otb: Creating a HiC/HiFi Pipeline to Assemble the *Prosapia bicincta* Genome

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Abstract

Two-lined spittlebug (Hemiptera: Cercopidae) is an insect herbivore that is widely distributed throughout the eastern portion of the United States. This insect is a significant pest of turf grasses and pasturelands, where feeding causes wilting and phytotoxemia, resulting in plant mortality. This pest is endemic to tropical and subtropical regions of the world and has a long-term impact on the composition of the plant community in grassland landscapes, altering plant communities in rangelands and posing a substantial threat to cattle production, citing a potential threat to livestock production. Using phased genomes to better understand the physiology, ecology, and evolution of this insect pest can yield novel targets to exploit for sustainable pest management. The introduction of a new HiC/HiFi phased genomics assembly pipeline called Only The Best (otb) has solved several data management problems that were prevalent in the creation and storage of genome assemblies.

Keywords: genome assembly; non-model organism; haplotype phasing; next-generation sequencing; assembly error correction

Introduction

The USDA-ARS AgPest 100 Initiative (Ag100pest) aims to generate high-quality genome assemblies of existing and/or emerging pest insect species that threaten agricultural production (Childers *et al.* 2021). High-quality genome assemblies can root and inform both basic and applied research. The time cost in production of multiple genomes assemblies can cause inefficiencies in projects such as the ag100pest; a HiFi/HiC assembly pipeline is required for efficiencies in these projects. In order to induce implementation of such a pipeline, it is required to utilize the pipeline in a test scenario. As the Ag100Pest often works with true bugs, and true bug genomics are relatively unexplored Jiang *et al.* (2021), an agriculturally significant true bug is the ideal test case for such a pipeline, Two-line spittlebugs (*Prosapia bicincta*) are such test case. Two-lined spittlebug (Hemiptera: Cercopidae) is an insect herbivore that is widely distributed throughout the eastern portion of the United States (Potter *et al.* 1991; Braman and Abraham 1995). In 2016, the two-lined spittlebug was first detected on Hawai'i Island (Thorne *et al.* 2017). The immature lifestages of this species are significant pests of turfgrass and pasturelands, where feeding causes wilting and phytotoxemia resulting in plant mortality (Byers and Wells 1966; Fagan and Kuitert 1969; Joseph and Jespersen 2021). Since its establishment in Hawai'i, two-lined spittlebugs have had significant cascading effects on plant communities in rangelands, altering the composition of plant communities of grass-dominated landscapes and posing a significant threat to cattle production (Bremer *et al.* 2021). Uncovering the behavioral and metabolic strategies insects employ to exploit plants is an important step in determining their pest status. Like other cercopids, the two-lined spittlebug eats a nutritionally impoverished diet in xylem sap (Mattson Jr 1980). The processing of this diluted diet produces the characteristic spittle masses at the base of the plant. Spittlebugs have some metabolic innovations to contend with these diluted diets. Like other hemipterans that feed on phloem and xylem, spittlebugs harbor endosymbiotic bacteria that reside in specialized structures called bacteriomes. Spittlebugs harbor two symbionts in independent organs. *Sulcia muelleri* is ubiquitous in Auchenorrhyncha, while the other symbiont can be *Zinderia insectola* or a *Sodalis*-like microorganism (Koga *et al.* 2013; Koga and Moran 2014). These symbionts help provide complementary sets of essential amino acids through complex and intertwined metabolic pathways (Ankrah *et al.* 2020).

Biological control of two-lined spittlebugs in grassland ecosystems is inherently challenging. Adults are long-lived and highly fecund (Peck 1998), few commercial grass cultivars exhibit resistance and / or tolerance to this insect (Braman *et al.* 2014; Joseph and Jespersen 2021), and nymphs feed in protected areas at the base of the plant, which facilitates their escape from natural enemies (Nachappa *et al.* 2006). In the case study we use here, a genome assembly of two-lined spittlebug is a critical first step towards understanding the physiology, ecology, and evolution of

this herbivorous pest and may yield novel targets to exploit for sustainable pest management.

