JOSH GREEN, M.D.
GOVERNOR | KE KIAʻĀINA

SYLVIA LUKE
LIEUTENANT GOVERNOR | KA HOPE KIAʻĀINA

STATE OF HAWAIʻI | KA MOKUʻĀINA ʻO HAWAIʻI
DEPARTMENT OF LAND AND NATURAL RESOURCES
KA ʻOIHANA KUMUWAIWAI ʻĀINA

P.O. BOX 621
HONOLULU, HAWAII 96809

DAWN N.S. CHANG
CHAIRPERSON
BOARD OF LAND AND NATURAL RESOURCES
COMMISSION ON WATER RESOURCE MANAGEMENT

LAURA H.E. KAAKUA
FIRST DEPUTY

M. KALEO MANUEL
DEPUTY DIRECTOR - WATER

AQUATIC RESOURCES
BOATING AND OCEAN RECREATION
BUREAU OF CONVEYANCES
COMMISSION ON WATER RESOURCE MANAGEMENT
CONSERVATION AND COASTAL LANDS
CONSERVATION AND RESOURCES ENFORCEMENT
ENGINEERING
FORESTRY AND WILDLIFE
HISTORIC PRESERVATION
KAHOOLAWE ISLAND RESERVE COMMISSION
LAND
STATE PARKS

May 1, 2023

VIA EMAIL

Lori Kato
Staff Attorney
Office of Information Practices

RE: Notice of Appeal of Sunshine Law Complaint (S APPEAL 23-9)

Dear Ms. Kato:

Please see the Board of Land and Natural Resources' response to the Sunshine Law Complaint from Tina Lia (S APPEAL 23-9) below.

Factual Background

On March 24, 2023, the Board of Land and Natural Resources ("Board") held a Board meeting pursuant to Haw. Rev. Stat. ("HRS") Chapter 92. The specific agenda item at issue was C-2: Request Approval of Final Environmental Assessment and Authorization for the Chairperson to Issue a Finding of No Significant Impact for the "Suppression of Invasive Mosquito populations to Reduce Transmission of Avian Malaria to Threatened and Endangered Forest Birds on East Maui" ("C-2"). The agenda is attached as Exhibit "1." The Board heard testimony on C-2 pursuant to HRS § 92-3. Ms. Lia submitted written testimony, attached hereto as Exhibit "2," and also provided oral testimony, beginning in at 1:18:04 in the audio file available at the link provided in Exhibit "3." At the end of her oral testimony, Ms. Lia requested a contested case hearing. Ex. 3 at 1:19:52. The Chair paused testimony and asked Ms. Lia to clarify if she was requesting the Board decide her

contested case hearing request prior to voting, and Ms. Lia confirmed she was requesting the Board vote on her oral request prior to voting. *Id.* at 1:20:20-1:20:36. The Chair asked Ms. Lia to clarify the basis for her contested case hearing request. *Id.* at 2:16:37. Board Member Yoon moved to deny Ms. Lia's oral request for contested case hearing because she failed to give a basis for her request. *Id.* at 2:18:22. The Board then voted to deny her oral request for contested case hearing. *Id.* at 2:19:57. Ms. Lia filed a written petition for contested case hearing on April 3, 2023, attached hereto as Exhibit "4." The Board has not yet decided the written petition.

Compliance with HRS Chapter 92

When a person makes an oral request for contested case hearing during a Chapter 92 meeting prior to decisionmaking, the Board stops the meeting to determine whether or not the person is entitled to the contested case hearing. Haw. Admin. R. ("HAR") § 13-1-29(a). The Board is not required hold a hearing prior to deciding if the request should be granted. HAR § 13-1-29.1. If the Board denies the contested hearing request, the person may follow-up in writing within 10-calendar days to request a contested case hearing. HAR § 13-1-29(a). That written petition is generally placed on a future agenda and the Board generally decides whether to grant the request based on the written petition and any testimony submitted (written or oral) for that meeting. But the Board is not required to hold a hearing. HAR § 13-1-29.1. Here, Ms. Lia made an oral request. The Board stopped the meeting and voted to deny her oral request. Ms. Lia followed up in writing within 10-business days with her written petition. The Board will decide that written request under a new agenda item at a future meeting.

Evidence

Exhibit "1" is a true and correct copy of the agenda containing item C-2 from the March 24, 2023, Board meeting.

Exhibit "2" is a true and correct copy of Tina Lia's written testimony on item C-2 from the March 24, 2023, Board meeting.

Exhibit "3" is a true and correct copy of the link to the official meeting minutes for the March 24, 2023, Board meeting.

Exhibit "4" is a true and correct copy of the written petition for contested case hearing filed by Tina Lia on April 3, 2023.

If you have any further questions, please contact me at (808)587-1496 or bin.c.li@hawaii.gov. Thank you.

Sincerely,

Bin C. Li
Administrative Proceedings Coordinator

cc: Dawn N.S. Chang, Chairperson, Board of Land and Natural Resources
Lainie Berry, Wildlife Manager, Department of Land and Natural Resources
Miranda C. Steed, Deputy Attorney General

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LAND
STATE PARKS

AGENDA
FOR THE MEETING OF THE
BOARD OF LAND AND NATURAL RESOURCES

DATE: MARCH 24, 2023
TIME: 9:15 AM
LOCATION: In person at 1151 Punchbowl St, Room 132 (Kalanimoku Building) online via ZOOM, livestream via YouTube

Board members, staff, applicants, and testifiers can choose to participate either in-person, via ZOOM or by telephone. Members of the public are encouraged to wear a mask if attending in-person.

Meeting materials are available for public review in advance of the meeting at:
http://www.dlnr.hawaii.gov/meetings

The meeting will be livestreamed at:
http://youtube.com/c/boardoflandandnaturalresourcesdlnr

To provide in person testimony:
Attend live at 1151 Punchbowl St. Room 132 (Kalanimoku Building), Honolulu, Hawaii

To provide video testimony:
Send your request to ***blnr.testimony@hawaii.gov***
Include your name and the agenda item on which you would like to testify. Once your request has been received,you will receive an email with the Zoom link. Requests may be made during the meeting.

To provide oral testimony by telephone: : +1 669 900 6833
Meeting ID: 840 8093 2937
Passcode: 0LB2Gu
Note: To unmute, press *6.

Written Testimony:
Interested persons can submit written testimony in advance of each meeting that will be distributed to Board Members prior to the meeting. Submit written testimony to ***blnr.testimony@hawaii.gov*** *or via postal mail to the Board of Land and Natural Resources at P.O. Box 621, Honolulu, Hawaii*

96809. We request written testimony be submitted no later than 24 hours prior to the meeting to ensure time for Board Member review. Late written testimony will be retained as part of the record and distributed to Board Members as soon as practicable, but we cannot ensure that Board Members will receive it with sufficient time for review prior to decision-making.

The Board may go into Executive Session pursuant to Section 92-5(a)(4), Hawaii Revised, Statutes, in order to consult with its attorney on questions and issues pertaining to the Board's powers, duties, privileges, immunities, and liabilities.

Individuals requiring special assistance or auxiliary aids or services (e.g., sign language interpreter), please contact staff at least 72 hours prior to the meeting at 808.587.0404 so that arrangements can be made.

In some of the matters before the Board, a person may wish to request a contested case hearing. If such a request is made before the Board's decision, then the Board will consider the request first - before considering the merits of the item before it. A person who wants a contested case may also wait until the Board decides the issue, then request the contested case after the decision. It is up to you. Any request must be made in writing within ten days. If no request for contested case is made, the Board will make a decision. The Department will treat the decision as final and proceed accordingly.

A. SUMMARY MINUTES

1. Approval of the January 13, 2023, Summary Meeting Minutes.

C. DIVISION OF FORESTRY AND WILDLIFE

1. Request Approval to Negotiate and Sign a Contract(s) between Department of Land and Natural Resources Division of Forestry and Wildlife, for the Management of the Department of Land and Natural Resources Hawai'i Island Trail Stewards Program.

2. Request Approval of Final Environmental Assessment and Authorization for the Chairperson to Issue a Finding of No Significant Impact for the "Suppression of Invasive Mosquito populations to Reduce Transmission of Avian Malaria to Threatened and Endangered Forest Birds on East Maui".

3. Acceptance of Hearing Master's Report, Set Aside Lands as Forest Reserves, Natural Area Reserves, and Wildlife Sanctuaries Statewide:

   FOREST RESERVES ON:

   KAUAI: (4) 4-4-001:002, (4) 5-6-002:001

OAHU: (1) 4-8-013:013, (1) 6-9-001:004, (1) 9-1-017:158, (1) 9-9-010:052 and (1) 9-9-011:002

MAUI: (2) 1-1-001:001, (2) 1-1-001:021, (2) 1-1-001:031, (2) 1-1-001:052, (2) 1-1-002:005, (2) 1-1-002:006, (2) 1-1-008:001, (2) 1-1-008:005, (2) 1-2-001:039, (2) 1-3-003:017, (2) 1-3-006:007, (2) 1-4-011:003, (2) 1-4-011:004, (2) 1-4-012:019, (2)1-5-002:004, (2) 1-5-008:004, (2) 1-5-010:008, (2) 1-5-011:007, (2) 1-5-011:012, (2) 1-5-011:014, (2) 1-5-011:015, (2) 1-5-011:029, (2) 1-6-002:009, (2) 1-7-002:011, (2) 1-7-002:044, (2) 1-7-003:013, (2) 2-4-016:001, (2) 2-9-001:020, (2) 2-9-001:033, (2) 2-9-002:012, (2) 2-9-010:008, (2) 2-9-010:009, (2) 2-9-010:012, (2) 2-9-010:021, (2)2-9-010:022, (2) 2-9-011:008, (2) 2-9-011:013, (2) 2-9-013:004, (2) 2-9-013:012, (2) 2-9-013:014, (2) 2-9-013:016, (2) 2-9-013:017, (2) 2-9-013:018, (2) 2-9-013:020, (2) 3-1-001:001, (2) 3-1-001:021, (2) 3-1-001:029, (2) 3-1-006:003, (2) 3-6-001:014 (por), (2) 4-4-004:002, (2) 4-4-004:006, (2) 4-4-004:009, (2) 4-4-004:011, (2) 4-4-004:019, (2) 4-4-007:006, (2) 4-5-021:004, (2) 4-5-021:023, (2) 4-8-001:001 (por), (2) 4-8-002:002, (2) 4-8-002:008, (2) 4-8-002:039, (2) 4-8-003:008 (por), (2) 4-8-003:040,

HAWAII: (3) 2-4-008:035, (3) 5-1-001:006, (3) 8-7-014:015, (3) 4-4:014:004, (3) 6-2:001:003 (por), and (3) 9-6-007:002

NATURAL AREA RESERVES ON:

MOLOKAI: (2) 6-1-001:002 (por)

MAUI: (2) 1-3-003:026, (2) 1-3-005:002, (2) 1-3-003:001, (2) 1-8-001:005, (2) 2-1-003:050 (por), (2) 2-1-004:075, (2) 2-1-004:110, (2) 2-1-006:010, (2) 2-1-006:077, (2) 2-1-006:078

WILDLIFE SANCTUARIES ON:

MAUI: (2) 3-1-001:014 and (2) 3-1-002:011

D. LAND DIVISION

1. Approve Evaluation Committee's Recommendation for Selection of Proposal Submitted by Savio SB Growth Venture LLC in Response to Request for Qualifications / Request for Proposals for Lease of Improved Public Lands; Issuance of Right-of-Entry Permit to Savio SB Growth Venture LLC for Purposes of Assessing the Physical Condition of the Property and Preparing Construction Documents; Waiakea, South Hilo, Hawaii, Tax Map Key: (3) 2-1-005:020.

2. Denial of Petition for Contested Case Hearing filed by 69 Railroad, LLC on September 30, 2022, Regarding the Board Action of September 23, 2022 Agenda Item D-2, Approved as Amended: *Amend Prior Board Action of February 11, 2022, Item D-2, Approved as Amended, Consent to Sublease General Lease No. S-3624, 69 Railroad, LLC, Lessee, to Self Storage Hilo LLC, Covan World-Wide Moving, Incorporated, C.A.R.S.S. LLC, Provision Solar, Inc., Tracey Gapol, Charles Wagner & Erin Wagner, Hawaii Behavioral Health, LLC, Whitney & Arnessa Iranon, Mr. & Mrs. Charles and Erin Wagner, McCully Works, Inc., and Lamar Pacheco, Sublessees, Waiakea, South Hilo, Hawaii, Tax Map Key: (3) 2-1-012:026.*

   Authorize the Chairperson to Approve and Execute a Development Agreement for a 30-Year Extension of Lease Term and to Execute the Lease Extension Document, General Lease No. S-3624, 69 Railroad, LLC, Lessee; Waiakea, South Hilo, Hawaii, Tax Map Key: (3) 2 -1-012: 026.

   Approve Mediated Settlement of Rent Reopening Dispute Pursuant to Mediation Agreement for the Periods of 2026-2046, General Lease No. S-3624, 69 Railroad, LLC, Lessee; Waiakea, South Hilo, Hawaii, Tax Map Key: (3) 2 -1-012: 026.

   The purposes of the amendments are to: (a) provide for the updating of certain lease extension terms and the development agreement for the 30-year extended lease term, and (b) extend the time for completion of improvements required under development agreement and lease extension from approximately 10 ½ months after the Board approval of February 11, 2022 to 18 months after the execution of the development agreement.

   Pursuant to Section 92-5(a) (4), Hawaii Revised Statutes (HRS), the Board may go into Executive Session in order to consult with its attorney on questions and issues pertaining to the Board's powers, duties, privileges, immunities and liabilities.

3. Issuance of Right-of-Entry Permit to Hawaii Explosives & Pyrotechnics, Inc. for Private Event Aerial Fireworks Display at Duke Kahanamoku Lagoon on April 18, 2023, Waikiki, Honolulu, Oahu, Tax Map Key: (1) 2-3-037: portion of 021.

4. Issuance of Direct Lease to Maunalua Fishpond Heritage Center for Conservation, Biological and Cultural Revitalization, Education and Scientific Research Purposes, Honolulu, Oahu, Tax Map Key: (1) 3-7-002:018 and 077.

5. Issuance of Direct Lease to Hui o Hau`ula for Community Services and Activities Purposes; Koolauloa, Oahu, Tax Map Key: (1) 5-4-014:003.

6. Report on Board of Land and Natural Resources' Questions to the Department of Agriculture and Hawaii Land & Livestock LLC Relating to the Cancellation of Governor's Executive Order No. 4584 to the Department of Agriculture for Agriculture Purposes, Honouliuli, Ewa, Oahu, Tax Map Key: (1) 9-1-031:001.

7. Grant of Perpetual Non-Exclusive Easement to the City and County of Honolulu on behalf of the Honolulu Authority for Rapid Transportation (HART) for Elevated Guideway Purposes; After-the-Fact Approval of Annual Renewal of Right-of-Entry to HART for Each Year Since the Board Approval of the Initial Issuance of the Right-of-Entry at Board's Meeting of February 10, 2012, Item D-6, Waipahu, Ewa, Oahu, Tax Map Key: (1) 9-4-008: Portion of 025.

F. DIVISION OF AQUATIC RESOURCES

1. Request for Approval of Policy for Using the Division of Aquatic Resources Logo on Informational Signs.

K. OFFICE OF CONSERVATION AND COASTAL LANDS

1. Denial of Contested Case Request HA 23-1 by Laulani Teale regarding the Approval of the Mauna Kea Comprehensive Management Plan 2022 Supplement: Management Actions Update located at the Mauna Kea Science Reserve, Kaʻohe, Hāmakua, Hawaiʻi Tax Map Keys: (3) 4-4-015:009, (3) 4-4-015:0012, and (3) 4-4-015:001.

   The Board may go into Executive Session pursuant to Section 92-5(a)(4), Hawaii Revised, Statutes, in order to consult with its attorney on questions and issues pertaining to the Board's powers, duties, privileges, immunities, and liabilities.

NON-ACTION ITEM

2. Informational Briefing by the City and County of Honolulu Department of Parks and Recreation regarding the Carrying Capacity Studies for the Hanauma Bay Nature Preserve located at the Hanauma Bay Nature Preserve, Oʻahu Tax Map Keys: (1) 3-9-012:002, (1) 3-9-012:010, (1) 3-9-012:012, (1) 3-9-012:014, (1) 3-9-012:016, and adjacent Submerged Lands.

3. Request to Amend Condition #12 of Conservation District Use Permit (CDUP) OA-2957 regarding Reporting Requirements for the Carrying Capacity Studies by the City and County of Honolulu Department of Parks and Recreation at the Hanauma Bay Nature Preserve located at Hanauma Bay Nature Preserve, Oʻahu Tax Map Keys: (1)

3-9-012:002, (1) 3-9-012:010, (1) 3-9-012:012, (1) 3-9-012:014, (1) 3-9-012:016, and adjacent Submerged Lands.

M. OTHERS

1. Issuance of a Revocable Permit for Commercial Use of a T-Hangar for Storage of Aircraft and a Maintenance Hangar to Support a Member-Based Aeronautical Flying Club, Belle Pacific Air, L.L.C., Daniel K. Inouye International Airport, Tax Map Key: (1) 1-1-076: Portion of 020

2. Issuance of a Revocable Permit for Aircraft Parking, Novictor Aviation LLC, Daniel K. Inouye International Airport, Tax Map Key: (1) 1-1-076: Portion of 023.

3. Issuance of a Revocable Permit for Paved, Improved Land to Store Above-Ground Storage Tanks to Provide Fuel to Airport Tenants, Signature Flight Support LLC, Daniel K. Inouye International Airport, Tax Map Key: (1) 1-1-003:001 (Portion).

4. Issuance of a Revocable Permit for Paved, Improved Land for U.S. Postal Mail Sortation and Ground Equipment Staging in Support of its Fixed-Based Operation, Signature Flight Support LLC, Daniel K. Inouye International Airport, Tax Map Key: (1) 1-1-072: 011 (Portion).

5. Issuance of Revocable Permit for Paved, Open Equipment Parking to Support Ground Handling Operations, Signature Flight Support LLC, Daniel K. Inouye International Airport, Tax Map Key: (1) 1-1-072: 069 (Portion) and (1) 1-1-072: 070.

6 . Issuance of a Revocable Permit for the Installation of Six (6) Avigilon Cameras at Gates E1, E3, E5, E6, E7 and E9 as Part of its Airline Operations, Southwest Airlines Co., Daniel K. Inouye International Airport, Tax Map Key: (1) 1-1-003: Portion 050 .

7. Issuance of a Revocable Permit for Aircraft Parking, Buddy R. Wilson, Lihue Airport, Tax Map Key: (4) 3-5-001: Portion of 008.

8. Issuance of a Revocable Permit for Commercial Use of a T-Hangar for Storage of Aircraft and a Maintenance Hangar to Support an Aeronautical Maintenance Business, PMX Aviation Services LLC, Daniel K. Inouye International Airport, Tax Map Key: (1) 1-1-076: Portion of 020.

9. Request for Authorization to Issue three Month-to-Month Revocable Permits to McCabe, Hamilton & Renny Company, Limited, for a Maintenance Shop, two Office Trailers, and a Work Area, situated at Pier 5, Kalaeloa Barbers Point Harbor, Island of Oahu, Tax Map Key Nos. (1) 9-1-014: Portion of 024, (1) 9-1-014:Portion of 036, and (1) 9-1-014: Portion of 062 (P), Governor's Executive Order No. 3383.

10. Request for Authorization to Conduct Public Auction for 65-year Lease for use of the former Hawaii Maritime Center and surrounding area, situated at Pier 7, to occupy and use the premises for a Museum and related facilities, with a Harbor and Wharfage Operation Component and Pier Space Availability for Harbor and Wharfage Operations, Honolulu Harbor, Island of Oahu, Tax Map Key Nos. (1) 2-1-001: Portion of 057 and (1) 2-1-001:Portion of 058 , Governor's Executive Order No. 3542; and Issuance of a Month-to-Month Revocable Permit (RP) to be replaced with an executed Lease.

From: Tina Lia
To: DLNR.BLNR.Testimony
Cc: DLNR.CO.PublicDLNR
Subject: [EXTERNAL] BLNR Meeting 3/24/23 9:15am Testimony Agenda Item C-2: Oppose
Date: Wednesday, March 22, 2023 11:21:23 PM
Attachments: 2023_0324_Testimony_Tina_Lia_Attachments.pdf

RE: C-2 Request Approval of Final Environmental Assessment and Authorization for the Chairperson to Issue a Finding of No Significant Impact for the "Suppression of Invasive Mosquito populations to Reduce Transmission of Avian Malaria to Threatened and Endangered Forest Birds on East Maui"

We're opposed to the request for approval of the Final Environmental Assessment for the planned biopesticide mosquito releases on Maui. This project is an experiment on our island home, and the outcome is admittedly unknown. The Final Environmental Assessment[1] does not adequately address the serious risks of this plan or the concerns of the public.

Sufficient research has not been conducted to assess the risks of horizontal transmission[2,3,4], increased pathogen infection[5], evolutionary events[2], population replacement[6], or accidental release of females[6]. The Final Environmental Assessment attempts to minimize the possibility of *Wolbachia* bacteria causing mosquitoes to become more capable of spreading diseases like avian malaria[5] and West Nile virus[7]. Scientific studies document these risks.

An Environmental Risk Assessment for this biopesticide has not been conducted by the EPA to determine the environmental, ecological, and human health risks; and the significant environmental consequences of the project have not been adequately studied. **This plan may actually cause the extinction of endangered native birds, and it could impact human health**.

Landscape level control of *Culex quinquefasciatus* mosquitoes using this Incompatible Insect Technique (IIT) has never been done before. Even with *Aedes* mosquitoes, the largest project area was 724 acres[8]. Federal documentation connected to this project confirms that "although used world-wide for human health, *Wolbachia* IIT is a novel tool for conservation purposes and its degree of efficacy in remote forest landscapes is unknown."[9] Additionally, the species planned for use in this project, *Culex quinquefasciatus*, has never been used for a stand-alone IIT field release.[8] It is inaccurate to state that *Wolbachia* IIT is being used for mosquito suppression globally. The majority of countries using *Wolbachia* mosquitoes through the World Mosquito Program[10] are using the method of population replacement, not suppression[11]. These are two entirely different techniques.

