococus[37]. According to various studies, bacteria in the digestive tract of insects have been shown to break down various pesticides and interfere with the effectiveness of pesticides used to control their targets [38].

Several families of Proteobacteria (Enterobacteria, Pseudomonads and Burkholderia) can break down acetate chlorpyrifos [10], trichlorfon [9], lambda-cyhalothrin [13], and spinosad, respectively [33]. Likewise, Actinobacteria and Firmicutes bacteria have also been shown to play a role in the process of removing toxins from the environment [39, 40]. Based on previous research, there are several symbionts in the digestive tract of insects which detoxify pesticides imidacloprid [41], neonicotinoid [42], organophosphate [43] and carboxylesterase [40]. The results of this study are the basis for further research to examine the effect of insecticide exposure on fitness responses and endosymbiont microbial profiles in *N. lugens*.

4 Conclusion

The highest abundance was obtained in the phylum Proteobacteria (50-62%), which was dominated by the species *A. nasoniae* with a species population presentation of ≤ 50%. Bacterial diversity in the four samples was classified as moderate, with a high level of dominance. The highest diversity and complexity index for endosymbiont microbial species

was found in the Karawang sample with an abundance value of 1.81 and an evenness value of 0.52.

References

1. S. Zhang, J. Shu, H. Xue, W. Zhang, Y. Zhang, Y. Liu, L. Fang, Y. Wang, and H. Wang, MSystems **5**, 1 (2020)
2. M. Akami, X. Ren, Y. Wang, A. Mansour, S. Cao, X. Qi, A. Ngakou, R. A. N. Ngane, and C. Y. Niu, Int. J. Trop. Insect Sci. **42**, 2127 (2022)
3. Y. Kikuchi, M. Hayatsu, T. Hosokawa, A. Nagayama, K. Tago, and T. Fukatsu, in *Proc. Natl. Acad. Sci. U. S. A.* (National Academy of Sciences, 2012), pp. 8618–8622
4. D. Cheng, Z. Guo, M. Riegler, Z. Xi, G. Liang, and Y. Xu, Microbiome **5**, 1 (2017)
5. M. Akami, N. Y. Njintang, O. A. Gbaye, A. A. Andongma, M. A. Rashid, C. Y. Niu, and E. N. Nukenine, Sci. Rep. **9**, 1 (2019)
6. G. C. Drew, E. J. Stevens, and K. C. King, Nat. Rev. Microbiol. **19**, 623 (2021)
7. P. Baumann, Annu. Rev. Microbiol. **59**, 155 (2005)
8. T. J. Hammer, J. G. Sanders, and N. Fierer, FEMS Microbiol. Lett. **366**, 1 (2019)
9. L. G. De Almeida, L. A. B. De Moraes, J. R. Trigo, C. Omoto, and F. L. Cônsoli, PLoS One **12**, 1 (2017)
10. Y. Kikuchi and I. Yumoto, Appl. Environ. Microbiol. **79**, 2088 (2013)
11. P. Suárez-Moo, M. Cruz-Rosales, E. Ibarra-Laclette, D. Desgarennes, C. Huerta, and A. Lamelas, Front. Microbiol. **11**, 1 (2020)
12. H. Salem and M. Kaltenpoth, Annu. Rev. Entomol. **67**, 201 (2022)
13. H. Itoh, K. Tago, M. Hayatsu, and Y. Kikuchi, Nat. Prod. Rep. **35**, 434 (2018)
14. Y. X. Liu, Y. Qin, T. Chen, M. Lu, X. Qian, X. Guo, and Y. Bai, Protein Cell **12**, 315 (2021)
15. P. D. Schloss and J. Handelsman, Curr. Opin. Biotechnol. **14**, 303 (2003)
16. P. H. Godwin, B. G. Xue, C. R. Kuske, and M. K. Sears, Ann. Appl. Biol. **124**, 27 (1994)
17. M. Tang, L. Lv, S. Jing, L. Zhu, and G. He, Appl. Environ. Microbiol. **76**, 1740 (2010)
18. R. R. Wick, L. M. Judd, and K. E. Holt, Genome Biol. **20**, 1 (2019)
19. W. De Coster, S. D'Hert, D. T. Schultz, M. Cruts, and C. Van Broeckhoven, Bioinformatics **34**, 2666 (2018)
20. A. B. Nygaard, H. S. Tunsjø, R. Meisal, and C. Charnock, Sci. Rep. **10**, 1 (2020)
21. D. Kim, L. Song, F. P. Breitwieser, and S. L. Salzberg, Genome Res. **26**, 1721 (2016)
22. G. M. Svendsen, M. O. Reinaldo, M. A. Romero, G. Williams, A. Magurran, S. Luque, and R. A. González, Mar. Ecol. Prog. Ser. **638**, 149 (2020)
23. J. H. Werren, S. W. Skinner, and A. M. Huger, Science (80-.). **231**, 990 (1986)
24. R. L. Gherna, J. H. Werren, W. Weisburg, R. Cote, C. R. Woese, L. Mandelco, and D. J. Brenner, Int. J. Syst. Bacteriol. **41**, 563 (1991)
25. L. Mouton, M. Thierry, H. Henri, R. Baudin, O. Gnankine, B. Reynaud, E. Zchori-Fein, N. Becker, F. Fleury, and H. Delatte, BMC Microbiol. **12**, 1 (2012)