Phased genomes, which determine both chromosomes of a diploid, are a valuable resource for analyzing genetic variation within populations (Snyder *et al.* 2015). This is particularly important in agricultural research, where understanding genetic variation is crucial to understanding the evolution and spread of resistance genes that can impact outbreaks of insect pests (Leftwich *et al.* 2015). The creation of a large number of phased arthropod genomes, a number of which could be enabled by more hands-off and accessible bioinformatic pipelines, has several applications (Tewhey *et al.* 2011). Having phased genomes to better understand the genetic variation of two-lined spittlebugs in an invasive population could lead to valuable insights into how the insect adapts to insectcides or adapts to new environments.

To create phased genomes of the two-lined spittlebug and other insects, we developed a new HiC / HiFi phased genomic assembly pipeline called otb, or Only The Best [genome assembly tools]. Our pipeline reduces the time spent organizing data, installing and calibrating bioinformatic tools, and, therefore, performing analysis. By implementing this pipeline, we reduced the amount of time required to produce a usable genome. The careful implementation of data management and standardization also significantly reduced team effort in genome assembly creation. otb is a software tool that utilizes the nextflow programming language (Tommaso *et al.* 2017) and is accessed through a bash script. To ensure a consistent compute environment across users, otb is implemented within a singularity container management software, which enables users to share containers with other users within the same environment. The use of nextflow provides the benefit of parallel task execution and efficient management of compute resources, while singularity ensures a consistent and reproducible compute environment. This also eliminates the need for software duplication across a high-performance computing cluster (HPC). The development of otb was primarily for the United States Department of Agriculture, Agricultural Research Services' ag100pest and Beenome projects, where large numbers of reference genomes needed to be created. However, otb can be used for any project that requires the automation of HiFi genomes until human involvement is necessary.

Materials and Methods

Sampling

Two-lined spittlebug were collected from the University of Hawai'i's Kona research station (79-7381 Hawai'i Belt Road, Holualoa, HI) were Shannon Wilson reared them. The samples were flash-frozen on site on August 13th, 2020.

DNA Extraction, PacBio Library Preparation, and Sequencing

High molecular weight DNA (HMW DNA) was extracted for the preparation of the PacBio HiFi library from a single adult male P. bicincta. The sample was cryoground using a Spex GenoGrinder 2010, and DNA was extracted from the ground tissue using the Qiagen MagAttract HMW DNA kit (Cat# 67563) following the kit protocol. The concentration of the extracted HMW DNA was quantified using the Qubit 1x dsDNA HS kit (Q33230), and DNA purity was assessed using UV-vis spectroscopy. The size distribution of the extracted HMW DNA was evaluated using an Agilent Femto Pulse instrument with the Genomic DNA 165kbp kit (Cat # FP-1002-0275).

Before preparation of the PacBio HiFi library, the extracted HMW DNA was sheared using the Diagenode Megaruptor 2 with the 20 kbp shearing program, to target a sheared DNA size of approximately 10-15kbp. Sheared DNA was used to prepare PacBio HiFi libraries, using PacBio's Express Template Prep Kit 2.0 (PN: 102-088-900) following the kit protocol, with the optional nuclease digestion step after library preparation. PacBio libraries were size selected with 40% diluted AMPure PB beads (PN: 102-182-500) to remove library molecules shorter than 3kbp following PacBio's protocol. The final libraries selected for size were quantified using the Qubit 1x dsDNA HS kit, and the library size was checked using the Agilent Femto Pulse with the Genomic DNA 165kbp kit. The PacBio libraries were sequenced on a PacBio Sequel IIe using a 30 hour movie time with 2 hours of pre-extension. Sequencing reaction was prepared using the Sequel II Binding Kit 2.2.