This project may have also been improperly segmented per HAR § 11-200-7[12] (replaced 2019). The revised rule, HAR § 11-200.1-10[13] – Multiple or phased actions, provides:

A group of actions shall be treated as a single action when:

(1) The component actions are phases or increments of a larger total program;

(2) An individual action is a necessary precedent to a larger action;

(3) An individual action represents a commitment to a larger action; or

(4) The actions in question are essentially identical and a single EA or EIS will adequately address the impacts of each individual action and those of the group of actions as a whole.

On June 17, 2022, Board of Land and Natural Resources Chairperson Suzanne D. Case signed an exemption notice for "Mosquito Control Research Using *Wolbachia*-based Incompatible Insect Technique."[14] The Final Environmental Assessment, dated March 24, 2023, states that the Department of Land and Natural Resources filed the exemption notice "to conduct limited import of male mosquitoes for preliminary transport trials and mark release recapture studies."[1]

The Hawaii Environmental Policy Act (HEPA) Citizen's Guide (2014)[15] states: "A proposed action must be described in its entirety and cannot be broken up into component parts, which if each is taken separately, may have minimal impact on the environment. Segmenting a project generally is forbidden." Because the project has been improperly segmented in this way, there have been no details or analysis of the preliminary trials or the mark release recapture studies. There has been no disclosure as to what type of mosquito is being transported, where the mosquitoes are being transported from, and whether or not the mosquitoes are being tested for pathogens prior to transport. We demand that all actions of the mosquito project – including trial imports, mark release recapture studies, and field releases – be addressed in one Environmental Impact Statement.

The Advisory Committee on Plants and Animals' recommendation to approve import and release of *Culex quinquefasciatus* mosquitoes[16] should be null and void due to the conflicts of interest of committee members pursuant to HRS 84-14[17]. The Ethics Guide for State Board and Commission Members[18] states that members must not take official action affecting a business in which they have "financial interest." "Financial interest" in a business includes "employment." Whether a business can be a government agency is unstated. The following members of the Advisory Committee on Plants and Animals unanimously voted (7/0) on June 9, 2022 to recommend approval of the import permit[16]:

- Darcy Oishi, Committee Chairperson, Hawaii Department of Agriculture (HDOA)
- Dr. Maria Haws, Professor of Aquaculture, Pacific Aquaculture & Coastal Research Center, University of Hawaii at Hilo
- Cynthia King, Entomologist, Division of Forestry & Wildlife, Department of Land & Natural Resources (DLNR), Ex Officio Member Designated Representative

- Gracelda Simmons, Environmental Management Program Manager, Hawaii Department of Health, Ex Officio Member Designated Representative

- Thomas Eisen, Planner, Environmental Review Program, Department of Business, Economic Development and Tourism, Ex Officio Member Designated Representative

- Joshua Fisher, Wildlife Biologist, U. S. Fish and Wildlife Service (USFWS)

- Dr. Samuel Ohu Gon III, Senior Scientist and Cultural Advisor, The Nature Conversancy – Hawaii (TNC)

Of the seven voting members' agencies, only those of Thomas Eisen and Darcy Oishi are not partner agencies in *Birds, Not Mosquitoes*. As employees of partner agencies, Dr. Maria Haws (University of Hawaii), Cynthia King (DLNR), Gracelda Simmons (Hawaii Department of Health), Joshua Fisher (USFWS), and Dr. Samuel Ohu Gon III (TNC) all have conflicts of interest.

Both Dr. Samuel Ohu Gon III[19] and Cynthia King[20] are also members of the *Birds, Not Mosquitoes* steering committee. The purpose of the steering committee, as stated in the National Fish and Wildlife Foundation Hawaii Conservation Business Plan[21], includes coordinating permits for this project. These are additional conflicts of interest, particularly for Dr. Samuel Ohu Gon III, who, with his vote, has taken official action affecting a business in which he has financial interest.

The Final Environmental Assessment (EA) does not address the concern of accidental pathogen introduction. The U.S. Department of the Interior Strategy for Preventing the Extinction of Hawaiian Forest Birds[9] confirms that The Nature Conservancy has contracted with mosquito lab Verily Life Sciences. There is no mention of this contract in the EA. No documented assurances have been made that Verily Life Sciences will be testing mosquitoes for human diseases or avian diseases to ensure that they are pathogen-free prior to shipping to Hawaii.

As this project involves the interstate transport of *Culex* mosquitoes, a known vector of poultry diseases, we are concerned about impacts to local poultry farms and egg production in Hawaii. Has the USDA inspected the Verily Life Sciences insectary? There is no mention in the EA of a USDA permit (e.g., OV VS 16-6 permit from APHIS) for the interstate transport of poultry pathogen vectors by a California shipper. The USDA Animal and Plant Health Inspection Service (APHIS)[22] states:

> "The Veterinary Services, Organisms and Vectors (OV) Permitting Unit regulates the importation into the United States, and interstate transportation, of organisms and vectors of **pathogenic diseases** of livestock and **poultry**.
>
> The Code of Federal Regulations, in 9 CFR, §122.2[23], mandates that '**no organisms or vectors shall be** imported into the United States or **transported from one State** or Territory or the District of Columbia **to another State** or

Territory or the District of Columbia **without a permit**.' "

Given that interstate transport of the vector (live *Culex)* is occurring from Maui to Verily Life Sciences' lab in South San Francisco, California[24], and those *Culex* may contain a highly contagious poultry pathogen, namely avian pox virus[25], this movement needs a federal permit. Additionally, the return trip from California to Hawaii[24] would require a federal permit. Lab mosquitoes are blood-fed from sources that are not identified in the EA, potentially including bird blood. These mosquitoes could be transporting avian pathogens back to Hawaii.

Even though male mosquitoes don't bite, male *Culex* mosquitoes are known to spread viruses to female mosquitoes through mating (e.g., St. Louis encephalitis virus[26]), as has been shown for dengue virus in *Aedes albopictus*[27].

The EA's assertion that released mosquitoes pose no risk to human health is based on unsound science. The 2010 article by Popovici et al.[28] cited in the EA has been discredited by the EPA. The EPA Human Studies Review Board met in 2018[29], and the following question was posed:

> "Is the research described in the published article 'Assessing key safety concerns of a *Wolbachia*-based strategy to control dengue transmission by Aedes mosquitoes' scientifically sound, providing reliable data for the purpose of contributing to a weight of evidence determination in EPA's assessment of the risks to human health associated with releasing *Wolbachia*-infected mosquitoes?"[30]

The Board's response states: "The Board concluded that the research described in the article by Popovici et al. was not scientifically sound and does not provide reliable data to contribute to a weight of evidence determination for assessment of human health risks due to release of *Wolbachia*-infected mosquitoes."[30]

The Hawaii Department of Agriculture has applied for an EPA Emergency Exemption[8] for use of the mosquitoes without going through regulatory safety processes. The EPA application is still under review, and the biopesticide mosquitoes have not been approved for emergency release. The Board of Land and Natural Resources cannot approve this Final Environmental Assessment and declare before the public that there is a Finding of No Significant Impact (FONSI) when there is still a possibility that the EPA will deny the Emergency Exemption due to safety concerns. This biopesticide cannot be approved for release when its safety is still under review by the EPA.

Additional concerns not adequately addressed in the Final Environmental Assessment: lack of adequate detail as required by HEPA[15]; failure to identify the *Wolbachia* strain planned for use in this project; failure to identify and describe the mark release recapture study as a proposed action; failure to adequately identify the mosquito packages planned for release into the environment; failure to adequately address the effects on the environment from the release of biodegradable packages with an unknown decay rate; failure to identify biosecurity protocols; failure to

adequately address viewscape impacts, noise disturbances to forest bird breeding and nesting, and significant environmental consequences, including impacts to the untrammeled, natural qualities of the wilderness character; failure to adequately address the potential negative impacts of introducing an invasive species to the islands; failure to identify the origin of biopesticide mosquitoes for this project as Palmyra Atoll[8]; failure to identify the origin of *Wolbachia* bacteria for the project as Kuala Lumpur in Malaysia[8]; failure to identify the strain of *Wolbachia* bacteria planned for import in connection with this project that does not exist on these islands[31,32]; failure to address the concerns of tropical disease and vector expert Dr. Lorrin Pang (private citizen) regarding the serious risks of this project[33]; failure to adequately study or address the impacts to endangered native Hawaiian hoary bats, native dragonflies, and endangered native damselflies; failure to study and address biopesticide wind drift; failure to adequately address Environmental Justice (human health impacts of this project have not been adequately studied, and the proposed action would impact ethnographic resources and traditional cultural practices); failure to conduct a feasibility study to provide a detailed analysis that considers all of the critical aspects of the proposed project in order to determine the likelihood of it succeeding; and failure to establish, under the precautionary principle, that the proposed activity will not result in significant harm.

Further, per HRS §171-4 (d)[34], BLNR Chair Dawn N.S. Chang and Board Member Vernon Char must recuse themselves from participating in any discussion or voting in this matter, given that they have clear conflicts of interest. Chang is employed by the DLNR[35], a lead agency in the mosquito project. Char is employed by a law firm[36] whose clients include The Nature Conservancy[37], another lead partner in the project.

Hawaii Unites has launched a petition to "Demand an Environmental Impact Statement for the Experimental Mosquito Release on Maui"[38] which has received more than 2,500 signatures. We have yet to receive a response from any of the decision makers.

We're opposed to the authorization for the Chairperson to issue a Finding of No Significant Impact (FONSI). The scope, risks, and experimental nature of the plan require detailed, comprehensive studies and documentation of the impacts to our native birds, wildlife, environment, and public health. The subject action will have a significant effect. **We demand an Environmental Impact Statement (EIS).**

Mahalo,
Tina Lia
Founder and President
Hawaii Unites
HawaiiUnites.org

REFERENCES:

"Suppression of Invasive Mosquito Populations to Reduce Transmission of Avian

Malaria to Threatened and Endangered Forest Birds on East Maui" (State of Hawaii Department of Land and Natural Resources, 3/24/23) https://dlnr.hawaii.gov/wp-content/uploads/2023/03/C-2-1.pdf

"*Wolbachia* infection in wild mosquitoes (Diptera: Culicidae): implications for transmission modes and host-endosymbiont associations in Singapore" – Huicong Ding, Huiqing Yeo, Nalini Puniamoorthy (BMC, 12/9/20) https://parasitesandvectors.biomedcentral.com/articles/10.1186/s13071-020-04466-8

"*Wolbachia* Horizontal Transmission Events in Ants: What Do We Know and What Can We Learn?" – Sarah J. A. Tolley, Peter Nonacs, Panagiotis Sapountzis (Frontiers in Microbiology, 3/6/19) https://www.frontiersin.org/articles/10.3389/fmicb.2019.00296/full

"The Intracellular Bacterium *Wolbachia* Uses Parasitoid Wasps as Phoretic Vectors for Efficient Horizontal Transmission" – Muhammad Z. Ahmed, Shao-Jian Li, Xia Xue, Xiang-Jie Yin, Shun-Xiang Ren, Francis M. Jiggins, Jaco M. Greeff, Bao-Li Qiu (National Center for Biotechnology Information, National Library of Medicine, 2/12/15) https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4347858/

"*Wolbachia* Can Enhance *Plasmodium* Infection in Mosquitoes: Implications for Malaria Control?" – Grant L. Hughes, Ana Rivero, Jason L. Rasgon (PLOS Pathogens, 9/4/14) https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4154766/

"*Wolbachia*-mediated sterility suppresses *Aedes aegypti* populations in the urban tropics" – The Project Wolbachia – Singapore Consortium, Ng Lee Ching (medRxiv, 6/17/21) https://www.medrxiv.org/content/10.1101/2021.06.16.21257922v1.full

"*Wolbachia* Enhances West Nile Virus (WNV) Infection in the Mosquito *Culex tarsalis*" – Brittany L. Dodson, Grant L. Hughes, Oluwatobi Paul, Amy C. Matacchiero, Laura D. Kramer, Jason L. Rasgon (PLOS Neglected Tropical Diseases, 7/10/14) https://journals.plos.org/plosntds/article?id=10.1371/journal.pntd.0002965

HDOA EPA Application for Emergency Exemption https://hawaiiunites.org/wp-content/uploads/2023/02/EPA-HQ-OPP-2022-0896-0002_content.pdf https://www.regulations.gov/document/EPA-HQ-OPP-2022-0896-0002

U.S. Department of the Interior Strategy for Preventing the Extinction of Hawaiian Forest Birds https://www.fws.gov/sites/default/files/documents/DOI%20Strategy%20for%20Preventing%20the%20Extinction%20of%20Hawaiian%20Forest%20Birds%20%28508%29.pdf

World Mosquito Program: Global progress https://www.worldmosquitoprogram.org/en/global-progress

World Mosquito Program: How our method

compares https://www.worldmosquitoprogram.org/en/learn/how-our-method-compares

. HAR § 11-200-7 https://casetext.com/regulation/hawaii-administrative-rules/title-11-department-of-health/subtitle-1-general-departmental-provisions/chapter-200-environmental-impact-statement-rules-repealed/subchapter-5-applicability-repealed/section-11-200-7-multiple-or-phased-applicant-or-agency-actions-repealed

. HAR § 11-200.1-10 https://casetext.com/regulation/hawaii-administrative-rules/title-11-department-of-health/subtitle-1-general-departmental-provisions/chapter-2001-environmental-impact-statement-rules/subchapter-6-applicability/section-11-2001-10-multiple-or-phased-actions

. HDOA Request to Import and Establish Permit Conditions for Southern House Mosquito (6/28/22) https://hawaiiunites.org/wp-content/uploads/2023/02/2022_0628_HDOA_Request_to_Import_and_Establish_Permit_Conditions_for_Southern_House_Mosquito.pdf

. Hawaii Environmental Policy Act (HEPA) Citizen's Guide (2014) https://files.hawaii.gov/dbedt/erp/OEQC_Guidance/2014-GUIDE-HEPA-Citizens-Guide.pdf

. Advisory Committee on Plants & Animals Meeting - June 9, 2022 https://www.youtube.com/watch?v=Wt_Jbygvek4

. HRS 84-14 https://www.capitol.hawaii.gov/hrscurrent/vol02_ch0046-0115/HRS0084/HRS_0084-0014.htm

. Ethics Guide for State Board and Commission Members https://ethics.hawaii.gov/wp-content/uploads/BCEthicsGuide.pdf

. The Constructed Environment: 2023 Conference: Plenary Speakers: Sam Gon https://constructedenvironment.com/2023-conference/program/sam-gon

. Facebook: Birds, Not Mosquitoes: Get to know Birds, Not Mosquitoes (4/22/22) https://www.facebook.com/BirdsNotMosquitoes/photos/a.106335221571876/315991167272946/

. National Fish and Wildlife Foundation Hawaii Conservation Business Plan (3/2021) https://www.nfwf.org/sites/default/files/2021-09/HI%20Business%20Plan%20%28August%202021%29.pdf

. USDA Animal and Plant Health Inspection Service (APHIS): Organisms and Vectors Guidance & Permitting https://www.aphis.usda.gov/aphis/ourfocus/animalhealth/animal-and-animal-product-import-information/organisms-vectors/ct_organisms_and_vectors

. 9 CFR, §122.2 https://www.ecfr.gov/current/title-9/chapter-I/subchapter-E/part-122

. Board of Land and Natural Resources Meeting

3/10/23 https://www.youtube.com/watch?v=u8oyjKaDTGg

5. Detection and molecular characterization of *Avipoxvirus* in *Culex* spp. (Culicidae) captured in domestic areas in Rio de Janeiro, Brazil – Carolina Soares van der Meer et al. (Nature, Scientific Reports, 8/5/22) https://www.nature.com/articles/s41598-022-17745-4

6. Venereal Transmission of St. Louis Encephalitis Virus by *Culex quinquefasciatus* Males (Diptera: Culicidae) – Donald A. Shroyer (Journal of Medical Entomology, 5/1990) https://academic.oup.com/jme/article-abstract/27/3/334/2220754?login=false

7. Sexual transmission of dengue viruses by Aedes albopictus – L. Rosen (NIH National Library of Medicine, 9/1987) https://pubmed.ncbi.nlm.nih.gov/3661831/

8. Assessing key safety concerns of a Wolbachia-based strategy to control dengue transmission by Aedes mosquitoes – Jean Popovici et al. (2010) https://www.epa.gov/sites/default/files/2018-04/documents/4g._popovici_article.pdf

9. April 24-26, 2018 Meeting of the Human Studies Review Board https://www.epa.gov/osa/april-24-26-2018-meeting-human-studies-review-board

10. April 24-26, 2018 EPA Human Studies Review Board Meeting Report https://www.epa.gov/sites/default/files/2018-07/documents/final_hsrb_report_from_april_2018.pdf

. HDOA Request to Import Southern House Mosquitoes for Immediate Field Release (6/9/22) https://hdoa.hawaii.gov/wp-content/uploads/2018/05/DLNR-Culex-quinquefasciatus-PA-All-Docs.pdf

12. University of Hawaii at Mānoa Request to: (1) Determine if the Establishment of the Southern House Mosquito, Culex quinquefasciatus, a Vector of Avian Influenza in Hawaii, Constitutes an Ecological Disaster;…(4) Determine the Probable Impact on the Environment if the Southern House Mosquito, Culex quinquefasciatus, an Unlisted Insect, Inoculated with a Foreign Wolbachia Bacteria Species, is Accidently Released;… (6/8/21) https://hdoa.hawaii.gov/wp-content/uploads/2018/05/HDOA-Mosquito-Request-PA_Final-6.8.21.pdf

13. Wolbachia Mosquitoes in Hawaii: Unsettled Science (Part 2) (7/21/22) https://mailchi.mp/12fb7ffe5f31/saturday-song-circle-in-paia-12pm-2pm-15015381

14. Hawaii Revised Statutes HRS §171-4 https://www.capitol.hawaii.gov/hrscurrent/vol03_ch0121-0200d/HRS0171/HRS_0171-0004.htm

15. Dawn N.S. Chang Financial Disclosure filed

1/2/23 https://hawaiiethics.my.site.com/public/s/hsecm-fd-public/a0i6R00000Y0Yv4QAF/fd2023010909

5. Vernon Char Financial Disclosure filed 6/1/22 https://hawaiiethics.my.site.com/public/s/hsecm-fd-public/a0i6R00000TQdsNQAT/fd2022010431

7. Char Sakamoto Ishii Lum & Ching Attorneys at Law (Present and Former Clients: The Nature Conservancy) http://lawcsilc.com/Clients.html

8. Petition: Demand an Environmental Impact Statement for the Experimental Mosquito Release on Maui https://www.change.org/Maui_Mosquito_Experiment_EIS

SECTION 166.20(a)(2): DESCRIPTION OF PESTICIDE REQUESTED

- **Common Chemical Name (Active Ingredients):** *Wolbachia pipientis*, wAlbB (DQB strain)

- **Trade Name:** DQB Males
  EPA Reg. No.: Unregistered

- **Confidential Statement of Formula:** Attached to this submission

- **Formulation:**
  wAlbB contained in live adult male *Culex quinquefasciatus* mosquitoes (DQB strain) active ingredient < 0.3%*
  *percent (w/w) of adult male mosquitoes

- **Mosquito and Wolbachia source:**

The DQB line of mosquitoes was developed through transfection of *Wolbachia pipientis* wAlb*B* isolated from *Ae. albopictus* KLP strain mosquitoes originating from Kuala Lumpur, Malaysia into *Culex quinquefasciatus* Palmyra strain mosquitoes originating from Palmyra Atoll. Prior to transfection, the naturally occurring wPip infection was removed from the Palmyra strain through antibiotic treatment using tetracycline and rifampicin as described in Pike & Kingcombe 2009 following the feeding protocol outlined in Dobson and Rattanadechakul 2001. Methods for DQB line generation are substantively similar to those outlined in MRID 51788911 with non-significant changes to account for Culex egg morphology. The DQB line was not created using genetic modification and the mosquitoes are not genetically modified organisms.

Table 1. Taxonomic designation of the *Wolbachia* present in the DAB line of *Ae. aegypti.*

Kingdom	Bacteria
Phylum	Proteobacteria
Class	Alphaproteobacteria
Order	Rickettsiales
Family	Rickettsiaceae
Genus	*Wolbachia*
Species	Pipientis
Clade	Supergroup: B
Strain	DQB: (**D**ebug) (*Culex* ***q****uinquefasciatus*) (wAlb**B**) DQB contains

Within *Culex quinquefasciatus*, the strain of incompatible bacterium will be *Wolbachia wAlbA, Wolbachia wAlbB,* or *Wolbachia wPip4.* These *Wolbachia* bacterium are not present within the corresponding species of Hawaii's established mosquito population. The presence of this bacterium will make these males sexually incompatible with the wild, established female mosquitoes. Once imported, the male, sexually incompatible males will be released according to EPA and HDOA label directions to suppress the population of the established mosquito populations. Based on the prior use of this technology in California, Florida, and Kentucky, there are no data to suggest releases of these male mosquitoes to have a negative impact on agriculture, the environment, or public health and safety. Existing wild-type bacteria strain that may be imported is wPipV, which is already found on all of the main Hawaiian islands.