26. E. F. Kirkness, B. J. Haas, W. Sun, H. R. Braig, M. A. Perotti, J. M. Clark, S. H. Lee, H. M. Robertson, R. C. Kennedy, E. Elhaik, D. Gerlach, E. V. Kriventseva, C. G. Elsik, D. Graur, C. A. Hill, J. A. Veenstra, B. Walenz, J. M. C. Tubío, J. M. C. Ribeiro, J. Rozas, J. S. Johnston, J. T. Reese, A. Popadic, M. Tojo, D. Raoult, D. L. Reed, Y. Tomoyasu, E. Krause, O. Mittapalli, V. M. Margam, H. M. Li, J. M. Meyer, R. M. Johnson, J. Romero-Severson, J. P. VanZee, D. Alvarez-Ponce, F. G. Vieira, M. Aguadé, S. Guirao-Rico, J. M. Anzola, K. S. Yoon, J. P. Strycharz, M. F. Unger, S. Christley, N. F. Lobo, M. J. Seufferheld, N. K. Wang, G. A. Dasch, C. J. Struchiner, G. Madey, L. I. Hannick, S. Bidwell, V. Joardar, E. Caler, R. Shao, S. C. Barker, S. Cameron, R. V. Bruggner, A. Regier, J. Johnson, L. Viswanathan, T. R. Utterback, G. G. Sutton, D. Lawson, R. M. Waterhouse, J. C. Venter, R. L. Strausberg, M. R. Berenbaum, F. H. Collins, E. M. Zdobnov, and B. R. Pittendrigh, Proc. Natl. Acad. Sci. U. S. A. **107**, 12168 (2010)

27. H. W. Fan, J. B. Lu, Y. X. Ye, X. P. Yu, and C. X. Zhang, Insect Sci. **23**, 478 (2016)

28. R. Pang, M. Chen, L. Yue, K. Xing, T. Li, K. Kang, Z. Liang, L. Yuan, and W. Zhang, PLOS Genet. **14**, 1 (2018)

29. C. Vorburger, L. Gehrer, and P. Rodriguez, Biol. Lett. **6**, 109 (2010)

30. J. Jaenike and R. Unckless, Science (80-.). (2010)

31. A. A. Lourthuraj, M. R. Hatshan, and D. S. Hussein, Environ. Res. **205**, 112553 (2022)

32. R. J. Russell, C. Scott, C. J. Jackson, R. Pandey, G. Pandey, M. C. Taylor, C. W. Coppin, J. W. Liu, and J. G. Oakeshott, Evol. Appl. **4**, 225 (2011)

33. A. F. F. Gomes, C. Omoto, and F. L. Cônsoli, J. Pest Sci. (2004). **93**, 833 (2020)

34. M. Hubbard, R. K. Hynes, M. Erlandson, and K. L. Bailey, Sustain. Chem. Process. **2**, 1 (2014)

35. L. R. P. Voirol, E. Frago, M. Kaltenpoth, M. Hilker, and N. E. Fatouros, Front. Microbiol. **9**, 1 (2018)

36. B. Chen, B. S. Teh, C. Sun, S. Hu, X. Lu, W. Boland, and Y. Shao, Sci. Reports 2016 61 **6**, 1 (2016)

37. M. F. Higuita Palacio, O. I. Montoya, C. I. Saldamando, E. García-Bonilla, H. Junca, G. E. Cadavid-Restrepo, and C. X. Moreno-Herrera, J. Insect Sci. **21**, 10 (2021)

38. S. L. Ramya, T. Venkatesan, K. S. Murthy, S. K. Jalali, and A. Varghese, J. Environ. Biol. **37**, 611 (2016)

39. J. A. Siddiqui, M. M. Khan, B. S. Bamisile, M. Hafeez, M. Qasim, M. T. Rasheed, M. A. Rasheed, S. Ahmad, M. I. Shahid, and Y. Xu, Front. Microbiol. **13**, 1 (2022)

40. S. L. Ramya, T. Venkatesan, K. S. Srinivasa Murthy, S. K. Jalali, and A. Verghese, Brazilian J. Microbiol. **47**, 327 (2016)

41. S. Pang, Z. Lin, Y. Zhang, W. Zhang, N. Alansary, S. Mishra, P. Bhatt, and S. Chen, Toxics **8**, 1 (2020)

42. C. J. A., D. B. A., B. J. P., and R. Gregor, MBio **10**, 1 (2019)

43. B. Chen, N. Zhang, S. Xie, X. Zhang, J. He, A. Muhammad, C. Sun, X. Lu, and Y. Shao, Environ. Int. **143**, 1 (2020)