HiC Library Preparation

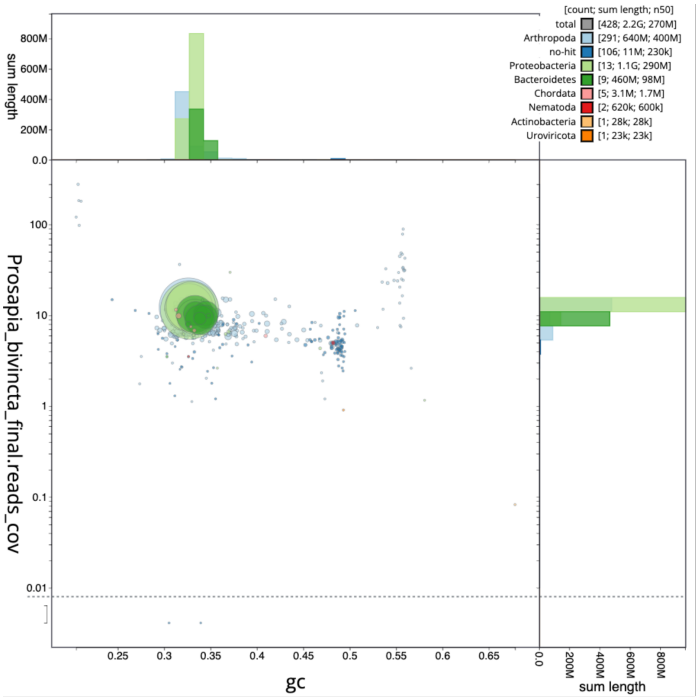
To prepare a HiC library, a single adult male P. bicincta was cryoground and then fixed in freshly prepared TC fixation buffer, following the low-input crosslinking protocol for the Arima HiC 2.0 kit. Proximity ligation was performed on cross-linked samples using the Arima HiC 2.0 kit following the manufacturer's protocol. Prior to preparation of the Illumina library, the proximity-ligated DNA was sheared using a Diagenode Biorupter and then size-selected to enrich the sheared DNA in the 200-600bp range. The size distribution of the selected size sheared DNA was checked using an Agilent TapeStation with the High Sensitivity D5000 ScreenTape (Cat # 5067-5588) before proceeding to library preparation. The Illumina HiC library was prepared using the Swift Accel NGS 2S Plus DNA Library kit following the protocol outlined in the Arima HiC 2.0 kit. Library amplification was performed using the KAPA Library Amplification Kit with Primer Mix (Cat# KK2620), with 8 cycles of PCR. The final libraries were quantified using the Qubit 1x dsDNA HS kit, and the library size distribution was checked using an Agilent TapeStation. The HiC library was sequenced on an Illumina NovaSeq 6000.

otb Genome Assembly

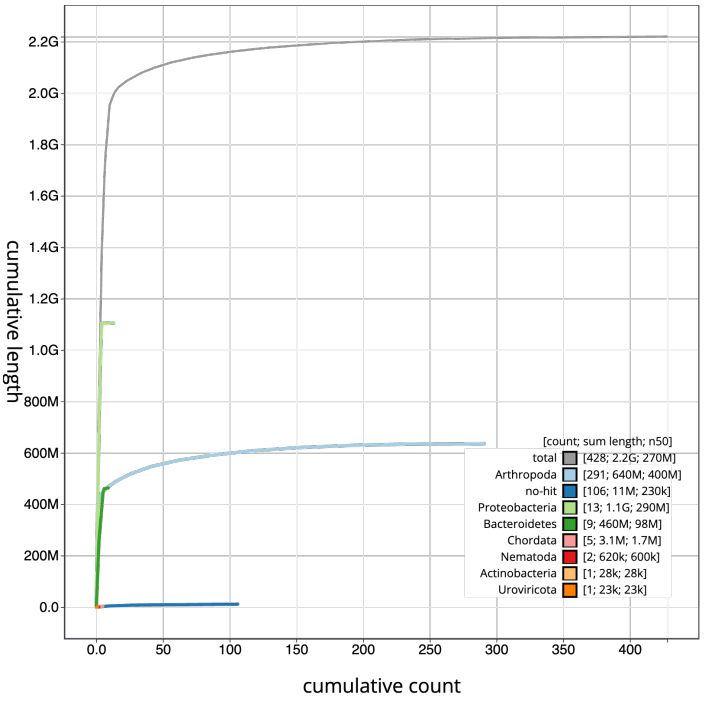
Genome assembly of Two-lined spittle-bug raw HiFi and HiC reads was completed with otb. otb was set to use the "merfin" polishing option, additionally, at the k-mer creation step, KMC3 was used. Juicebox was then utilized for HiC correction and assembly was modified accordingly. shhquis.jl was utilized post assembly to rearrange chromosomes for hic mapping, reducing the manual time required in the hic rearrangement step. Manual hic rearrangement, which was completed with Yahs exported HiC data was exported to Juicebox and final genome assembly report which was completed with Blobtools. otb was written for this project in Nextflow, in addition to a functionalized bash script, which pre-downloads and checks software containers of the constituent assembly tools. As a nextflow pipeline was written so that if errors occur in the pipeline, otb can be rerun from that point. otb also was written so that software versions of all tools are exported into a final reports folder (see Supplement for software versions). otb was also written with configs for slurm, local, and sge cluters, and comes with an optional slurm template script to run the pipeline.