DISCUSSION:

1. Persons Responsible:

DLNR Chairperson, Suzanne Case
DOFAW Administrator, David Smith
DOFAW Entomologist, Cynthia King
Department of Land and Natural Resources – Oahu
1151 Punchbowl Street, Honolulu, HI 96813

DLNR-DOFAW, Hawaii Invertebrate Program Captive Propagation Facility - Oahu
779 Ulukahiki Street, Kailua, Honolulu, HI 96813, (808) 266-7989

DLNR Waimano Baseyard – Oahu
2680 Waimano Home Road, Pearl City, HI 96782, (808) 266-7989

Kaua'i Branch Manager, Sheri Mann, Division of Forestry & Wildlife, 3060 Eiwa Street Rm. 306, Lihue, HI 96766. (808) 274-3433

O'ahu Branch, Division of Forestry & Wildlife, 2135 Makiki Heights Drive, Honolulu, HI 96822. (808) 973-9778

Maui (& Moloka'i) Branch, Division of Forestry & Wildlife, 1955 Main Street, Room 301, Wailuku, HI 96793. (808) 984-8100

Hawai'i Branch, Division of Forestry & Wildlife, 19 E. Kawili Street, Hilo, HI 96720. (808) 974-4221

2. Locations and Safeguards:

All mosquitoes for import will originate from Hawaii biotypes collected from

- *Wolbachia albopictus A* (*wAlbA*) imported in *C. quinquefasciatus*. In Hawaii, this strain already exists in *Aedes albopictus*.
- *Wolbachia albopictus B* (*wAlbB*) imported in *C. quinquefasciatus*. In Hawaii, this strain already exists in *Aedes albopictus*.
- *Wolbachia wPip4* imported in *C. quinquefasciatus*. This strain does not currently exist in Hawaii. It naturally exists in parts of Europe, Asia, the Middle East, and Africa, and is bidirectionally incompatible with strain *wPip5*. Strain *wPip5* is the most common strain in *C. quinquefasciatus* in Hawaii (Atkinson, C. T., W. Watcher-Weatherwax, and D. A. LaPointe. (2016) Genetic diversity of *Wolbachia* endosymbionts in *C. quinquefasciatus* from Hawaii, Midway Atoll and American Samoa. Technical Report HCSU-074).

Once imported, we will rear the imported mosquitoes to the maximum capacity of our facilities. Male mosquitoes with one or more of the imported strains (*wAlbA / wAlbB / wPip4*) could then be used for incompatible crosses to females that carry *wPip5.* The attached letter from the DLNR describes how there is an ecological disaster occurring (*i.e.* Hawaii's native birds going extinct). The imported mosquito[e]s are intended for release (only males are intended for release) to mitigate this disaster. Based on the prior use of this technology in California, Florida, and Kentucky, we do not expect releases of these male mosquitoes to have a negative impact on agriculture, the environment, or public health and safety.

PQB NOTES: *In addition to this request, the applicants have submitted a request to import the aforementioned species of unlisted Wolbachia bacteria. The import request for the Wolbachia species was submitted to the PQB Advisory Subcommittee on Bacteria for review and recommendation. The Advisory Subcommittee on Bacteria unanimously deemed these Wolbachia species to be low risk, and recommended approval of the import request via a letter of authorization. Hawaii Administrative Rules §4-71A-25(b) states: "An unlisted microorganism that is determined by the department to be a low risk microorganism may be allowed import by a letter of authorization issued by the Chief without advisory committee review or board approval."*

DISCUSSION:

1. <u>Persons Responsible</u>:

1) Floyd A. Reed, UHM, 2538 McCarthy Mall, Edmondson Hall 216, Honolulu, Hawaii 96822, (808) 956-6489.

2) Matthew Medeiros, University of Hawaii at Mānoa, 1993 East-West Road, Honolulu, Hawaii 96822 Ph: (808) 956-8187

DAVID Y. IGE
GOVERNOR OF
HAWAI'I

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LAND
STATE PARKS

STATE OF HAWAI'I
DEPARTMENT OF LAND AND NATURAL RESOURCES

POST OFFICE BOX 621
HONOLULU, HAWAI'I 96809

EXEMPTION NOTICE

Regarding the preparation of an environmental assessment under the authority of Chapter 343, HRS and Section 11-200.1-17, HAR

Project Title:	Mosquito Control Research Using *Wolbachia*-based Incompatible Insect Technique
Project Location:	Maui (2) 2-3-005:004: Waikamoi Preserve (2) 2-4-016:004: Waikamoi Preserve (2) 1-2-004:013: Hanawi Natural Area Reserve (2) 2-3-005:001: Haleakala National Park (2) 1-8-001:007: Haleakala National Park (2) 1-3-001:003: Haleakala National Park (2) 1-7-004:016: Haleakala National Park (2) 1-6-001:001: Haleakala National Park (2) 1-6-001:002: Haleakala National Park (2) 1-2-010:001: Haleakala National Park Kauai (4) 1-4-001:003: Alakai Wilderness Preserve (4) 1-4-001:013: Kokee State Park
Chapter 343 Trigger(s):	Use of State Funds and Lands
Project Description:	The main objective of this project is to initiate research to inform incompatible insect technique applications for the control of invasive *Culex quinquefasciatus* mosquitoes which are the primary vector of avian malaria. The disease threatens the survival of remaining endangered forest bird species where they persist in high elevation montane forest habitat on Maui and Kauai. Male mosquitoes which have been given an incompatible strain of *Wolbachia* bacteria are to be released on the landscape, and upon release those males will breed with wild female mosquitoes. As a result of those pairings, the wild female mosquitoes will lay eggs which will not hatch, and no offspring will be produced. When releases of incompatible male mosquitoes are completed consecutively, the approach results in the suppression of mosquito populations at a landscape-scale. If releases are halted, mosquito

	populations will gradually return to pre-release levels as wild female and male mosquitoes migrate back into the treated area from surrounding forest habitat. Initial research will contribute to EPA registration of male *Culex quinquefasciatus* mosquitoes with *Wolbachia* as a biopesticide, as well as determine the minimum number of male mosquitoes that must be released in each area to ensure population suppression. This project may be funded by Federal sources.
Consulted Parties:	U.S. Fish and Wildlife Service
Authorization:	November 13, 2015, Land Board submittal (C-6). Delegation of Authority to the Chairperson or their authorized representative to declare exempt from the preparation of an Environmental Assessment those Department actions which are included in the Department-wide exemption list when the Board of Land and Natural Resources has delegated the authority to conduct those actions.
Exemption Class & Description:	**Exemption Classes:** **General Exemption Type 5** *Basic data collection, research, experimental management, and resource and infrastructure testing and evaluation activities that do not result in a serious or major disturbance to an environmental resource.* PART 1 13. Research that the Department declares is designed specifically to monitor, conserve, or enhance native species or native species' habitat. 16. Research to identify, monitor, control, or eradicate introduced species. Date of Agency Exemption List: November 10, 2020.
Determination:	The Department of Land and Natural Resources declares that this project will likely have minimal or no significant impact on the environment and is therefore exempt from the preparation of an environmental assessment under the above exemption classes.

DLS Suzanne Q. Case Jun 17, 2022

Suzanne D. Case, Chairperson
Board of Land and Natural Resources

Date

Signature:

Email: david.g.smith@hawaii.gov

From: **Fretz, Scott** scott.fretz@hawaii.gov
Subject: RE: MRR Study: Makawao Forest Reserve
Date: February 9, 2023 at 2:30 PM
To: Tina Lia tinalia@live.com

SF

Aloha Ms. Lia:

Thank you for your follow up inquiry. You are correct that an exemption was filed for the MRR study. However, after further review and scheduling, it is our intention to carry out the MRR study as part of the actions described and analyzed in the EA. The MRR study will be done using IIT mosquitoes, as described in the EA.

Scott

J. Scott Fretz, PhD
Maui Branch Manager
Hawaii Department of Land and Natural Resources
Division of Forestry and Wildlife
685 Haleakala Highway
Kahului, Hawaii 96732
Phone (808) 984-8107
Cell (808) 227-3403
FAX (808) 984-8114
email: Scott.Fretz@hawaii.gov

From: Tina Lia <tinalia@live.com>
Sent: Thursday, February 2, 2023 3:04 PM
To: Fretz, Scott <scott.fretz@hawaii.gov>
Subject: [EXTERNAL] MRR Study: Makawao Forest Reserve

Aloha Dr. Fretz,

Thank you for your message explaining that the DLNR does not intend to initiate the mark-release-recapture (MRR) study until the EA has received final approval. It had been my understanding that the MRR study was not part of the proposed action in the EA. It was not mentioned nor described as part of the proposed action. Rather, the EA states that "DLNR filed an **exemption notice** regarding the preparation of an environmental assessment under the authority of Chapter 343, Hawaii Revised Statutes (HRS) and Section 11-200.1-17. HAR, to conduct limited import of male mosquitoes for preliminary transport trials and **mark release recapture studies**."

When I asked about the MRR study at the virtual public meeting for the EA on January 5, 2023, Chris Warren said that the study would happen in the western project area. The project area map shows Makawao Forest Reserve to be the westernmost parcel.

Following is the question I posed and the response (26:25 marker):

Q: (Tina Lia) “Regarding the mark-release-recapture study mentioned in the environmental assessment, why is the study necessary, and when and where will it be occurring? Will incompatible mosquitoes be released as a part of that study?”

A: (Chris Warren) "Yeah, that's great. You know, the mark-release-recapture study is part of the initial field trials, and we would learn really critical things during those trials that would make sure that this method is as efficient as it possibly can be. And at the moment, **we are discussing not using IIT mosquitoes for this at all**. It would be, you

know, again only male mosquitoes released in a small area, likely in the **western portion of the project area** that is more readily accessible but still away from places that people access on a regular basis."

I found his answer concerning because the release of compatible male mosquitoes, rather than the incompatible ones, is something that is not mentioned or evaluated in the EA. Providing potential male mates could increase the mosquito population, which could have adverse impacts to forest birds. This is at odds with the EA which specifically states, "This project would release only male mosquitoes with a different strain of *Wolbachia* bacteria to that occurring in southern house mosquitoes in East Maui."

Could you please clarify which is the environmental review document that covers the mark-release-recapture study? Is it the EA exemption notice or the draft EA? The draft EA makes it seem that the exemption notice covers the MRR study, but your answer implies that the MRR study is covered by the EA. Also, the EA is only for the release of incompatible mosquitoes, whereas compatible mosquitoes are being discussed for release in the western project area as part of the MMR.

Thank you for taking the time to respond to these concerns.

Aloha,
Tina Lia
tinalia@live.com
(808) 298-6335

> On Feb 2, 2023, at 11:06 AM, Fretz, Scott <scott.fretz@hawaii.gov> wrote:
>
> Aloha Ms. Lia:
>
> Thank you for your inquiry. The actions proposed for the mark-release-recapture study are covered in the Environmental Assessment (EA) that was published on December 23, 2022. We do not intend to initiate the study until the EA has received final approval. Therefore, no decisions have been made regarding the Makawao Forest Reserve as a study site.
>
> Scott
>
> _____
> J. Scott Fretz, PhD
> Maui Branch Manager
> Hawaii Department of Land and Natural Resources
> Division of Forestry and Wildlife
> 685 Haleakala Highway
> Kahului, Hawaii 96732
> Phone (808) 984-8107
> Cell (808) 227-3403
> FAX (808) 984-8114
> email: Scott.Fretz@hawaii.gov
> ---
>
> **From:** Tina Lia <tinalia@live.com>
> **Sent:** Monday, January 30, 2023 1:39 PM
> **To:** Fretz, Scott <scott.fretz@hawaii.gov>
> **Subject:** [EXTERNAL] MRR Study: Makawao Forest Reserve

Aloha Mr. Fretz,

I'm writing inquire about the Mark-Release-Recapture (MRR) study for the State of Hawaii's multi-agency *Birds, Not Mosquitoes* project "Mosquito Control Research Using Wolbachia-based Incompatible Insect Technique." Can you confirm that the Makawao Forest Reserve is a release site for the MRR study? If so, have signs been posted notifying the public of the MRR study being conducted?

Mahalo,
Tina Lia
tinalia@live.com
(808) 298-6335

Parasites & Vectors

RESEARCH Open Access

Wolbachia infection in wild mosquitoes (Diptera: Culicidae): implications for transmission modes and host-endosymbiont associations in Singapore

Huicong Ding†, Huiqing Yeo† and Nalini Puniamoorthy*

Abstract

Background: *Wolbachia* are intracellular bacterial endosymbionts found in most insect lineages. In mosquitoes, the influence of these endosymbionts on host reproduction and arboviral transmission has spurred numerous studies aimed at using *Wolbachia* infection as a vector control technique. However, there are several knowledge gaps in the literature and little is known about natural *Wolbachia* infection across species, their transmission modes, or associations between various *Wolbachia* lineages and their hosts. This study aims to address these gaps by exploring mosquito-*Wolbachia* associations and their evolutionary implications.

Methods: We conducted tissue-specific polymerase chain reaction screening for *Wolbachia* infection in the leg, gut and reproductive tissues of wild mosquitoes from Singapore using the *Wolbachia* surface protein gene (*wsp)* molecular marker. Mosquito-*Wolbachia* associations were explored using three methods—tanglegram, distance-based, and event-based methods—and by inferred instances of vertical transmission and host shifts.

Results: Adult mosquitoes (271 specimens) representing 14 genera and 40 species were screened for *Wolbachia*. Overall, 21 species (51.2%) were found positive for *Wolbachia*, including five in the genus *Aedes* and five in the genus *Culex*. To our knowledge, *Wolbachia* infections have not been previously reported in seven of these 21 species: *Aedes* nr. *fumidus*, *Aedes annandalei*, *Uranotaenia obscura*, *Uranotaenia trilineata*, *Verrallina butleri*, *Verrallina* sp. and *Zeugnomyia gracilis*. *Wolbachia* were predominantly detected in the reproductive tissues, which is an indication of vertical transmission. However, *Wolbachia* infection rates varied widely within a mosquito host species. There was no clear signal of cophylogeny between the mosquito hosts and the 12 putative *Wolbachia* strains observed in this study. Host shift events were also observed.

Conclusions: Our results suggest that the mosquito-*Wolbachia* relationship is complex and that combinations of transmission modes and multiple evolutionary events likely explain the observed distribution of *Wolbachia* diversity across mosquito hosts. These findings have implications for a better understanding of the diversity and ecology of *Wolbachia* and for their utility as biocontrol agents.

Keywords: *Wolbachia*, *Wolbachia* surface protein gene, Reproductive endosymbiont, Tissue-specific polymerase chain reaction, Transmission modes, Host-endosymbiont association

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Background

Wolbachia are intracellular endosymbiotic bacteria that alter host reproduction [1]. They are widespread in arthropods, infecting a wide range of insect, crustacean, and nematode species [2, 3]. In some cases, *Wolbachia* exist in a mutualistic relationship with their hosts [4–6]. However, *Wolbachia* are most often recognised as reproductive manipulators that bias the sex ratio of the host offspring towards the production of more infected females [7, 8]. This reproductive manipulation is commonly achieved through four phenotypes—male killing [9], feminisation [10, 11], parthenogenesis [12, 13], and cytoplasmic incompatibility [14, 15]—which increase the endosymbiont's reproductive success [16]. Owing to their strong influence on host reproduction, an increasing amount of research is being dedicated to exploring the impacts of reproductive endosymbionts on host population dynamics and evolution [17, 18], especially in medically important insects such as mosquitoes. The promising use of *Wolbachia* to alter both mosquito reproduction [19] and arboviral transmission [20] has prompted the deployment of novel *Wolbachia*-infected mosquitoes for population replacement and suppression [21].

Several countries, including Singapore, have started to employ *Wolbachia* as biocontrol agents of mosquitoes by releasing infected mosquitoes [22–24]. However, the presence of naturally occurring endosymbionts in wild mosquito populations has not been adequately assessed. The release of mosquitoes artificially infected with *Wolbachia* might have a profound impact on closely interacting wild mosquito populations through various transmission modes. For instance, horizontal transmission of an introduced *Wolbachia* strain may result in manipulation of the reproductive biology of non-target species, which could potentially result in an unintentional population crash, opening up niches for other vector species [25]. Another possible effect of this type of biocontrol method is the increased likelihood of co-infections with other naturally occurring *Wolbachia* strains or other endosymbionts, such as *Cardinium*, *Rickettsia*, and *Spiroplasma*. These co-infections may result in a synergistic effect on mosquito host fitness and future transmission of endosymbionts [26–29]. Without a detailed characterisation of *Wolbachia* prevalence and diversity among wild mosquitoes, the ecological risk of releasing artificially infected mosquitoes might be overlooked. Therefore, bearing the precautionary principle in mind, it is important to investigate the natural occurrences of *Wolbachia*.

There is also a need to discern the main mode of infection transmission among mosquitoes. Although *Wolbachia* are mainly thought to be vertically transmitted [15, 30], there have been accounts of horizontal transmissions into wild populations through parasitism [31, 32], or through proximity to infected individuals [33]. *Wolbachia* may not be strictly localised in germline tissues, as they have also been detected in somatic tissues such as the gastrointestinal tract and haemolymph [34–36]. The detection of *Wolbachia* in the gastrointestinal tract suggests that they could be horizontally transmitted through uptake from the environment or host sharing [34, 37, 38], whereas their detection in non-gastrointestinal somatic tissues, such as those of jointed appendages, could indicate horizontal bacterial genome integration into the host genome [36]. Currently, detection of *Wolbachia* in mosquitoes is mostly achieved through conventional polymerase chain reaction (PCR) methods using DNA extracted from an entire individual or its abdomen [39–47]. This limits our ability to identify the site of endosymbiont infection within an individual (tissue tropism). Tissue-specific screening of *Wolbachia* is necessary to provide insights and infer the extent of vertical and horizontal transmission.

It has been proposed that host mitochondrial DNA (mtDNA) and *Wolbachia* are maternally co-transmitted within the cytoplasm [17, 48], which suggests a congruency between host mtDNA and *Wolbachia* phylogenies—a consequence of cytoplasmic hitchhiking driven by endosymbiont transmission [17]. In insect systems such as bedbugs where vertical transmission has been established to be the main mode of transmission, *Wolbachia* exhibit clear patterns of cophylogeny with their hosts, with few instances of host shifting or multiple infections within a single host species [49, 50]. In contrast, cophylogeny is not apparent among nematodes and bees, and numerous acquisitions of *Wolbachia* infections through horizontal transmission as well as losses have been shown in these diversified host lineages [51, 52]. The modes of *Wolbachia* transmission among mosquitoes have not been well established, nor has the extent of multiple infections within mosquito hosts or host shifting of these bacteria.

There is presently no comprehensive analysis of the evolutionary associations between *Wolbachia* and their mosquito host species. An understanding of host-endosymbiont associations will not only further our ability to discern the mode of transmission which influences *Wolbachia* diversity, but will also allow for an evaluation of *Wolbachia* host specificity, speciation, and their ability to establish in new hosts. All of this is key to understanding the diversity and ecology of *Wolbachia*, and their utility in biocontrol methods.

This study has three major research objectives. First, to examine the prevalence and diversity of *Wolbachia* among wild mosquitoes from Singapore. Second, to determine the tissue tropism of *Wolbachia* infection

in mosquitoes using a tissue-specific PCR screening method. Finally, to reconstruct the evolutionary associations between *Wolbachia* and their mosquito hosts to provide a basis for an understanding of host-endosymbiont evolution.

Methods

Adult mosquito collection and identification

Mosquito samples were collected from 12 localities across Singapore between March 2018 and November 2019 (Fig. 1a). Three methods were employed to collect the samples: CO_2-baited Centers for Disease Control and Prevention traps, sweep-netting using hand-held fan traps, and larval sampling [53]. For the latter, dipping was carried out at streams and ponds and pipettes were used to collect larvae from various microhabitats, including tree holes, plant axils, and artificial containers. Thereafter, the field-collected larvae were reared to adults in an incubator maintained at 26 °C and 70% relative humidity, under a 12:12-h (day:night) photoperiod. Larvae were fed with pulverised fish food (TetraMin Granules) daily. Mosquitoes were identified using relevant taxonomic keys and descriptions [54–59]. A subset of individuals from commonly sampled species was selected and preserved in phosphate-buffered saline solution at – 80 °C for subsequent dissection step.

Tissue-specific dissection

Tissue-specific dissection was carried out on each adult mosquito sample to isolate the leg, gut, and reproductive tissues (Fig. 1b–d). To prevent the contamination of tissues with bacteria on the external surface of the mosquito, the leg was removed first before isolating the gut and reproductive tissues. All dissection equipment and microscope slides were thoroughly wiped with 70% ethanol before commencing dissection of the next sample. Dissected tissues were individually placed into a 96-well plate on ice to prevent DNA degradation.

DNA extraction, PCR amplification, and sequencing

DNA extraction of each dissected tissue was performed using 7 µl of QuickExtract DNA Extraction Solution (Lucigen, Madison, USA) in a thermocycler (Eppendorf, Hamburg, Germany) with the following protocol: 65 °C for 18 min, followed by 98 °C for 2 min, ending with cooling on ice for at least 10 min. All dissected tissues were screened for *Wolbachia* infections following single-primer PCR protocols described by Martin et al. [26] with slight modifications to the cycle conditions. The *Wolbachia* surface protein gene (*wsp*) general primers, wsp81F (5′-TGGTCCAATAAGTGATGAAGAAAC TAGCT-3′) and wsp691R (5′-AAAAATTAAACGCTA CTCCAGCTTCTGCAC-3′), were used in this study [60]. In addition, a fragment of the cytochrome c oxidase subunit I (*cox*1) gene of the mosquito hosts was also amplified using primers LCO1498 (5′-GGTCAACAA ATCATAAAGATATTGG-3′) and HCO2198 (5′-TAA ACTTCAGGGTGACCAAAAAATCA-3′) [61]. This served to confirm host identity and acted as an internal control. We used DNA from known *Wolbachia*-infected *Nasonia* specimens as positive controls for this study.

