1. Import supporting documents from Github.
   * File **CSV-ImportAndOrganize.xlsm** should be saved to a location external to Geneious and will be used to organize classified 16S rRNA sequences.
   * File **Results Folders.geneious** should be saved to a location external to Geneious and will need to be imported each time the workflow is run (instructions below).
   * Open Geneious and import files **Reference Sequences.geneious** and **Workflows.geneious**
   * **Reference Sequences.geneious** should be placed within the Geneious program in a reference sequence folder, and used to direct the workflow to necessary reference sequences at outlined in the manuscript.
   * File **Workflows.geneious** will automatically be added to the Geneious workflows
2. Open each workflow and direct map to reference functions to the appropriate reference sequences imported with file **Reference Seqeuences.geneious**
3. Create a folder in Geneious for your project. Import libraries to be analyzed, and merge paired reads using the **Geneious FASTQ** import function, with options for paired ends (inward pointing) and insert size 250 bp.
   * The starting format library names is presumed to be: 1\_S1\_R\_001
4. Select merged library reads from above and run workflow: **BHS Trim with BBDuk**, which will also rename the files to 1, 2, 3 … if starting with format 1\_S1\_R\_001. Otherwise you will need to adjust batch rename step in the BHS Trim with BBDuk workflow.
   * The resulting format for library names must be: 1, 2, 3 … (you must use this format for renaming commands to work in downstream workflows)
   * Trimmed libraries will be saved in a subfolder named **Trimmed**
5. Import Geneious file **BHS Results Folders.geneious** (**File 🡪 Import🡪Files🡪Select Geneious formats**) from a location where they have been saved external to Geneious. You will need to **move the six folders (LKT, Local 16S Database, MLST B trehalosi, MLST M ovipneumoniae, MLST Mannheimia species, MLST P multocida)** into the Trimmed folder and **delete** the folder “BHS Multiplex Folders”.
   * These folders will be used to create phylogenetic trees and classify 16S sequences.
   * The folders must be saved external to Geneious and imported. You cannot copy and paste folders within the Geneious program.
6. Select trimmed libraries in the Trimmed folder and run workflow **BHS Multiplex MLST**
7. Open each MLST folder and examine data for quality. May need to delete duplicate sequences if the same Mannheimia sequence mapped to multiple Mannheimia reference sequences (assess in alignment step below)
8. In **each MLST folder**, Create an **MAFFT** **alignment** of sample MLST sequences and reference MLST sequences.
9. Select alignment and create phylogenetic tree.
   * For species MLST trees use:

|  |  |
| --- | --- |
| **Tree Building Software** | RAxML v 8.2.11 |
| Nucleotide model | GTR GAMMA |
| Algorithm | Rapid bootstrapping and search for best-scoring maximum likelihood tree  (-f a-x 1) |
| Bootstrap replicates | 100 |
| Parsimony random seed | 1 |
| Additional command line options | None |

* + For lkt tree use:

|  |  |
| --- | --- |
| **Tree Building Software** | Geneious Tree Builder v 2022.2.2 |
| Genetic distance model | Tamura-Nei |
| Tree build method | Neighbor-joining |
| Outgroup | No outgroup |
| Consensus tree options | None |

1. If you would like different labels for sample IDs, create a spreadsheet with the old and new sample Names/IDs and **Import Metadata** from spreadsheet.
2. You can toggle Sample name (1,2,3…) with new Sample IDs in the alignment and tree views.
3. Open Folder **Local 16S Database** and group sequences produced by the workflow with any predetermined 16S sequences you imported with this folder, creating a single sequence list. Delete other entries so the folder contains only one sequence list.
4. Select trimmed libraries in **Trimmed** folder and run workflow: **BHS Classify Sequences**
   * When prompted, select the folder with the **Local 16S Database** you would like to use for sequence classification. Typically this will be the **Local 16S Database** folder referenced in step 10 above and located as a subfolder in your **Trimmed** folder.
5. Select each result in the **16S Classification** folder
6. For each result, **Export Table** to a location external to Geneious using CSV format.
   * Must be done for each sample, and it takes forever for the tables to load. I have tried to work with Geneious on this but they don’t have a solution. If you have another 16S Classification Tool you could use it here instead of the Geneious Classify Sequences Tool, used by this workflow.
7. Open **CSV-ImportandOrganize**, open Macros Function and run program. Program should direct you to upload the tables exported in step 15 above.