Results and Discussion

The goal of otb is to deliver a genome assembly as close to a polished genome as possible (i.e. reduce manual task time). otb takes several steps in order to reach this point (see Fig. 2, Table 1). Starting with a setup, first otb will check its environment and any set environmental variables, as well as called flags and any modes it should be running int, then otb will check if all required



A



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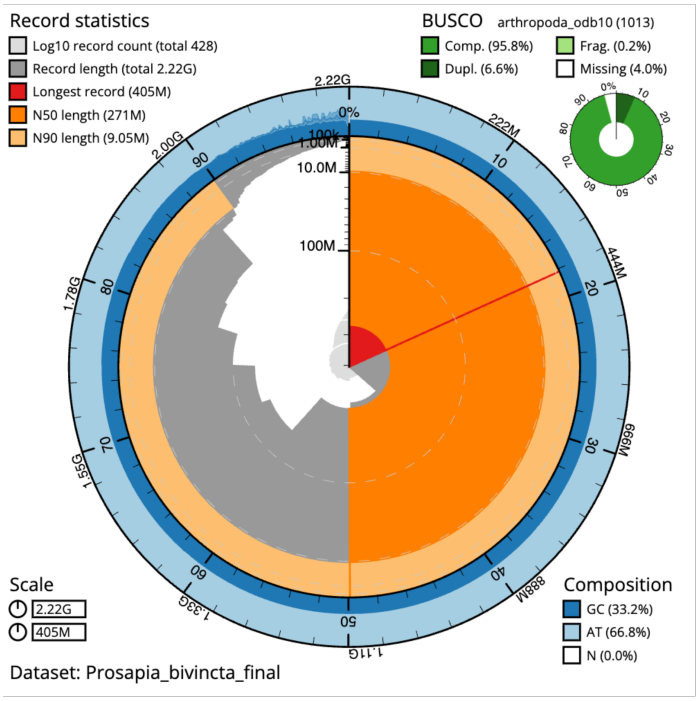