All PCR procedures were performed in reaction mixtures consisting of 12.5 µl of GoTaq G2 Green Mastermix (Promega, Madison, USA), 1 µl of 1 mg ml^{-1} bovine

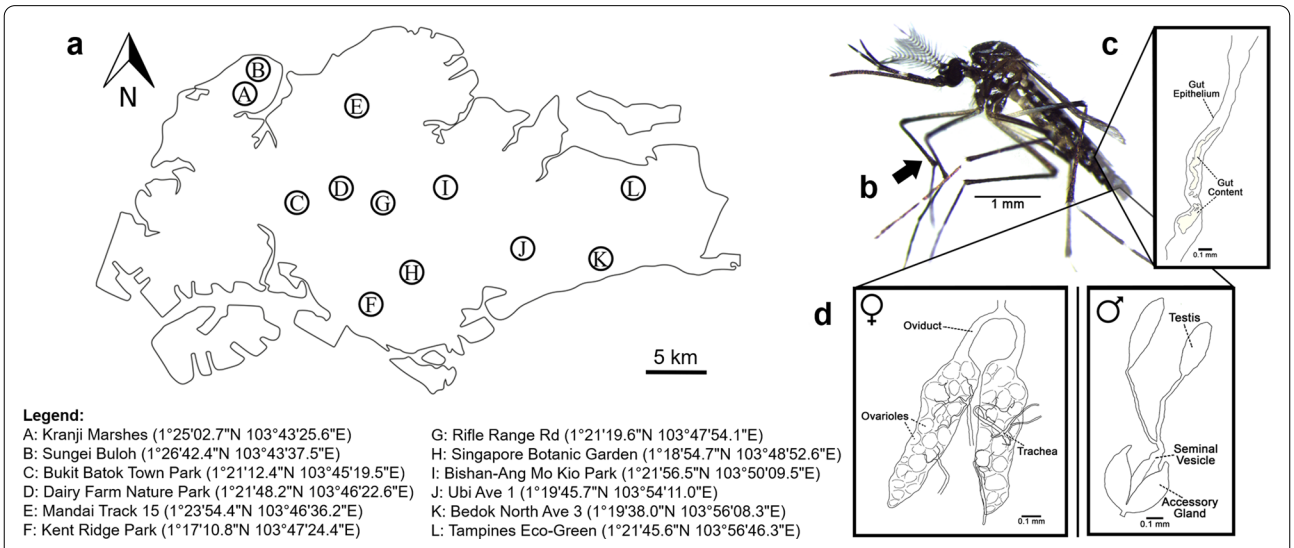

Fig. 1a–d Map of sampling sites and diagrammatic images of *Aedes aegypti* and its dissected tissues. **a** Various mosquito collection localities across Singapore and their respective coordinates, **b** mosquito leg, **c** gut, **d** female reproductive tissue (*left*) and male reproductive tissue (*right*)

serum albumin, 0.184 µl of 25 mM magnesium chloride, 1.5 µl of extracted DNA, and 1.5 µl each of 5 µM *wsp* forward and reverse primers for *Wolbachia* PCR screens or 1.0 µl each of 5 µM LCO1498 and HCO2198 primers for *cox*1 PCRs. Double-distilled water was used to top up the reaction mixture to a final volume of 25 µl. PCR amplification of positive and negative controls was also conducted simultaneously.

PCR conditions were as follow: 94 °C for 5 min, followed by 35 cycles of 95 °C for 30s, 55 °C for 45s, and 72 °C for 1 min, with a final elongation step of 72 °C for 10 min. Amplicons were separated by gel electrophoresis on 2% agarose gel stained with GelRed (Biotium, Fremont, USA) and visualised under a ultraviolet transilluminator (Syngene, Cambridge, UK). PCR products were purified using SureClean Plus (Bioline, London, UK) following the manufacturer's protocol. Samples were sequenced by First Base Laboratories (Axil Scientific, Singapore), using a 3730XL DNA Analyzer (Applied Biosystems, Waltham, USA). Obtained sequences were edited and aligned using Geneious Prime (version 2019.2.3) (https://geneious.com). Similarities with publicly available sequences were assessed using the Basic Local Alignment Search Tool (BLAST) [62].

Statistical analyses

To test if there were significant differences in *Wolbachia* infection across the different mosquito tissues, Cochran's Q-test was carried out. As a follow-up, McNemar's post hoc test was employed to identify the tissue pairs that differed significantly in infection. Individuals for which the internal control (*cox*1 gene) was not amplified successfully for any of the three dissected tissues were excluded from this statistical analysis. The effect of sex on host infection was also tested using binary logistics regression with sex as a categorical dependent variable and infection outcome as a binary independent variable. Logistic regression was conducted on a subset that only included species that had a roughly similar representation of both sexes, i.e. for every species included, the number of individuals of the less common sex was proportionally at least 60% of the number of individuals of the more common sex. This was to prevent a biased analysis due to a dataset with unequal representation of the sexes. Statistical significance was determined as $P < 0.05$. All statistical analyses were performed in R version 3.6.2 [63] with packages nonpar [64], rcompanion [65], and ISLR [66].

Sequence analyses

Multiple alignment of consensus sequences was carried out using the ClustalW algorithm with default settings (gap penalty = 15, gap extension penalty = 6.66) [67], in software MEGA X [68]. Mosquito *cox*1 sequences generated in this study were aligned with 61 reference *cox*1 barcodes of identified local mosquitoes from Chan et al. [53]. For *wsp* sequences, the generated sequences were aligned with 54 available *wsp* sequences of known *Wolbachia* strains obtained from GenBank [69]. Short sequence reads (< 500 base pairs) were excluded.

Neighbour-joining (NJ) phylogenetic trees for mosquito hosts and *Wolbachia* were reconstructed using the sequenced *cox*1 gene fragment and the *wsp* gene, respectively. *cox*1 sequences from previous publications were not included because a comparison of the genetic relationships between the hosts was not the aim of this research. Instead, 54 *wsp* sequences from GenBank were included in the construction of the *Wolbachia* NJ tree. The NJ tree reconstruction was performed with the Kimura two-parameter model as the nucleotide substitution model in MEGA X [68]. Internal gaps were treated as indels and terminal gaps as missing for *wsp* sequences. Bootstrap probabilities were estimated by generating 1000 bootstrap replicates. We designated two biting midge species, *Culicoides asiana* (KJ162955.1) and *Culicoides wadai* (KT352425.1), as outgroups for the host NJ tree construction. Due to the lack of an appropriate endosymbiont outgroup [51], the *Wolbachia* NJ tree was midpoint rooted.

When possible, *Wolbachia* strains were classified into supergroups and putative strains using 97% bootstrap probability as a threshold [60]. *Wolbachia* surface protein sequences that did not have 97% bootstrap support were evaluated on a case-by-case basis. For example, sequences which clustered closely together and had a relatively high support value (> 90%) were deemed as originating from the same putative strain.

Putative strains which were infectious to only one host species were categorized as 'specialists' and those which infected two or more hosts as 'generalists'. Then, the standardised phylogenetic host specificity (SPS) score of each generalist strain was calculated by adapting the method outlined by Poulin et al. [70] and Kembel et al. [71]. SPS measures the degree of phylogenetic relatedness among host species infected by the same endosymbiont strain. It also tests for significance by comparing it with null models generated with 999 replicates of random host-endosymbiont associations. A positive SPS value with a high *P*-value ($P > 0.95$) indicates a high degree of host flexibility where *Wolbachia* infect hosts which are phylogenetically even. A negative SPS value with low *P*-value ($P < 0.05$) suggests a low degree of host flexibility where the infected hosts are phylogenetically clustered together. SPS scores were calculated using R package picante [71].

Evolutionary analyses of the mosquito-*Wolbachia* relationship

Three distinct methods were used to explore the evolutionary associations between mosquito hosts and their *Wolbachia* endosymbionts. The analyses were carried out using pruned phylogenies where each species is represented by a single individual.

First, using the software TreeMap 3.0 [72], a tanglegram was created between host and endosymbiont NJ trees to visualise mosquito-*Wolbachia* associations. A tanglegram is useful as a pictorial representation of the interactions between two phylogenies [73]. TreeMap also seeks to minimise the entanglement between the two trees to provide a clearer visualisation of the phylogenetic relationship between host and endosymbiont [72].

Second, ParaFit Global test, a distance-based method, was employed to quantitatively estimate congruence between the host and endosymbiont phylogenetic trees by comparing genetic distances among infected host species and the *Wolbachia* strains [74]. The null hypothesis for this test states that the associations between host and endosymbiont trees are random, whereas the alternative hypothesis suggests that there are strong associations between hosts and parasites, which are indicated by phylogenetic distances. Significance was tested by comparing the observed associations between host and endosymbiont with randomised associations generated with 5000 permutations. The respective host-endosymbiont associations which contributed significantly to the ParaFit Global statistics were also identified by performing a Parafit Link test. ParaFit tests were performed with the Cailliez correction to correct for negative eigenvalues generated [75] using R package ape [76].

Third, an event-based analysis was performed in Jane 4.0 [77] to map out potential evolutionary events of the endosymbiont in relation to the host phylogeny [78]. Five evolutionary events were considered: co-speciation (host and endosymbiont speciate simultaneously), duplication (intra-host speciation), duplication with host shift (endosymbiont host shifts), loss (host speciates but endosymbiont fails to establish in one of the new lineages), failure to diverge (host speciates and endosymbiont remains in both lineages). As each event is expected to have differing likelihoods, default cost values were attached to each of the events. Jane 4.0 determined the best reconstruction of evolutionary events by minimising the overall cost. The following cost-scheme regime was used with 100 generations and a population size of 300: co-speciation = 0, duplication = 1, duplication with host shift = 2, loss = 1, and failure to diverge = 1 [79]. As a follow-up, random tip mapping (randomisation of host-endosymbiont associations) was carried out for 50 iterations, to determine if the overall cost of reconstruction was significantly lower than expected by chance. If 5% or fewer of the random solutions have costs lower than the reconstructed coevolution phylogeny, there is support for the coevolution of the hosts and endosymbionts through co-speciation.

Results

Prevalence of *Wolbachia* in wild-caught mosquitoes

A total of 271 adult mosquitoes, representing 40 species and 14 genera, were collected from 12 localities in Singapore (Fig. 1a). Overall, infection prevalence was moderate with 119 out of 271 (43.9%) individuals screening positive for *Wolbachia* (Table 1). In total, 21 (51.2%) species were positive for *Wolbachia*. According to our knowledge, *Wolbachia* infection in seven of these species is reported here for the first time (Table 1). *Wolbachia* were detected in all genera except for *Aedeomyia*, *Anopheles* and *Mimomyia* (i.e. 11 out of 14 genera; 78.6%). Five out of the seven *Aedes* species collected (71.4%) were positive for *Wolbachia*, while in the genus *Culex*, five out of 16 species (31.3%) were positive. Some of the screened species in the genera *Aedes* and *Culex* that were positive for *Wolbachia*, such as *Aedes albopictus* and *Culex quinquefasciatus*, are medically important vector species.

The infection rates varied across the mosquito species. Notably, there was variation in the percentage of infection between species that are epidemiologically related. For instance, *Wolbachia* infection was not detected in *Aedes aegypti*. However, infection was moderately high (56.8%) for *Aedes albopictus*. There was also a difference in the infection rate of two closely related species, *Culex pseudovishnui* (86.4%) and *Culex vishnui* (0%) [53].

Locality did not seem to play a role in the *Wolbachia* infection of mosquito hosts. Among species that have a wide range across Singapore, the percentage of infection was consistent in populations across different habitats. For example, the infection percentage was consistently high for *Cx. pseudovishnui*, while consistently low for *Malaya genurostris*. Based on our results, species identity was a better predictor of infection status than locality.

Based on a data subset containing 153 individuals (45.8% males) representing 12 mosquito species, sex was a significant explanatory variable, and there was a significantly lower infection prevalence in males than females (odds ratio 0.434; binary logistics regression: $Z = -2.48$, $df = 151$, $P = 0.013$).

Tissue tropism of *Wolbachia* infection in mosquitoes

Among the 159 successfully amplified *cox*1 sequences, *Wolbachia* infection was mainly observed in the reproductive tissues. Among the reproductive tissues of 159 dissected individuals, 42.1% ($n = 67$) were infected. Percentage infection was lower in the gut (5.7%, $n = 9$) and leg (3.1%, $n = 5$) tissues. The difference in

Table 1 Percentage infection of *Wolbachia* in 40 mosquito species collected from 12 Singapore localities

Mosquito species	Localities												Total	Infection (%)	Supergroup
	BN	BA	BB	DF	KR	KJ	M	RR	SBG	SBL	T	U			
Aedeomyia catastica	–	0/1	–	–	–	–	–	–	–	–	–	–	0/1	0.0	–
Aedes aegypti	0/1	–	–	–	–	–	–	–	–	–	–	0/13	0/14	0.0	–
Aedes albolineatus	–	–	–	–	–	–	0/3	–	–	–	–	–	0/3	0.0	–
Aedes albopictus	–	–	–	6/10	6/10	3/6	6/11	–	–	–	–	–	21/37	56.8	A, B
Aedes annandalei[a]	–	–	–	–	3/4	–	8/9	–	–	–	–	–	11/13	84.6	A
Aedes nr. *fumidus*[a]	–	–	–	–	–	–	–	–	–	6/10	–	–	6/10	60.0	A
Aedes gardnerii	–	–	–	–	–	–	1/1	–	–	–	–	–	1/1	100.0	A
Aedes malayensis	–	–	–	1/2	13/16	0/2	–	–	–	–	–	–	14/20	70.0	A
Anopheles barbirostris complex	–	–	–	0/2	–	–	0/2	–	–	–	–	–	0/4	0.0	–
Anopheles lesteri	–	–	–	–	–	0/2	–	–	–	–	–	–	0/2	0.0	–
Anopheles sinensis	–	0/12	–	–	–	–	–	–	–	–	–	–	0/12	0.0	–
Armigeres kesseli	–	–	–	–	3/3	–	–	–	–	–	–	–	3/3	100.0	B
Coquillettidia crassipes	–	–	–	2/2	6/7	4/4	–	–	–	–	–	–	12/13	92.3	B
Culex (*Lophoceramyia*) spp.[c]	–	–	–	–	0/1	0/2	1/9	–	–	–	0/2	–	1/14	7.1	B
Culex bitaeniorhynchus	–	–	–	–	0/1	–	–	–	–	–	–	–	0/1	0.0	–
Culex brevipalpis	–	–	–	0/1	–	–	0/2	–	–	–	–	–	0/3	0.0	–
Culex nigropunctatus	–	–	–	–	–	0/1	0/2	–	–	–	–	–	0/3	0.0	–
Culex pseudovishnui	–	–	–	–	11/12	–	4/4	–	3/5	1/1	–	–	19/22	86.4	B
Culex quinquefasciatus	–	5/8	–	–	–	–	–	–	–	–	–	–	5/8	62.5	B
Culex sitiens	–	–	–	–	–	–	–	–	–	2/4	–	–	2/4	50.0	B
Culex sp.	–	–	–	–	–	–	0/2	–	–	–	–	–	0/2	0.0	–
Culex tritaeniorhynchus	–	–	–	–	–	2/5	–	–	–	0/1	0/1	–	2/7	28.6	UC[b]
Culex vishnui	–	–	–	–	–	–	0/2	–	–	–	0/3	–	0/5	0.0	–
Malaya genurostris	–	–	2/4	–	0/1	4/13	–	–	0/1	–	–	–	6/19	31.6	B
Mansonia dives	–	–	–	–	–	–	0/2	–	–	–	–	–	0/2	0.0	–
Mansonia indiana	–	–	–	–	–	3/3	–	–	–	–	–	–	3/3	100.0	B
Mimomyia luzonensis	–	–	–	–	–	0/1	–	–	–	–	–	–	0/1	0.0	–
Tripteroides sp.	–	–	–	–	0/7	–	½	–	–	–	–	–	1/9	11.1	UC[b]
Uranotaenia obscura[a]	–	–	–	2/4	–	–	2/2	1/1	–	–	–	–	5/7	71.4	A
Uranotaenia sp.	–	–	–	1/2	–	–	–	–	–	–	–	–	1/2	50.0	A
Uranotaenia trilineata[a]	–	–	–	–	–	–	1/1	–	–	–	–	–	1/1	100.0	B
Verrallina butleri[a]	–	–	–	–	–	1/1	–	–	–	–	–	–	1/1	100.0	UC[b]
Verrallina sp.[a]	–	–	–	–	–	–	–	1/5	–	–	–	–	1/5	20.0	UC[b]
Zeugnomyia gracilis[a]	–	–	–	1/2	–	–	1/13	1/4	–	–	–	–	3/19	15.8	B
Total	0/1	5/21	2/4	13/25	42/62	17/40	25/67	3/10	3/6	9/16	0/6	0/13	119/271	43.9	

BN Bedok North Avenue 3, *BA* Bishan-Ang Mo Kio Park, *BB* Bukit Batok Town Park, *DF* Dairy Farm Nature Park, *KR* Kent Ridge Park, *KJ* Kranji Marshes, *M* Mandai Track 15, *RR* Rifle Range Road, *SBG* Singapore Botanic Garden, *SBL* Sungei-Buloh, *T* Tampines Eco-Green, *U* Ubi Avenue 1

[a] Species in which, according to our knowledge, *Wolbachia* infection has not been previously reported

[b] *Wolbachia* infections that were unclassified (*UC*) with respect to supergroup [60] because their DNA sequences were either too short (< 400 base pairs), or there were alignment issues during the phylogenetic analyses

[c] *Culex* (*Lophoceramyia*) comprises seven unique species, which were not identified here

percentage infection across the three dissected tissues was statistically significant (Cochran's Q-test: $Q = 109.5$, $df = 2$, $P < 0.0001$). The percentage of infection in the reproductive tissues was significantly higher than in the gut (McNemar's post hoc test: $P < 0.0001$) and leg tissues (McNemar's post hoc test: $P < 0.0001$), but the difference in percentage of infection between the gut and leg tissues was not significant (McNemar's post hoc test: $P = 1.0$). Notably, the amplicon size of *wsp* in the gut and leg tissues tended to be shorter than 400 base pairs.

Wolbachia diversity among mosquito fauna from Singapore

Following Zhou et al. [60], all *wsp* sequences obtained in this study can be broadly classified into A and B *Wolbachia* supergroups. Out of 21 infected species, six were infected with supergroup A, ten with supergroup B, and one species, *Ae. albopictus*, was infected with both supergroups (Fig. 2). Infection of the remaining four species (*Culex tritaeniorhynchus*, *Tripteroides* sp., *Verrallina butleri*, and *Verrallina* sp.) was unclassified due to short sequences (< 400 base pairs) or sequence alignment issues during sequences analyses. The analysed *wsp* sequences were also clustered into 12 putative strains: 'Wol 1' to 'Wol 12'. Four (Wol 1, Wol 2, Wol 3, and Wol 8) out of the 12 putative strains could be matched to previously typed strains [60, 80]. *Wolbachia* strains from this study are also closely related to those isolated from other insect groups (Fig. 2). For instance, Wol 9 and Wol 10 are closely related to the *Wolbachia* strains harboured by *Drosophila* spp. (bootstrap value > 99%).

Host specificity of *Wolbachia* strains

The degree of host specificity varied across the 12 putative strains. Seven out of the 12 strains (Wol 2, Wol 4, Wol 5, Wol 6, Wol 8, Wol 10, and Wol 12) were considered as specialists. These strains were host specific and were only detected in one host species each (Fig. 3). The remaining five strains were considered as generalists as they were found in more than one host. Amongst the generalists, Wol 3 was found in the highest number of host species, i.e. three, *Coquillettidia crassipes*, *Mansonia indiana*, and *Culex sitiens*. The SPS scores revealed that Wol 1 had the lowest degree of host flexibility (SPS test: $Z = -1.41$, $P = 0.049$). Wol 7 had the highest degree of host flexibility, but this was not statistically significant (SPS test: $Z = 0.07$, $P = 0.779$) (Table 2).

Evolutionary relationship between mosquitoes and *Wolbachia*

We recorded 18 counts of mosquito-*Wolbachia* associations in wild-caught mosquitoes from Singapore. A visualisation of these associations using a tanglegram showed patterns of broad associations (Fig. 3). For instance, the clade which consists of *Aedes* species was observed to be mostly associated with *Wolbachia* supergroup A. In contrast, other species, especially the clade representing various *Culex* species, had numerous associations with *Wolbachia* supergroup B.