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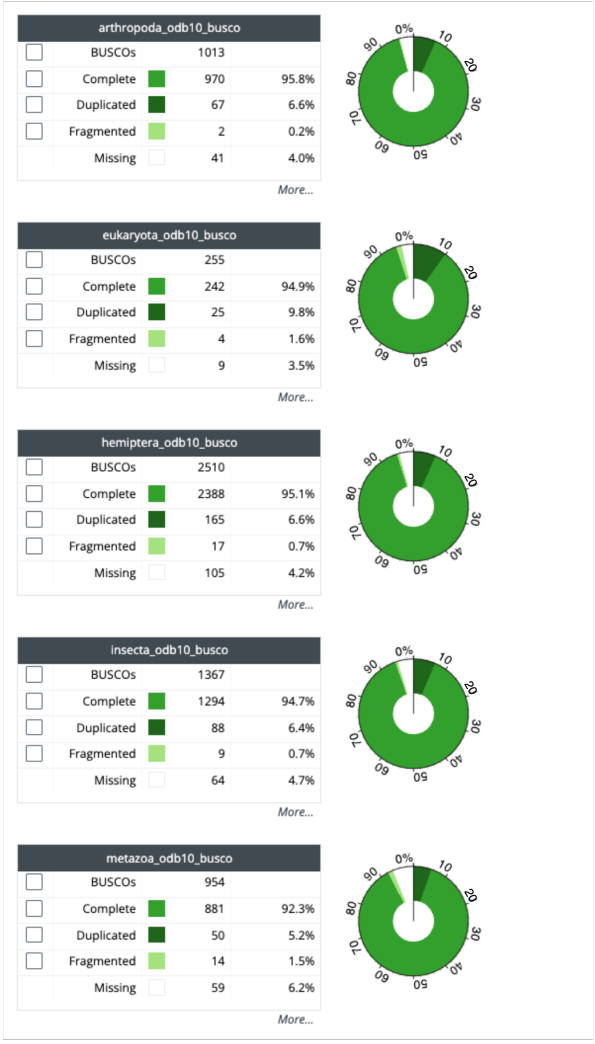

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Figure 1 Blobtools reports: A: blobplot, showing length and GC content B: snail plot showing record statistics and Busco C: cumulative plots showing lenth and assignment of reads D: Busco plots showing complete buscos of several taxonomic classifications

Table 1 Software Tools Utilized by otb

Software	References	Latest Title
BamTools	(Barnett *et al.* 2011)	BamTools: a C++ API and toolkit for analyzing and managing BAM files
BBTools	(Bushnell *et al.* 2017)	BBMerge – Accurate paired shotgun read merging via overlap
BCFTools	(Li 2011; Danecek *et al.* 2021)	Twelve years of SAMtools and BCFtools
bwa	(Li and Durbin 2009)	Fast and accurate short read alignment with Burrows–Wheeler transform
fcs-adaptor	(CGR 2022a,b)	Foreign Contamination Screen (FCS) tool for GenBank submissions
GFAstats	(Formenti *et al.* 2022a)	Gfastats: conversion, evaluation and manipulation of genome sequences using assembly graphs
BUSCO	(Manni *et al.* 2021b,a; Seppey *et al.* 2019) (Waterhouse *et al.* 2018, 2017; Simão *et al.* 2015)	BUSCO Update: Novel and streamlined workflows along with a wider and deeper phylogenetic coverage for the scoring of eukaryotic, prokaryotic, and viral genomes
DeepVariant	(Poplin *et al.* 2018)	A universal SNP and small-indel variant caller using deep neural networks
GenomeScope2	(Ranallo-Benavidez *et al.* 2020; Vurture *et al.* 2017)	GenomeScope 2.0 and Smudgeplot for reference-free profiling of polyploid genomes
hicstuff	(Matthey-Doret *et al.* 2020, 2021)	koszullab/hicstuff: Use miniconda layer for docker and improve P(s) normalization.
HiFiAdapterFilt	(Sim *et al.* 2022)	HiFiAdapterFilt, a memory efficient read processing pipeline, prevents occurrence of adapter sequence in PacBio HiFi reads and their negative impacts on genome assembly
hifiasm	(Cheng *et al.* 2021)	Haplotype-resolved de novo assembly using phased assembly graphs with hifiasm
Jellyfish	(Marçais and Kingsford 2011)	A fast, lock-free approach for efficient parallel counting of occurrences of k-mers
KMC 3	(Kokot *et al.* 2017)	KMC 3: counting and manipulating k-mer statistics
Merfin	(Formenti *et al.* 2022b)	Merfin: improved variant filtering, assembly evaluation and polishing via k-mer validation
RagTag	(Alonge *et al.* 2021, 2019)	Automated assembly scaffolding elevates a new tomato system for high-throughput genome editing
SAMTools	(Li 2011; Danecek *et al.* 2021)	Twelve years of SAMtools and BCFtools
Shhquis.jl	(Molik 2022)	molikd/Shhquis.jl: Inital Release
VCFTools	(Danecek *et al.* 2011)	The variant call format and VCFtools
Yahs	(Kokot *et al.* 2017; Deorowicz *et al.* 2015, 2013)	KMC 3: counting and manipulating k-mer statistics

containers are available and working, if not, otb will download the required singularity containers and proceed to calling the nextflow run, the main body of the analysis. The sequencing data will be filtered and then assembled with HiFiASM and hicstuff (Cheng *et al.* 2021; Matthey-Doret *et al.* 2020). If the user requests it, Busco can be optionally run at this point. Shhquis.jl, an in-house script is run after the initial assembly to cluster and orient the contigs. Optionally, otb can undergo genome assembly polishing, and it provides this in three ways: Merfin, DeepVariant (Formenti *et al.* 2022b; Poplin *et al.* 2018), or a 'Simple'. In "Simple" the error corrected reads are used to scaffold, both Merfin and DeepVariant do this as well. Busco can optionally be run at this point. The user can then optionally run KMC/Yahs, and Busco can optionally run at that point as well (Kokot *et al.* 2017; Manni *et al.* 2021a). The result is that the maximum number of assembly steps are carried out, saving the user from having to perform each step individually. By including Hi-C contact map rearranging in the pipeline, and steps to reduce the number of contigs in the draft assembly, less work is needed in the scaffolding step (Molik 2022).

otb was tested with Two-lined spittle bug data. The resultant genome was fairly typical of a true bug. The genome assembly had a N50 of 270.86 Megabases, a Total scaffold length of 2.22 Gigabases, a GC content of 33.22%, had an average scaffold length 5.19 Megabases, had a Total scaffold length 2.22 Gigabases, had 33.22% GC Content, and had a N50 270.86 Megabases. Blobtools hemiptera showed a 95.1% Buscos found (see Fig. 1. The genome assembly of the two-lined spittle bug will provide a basis for further work into its interaction with its obligate hosts, and no doubt into the control of the pest.

Nextflow and Singularity are two software infrastructure tools commonly used in scientific computing workflows. Nextflow is an open-source software solution that enables users to define pipelines and processes for data analysis in a concise and reproducible manner, supporting various languages and technologies. This versatile platform is built on a reactive programming model, allowing dynamic, parallel processing, and data flow management. This makes Nextflow an ideal tool for managing large-scale, multi-step data analysis processes in a scalable and efficient manner.

Singularity is a container technology specifically designed for high-performance and scientific computing environments. This technology provides a way to bundle applications, dependencies, and the environment into a single executable package, ensuring reproducibility, compatibility with existing Linux container technologies, and security. Singularity offers several key features, such as its ability to run containers with root-level access on any system while ensuring that the host system remains isolated and protected. These features make Singularity an attractive solution for many scientific and engineering workflows.

Together, Nextflow and Singularity provide a powerful and versatile solution for managing complex scientific computing workflows. They enable users to share and run applications, tools, and workflows in a consistent and reproducible manner, across different HPC environments and operating systems. As such, they have become essential tools for managing the large-scale and complex data analysis processes required in modern scientific research.

The introduction of otb, a new HiC/HiFi phased genomics assembly pipeline, has solved several data management problems that were prevalent in the creation and storage of genome assemblies. otb is written in nextflow and utilizes singularity to ensure uniformity of the computing environment. Offers parallel task execution and resource management, while also reducing the time spent organizing data, installing tools, and performing analysis. With the implementation of otb, genome creation can be automated, especailly in regards to projects such as the Ag100pest.

Data availability

Code used in the creation of this genome is in the public domain per United States 17 U.S.C. § 105. The code is freely available for

use and modification:

	github	DOI
otb	molikd/otb	10.5281/zenodo.6689816
Shhquis.jl	molikd/Shhquis.jl	10.5281/zenodo.6315237

Data published for this article are in the public domain per United States 17 U.S.C. § 105. The data are freely available for use and modification:

	Identifier
BioProject	PRJNA987615
BioSample	SAMN35984262

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Conflicts of interest

none declared.