The distance-based quantitative test showed that mosquito and *Wolbachia* phylogenies were weakly congruent at the global level (ParaFit Global test: ParaFit Global = 0.006, $P = 0.048$). Among the numerous

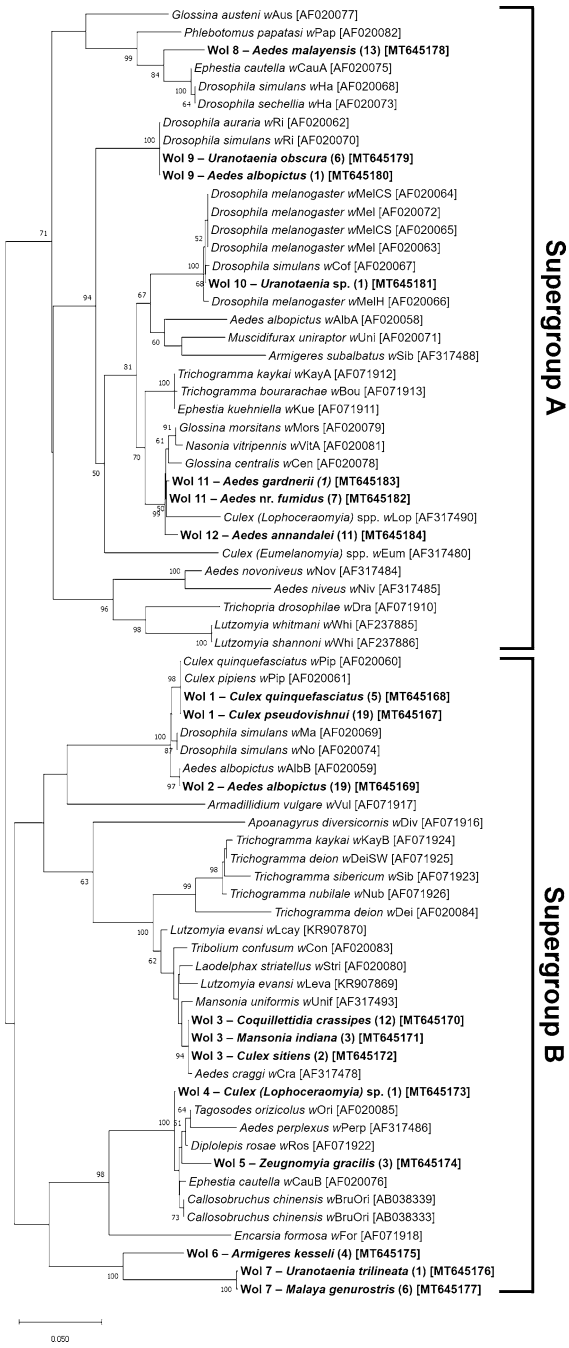

Fig. 2 *Wolbachia* neighbour-joining (NJ) tree constructed with the *Wolbachia* surface protein gene (*wsp*). All analysed sequences generated from this study (*bold*) were broadly classified into *Wolbachia* supergroups A or B and clustered into 12 putative strains ('Wol 1'–'Wol 12'). The number of sequences of each putative strain is indicated *within parentheses*. Also included are 54 sequences obtained from GenBank. Taxa are labelled as the host from which the *Wolbachia* strain was isolated, followed by the strain name. The NJ tree was mid rooted due to a lack of appropriate outgroups [45]. Bootstrap probability (generated with 1000 replicates) higher than 50% is indicated on the tree. Genbank accession number of each sequence is indicated *within brackets*

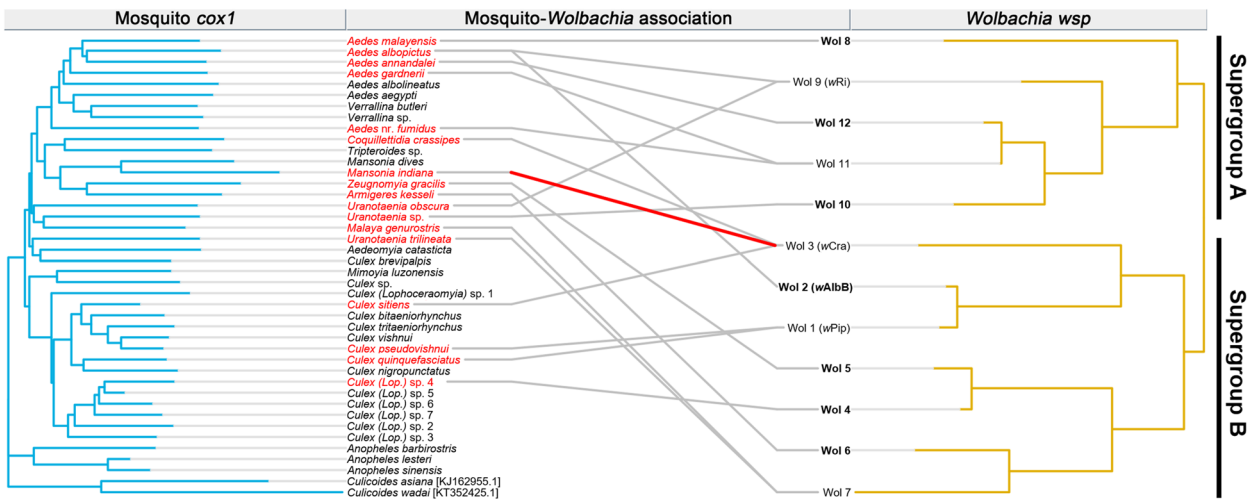

Fig. 3 Tanglegram of mosquito *cox*1 NJ tree compared to the *Wolbachia* endosymbiont NJ tree. Mosquito host species that harboured *Wolbachia* infection are indicated in *red*. Specialist *Wolbachia* strains are in *bold*. *Grey lines* represent the associations between hosts and endosymbionts. A *red line* indicates the host-endosymbiont association that was significant in the Global ParaFit test of congruence between host and endosymbiont phylogenies (Parafit Link test: ParaFit Link = 0.045, $P = 0.029$)

Table 2 Standardised phylogenetic host-specificity (*SPS*) scores of putative *Wolbachia* generalists

Putative *Wolbachia* strain	No. of infected hosts	Phylogenetic host-specificity score	SPS score	*P*-value
Wol 1	2	0.281	− 1.41	0.049*
Wol 3	3	0.391	− 0.162	0.421
Wol 7	2	0.281	0.068	0.779
Wol 9	2	0.281	− 0.234	0.249
Wol 11	2	0.281	− 0.817	0.157

* $P < 0.05$

host-endosymbiont links, only the association between *Mansonia indiana* and Wol 3 was statistically significant (ParaFit Link test: ParaFit Link = 0.045, $P = 0.029$) (Fig. 3).

The event-based analysis between mosquito and *Wolbachia* phylogenies resulted in a reconstructed output of one co-speciation event, three counts of duplication, seven counts of duplication with host shift, 29 losses, and six counts of failure to diverge, amounting to a total cost of 52 (Fig. 4). Interestingly, the number of duplications with a host shift and losses was much greater than co-speciation events. Notably, multiple host shift events tend to follow after loss events occurring earlier in the evolutionary history of the endosymbiont. For example, we see instances of consecutive host shifts to new hosts that were not previously infected (Fig. 4, red arrows). Additionally, based on random tip mapping, 14% of the random solutions had lower costs than the reconstructed output. Overall, there was support for multiple host shift events and losses of *Wolbachia* among the mosquitoes, and no clear signal for mosquito-*Wolbachia* cophylogeny.

Discussion

Detection of *Wolbachia* infection and distribution in wild mosquitoes

In this study, the PCR-based *Wolbachia* screening method had a high positive detection rate with 86.3% of all sequenced amplicons having successful BLAST matches to *Wolbachia*. This suggests that the conventional PCR method used is adequate for *Wolbachia* detection. Even if the study had been carried out without the additional DNA sequencing step, observed amplicon bands would likely have indicated true positives.

Our results indicate that *Wolbachia* are widespread across members of the family Culicidae. To our knowledge, *Wolbachia* infections have not been previously reported in seven of the mosquito species collected in this study. Overall, the percentage infection of screened individuals was 43.9%, which was largely congruent with percentages reported in past studies from the Oriental region, i.e. 31% infection in Malaysia [81], 26.4% in Sri Lanka [39], and 61.6% in Thailand [82]. At the species level, previous studies reported *Wolbachia* infection in 40% of all tested mosquito species in India [83], 18.2% in Sri Lanka [39], 51.7% in Taiwan [84], and between 28.1% and 37.8% in Thailand [82, 85]. Our study showed that 51.2% of all tested species were infected with *Wolbachia*,

Fig. 4 Least-cost evolutionary reconstruction between mosquito (*black*) and *Wolbachia* (*blue*) phylogenies achieved using Jane 4.0. In total, one co-speciation event (*open circle*), three counts of duplication (*grey dot*), seven counts of duplication with host shift (*black dot* with an *arrow* pointing outwards), 29 losses (*dotted line*), and six counts of failure to diverge (*squiggly line*) were mapped out. *Red arrows* indicate periods where multiple host shifts occurred in succession

which is generally higher than the percentage reported in most studies. This was likely due to the broad range of species tested, including those from the genera *Malaya*, *Verrallina*, and *Zeugnomyia* [85]. It is also possible that infection prevalence may vary across geographical regions.

Wolbachia detection in three medically important mosquito genera, *Culex*, *Anopheles*, and *Aedes*, was highly consistent with that of past studies. These genera are responsible for the transmission of vector-borne diseases such as filariasis, malaria and arboviral diseases [86]. Within the genus *Culex*, *Wolbachia* infection has been reported to be variable across its member species [39, 46, 82, 84]. In this study, infections were observed only in five out of 16 *Culex* species. We noticed moderately high *Wolbachia* infection in *Cx. quinquefasciatus* (62.5%), which is a member of the *Culex pipiens* complex responsible for the transmission of filariasis in Singapore [86, 87]. Surprisingly, no *Wolbachia* infection was observed in *Cx. vishnui*–which has been found to harbour Japanese encephalitis virus in Southeast Asia [89]–although it is closely related to *Cx. pseudovishnui* [88] in which the rate of *Wolbachia* infection was high. However, studies in India and Thailand showed a reverse pattern, with *Wolbachia* infection present in *Cx. vishnui* but not in *Cx. pseudovishnui* [39, 85]. As the two species are morphologically similar [53], DNA barcoding was conducted to aid morphological identification, and thus avoid any misidentification. The results lend further support to possible variation in infection prevalence between geographically distant populations.

We did not detect *Wolbachia* in any of the wild-caught *Anopheles* species (18 individuals representing three species), many of which are potential malaria vectors [86]. This is largely consistent with previous reports from different countries [39, 90, 91]. The absence of *Wolbachia* in *Anopheles* mosquitoes is thought to be due to the unsuitability of *Anopheles* reproductive tissues for *Wolbachia* establishment [84, 85]. However, there have been recent reports of *Wolbachia* detected in wild *Anopheles* mosquitoes from West Africa [42, 92, 93] and Malaysia [94]. Knowledge of natural *Wolbachia* infections in *Anopheles* mosquitoes is important for malaria control strategies [93], hence more wild-caught *Anopheles* samples should be screened in Singapore to determine more accurately their infection status.

Wolbachia were not detected in *Ae. aegypti*, the primary vector of dengue in Southeast Asia [87]. Conversely, *Wolbachia* infection was moderately high in the secondary vector *Ae. albopictus*. These results are highly consistent with those of past studies, which reported an absence of infection in wild *Ae. aegypti* [21, 95], but found stable infection in wild *Ae. albopictus* [96]. Although *Ae. aegypti* and *Ae. albopictus* belong to the same subgenus, *Stegomyia*, and occupy similar ecological niches [97], they are rarely found in the same locality, [43, 98, 99], as also observed in this study. This could imply a certain degree of competitive exclusion between the two species, preventing them from occupying the same space. There is evidence that symbionts may influence a host's resource acquisition and specificity, which may ultimately lead to competitive exclusion between closely related host species with differing symbiont infections [100, 101]. However, research on *Wolbachia*-induced competitive exclusion is scarce except for a few studies on heterogonic gall wasps [102], grasshoppers [103], and gall-inducing aphids [104]. Given the widespread influence of *Wolbachia*, future research should explore potential cases of *Wolbachia*-induced competitive exclusion between closely related species of mosquitoes as this may have major implications for an understanding of their symbioses and speciation.

Additionally, although *Ae. aegypti* is frequently artificially infected with *Wolbachia* for biocontrol purposes [105–109], our findings suggest that infected *Ae. aegypti* might not be stably maintained in the wild. This may be advantageous for vector population suppression as the cytoplasmic-incompatibility effect of any artificially introduced *Wolbachia* strain will likely be fully manifested in the uninfected native population [21]. However, this also implies that this type of biocontrol method may have low long-term effectiveness if the infection cannot be naturally sustained in the wild population. The detection of natural *Wolbachia* infection in wild *Ae. aegypti*, therefore, has huge implications for vector control programmes [21]. Not only does it inform the selection of a suitable *Wolbachia* strain prior to its field release, but it can also be used to gauge the long-term effectiveness of a specific vector control programme.

Interestingly, the sex of the mosquitoes had an effect on their *Wolbachia* infection status. This could be an artefact of various *Wolbachia*-induced reproductive phenotypes, such as parthenogenetic and male-killing ones, resulting in offspring that are largely female [15]. If this were true, over multiple generations with vertical *Wolbachia* transmission, one should observe an increasing proportion of females that are infected. Hence, the phenomenon observed here could be a consequence of reproductive manipulation by *Wolbachia* and vertical transmission.

While we were unable to statistically test for the effects of locality on infection status due to uneven and small sample sizes of the respective species across different localities, our results suggest that mosquitoes found in localities across Singapore have roughly equal chances of harbouring *Wolbachia*. This also suggests that underlying physiological factors and phylogenetic relatedness in mosquitoes contribute more to their infection by *Wolbachia* than the habitat in which they are found.

The reproductive effect of *Wolbachia* can be masked or enhanced by other reproductive endosymbionts such as *Cardinium*, *Rickettsia*, and *Spiroplasma* [7, 26–29]. Unfortunately, we were unable to detect these

endosymbionts due to a high degree of false positives with the PCR-based screening methods used here (Additional file 1). This was likely due to using primers that are not optimised for screening mosquito-specific endosymbionts [110–112]. As a result, co-infections with various reproductive endosymbionts, which would have provided greater insights into the synergistic effects of co-infections on mosquito evolution, could not be identified among the wild mosquitoes examined here. There is, hence, a need to develop and optimise alternative screening methods, such as multilocus sequence typing (MLST) techniques, especially for the detection of *Cardinium*, *Rickettsia*, and *Spiroplasma* in mosquitoes.

Tissue tropism of *Wolbachia* infection in mosquitoes

Wolbachia were detected mainly in the reproductive tissues, which agrees with results from studies across multiple insect groups [15, 84, 113], and suggests that *Wolbachia* are mainly vertically transmitted. Interestingly, through the course of this study, there was significant variation in reproductive traits (testis and ovary length) across and within species. These reproductive traits did not vary significantly with *Wolbachia* infection status, even after accounting for phylogenetic relatedness (see Additional file 2).

Infection in the gut and leg tissues was detected, albeit infrequently. This is not surprising, as previous studies have also detected *Wolbachia* in those tissues [34–36, 114]. Interestingly, the nucleotide sequences from gut and leg infections tend to be shorter in length. Considering that *Wolbachia* are unlikely to survive extracellularly for a long period of time [35], the small amplicon size suggests potential horizontal integration of the *Wolbachia* genome into the host genome for a few species. This phenomenon has been observed in several *Wolbachia* hosts [115, 116], and mosquito species such as *Ae. aegypti* and *Cx. quinquefasciatus* [117, 118]. A recent study showed that horizontal integration of the *Wolbachia* genome into the host genome can have implications for sex determination and evolution. This is evident in the common pillbug *Armadillidium vulgare*, and results in the formation of a new sex chromosome [119]. Researchers have also proposed that horizontal gene transfer between an endosymbiont and host can result in evolutionary innovation where new functional genes arise in both host and bacteria [117, 118].

Future research should explore the relative importance of each transmission method with relation to host-endosymbiont ecology and evolution. Tissue-specific screening methods such as those used here can be used in other arthropods, especially when the mode of transmission is not clear. Currently, most *Wolbachia* screening is conducted on ground specimens or specimens in their entirety [39–41]. In these cases, researchers are unable to determine tissue tropism of *Wolbachia* infection, which could provide clues to its mode of transmission. Thus, adopting tissue-specific screening methods would enable researchers to verify or refute the commonly reported assumption that *Wolbachia* is transmitted vertically [15, 30].

Diversity and host-specificity of *Wolbachia* strains

Not only does the *wsp* molecular marker allow successful detection of *Wolbachia* infection across numerous taxa, it also enables strain genotyping and evolutionary comparison between detected *Wolbachia* strains [60]. In this study, *Wolbachia wsp* sequences were clustered into 12 putative *Wolbachia* strains falling within supergroup A or B. This is consistent with the results of previous studies that looked at *Wolbachia* infections in mosquitoes [39, 80, 85]. Each mosquito host species was only infected by strains belonging to supergroups A or B, with the exception of *Ae. albopictus*, which harboured both. Infection with more than one strain (superinfection of wild *Ae. albopictus* with *Wolbachia* supergroups A and B) has been previously reported, and this phenomenon was commonly observed to be fixed in the examined populations due to strong cytoplasmic incompatibility effects [120, 121]. This suggests stable vertical transmission of both strains in *Ae. albopictus*. Additionally, only four out of 12 putative strains were identified as previously typed *Wolbachia* strains reported by Zhou et al. [60] and Ruang-Areerate et al. [80]—Wol 1, Wol 2, Wol 3, and Wol 8 were identified as *w*Pip, *w*AlbB, *w*Cra, and *w*Ri strain, respectively.

Host specificity is thought to be a characteristic of the ancestral *Wolbachia* strain, with host flexibility reported mainly in *Wolbachia* supergroups A and B [122]. In our study, we found a combination of specialists and generalists, with more of the former. A study of mosquitoes from Taiwan showed a similar pattern [84]. In beetles, a mixture of *Wolbachia* supergroup A host-specific and host-flexible strains within a population has also been reported [49]. While our estimates of specialists and generalists might vary with greater sampling effort, the higher numbers of specialists observed can be explained by the process of reciprocal selection between host and endosymbiont over evolutionary time [123]. This is also known as Red Queen dynamics, where the endosymbiont constantly adapts to its host to ensure continued establishment in the same host [124]. An alternative, generalist strategy can also be maintained in a population. It ensures survival in an environment where resources (i.e. hosts) are rarely found [123]. However, there are generally more instances of

host specialists than generalists across numerous parasitic and endosymbiotic taxa [125–127].

The SPS scores revealed that host flexibility among the generalists varied greatly. Understanding *Wolbachia* host specificity has huge implications, especially for the optimisation of *Wolbachia* biocontrol strategies. Not only should researchers select strains that can effectively limit pathogen replication [128], they should also select strains for their host specificity. This is not possible without the screening of a wide variety of species or closely related species, which was achieved in this study. Using a host-specific strain will decrease the likelihood of host shift to non-target species, and thereby minimise the overall ecological risk of a strategy.

Evolutionary relationships between mosquitoes and *Wolbachia*

Host-*Wolbachia* relationships are often understudied and limited to a few taxa [52]. Studies have shown that the evolutionary associations between *Wolbachia* and their insect hosts do vary across taxa [49–52, 129]. Likewise, our exploratory analyses of mosquito hosts and their *Wolbachia* infection support such a complex relationship, with neither co-speciation nor host shifting fully accounting for evolutionary association in these lineages.

Based on the tanglegram, a broad association pattern between mosquitoes and *Wolbachia* strains was observed (Fig. 3). *Aedes* mosquitoes tended to be associated with *Wolbachia* supergroup A, while other mosquito species, particularly of the genus *Culex*, were largely associated with *Wolbachia* supergroup B. This showed that closely related *Wolbachia* strains are likely to establish themselves in related hosts. There might have been radiation of *Wolbachia* in these clades after their respective initial establishment. Nevertheless, the observed variations in host-endosymbiont associations make us question the mosquito-*Wolbachia* association pattern.

The ParaFit analysis showed weak support for congruency between host and endosymbiont phylogenies. Among the 18 host-*Wolbachia* associations, only the link between *Mansonia indiana* and Wol 3 showed a significant association (Fig. 3). This was interesting considering that Wol 3 was largely host flexible. Given that this was the only significant association, it is worth carrying out further genus-specific study on *Mansonia* spp. to elucidate coevolutionary patterns within a group of closely related mosquito species. It is possible that the degree to which *Wolbachia* co-evolve with their mosquito hosts varies across different taxonomic levels [74]. The analyses carried out thus far suggest that mosquito-*Wolbachia* associations are likely random at higher taxonomic levels, and that mosquito-*Wolbachia* co-speciation occurs at finer phylogenetic resolution (i.e. similar to patterns seen in diffuse coevolution).

The event-based analysis performed in Jane 4.0 (Fig. 4) indicated that co-speciation events were infrequent as compared to other evolutionary events. We noticed a greater proportion of host shifts and numerous losses. Interestingly, the least cost coevolutionary reconstruction indicated multiple consecutive host shifts occurring near the tips of the cladogram. This suggests that co-speciation does not fully explain the evolutionary associations between mosquito hosts and *Wolbachia*. Instead, recent host shifting through horizontal transmission seems to promote *Wolbachia* diversification. This lends greater support to the idea that horizontal transmission between distantly related species is possible [32, 33, 130].

Furthermore, losses, which represent endosymbiont extinction events that occurred upon host speciation, seem to dominate the evolutionary history of *Wolbachia*. Extinction events are believed to be frequent in host-endosymbiont systems [123], due to either evolution of resistance in the host or declining host population size, which result in the inability of highly specialised endosymbionts to establish themselves [131, 132]. Additionally, losses could potentially influence endosymbiont evolution through the creation of vacant niches [131]. The observed losses followed by host shifts in the mosquito-*Wolbachia* relationship are possible consequences of vacant niche exploitation by generalists. Perhaps this enabled successful endosymbiont invasion due to minimal intra-strain competition. If this were true, horizontal *Wolbachia* transmission and losses may play a bigger role in accounting for *Wolbachia* diversity than previously thought.

As this was an exploratory study, we were unable to determine the exact mechanism behind the diversity and evolutionary associations of *Wolbachia*. The presence of numerous specialists could be a sign of mosquito-*Wolbachia* coevolution since coevolution is fundamentally reciprocal selection between host and endosymbiont which gives rise to micro-evolutionary changes [133]. The numerous host shifts and losses might have, however, blurred the effects of vertical transmission over a long evolutionary period [52]. Thus, co-speciation might have occurred within smaller clades of *Wolbachia* and mosquitoes, but at higher taxa levels, horizontal transmission and loss events are more likely the prominent force driving *Wolbachia* evolution.

Strengths, limitations, and future directions

The three distinct methods employed here to explore evolutionary associations have both strengths and limitations. The tanglegram allows for clear visualisation of host-endosymbiont association without taking into

account any evolutionary relationships, but there have been calls for careful interpretation of the results generated using this method as the degree of entanglement may not necessarily represent phylogenetic congruence [134]. The Global ParaFit test seeks to address this limitation by testing for global congruency with an unbiased, statistical approach [74]. The event-based method enables the evaluation of potential evolutionary events that might have occurred throughout an endosymbiont's evolutionary history such as co-speciation, duplication, and host shifting. This last method, however, cannot fully differentiate a topological congruence from an evolutionary event [135]. Without knowledge of the time of divergence for both symbiont and host, a co-phylogenetic pattern may be better explained by ecological factors (as compared to co-speciation) given that bacterial lineages often evolve faster than their hosts [136, 137], and the high likelihood of host shifts among closely related species [133].

The *Wolbachia wsp* gene has been shown to provide well-resolved phylogenies [60], and this study provides an exploratory snapshot of the evolutionary associations between mosquito hosts and their *Wolbachia* endosymbionts. There is, of course, a potential caveat, since only a single gene was used to construct the respective phylogenetic trees. To obtain a more accurate phylogeny, future studies could adopt MLST [17, 51], or whole-genome shotgun sequencing [52]. The former could potentially characterise putative *Wolbachia* strains that cannot be distinguished with *wsp* gene primers.

Notwithstanding their limitations, the employment of various analytical methods allows for a comprehensive examination of the evolutionary associations between *Wolbachia* and mosquito hosts which are presently lacking in the literature. The scope of future studies that examine the evolution of medically important vector species could be narrowed to the Aedini tribe, as this would provide greater statistical power for the examination of mosquito-endosymbiont associations.

Conclusion

To our knowledge, this is the first study to examine *Wolbachia* infections in wild mosquitoes in Singapore. We detected 12 putative strains of *Wolbachia* among 40 mosquito species, and recorded infections in seven species for which, to our knowledge, *Wolbachia* infections have not been previously reported. By employing a tissue-specific PCR screening method, we were able to observe that the *Wolbachia* infections were preferentially located in the reproductive tissues, which provides support for vertical transmission as the main mode of infection transmission. However, even if *Wolbachia* infection is mainly transmitted vertically, this is unlikely to fully explain the observed diversity of *Wolbachia* and why closely related *Wolbachia* lineages were found in distantly related mosquito species. Hence, this study also served as an exploratory study which examined mosquito-*Wolbachia* evolutionary associations across a wide range of host mosquito species through three evolutionary analyses. Overall, we propose that the evolutionary associations between mosquito hosts and *Wolbachia* are consequences of both vertical and horizontal transmission and various evolutionary events.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13071-020-04466-8.

Additional file 1: Table S1. Polymerase chain reaction (PCR) screening of *Cardinium*, *Rickettsia*, and *Spiroplasma* in wild mosquitoes from Singapore.

Additional file 2: Figure S1. Weighted reproductive tissue length across various mosquito species.

Abbreviations

BLAST: Basic Local Alignment Search Tool; *cox*1: Cytochrome c oxidase subunit I gene; MLST: Multilocus sequence typing; mtDNA: Mitochondrial DNA; NJ: Neighbour joining; PCR: Polymerase chain reaction; SPS: Standardised phylogenetic host specificity; *wsp*: *Wolbachia* surface protein gene.

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Authors' contributions

HY and NP designed the research. HD and HY collected the mosquitoes from the field. HY identified the mosquito samples. HD performed the DNA extraction and PCR. HD and HY carried out the sequence analyses. HD, HY, and NP interpreted the results and wrote the manuscript. All the authors read and approved the final draft of the manuscript.

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Availability of data and materials

The datasets generated and/or analysed during this study are available in the Dryad repository, https://doi.org/10.5061/dryad.zs7h44j63. Sequence data that support the findings of this study have been deposited in Genbank with the accession codes MT645167–MT645184.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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References

1. Weeks AR, Reynolds KT, Hoffmann AA, Tracy K, Ary R, Hoffmann A. *Wolbachia* dynamics and host effects: what has (and has not) been demonstrated? Trends Ecol Evol. 2002;17:257–62.
2. Duron O, Bouchon D, Boutin S, Bellamy L, Zhou L, Engelstädter J, et al. The diversity of reproductive parasites among arthropods: *Wolbachia* do not walk alone. BMC Biol. 2008;6:1–12.
3. Zug R, Hammerstein P. Still a host of hosts for *Wolbachia*: analysis of recent data suggests that 40% of terrestrial arthropod species are infected. PLoS ONE. 2012;7:7–9.
4. Zchori-Fein E, Borad C, Harari AR. Oogenesis in the date stone beetle, *Coccotrypes dactyliperda*, depends on symbiotic bacteria. Physiol Entomol. 2006;31:164–9.
5. Moran NA, Mccutcheon JP, Nakabachi A. Genomics and evolution of heritable bacterial symbionts. Annu Rev Genet. 2008;42:165–90.
6. Fenn K, Blaxter M. Are filarial nematode *Wolbachia* obligate mutualist symbionts? Trends Ecol Evol. 2004;19:163–6.
7. Zchori-Fein E, Perlman SJ. Distribution of the bacterial symbiont *Cardinium* in arthropods. Mol Ecol. 2004;13:2009–16.
8. Weeks AR, Turelli M, Harcombe WR, Reynolds KT, Hoffmann AA. From parasite to mutualist: rapid evolution of *Wolbachia* in natural populations of *Drosophila*. PLoS Biol. 2007;5:e114.
9. Jiggins FM, Hurst GDD, Majerus MEN. Sex ratio distortion in *Acraea encedon* (Lepidoptera: Nymphalidae) is caused by a male-killing bacterium. Heredity (Edinb). 1998;81:87–91.
10. Rousset F, Bouchon D, Pintureau B, Juchault P, Solignac M. *Wolbachia* endosymbionts responsible for various alterations of sexuality in arthropods. Proc R Soc B Biol Sci. 1992;250:91–8.
11. Richard FJ. Symbiotic bacteria influence the odor and mating preference of their hosts. Front Ecol Evol. 2017;5:143.
12. Weeks AR, Breeuwer JAJ. *Wolbachia*-induced parthenogenesis in a genus of phytophagous mites. Proc R Soc B Biol Sci. 2001;268:2245–51.
13. Ma WJ, Schwander T. Patterns and mechanisms in instances of endosymbiont-induced parthenogenesis. J Evol Biol. 2017;30:868–88.
14. Moretti R, Calvitti M. Male mating performance and cytoplasmic incompatibility in a *w*Pip *Wolbachia* trans-infected line of *Aedes albopictus* (*Stegomyia albopicta*). Med Vet Entomol. 2013;27:377–86.
15. Werren J, Baldo L, Clark ME. *Wolbachia*: master manipulators of invertebrate biology. Nat Rev Microbiol. 2008;6:741–51.
16. Tseng SP, Wetterer JK, Suarez AV, Lee CY, Yoshimura T, Shoemaker DW, et al. Genetic diversity and *Wolbachia* infection patterns in a globally distributed invasive ant. Front Genet. 2019;10:1–15.
17. Atyame CM, Delsuc F, Pasteur N, Weill M, Duron O. Diversification of *Wolbachia* endosymbiont in the *Culex pipiens* mosquito. Mol Biol Evol. 2011;28:2761–72.
18. Kajtoch Ł, Kotásková N. Current state of knowledge on *Wolbachia* infection among Coleoptera: a systematic review. PeerJ. 2018;6:e4471.
19. Bourtzis K, Dobson SL, Xi Z, Rasgon JL, Calvitti M, Moreira LA, et al. Harnessing mosquito–*Wolbachia* symbiosis for vector and disease control. Acta Trop. 2014;132:150–63.
20. Blagrove MSC, Arias-goeta C, Di GC, Failloux A, Sinkins SP. A *Wolbachia w*Mel transinfection in *Aedes albopictus* is not detrimental to host fitness and inhibits chikungunya virus. PLoS Negl Trop Dis. 2013;7:e2152.
21. Ross P. An elusive endosymbiont: does *Wolbachia* occur naturally in *Aedes aegypti*? Ecol Evol. 2020;10:1581–91.
22. Nguyen TH, Le NH, Nguyen TY, Vu SN, Tran ND, Le TN, et al. Field evaluation of the establishment potential of *w*melpop *Wolbachia* in Australia and Vietnam for dengue control. Parasites Vectors. 2015;8:1–14.
23. Nazni WA, Hoffmann AA, NoorAfizah A, Cheong YL, Mancini MV, Golding N, et al. Establishment of *Wolbachia* strain *w*AlbB in Malaysian populations of *Aedes aegypti* for dengue control. Curr Biol. 2019;29:4241–8.
24. National Environment Agency. *Wolbachia-Aedes* mosquito suppression strategy. 2018. https://www.nea.gov.sg/corporate-functions/resources/research/wolbachia-aedes-mosquito-suppression-strategy/frequently-asked-questions. Accessed 13 Sept 2020.
25. Iturbe-Ormaetxe I, Walker T, O'Neill SL. *Wolbachia* and the biological control of mosquito-borne disease. EMBO Rep. 2011;12:508–18.
26. Martin OY, Puniamoorthy N, Gubler A, Wimmer C, Bernasconi MV. Infections with *Wolbachia*, *Spiroplasma*, and *Rickettsia* in the Dolichopodidae and other Empidoidea. Infect Genet Evol. 2013;13:317–30.
27. White JA, Kelly SE, Cockburn SN, Perlman SJ, Hunter MS. Endosymbiont costs and benefits in a parasitoid infected with both *Wolbachia* and *Cardinium*. Heredity. 2011;106:585–91.
28. Zhang YK, Chen YT, Yang K, Qiao GX, Hong XY. Screening of spider mites (Acari: Tetranychidae) for reproductive endosymbionts reveals links between co-infection and evolutionary history. Sci Rep. 2016;6:1–9.
29. Engelstädter J, Telschow A, Yamamura N. Coexistence of cytoplasmic incompatibility and male-killing-inducing endosymbionts, and their impact on host gene flow. Theor Popul Biol. 2008;73:125–33.
30. Engelstädter J, Hurst GDD. The ecology and evolution of microbes that manipulate host reproduction. Annu Rev Ecol Evol Syst. 2009;40:127–49.
31. Heath BD, Butcher RDJ, Whitfield WGF, Hubbard SF. Horizontal transfer of *Wolbachia* between phylogenetically distant insect species by a naturally occurring mechanism. Curr Biol. 1999;9:313–6.
32. Ahmed MZ, Li S, Xue X, Yin X, Ren S. The intracellular bacterium *Wolbachia* uses parasitoid wasps as phoretic vectors for efficient horizontal transmission. PLoS Pathog. 2015;11:e1004672.
33. Li S, Ahmed MZ, Lv N, Shi P, Wang X, Huang J-L, et al. Plant-mediated horizontal transmission of *Wolbachia* between whiteflies. ISME J. 2017;11:1019–28.
34. Frost CL, Pollock SW, Smith JE, Hughes WOH. *Wolbachia* in the flesh: symbiont intensities in germ-line and somatic tissues challenge the conventional view of *Wolbachia* transmission routes. PLoS ONE. 2014;9:e95122.
35. Pietri JE, DeBruhl H, Sullivan W. The rich somatic life of *Wolbachia*. Microbiol Open. 2016;5:923–36.
36. Dobson SL, Bourtzis K, Braig HR, Jones BF, Zhou W, Rousset F, et al. *Wolbachia* infections are distributed throughout insect somatic and germ line tissues. Insect Biochem Mol Biol. 1999;29:153–60.
37. Espino CI, Gómez T, González G, Brazil Do Santos MF, Solano J, Sousa O, et al. Detection of *Wolbachia* bacteria in multiple organs and feces of the triatomine insect *Rhodnius pallescens* (Hemiptera, Reduviidae). Appl Environ Microbiol. 2009;75:547–50.
38. Andersen SB, Boye M, Nash DR, Boomsma JJ. Dynamic *Wolbachia* prevalence in *Acromyrmex* leaf-cutting ants: potential for a nutritional symbiosis. J Evol Biol. 2012;25:1340–50.
39. Nugapola NWNP, De Silva WAPP, Karunaratne SHPP. Distribution and phylogeny of *Wolbachia* strains in wild mosquito populations in Sri Lanka. Parasites Vectors. 2017;10:1–8.
40. Sunish IP, Rajendran R, Paramasivan R, Dhananjeyan KJ, Tyagi BK. *Wolbachia* endobacteria in a natural population of *Culex quinquefasciatus* from filariasis endemic villages of south India and its phylogenetic implication. Trop Biomed. 2011;28:569–76.
41. Thongsripong P, Chandler JA, Green AB, Kittayapong P, Wilcox BA, Kapan DD, et al. Mosquito vector-associated microbiota: metabarcoding bacteria and eukaryotic symbionts across habitat types in Thailand endemic for dengue and other arthropod-borne diseases. Ecol Evol. 2018;8:1352–68.
42. Niang EHA, Bassene H, Makoundou P, Fenollar F, Weill M, Mediannikov O. First report of natural *Wolbachia* infection in wild *Anopheles funestus* population in Senegal. Malar J. 2018;17:1–6.
43. Kulkarni A, Yu W, Jiang J, Sanchez C, Karna AK, Martinez KJL, et al. *Wolbachia pipientis* occurs in *Aedes aegypti* populations in New Mexico and Florida, USA. Ecol Evol. 2019;9:6148–56.
44. Leggewie M, Krumkamp R, Badusche M, Heitmann A, Jansen S, Schmidt-Chanasit J, et al. *Culex torrentium* mosquitoes from Germany are negative for *Wolbachia*. Med Vet Entomol. 2018;32:115–20.
45. Bozorg-Omid F, Oshaghi MA, Vahedi M, Karimian F, Seyyed-Zadeh SJ, Chavshin AR. *Wolbachia* infection in West Nile Virus vectors of northwest Iran. Appl Entomol Zool. 2020;55:105–13.
46. Jeffries CL, Tantely LM, Raharimalala FN, Hurn E, Boyer S, Walker T. Diverse novel resident *Wolbachia* strains in culicine mosquitoes from Madagascar. Sci Rep. 2018;8:1–15.
47. Shaikevich E, Bogacheva A, Rakova V, Ganushkina L, Ilinsky Y. *Wolbachia* symbionts in mosquitoes: intra- and intersupergroup recombinations,

horizontal transmission and evolution. Mol Phylogenet Evol. 2019;134:24–34.
48. Hurst GDD, Jiggins FM. Problems with mitochondrial DNA as a marker in population, phylogeographic and phylogenetic studies: the effects of inherited symbionts. Proc R Soc B Biol Sci. 2005;272:1525–34.
49. Sontowski R, Bernhard D, Bleidorn C, Schlegel M, Gerth M. *Wolbachia* distribution in selected beetle taxa characterized by PCR screens and MLST data. Ecol Evol. 2015;5:4345–53.
50. Balvín O, Roth S, Talbot B, Reinhardt K. Co-speciation in bedbug *Wolbachia* parallel the pattern in nematode hosts. Sci Rep. 2018;8:1–9.
51. Lefoulon E, Bain O, Makepeace BL, D'Haese C, Uni S, Martin C, et al. Breakdown of coevolution between symbiotic bacteria *Wolbachia* and their filarial hosts. PeerJ. 2016;4:e1840.
52. Gerth M, Röthe J, Bleidorn C. Tracing horizontal *Wolbachia* movements among bees (Anthophila): a combined approach using multilocus sequence typing data and host phylogeny. Mol Ecol. 2013;22:6149–62.
53. Chan A, Chiang L-P, Hapuarachchi HC, Tan C-H, Pang S-C, Lee R, et al. DNA barcoding: complementing morphological identification of mosquito species in Singapore. Parasites Vectors. 2014;7:1–12.
54. Rattanarithikul R, Harbach RE, Harrison BA, Panthusiri P, Coleman RE, Richardson JH. Illustrated keys to the mosquitoes of Thailand. VI. Tribe Aedini. Southeast Asian J Trop Med Public Health. 2010;41:1–225.
55. Rattanarithikul R, Harbach RE, Harrison BA, Panthusiri P, Coleman RE. Illustrated keys to the mosquitoes of Thailand. V. Genera *Orthopodomyia*, *Kimia*, *Malaya*, *Topomyia*, *Tripteroides*, and *Toxorhynchites*. Southeast Asian J Trop Med Public Health. 2007;38:1.
56. Rattanarithikul R, Harrison BA, Panthusiri P, Peyton EL, Coleman RE. Illustrated keys to the mosquitoes of Thailand. III. Genera *Aedeomyia*, *Ficalbia*, *Mimomyia*, *Hodgesia*, *Coquillettidia*, *Mansonia*, and *Uranotaenia*. Southeast Asian J Trop Med Public Health. 2006;37:1–85.
57. Rattanarithikul R, Harrison BA, Panthusiri P, Coleman RE. Illustrated keys to the mosquitoes of Thailand. I. Background; geographic distribution; lists of genera, subgenera, and species; and a key to the genera. Southeast Asian J Trop Med Public Health. 2005;36:1–80.
58. Rattanarithikul R, Harbach RE, Harrison BA, Panthusiri P, Jones JW. Illustrated keys to the mosquitoes of Thailand. II. Genera *Culex* and *Lutzia*. Southeast Asian J Trop Med Public Health. 2005;36:1–97.
59. Rattanarithikul R, Harrison BA, Harbach RE, Panthusiri P, Coleman RE. Illustrated keys to the mosquitoes of Thailand. IV. *Anopheles*. Southeast Asian J Trop Med Public Health. 2006;37:1–128.
60. Zhou W, Rousset F, O'Neill S. Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences. Proc R Soc Lond B. 1998;265:509–15.
61. Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol. 1994;3:294–9.
62. Madden T. The BLAST sequence analysis tool. NCBI Handbook. 2nd Ed. Bethesda: National Center for Biotechnology Information (US); 2013.
63. R Core Team. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. 2019.
64. Sweet L. Nonpar: a collection of nonparametric hypothesis tests. R package version 1.0.1. 2017. https://CRAN.R-project.org/package=nonpar. Accessed 23 Jun 2020.
65. Mangiafico S. Rcompanion: functions to support extension education program evaluation. R package version 2.3.7. 2019. https://CRAN.R-project.org/package=rcompanion. Accessed 23 Jun 2020.
66. James G, Witten D, Hastie T, Tibshirani R. ISLR: data for an introduction to statistical learning with applications in R. R package version 1.2. 2017. https://CRAN.R-project.org/package=ISLR. Accessed 23 Jun 2020.
67. Thompson JD, Higgins DG, Gibson TJ. ClustalW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 1994;22:4673–80.
68. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol. 2018;35:1547–9.
69. NCBI Resource Coordinators. Database resources of the National Center for Biotechnology Information. Nucleic Acids Res. 2016;44:D7-19.
70. Poulin R, Krasnov BR, Mouillot D. Host specificity in phylogenetic and geographic space. Trends Parasitol. 2011;27:355–61.
71. Kembel SW, Cowan PD, Helmus M, Cornwell W, Morlon H, Ackerly D, et al. Picante: R tools for integrating phylogenies and ecology. Bioinformatics. 2010;26:1463–4.
72. Charleston M. TreeMap 3b. 2011. https://sites.google.com/site/cophylogeny. Accessed 23 Jun 2020.
73. Matsen FA, Billey SC, Kas A, Konvalinka M. Tanglegrams: a reduction tool for mathematical phylogenetics. IEEE/ACM Trans Comput Biol Bioinforma. 2018;15:343–9.
74. Legendre P, Desdevises Y, Bazin E. A statistical test for host-parasite coevolution. Syst Biol. 2002;51:217–34.
75. Balbuena JA, Míguez-Lozano R, Blasco-Costa I. PACo: a novel Procrustes application to cophylogenetic analysis. PLoS ONE. 2013;8:e61048.
76. Paradis E, Schliep K. Ape 5.0: an environment for modern phylogenetics and evolutionary analyses in R. Bioinformatics. 2019;35:526–8.
77. Conow C, Fielder D, Ovadia Y, Libeskind-Hadas R. Jane: a new tool for the cophylogeny reconstruction problem. Algorithms Mol Biol. 2010;5:1–10.
78. Charleston M. Jungles: a new solution to the host/parasite phylogeny reconciliation problem. Math Biosci. 1998;149:191–223.
79. Li YM, Shivas RG, Cai L. Cryptic diversity in *Tranzscheliella* spp. (Ustilaginales) is driven by host switches. Sci Rep. 2017;7:43549.
80. Ruang-Areerate T, Kittayapong P, Baimai V, O'Neill SL. Molecular phylogeny of *Wolbachia* endosymbionts in Southeast Asian mosquitoes (Diptera: Culicidae) based on *wsp* gene sequences. J Med Entomol. 2003;40:1–5.
81. Noor-Shazleen-Husnie MM, Emelia O, Ahmad-Firdaus MS, Zainol-Ariffin P, Aishah-Hani A. Detection of *Wolbachia* in wild mosquito populations from selected areas in Peninsular Malaysia by loop-mediated isothermal amplification (LAMP) technique. Trop Biomed. 2018;35:330–46.
82. Wiwatanaratanabutr I. Geographic distribution of wolbachial infections in mosquitoes from Thailand. J Invertebr Pathol. 2013;114:337–40.
83. Ravikumar H, Ramachandraswamy N, Sampathkumar S, Prakash BM, Huchesh HC, Uday J, et al. A preliminary survey for *Wolbachia* and bacteriophage *WO* infections in Indian mosquitoes (Diptera: Culicidae). Trop Biomed. 2010;27:384–93.
84. Tsai K-H, Lien J-C, Huang C-G, Wu W-J, Chen W-J. Molecular (sub) grouping of endosymbiont *Wolbachia* infection among mosquitoes of Taiwan. J Med Entomol. 2004;41:677–83.
85. Kittayapong P, Baisley KJ, Baimai V, O'Neill SL. Distribution and diversity of *Wolbachia* infections in Southeast Asian mosquitoes (Diptera: Culicidae). J Med Entomol. 2000;37:340–5.
86. Lam-Phua SG, Yeo H, Lee RML, Chong CS, Png AB, Foo SY, et al. Mosquitoes (Diptera: Culicidae) of Singapore: updated checklist and new records. J Med Entomol. 2019;56:103–19.
87. Foster WA, Walker ED. Mosquitoes (Culicidae). In: Mullen G, Durden L, editors. Medical and Veterinary Entomology. New York: Academic Press; 2018. p. 261–325.
88. Yeo G, Wang Y, Chong SM, Humaidi M, Lim XF, Mailepessov D, et al. Characterization of fowlpox virus in chickens and bird-biting mosquitoes: a molecular approach to investigating avipoxvirus transmission. J Gen Virol. 2019;100:838–50.
89. Vythilingam I, Oda K, Chew TK, Mahadevan S, Vijayamalar B, Morita K, et al. Isolation of Japanese encephalitis virus from mosquitoes collected in Sabak Bernam, Selangor, Malaysia in 1992. J Am Mosq Control Assoc. 1995;11:94–8.
90. de Oliveira CD, Gonçalves DS, Baton LA, Shimabukuro PHF, Carvalho FD, Moreira LA. Broader prevalence of *Wolbachia* in insects including potential human disease vectors. Bull Entomol Res. 2015;105:305–15.
91. Shaikevich E, Bogacheva A, Ganushkina L. *Dirofilaria* and *Wolbachia* in mosquitoes (Diptera: Culicidae) in central European Russia and on the Black Sea coast. Parasite. 2019;26:1–12.
92. Baldini F, Segata N, Pompon J, Marcenac P, Robert Shaw W, Dabiré RK, et al. Evidence of natural *Wolbachia* infections in field populations of *Anopheles gambiae*. Nat Commun. 2014;5:1–7.
93. Gomes FM, Hixson BL, Tyner MDW, Ramirez JL, Canepa GE, Alves e Silva TL, et al. Effect of naturally occurring Wolbachia in *Anopheles gambiae* s.l. mosquitoes from Mali on *Plasmodium falciparum* malaria transmission. Proc Natl Acad Sci. 2017;114:12566–71.
94. Wong ML, Liew JWK, Wong WK, Pramasivan S, Mohamed Hassan N, Wan Sulaiman WY, et al. Natural *Wolbachia* infection in field-collected