Footnotes

The U.S. Department of Agriculture is an equal opportunity lender, provider, and employer.

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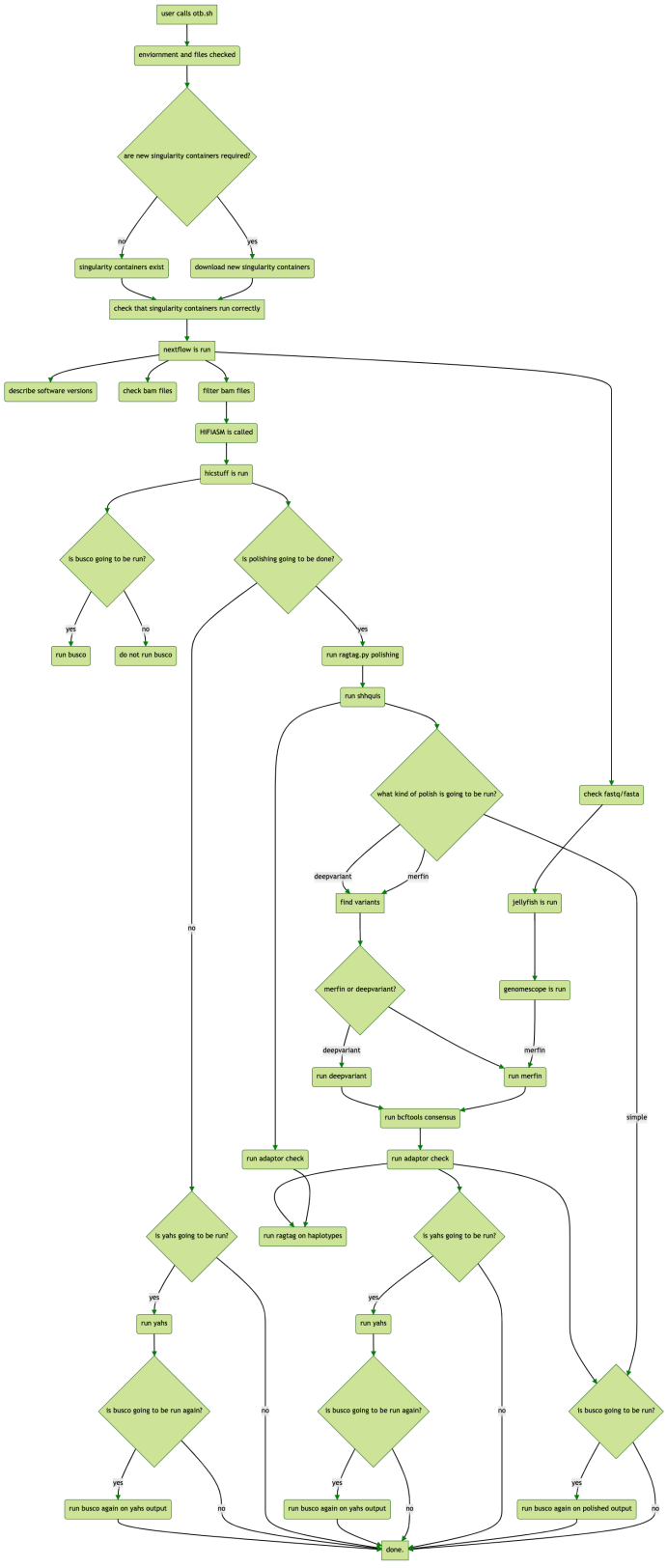

Figure 2 otb flowchart. Workflow diagram of otb showing the provess of otb running, otb.sh the entry point for otb run software and container checks, followed by the assessment of the type of hifiasm assembly to be created, otb allows also allows for multiple types of a sequence based polishing run, including a "simple" or reuse of error correct reads remapped using ragtag.py, a "deep variant" which uses deepvariant, and "merfin". Busco and sequence stats are run at multipe points in the pipeline. Yahs is run to produce HiC maps which can be utilize in JuiceBox Robinson *et al.* (2018).