Anopheles and other mosquito species from Malaysia. Parasites Vectors. 2020;13:1–15.
95. Gloria-Soria A, Chiodo TG, Powell JR. Lack of evidence for natural *Wolbachia* infections in *Aedes aegypti* (Diptera : Culicidae). J Med Entomol. 2018;55:1354–6.
96. Kittayapong P, Baimai V, O'Neill SL. Field prevalence of *Wolbachia* in the mosquito vector *Aedes albopictus*. Am J Trop Med Hyg. 2002;66:108–11.
97. Lounibos LP, Juliano SA. Where vectors collide: the importance of mechanisms shaping the realized niche for modeling ranges of invasive *Aedes* mosquitoes. Biol Invasions. 2018;20:1913–29.
98. Chan KL, Chan YC, Ho BC. *Aedes aegypti* (L.) and *Aedes albopictus* (Skuse) in Singapore city. 4. Competition between species. Bull World Health Organ. 1971;44:643–9.
99. Coon KL, Brown MR, Strand MR. Mosquitoes host communities of bacteria that are essential for development but vary greatly between local habitats. Mol Ecol. 2016;25:5806–26.
100. Brucker RM, Bordenstein SR. Speciation by symbiosis. Trends Ecol Evol. 2012;27:443–51.
101. Janson EM, Stireman JO, Singer MS, Abbot P. Phytophagous insect-microbe mutualisms and adaptive evolutionary diversification. Evol Int J Org Evol. 2008;62:997–1012.
102. Schuler H, Egan SP, Hood GR, Busbee RW, Driscoe AL, Ott JR. Diversity and distribution of *Wolbachia* in relation to geography, host plant affiliation and life cycle of a heterogonic gall wasp. BMC Evol Biol. 2018;18:1–15.
103. Martínez-Rodríguez P, Bella JL. *Chorthippus parallelus* and *Wolbachia*: overlapping orthopteroid and bacterial hybrid zones. Front Genet. 2018;9:604.
104. Amit L, Ben-Shlomo R, Chiel E. Are microbial symbionts involved in the speciation of the gall-inducing aphid, *Slavum wertheimae*? Arthropod Plant Interact. 2017;11:475–84.
105. Hancock PA, White VL, Callahan AG, Godfray CHJ, Hoffmann AA, Ritchie SA. Density-dependent population dynamics in *Aedes aegypti* slow the spread of *w*Mel *Wolbachia*. J Appl Ecol. 2016;53:785–93.
106. Walker T, Johnson PH, Moreira LA, Iturbe-Ormaetxe I, Frentiu FD, McMeniman CJ, et al. The *w*Mel *Wolbachia* strain blocks dengue and invades caged *Aedes aegypti* populations. Nature. 2011;476:450–5.
107. McMeniman CJ, Lane RV, Cass BN, Fong AWC, Sidhu M, Wang YF, et al. Stable introduction of a life-shortening *Wolbachia* infection into the mosquito *Aedes aegypti*. Science. 2009;323:141–4.
108. Ant TH, Herd CS, Geoghegan V, Hoffmann AA, Sinkins SP. The *Wolbachia* strain *w*Au provides highly efficient virus transmission blocking in *Aedes aegypti*. PLoS Pathog. 2018;14:1–19.
109. Fraser JE, De Bruyne JT, Iturbe-Ormaetxe I, Stepnell J, Burns RL, Flores HA, et al. Novel *Wolbachia*-transinfected *Aedes aegypti* mosquitoes possess diverse fitness and vector competence phenotypes. PLoS Pathog. 2017;13:1–19.
110. Davis MJ, Ying Z, Brunner BR, Pantoja A, Ferwerda FH. Rickettsial relative associated with papaya bunchy top disease. Curr Microbiol. 1998;36:80–4.
111. Majerus TMO, Schulenburg JH, Majerus MEN, Hurst GDD. Molecular identification of a male-killing agent in the ladybird *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae). Insect Mol Biol. 1999;8:551–5.
112. Weeks AR, Velten R, Stouthamer R. Incidence of a new sex-ratio-distorting endosymbiotic bacterium among arthropods. Proc R Soc Lond B. 2003;270:1857–65.
113. Goodacre SL, Martin OY. Modification of insect and arachnid behaviours by vertically transmitted endosymbionts: infections as drivers of behavioural change and evolutionary novelty. Insects. 2012;3:246–61.
114. Minard G, Mavingui P, Moro CV. Diversity and function of bacterial microbiota in the mosquito holobiont. Parasites Vectors. 2013;6:1–12.
115. McNulty SN, Abubucker S, Simon GM, Mitreva M, McNulty NP, Fischer K, et al. Transcriptomic and proteomic analyses of a *Wolbachia*-free filarial parasite provide evidence of trans-kingdom horizontal gene transfer. PLoS ONE. 2012;7:1–12.
116. Kondo N, Nikoh N, Ijichi N, Shimada M, Fukatsu T. Genome fragment of *Wolbachia* endosymbiont transferred to X chromosome of host insect. Proc Natl Acad Sci. 2002;99:14280–5.
117. Klasson L, Kambris Z, Cook PE, Walker T, Sinkins SP. Horizontal gene transfer between *Wolbachia* and the mosquito *Aedes aegypti*. BMC Genomics. 2009;10:1–9.
118. Woolfit M, Iturbe-Ormaetxe I, McGraw EA, O'Neill SL. An ancient horizontal gene transfer between mosquito and the endosymbiotic bacterium *Wolbachia pipientis*. Mol Biol Evol. 2009;26:367–74.
119. Leclercq S, Thézé J, Chebbi MA, Giraud I, Moumen B, Ernenwein L, et al. Birth of a W sex chromosome by horizontal transfer of *Wolbachia* bacterial symbiont genome. Proc Natl Acad Sci. 2016;113:15036–41.
120. Afizah AN, Roziah A, Nazni WA, Lee HL. Detection of *Wolbachia* from field collected *Aedes albopictus* Skuse in Malaysia. Indian J Med Res. 2015;142:205–10.
121. Kittayapong P, Baisley KJ, Sharpe RG, Baimai V, O'Neill SL. Maternal transmission efficiency of *Wolbachia* superinfections in *Aedes albopictus* populations in Thailand. Am J Trop Med Hyg. 2002;66:103–7.
122. Gerth M, Gansauge MT, Weigert A, Bleidorn C. Phylogenomic analyses uncover origin and spread of the *Wolbachia* pandemic. Nat Commun. 2014;5:1–7.
123. de Vienne DM, Refrégier G, López-Villavicencio M, Tellier A, Hood ME, Giraud T. Cospeciation vs. host-shift speciation: methods for testing, evidence from natural associations and relation to coevolution. New Phytol. 2013;198:347–85.
124. Price PW, Westoby M, Rice B, Atsatt PR, Fritz RS, Thompson JN, et al. Parasite mediation in ecological interactions. Annu Rev Ecol Syst. 1986;17:487–505.
125. Ikeda-Ohtsubo W, Brune A. Cospeciation of termite gut flagellates and their bacterial endosymbionts: *Trichonympha* species and "*Candidatus* Endomicrobium trichonymphae". Mol Ecol. 2009;18:332–42.
126. Drès M, Mallet J. Host races in plant-feeding insects and their importance in sympatric speciation. Philos Trans R Soc B Biol Sci. 2002;357:471–92.
127. Giraud T, Refrégier G, Le Gac M, de Vienne DM, Hood ME. Speciation in fungi. Fungal Genet Biol. 2008;45:791–802.
128. Bian G, Xu Y, Lu P, Xie Y, Xi Z. The endosymbiotic bacterium *Wolbachia* induces resistance to dengue virus in *Aedes aegypti*. PLoS Pathog. 2010;6:e1000833.
129. Ahmed MZ, Breinholt JW, Kawahara AY. Evidence for common horizontal transmission of *Wolbachia* among butterflies and moths. BMC Evol Biol. 2016;16:1–16.
130. Le Clec'h W, Chevalier FD, Genty L, Bertaux J, Bouchon D, Sicard M. Cannibalism and predation as paths for horizontal passage of *Wolbachia* between terrestrial isopods. PLoS ONE. 2013;8:e60232.
131. Ricklefs RE. Evolutionary diversification, coevolution between populations and their antagonists, and the filling of niche space. Proc Natl Acad Sci. 2010;107:1265–72.
132. de Castro F, Bolker BM. Parasite establishment and host extinction in model communities. Oikos. 2005;111:501–13.
133. de Vienne DM, Giraud T, Shykoff JA. When can host shifts produce congruent host and parasite phylogenies? A simulation approach. J Evol Biol. 2007;20:1428–38.
134. de Vienne DM. Tanglegrams are misleading for visual evaluation of tree congruence. Mol Biol Evol. 2019;36:174–6.
135. Chen R, Wang Z, Chen J, Jiang LY, Qiao GX. Insect-bacteria parallel evolution in multiple-co-obligate-aphid association: a case in Lachninae (Hemiptera: Aphididae). Sci Rep. 2017;7:1–9.
136. Degnan PH, Lazarus AB, Brock CD, Wernegreen JJ. Host-symbiont stability and fast evolutionary rates in an ant-bacterium association: cospeciation of *Camponotus* species and their endosymbionts *Candidatus blochmannia*. Syst Biol. 2004;53:95–110.
137. Moran NA. Accelerated evolution and Muller's rachet in endosymbiotic bacteria. Proc Natl Acad Sci. 1996;93:2873–8.

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Opinion

Wolbachia Can Enhance *Plasmodium* Infection in Mosquitoes: Implications for Malaria Control?

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The symbiotic bacterium *Wolbachia* is an attractive agent for vector-borne pathogen control. It has long been studied for its ability to manipulate host reproduction and spread into arthropod populations [1]. These properties, coupled with the recently identified ability to inhibit diverse pathogens [2–6], open avenues for its use in controlling vector-borne disease. Numerous *Wolbachia*-based control strategies are being investigated (reviewed in [7–9]), with some studies having progressed to field trials [10,11]. However, a worrying trend is emerging whereby *Wolbachia* infections have been demonstrated to enhance rather than suppress pathogens in some systems [12–18]. *Plasmodium* parasites, which are the causal agent of malaria, seem particularly prone to *Wolbachia*-mediated pathogen enhancement [13–16].

Wolbachia-based strategies have been proposed to control malaria [19]. *Anopheles* mosquitoes (the vectors of human malaria parasites) are not naturally infected by *Wolbachia* [20,21], but artificial transfer of this bacterium between species can be accomplished in the laboratory (reviewed in [22]). Pathogen interference phenotypes appear to be most prominent when *Wolbachia* is transferred into a novel host [16,23]. Given that *Anopheles* are for the most part naturally uninfected by *Wolbachia* (but see [24]), they can be considered an open niche for infection and a prime mosquito genus for *Wolbachia*-based control strategies. However, the main impediment for developing a control strategy is the difficulty in creating a stable artificial infection in *Anopheles* [19]. While examining *Plasmodium* interference in a stably infected host is the gold standard, a more convenient system is to transiently infect mosquitoes by intrathoracic microinjection. Using this system, the infection persists during the lifetime of the transinfected individual but is not transmitted to its offspring. Transient infection allows the rapid assessment of *Wolbachia*-host interactions without the need for generating stable artificial infections [5]. It is uncertain how representative transient infections are of stable inherited associations; however, similarities in tissues tropism and fitness costs incurred upon the host between stable and transiently infected *Anopheles* mosquitoes are evident [5,14,25]. Furthermore, both types of infection have been shown to inhibit the human malaria parasite *Plasmodium falciparum* [5,25]. However, studies using transient infection models have found that *Wolbachia* can enhance certain *Plasmodium* species [13,14].

The *Plasmodium* interference phenotype is therefore not universal, but context dependent. While *P. falciparum* is suppressed by the *w*AlbB strain of *Wolbachia* from *Aedes albopictus* [5,25], transient infections have shown the opposite effect on rodent malaria parasites. *Anopheles gambiae* transiently infected with *w*AlbB exhibited enhanced *P. berghei* development at the oocyst stage [14]. Similarly, *w*AlbB increased the number of *P. yoelii* oocysts in *An. stephensi*, although the phenotype was modulated by temperature [13]. At a temperature optimal for parasite development, *Wolbachia* increased parasite intensity compared to uninfected controls, but at warmer temperatures, *Wolbachia* inhibited *Plasmodium* development [13].

While *P. falciparum* is a major parasite in sub-Saharan Africa, four other parasites also cause human malaria worldwide: *P. malariae*, *P. ovale*, *P. knowlesi,* and *P. vivax* (the etiological agent of the most prevalent form of relapsing malaria). To our knowledge, the effect of *Wolbachia* on these other human *Plasmodium* parasites is unknown. The question is relevant for two reasons. First, the precedent that a particular *Wolbachia* strain can inhibit one parasite yet enhance another has already been documented [5,14], indicating that effects on parasites can be species-specific. Troublingly, *P. malariae*, *P. ovale*, *P. knowlesi*, and *P. vivax* are phylogenetically more closely related to rodent malaria parasites, which are enhanced by *Wolbachia* infections [13,14], than they are to *P. falciparum* (Figure 1) [26,27]. Second, many human *Plasmodium* parasites occur in sympatry and are transmitted by the same vectors. A case in point is *P. falciparum* and *P. vivax*, both of which occur in sympatry over large stretches of the Asian continent where they are both transmitted by *An. stephensi* [28,29]. Any potential control strategy devised in regions where more than one parasite species occurs needs to thoroughly investigate the effect of *Wolbachia* on all parasite species transmitted by the vector, as well as other pathogens such as filarial worms or arboviruses (both as single infections and in the context of coinfections) to ensure that *Wolbachia*-infected mosquitoes do not inadvertently enhance transmission of secondary pathogens.

While difficult, forecasting the long-term evolutionary response in this tripartite relationship between *Wolbachia*, *Plasmodium,* and *Anopheles* is very important. Natural *Wolbachia*–mosquito associations in which the symbiont and the host have tightly coevolved exist and may provide powerful models for studying the long-term evolutionary effects of *Wolbachia*

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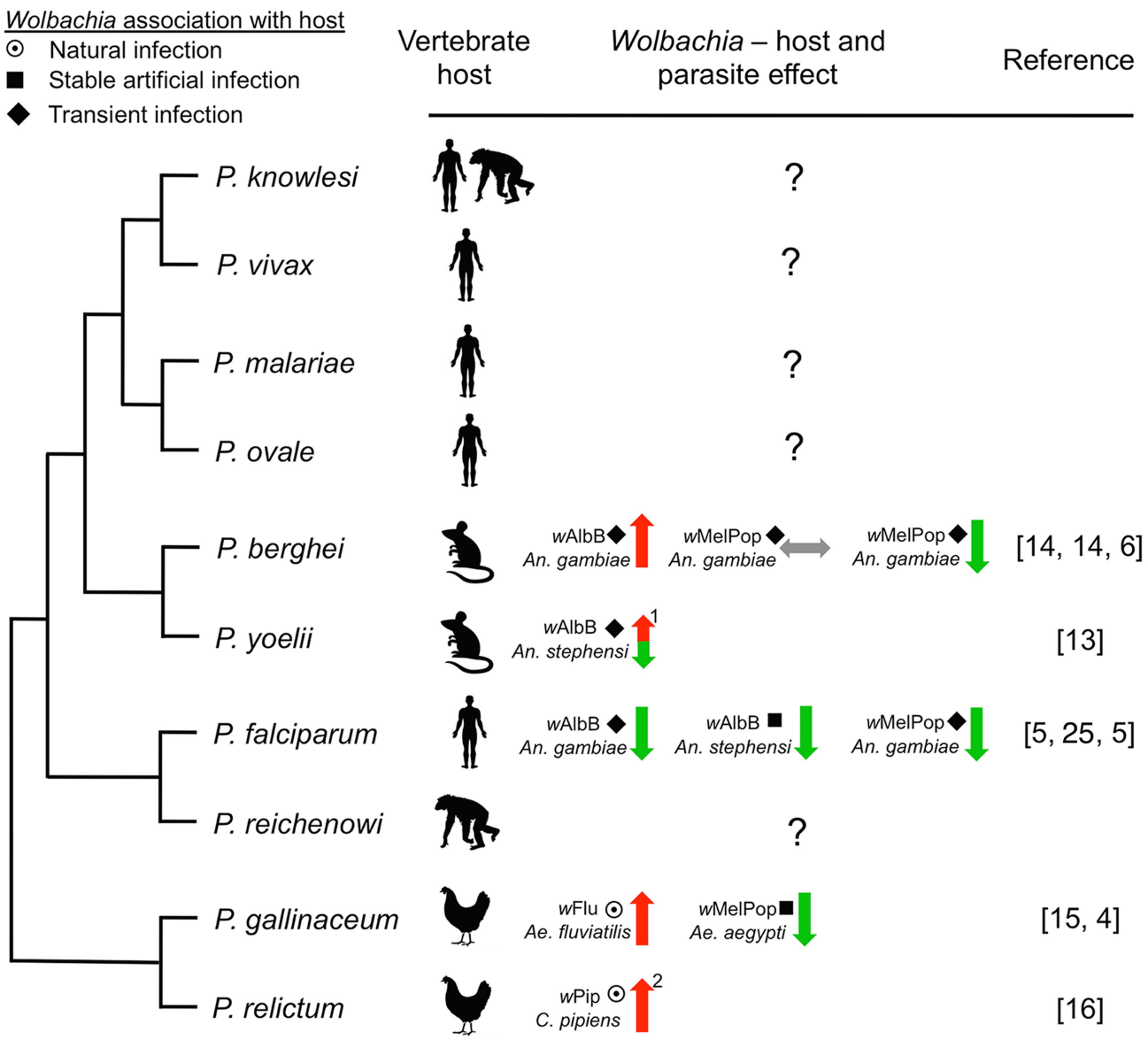

Figure 1. Representative phylogenetic dendrogram of *Plasmodium* parasites, their vertebrate hosts, and the influence of *Wolbachia* infection on parasite development within the mosquito vector. The protective effect of *Wolbachia* is variable and dependent on the *Wolbachia* strain and the insect host background, suggesting that complex tripartite interactions influence the effect on *Plasmodium*. The type of association between *Wolbachia* with the vector may also influence *Plasmodium*. Only one human malaria parasite (*P. falciparum*) has been assessed, while the effect of *Wolbachia* infection on the other four human parasites is unknown. Arrows indicate suppression (green), enhancement (red), or no effect (grey) of *Plasmodium*. The type of association within the host is depicted by symbols (target: natural infection, square: stable artificial infection, diamond: transient artificial infection). Numbers indicate: (1) the phenotype is temperature sensitive, (2) *Wolbachia* infection also increases insect life span [31], which has implications for pathogen transmission. Phylogeny was reconstructed based on work from Carlton et al. [26] and Martinsen et al. [27].
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infections. The evidence currently available suggests that natural *Wolbachia* infections can also enhance malaria parasite development within the mosquito. *Aedes fluviatilis* naturally infected with the *w*Flu *Wolbachia* strain had a significantly higher number of *P. gallinaceum* oocysts compared to an *Ae. fluviatilis* line which had been cleared of the *Wolbachia* infection [15]. *Ae. fluviatilis* is not, however, a natural vector of *P. gallinaceum*, and it is well known that the outcome of experiments using such laboratory models can differ significantly from those of natural mosquito–*Plasmodium* combinations (e.g., Boete [30]). Recent studies carried out in *Culex pipiens* mosquitoes, which are naturally infected with the *w*Pip *Wolbachia* strain and transmit the avian malaria parasite *P. relictum*, have also demonstrated *Plasmodium* enhancement. In this natural system, *Wolbachia* protects the mosquito host against the detrimental fitness effects incurred by *Plasmodium* infection [31] and increases the susceptibility of *C. pipiens* to *P. relictum*, with *w*Pip-infected mosquitoes having a higher prevalence of *Plasmodium* sporozoites in the salivary glands [16]. These studies show that the

Plasmodium-inhibiting properties of *Wolbachia* are far from universal; certain mosquito–*Wolbachia*–*Plasmodium* combinations and experimental conditions transform *Wolbachia*-infected mosquitoes into better vectors of malaria parasites. This is worrisome for the general implementation of *Wolbachia*-based control strategies.

Given that *Wolbachia*-based control strategies will use stable transinfected mosquitoes, the key question is whether stable and natural infections will behave in the same way. The stable transfer of *Wolbachia* into the host likely alters many aspects of host homeostasis, as evidenced by the novel phenotypes induced by infection [32–34], and as such, these associations likely differ from natural associations where *Wolbachia* and its host have coevolved. Another question is whether stable artificial infections will evolve over time. Theory and empirical studies show that these maternally transmitted bacteria will tend to evolve towards mutualistic associations with their host [35–38]. However, the evolutionary outcomes of pathogen interference or enhancement are harder to predict. A more complete mechanistic understanding of how *Wolbachia* infection modulates *Plasmodium* parasites is critical to address these important evolutionary questions and to evaluate if they are likely to occur in timescales relevant for disease control.

To date, two stable artificial *Wolbachia* transinfections have been assessed for their effect on *Plasmodium*. First, an *Aedes aegypti* line infected with *w*MelPop had inhibited *P. gallinaceum* infection [4]; *Ae. aegypti* is not, however, the natural vector of this parasite. Second, and more recently, the *w*AlbB strain was stably transferred into *An. stephensi*, one of the main vectors of human malaria in Asia [25]. This groundbreaking work demonstrated that stable artificial infections in epidemiologically relevant malaria vectors are feasible, and that *P. falciparum* can be inhibited by *Wolbachia* within its natural vector. If the severe fitness effects induced by *Wolbachia* in *Anopheles* can be overcome [25], then this approach is highly promising.

The work by Bian and colleagues [25] dramatically enhances the prospect for the use of *Wolbachia* in a malaria control strategy, but many questions still remain. What are the effects of *Wolbachia* on the other four species of *Plasmodium* parasites that infect humans? How relevant are transient infection models? Do some strains of *Wolbachia* enhance pathogens in a field context? What are the long-term evolutionary consequences of novel *Wolbachia*-host associations on *Plasmodium* development within the insect host? What are the mechanisms behind pathogen interference and enhancement of *Wolbachia* on *Plasmodium* parasites, and are the mechanisms of enhancement seen in rodent and avian model systems relevant to human malaria parasites? How influential are environmental variables on pathogen inhibition phenotypes? While many of these questions may be difficult to answer in the short term, assessing the relevance of transient infections would seem within the grasp of the scientific community. Although challenging, understanding the evolutionary consequences of novel *Wolbachia* associations on pathogen transmission and identifying the mechanisms behind *Wolbachia* modulation of *Plasmodium* is critical for developing effective control strategies and assessing their long-term feasibility. Insights from non-Anopheline systems where *Wolbachia* naturally infects the vector may be useful in this regard [16,31,39].

In conclusion, *Wolbachia*-based control of vector-borne pathogens is a promising novel strategy that has many advantages over other conventional and contemporary control methods. The development of a stable infection in *Anopheles* means the prospect of *Wolbachia*-based control of malaria can now be entertained [25], but many important questions need to be resolved before this idea can become a reality. While the concerns raised here focus on *Plasmodium*, these issues are relevant for *Wolbachia* control of any vector-borne pathogen [18]; we suggest that transinfected mosquitoes intended for release into nature should be assessed for inhibition (or lack thereof) of all relevant pathogens circulating in the system.

References

1. Werren JH, Baldo L, Clark ME (2008) *Wolbachia*: master manipulators of invertebrate biology. Nat Rev Microbiol 6: 741–751.
2. Hedges LM, Brownlie JC, O'Neill SL, Johnson KN (2008) *Wolbachia* and virus protection in insects. Science 322: 702.
3. Kambris Z, Cook PE, Phuc HK, Sinkins SP (2009) Immune activation by life-shortening Wolbachia and reduced filarial competence in mosquitoes. Science 326: 134–136.
4. Moreira LLA, Iturbe-Ormaetxe I, Jeffery JA, Lu G, Pyke AAT, et al. (2009) A *Wolbachia* symbiont in *Aedes aegypti* limits infection with Dengue, Chikungunya, and *Plasmodium*. Cell 139: 1268–1278.
5. Hughes GL, Koga R, Xue P, Fukastu T, Rasgon JL (2011) *Wolbachia* infections are virulent and inhibit the human malaria parasite *Plasmodium falciparum* in *Anopheles gambiae*. PLoS Pathog 7: e1002043.
6. Kambris Z, Blagborough AM, Pinto SB, Blagrove MSC, Godfray HCJ, et al. (2010) *Wolbachia* stimulates immune gene expression and inhibits *Plasmodium* development in *Anopheles gambiae*. PLoS Pathog 6: e1001143.
7. McGraw EA, O'Neill SL (2013) Beyond insecticides: new thinking on an ancient problem. Nat Rev Microbiol 11: 181–193.
8. Bourtzis K, Dobson SL, Xi Z, Rasgon JL, Calvitti M, et al. (2014) Harnessing mosquito-*Wolbachia* symbiosis for vector and disease control. Acta Trop 132S: S150–S163.
9. Iturbe-Ormaetxe I, Walker T, O' Neill SL (2011) *Wolbachia* and the biological control of mosquito-borne disease. EMBO Rep 12: 508–518.
10. Hoffmann AA, Montgomery BL, Popovici J, Iturbe-Ormaetxe I, Johnson PH, et al. (2011) Successful establishment of *Wolbachia* in *Aedes* populations to suppress dengue transmission. Nature 476: 454–457.
11. Walker T, Johnson PH, Moreira LA, Iturbe-Ormaetxe I, Frentiu FD, et al. (2011) The *w*Mel Wolbachia strain blocks dengue and invades caged *Aedes aegypti* populations. Nature 476: 450–453.
12. Graham RI, Grzywacz D, Mushobozi WL, Wilson K (2012) *Wolbachia* in a major African crop pest increases susceptibility to viral disease rather than protects. Ecol Lett 15: 993–1000.
13. Murdock CC, Blanford S, Hughes GL, Rasgon JL, Thomas MB (2013) Temperature alters malaria transmission blocking by *Wolbachia*. Sci Rep 4: 3932.
14. Hughes GL, Vega-Rodriguez J, Xue P, Rasgon JL (2012) *Wolbachia* strain *w*AlbB enhances infection by the rodent malaria parasite *Plasmodium* berghei in *Anopheles gambiae* mosquitoes. Appl Environ Microbiol 78: 1491–1495.
15. Baton LA, Pacidônio EC, Gonçalves DDS, Moreira LA (2013) *w*Flu: characterization and evaluation of a native *Wolbachia* from the mosquito *Aedes fluviatilis* as a potential vector control agent. PLoS ONE 8: e59619.
16. Zélé F, Nicot A, Berthomieu A, Weill M, Duron O, et al. (2013) *Wolbachia* increases susceptibility to *Plasmodium* infection in a natural system. Proc Biol Sci 281: 20132837.
17. Hussain M, Lu G, Torres S, Edmonds JH, Kay BH, et al. (2013) Effect of *Wolbachia* on replication of West Nile virus in a mosquito cell line and adult mosquitoes. J Virol 87: 851–858.
18. Dodson BL, Hughes GL, Paul O, Matacchiero AC, Kramer LD, et al. (2014) *Wolbachia* Enhances West Nile Virus (WNV) Infection in the Mosquito *Culex tarsalis*. PLoS Negl Trop Dis 8: e2965.
19. Walker T, Moreira LA (2011) Can *Wolbachia* be used to control malaria? Mem Inst Oswaldo Cruz 106 (Suppl. I): 212–217.
20. Ricci I, Cancrini G, Gabrielli S, D'Amelio S, Favi G (2002) Searching for *Wolbachia* (Rickettsiales: Rickettsiaceae) in mosquitoes (Diptera: Culicidae): large polymerase chain reaction survey and new identifications. J Med Entomol 39: 562–567.
21. Rasgon JL, Scott TW (2004) An initial survey for *Wolbachia* (Rickettsiales: Rickettsiaceae) infections in selected California mosquitoes (Diptera: Culicidae). J Med Entomol 41: 255–257.
22. Hughes GL, Rasgon JL (2014) Transinfection: a method to investigate *Wolbachia*-host interactions and control arthropod-borne disease. Insect Mol Biol 23: 141–151.
23. Bian G, Xu Y, Lu P, Xie Y, Xi Z (2010) The endosymbiotic bacterium *Wolbachia* induces resistance to Dengue virus in *Aedes aegypti*. PLoS Pathog 6: e1000833.
24. Baldini F, Segata N, Pompon J, Marcenac P, Robert Shaw W, et al. (2014) Evidence of natural

Wolbachia infections in field populations of *Anopheles gambiae*. Nat Commun 6: 3985.
25. Bian G, Joshi D, Dong Y, Lu P, Zhou G, et al. (2013) *Wolbachia* invades *Anopheles stephensi* populations and induces refractoriness to *Plasmodium* infection. Science 340: 748–751.
26. Carlton JM, Escalante AA, Neafsey D, Volkman SK (2008) Comparative evolutionary genomics of human malaria parasites. Trends Parasitol 24: 545–550.
27. Martinsen ES, Perkins SL, Schall JJ (2008) A three-genome phylogeny of malaria parasites (*Plasmodium* and closely related genera): evolution of life-history traits and host switches. Mol Phylogenet Evol 47: 261–273.
28. Korgaonkar NS, Kumar A, Yadav RS, Kabadi D, Dash AP (2012) Mosquito biting activity on humans & detection of *Plasmodium falciparum* infection in *Anopheles stephensi* in Goa, India. Indian J Med Res 135: 120–126.
29. Adak T, Singh OP, Das MK, Wattal S, Nanda N (2005) Comparative susceptibility of three important malaria vectors *Anopheles stephensi*, *Anopheles fluviatilis*, and *Anopheles sundaicus* to *Plasmodium vivax*. J Parasitol 91: 79–82.
30. Boëte C (2005) Malaria parasites in mosquitoes: laboratory models, evolutionary temptation and the real world. Trends Parasitol 21: 445–447.
31. Zélé F, Nicot A, Duron O, Rivero A (2012) Infection with *Wolbachia* protects mosquitoes against *Plasmodium*-induced mortality in a natural system. J Evol Biol 25: 1243–1252.
32. Clancy DJ, Hoffmann AA (1997) Behavior of *Wolbachia* endosymbionts from *Drosophila simulans* in *Drosophila serrata*, a novel host. Am Nat 149: 975–988.
33. Suh E, Mercer D, Fu Y, Dobson SL (2009) Pathogenicity of life-shortening *Wolbachia* in *Aedes albopictus* after transfer from *Drosophila melanogaster*. Appl Env Microbiol 75: 7783–7788.
34. Bouchon D, Rigaud T, Juchault P (1998) Evidence for widespread *Wolbachia* infection in isopod crustaceans: molecular identification and host feminization. Proc Biol Sci 265: 1081–1090.
35. McGraw EA, Merritt DJ, Droller JN, O'Neill SL (2002) *Wolbachia* density and virulence attenuation after transfer into a novel host. Proc Natl Acad Sci U S A 99: 2918–2923.
36. Weeks AR, Turelli M, Harcombe WR, Reynolds KT, Hoffmann AA (2007) From parasite to mutualist: rapid evolution of *Wolbachia* in natural populations of *Drosophila*. PLoS Biol 5: E114.
37. Turelli M (1994) Evolution of incompatibility-inducing microbes and their hosts. Evolution 48: 1500–1513.
38. Carrington LB, Hoffmann AA, Weeks AR (2010) Monitoring long-term evolutionary changes following *Wolbachia* introduction into a novel host: the *Wolbachia* popcorn infection in *Drosophila simulans*. Proc Biol Sci. 277: 2059–2068.
39. Hughes GL, Samuels SK, Shaikh K, Rasgon JL, Vardo-Zalik AM (2014) Discrimination of the *Plasmodium mexicanum* vectors *Lutzomyia stewarti* and *Lutzomyia vexator* by a PCR-RFLP assay and *Wolbachia* infection. J Vector Ecol 39: 224–227.

https://files.hawaii.gov/dlnr/meeting/audio/Audio-LNR-230324-1.m4a

Exhibit “3”

STATE OF HAWAII
BOARD OF LAND AND NATURAL RESOURCES

PETITION FOR A CONTESTED CASE HEARING

OFFICIAL USE ONLY	
Case No.	**Date Received**
Board Action Date / Item No.	**Division/Office**

INSTRUCTIONS:

1. File (deliver, mail or fax) this form within ten (10) days of the Board Action Date to:

   Department of Land and Natural Resources
   Administrative Proceedings Office
   1151 Punchbowl Street, Room 130
   Honolulu, Hawaii 96813
   Phone: (808) 587-1496, Fax: (808) 587-0390

2. DLNR's contested case hearing rules are listed under Chapter 13-1, HAR, and can be obtained from the DLNR Administrative Proceedings Office or at its website (http://dlnr.hawaii.gov/forms/contested-case-form/). Please review these rules before filing a petition.

3. If you use the electronic version of this form, note that the boxes are expandable to fit in your statements. If you use the hardcopy form and need more space, you may attach additional sheets.

4. Pursuant to §13-1-30, HAR, a petition that involves a Conservation District Use Permit must be accompanied with a $100.00 non-refundable filing fee (payable to "DLNR") or a request for waiver of this fee. A waiver may be granted by the Chairperson based on a petitioner's financial hardship.

5. All materials, including this form, shall be submitted in **three (3)** photocopies.

A. PETITIONER (If there are multiple petitioners, use one form for each.)		
1. Name Hawaii Unites	**2. Contact Person** Tina Lia	
3. Address P.O. Box 1773	**4. City** Kihei	**5. State and ZIP** HI 96753
6. Email tinalia@live.com	**7. Phone** (808) 298-6335	**8. Fax**

B. ATTORNEY (if represented)		
9. Attorney Name	**10. Firm Name**	
11. Address	**12. City**	**13. State and ZIP**
14. Email	**15. Phone**	**16. Fax**

C. SUBJECT MATTER	
17. Board Action Being Contested	
18. Board Action Date	**19. Item No.**
20. Any Specific Statute or Rule That Entitles Petitioner to a Contested Case	
21. Any Specific Property Interest of Petitioner That Is Entitled to Due Process Protection	
22. Any Disagreement Petitioner May Have with an Application before the Board	
23. Any Relief Petitioner Seeks or Deems Itself Entitled to	
24. How Petitioner's Participation in the Proceeding Would Serve the Public Interest	
25. Any Other Information That May Assist the Board in Determining Whether Petitioner Meets the Criteria to Be a Party under Section 13-1-31, HAR	

☒ Check this box if Petitioner is submitting supporting documents with this form.

☒ Check this box if Petitioner will submit additional supporting documents after filing this form.

Tina Lia
Petitioner or Representative (Print Name)

Signature

03/30/2023
Date

PETITION FOR A CONTESTED CASE HEARING
C. SUBJECT MATTER (Supporting Documents)

17. Board Action Being Contested

We are contesting the Board of Land and Natural Resources' approval of Agenda Item C-2, DIVISION OF FORESTRY AND WILDLIFE: Request Approval of Final Environmental Assessment and Authorization for the Chairperson to Issue a Finding of No Significant Impact for the "Suppression of Invasive Mosquito populations to Reduce Transmission of Avian Malaria to Threatened and Endangered Forest Birds on East Maui."

18. Board Action Date

March 24, 2023

19. Item No.

C-2

20. Any Specific Statute or Rule That Entitles Petitioner to a Contested Case

Relevant statutes and constitutional provisions covered in this request are: HRS 343; Hawaii Constitution Article XI, section 1, 2, 7, and 9; HRS 92-7; HAR 13-1-29

21. Any Specific Property Interest of Petitioner That Is Entitled to Due Process Protection

Hawaii Unites is a 501(c)(3) nonprofit organization dedicated to the conservation and protection of our environment and natural resources. Our mission is honoring and protecting our sacred connection to the natural world. Formed in 2023, Hawaii Unites launched a petition through Change.org to "Demand an Environmental Impact Statement for the Experimental Mosquito Release on Maui" which has received more than 2,700 signatures. Our nonprofit officers and all petition signatories residing in Hawaii, particularly those in East Maui, are directly affected by the actions of the Board on item C-2, which seeks to approve a landscape-scale biopesticide experiment with a project area covering 64,666 acres of East Maui.

The rights of our officers and signatories relevant to these natural areas are protected by the Hawaii State Constitution and state law. Hawaii Unites' officers and signatories have rights to a clean and healthful environment under article XI, section 9 of the Constitution, which mandates a contested case hearing whenever the State makes binding decisions under "laws relating to environmental quality, including control of pollution and conservation, protection and enhancement of natural resources."

22. Any Disagreement Petitioner May Have with an Application before the Board

Hawaii Unites opposes the approval of the Final Environmental Assessment and the authorization for the Chairperson to issue a Finding of No Significant Impact for the "Suppression of Invasive Mosquito populations to Reduce Transmission of Avian Malaria to Threatened and Endangered Forest Birds on East Maui" because:

(a) The Final Environmental Assessment lacks adequate detail as required by HEPA.

(b) The Final Environmental Assessment fails to identify the *Wolbachia* strain planned for use in this project.

(c) The Final Environmental Assessment fails to identify and describe the mark release recapture study as a proposed action, and this project may have been improperly segmented.

(d) The Final Environmental Assessment fails to adequately identify the mosquito packages planned for release into the environment, and the effects on the environment from the release of biodegradable packages with an unknown decay rate are not adequately addressed.

(e) The Final Environmental Assessment fails to identify biosecurity protocols

(f) The Final Environmental Assessment does not address the concern of accidental pathogen introduction and does not specify required permits for interstate transport of pathogens

(g) Viewscape impacts, noise disturbances to forest bird breeding and nesting, and significant environmental consequences, including impacts to the untrammeled, natural qualities of the wilderness character, have not been adequately addressed.

(h) The potential negative impacts of introducing an invasive species to the islands have not been adequately addressed.

(i) Biopesticide mosquitoes for this project originate from Palmyra Atoll. *Wolbachia* bacteria for the project originates from Kuala Lumpur in Malaysia. At least one strain of *Wolbachia* planned for import in connection with the project does not exist on these islands.

(j) Landscape level control of *Culex quinquefasciatus* mosquitoes using the Incompatible Insect Technique (IIT) has never been done before.

(k) The mosquito species planned for use in this project, *Culex quinquefasciatus*, has never been used for a stand-alone IIT field release.

(l) Peer-reviewed studies confirm that *Wolbachia* bacteria can cause mosquitoes to become more capable of spreading diseases like avian malaria and West Nile virus (bird and human). The Final Environmental Assessment fails to adequately address these risks.

(m) Tropical disease expert Dr. Lorrin Pang (private citizen) has expressed concerns about horizontal transmission of the lab bacteria to wild mosquitoes and other insect vectors of disease. The Final Environmental Assessment fails to adequately address these concerns.

(n) Scientific studies document the risks of horizontal transmission, increased pathogen infection, evolutionary events, population replacement, and accidental release of females (who bite and breed). The Final Environmental Assessment fails to adequately address these risks.

(o) This project has the potential to cause the extinction of endangered native birds, and it could impact human health.

(p) Impacts to endangered native Hawaiian hoary bats, native dragonflies, and endangered native damselflies have not been adequately studied or addressed in the Final Environmental Assessment.

(q) Biopesticide wind drift has not been studied and is not addressed in the Final Environmental Assessment.

(r) Environmental Justice is not adequately addressed in the Final Environmental Assessment. Human health impacts of this project have not been adequately studied, and the proposed action would impact ethnographic resources and traditional cultural practices.

(s) The Final Environmental Assessment's assertion of released mosquitoes posing no risk to human health is based on unsound science. The 2010 article by Popovici et al. cited in the Final Environmental Assessment has been discredited by the EPA.

(t) The EPA has not conducted an Environmental Risk Assessment for this mosquito biopesticide to determine the environmental, ecological, and human health risks.

(u) The Hawaii Department of Agriculture has applied for an EPA Emergency Exemption for use of the mosquitoes without going through regulatory safety processes. The EPA application is still under review, and the biopesticide mosquitoes have not been approved for emergency release.

(v) A feasibility study has not been conducted to provide a detailed analysis that considers all of the critical aspects of the proposed project in order to determine the likelihood of it succeeding.

(w) The U.S. Department of the Interior states that "although used world-wide for human health, *Wolbachia* IIT is a novel tool for conservation purposes and its degree of efficacy in remote forest landscapes is unknown."

(x) Under the precautionary principle, it is the responsibility of the proponents of this project to establish that the proposed activity will not result in significant harm.

(y) The subject action will have a significant effect and, therefore, requires the preparation of an Environmental Impact Statement.

(z) Conflicts of interest have not been disclosed or addressed.

23. Any Relief Petitioner Seeks or Deems Itself Entitled to

Hawaii Unites requests that the approval of the Final Environmental Assessment and the authorization for the Chairperson to issue a Finding of No Significant Impact for the "Suppression of Invasive Mosquito populations to Reduce Transmission of Avian Malaria to Threatened and Endangered Forest Birds on East Maui" be denied. The subject action will have a significant effect and, therefore, requires the preparation of an Environmental Impact Statement.

Hawaii Unites also requests that State of Hawaii Board of Land and Natural Resources Chairperson Dawn N.S. Chang and Board Member Vernon Char recuse themselves from participating in any discussion or voting in this matter, given that they have conflicts of interest per HRS §171-4 (d).

Any action taken by the Board of Land and Natural Resources on this Petition for a Contested Case Hearing prior to receipt of said Petition shall be null and void, as any such action is in violation of the Sunshine Law HRS §92-7 and of HAR §13-1-29. Receipt of this Petition shall serve as notice to the Board of Land and Natural Resources that the Petition remains active. Any action taken by the Board of Land and Natural Resources on the March 24, 2023 Agenda Item C-2, a subject within the adjudicatory jurisdiction of the Board, shall be subsequently null and void.

24. How Petitioner's Participation in the Proceeding Would Serve the Public Interest

Hawaii Unites has provided peer-reviewed studies documenting the serious risks of the proposed project. We have described the concerns of tropical disease and vector expert Dr. Lorrin Pang. In a contested case hearing, we will provide the Board with additional peer-reviewed studies. We will give a detailed description of Dr. Pang's concerns regarding horizontal transmission of the introduced bacteria strain, which will include information that has not yet been submitted in previous testimony or comments. We will provide a statement by a retired scientist from the EPA Office of Pesticide Programs strongly advising that a full Environmental Impact Statement be conducted. We will provide documentation of petition signatories and public testimony. Our evidence will demonstrate that the project risks and the concerns of the public in opposition to this proposed experiment have not been adequately studied or addressed. Our participation in a contested case hearing will help to ensure that this Board has all the information it needs to make a decision that fully protects the public's interests and satisfies the Board's public trust obligations per the Hawaii State Constitution.

25. Any Other Information That May Assist the Board in Determining Whether Petitioner Meets the Criteria to Be a Party under Section 13-1-31, HAR

Per HAR §13-1-31 (b) (2), Hawaii Unites represents all petition signatories who have some property interest in the land, who lawfully reside on the land, who are adjacent property owners, or who otherwise can demonstrate that they will be so directly and immediately affected by the requested action that their interest in the proceeding is clearly distinguishable from that of the general public.

Per HAR §13-1-31 (c), as a 501(c)(3) nonprofit organization dedicated to the conservation and protection of our environment and natural resources, Hawaii Unites can show a substantial interest in the matter.