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GetOrganelle: a fast and versatile toolkit for accurate *de novo* assembly of organelle genomes

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Abstract

GetOrganelle is a state-of-the-art toolkit to accurately assemble organelle genomes from WGS data. This toolkit recruits organelle-associated reads using a modified "baiting and iterative mapping" approach, conducts *de novo* assembly, filters and disentangles the assembly graph, and produces all possible configurations of circular organelle genomes. For 50 published plant datasets, we were able to reassemble the circular plastomes from 47 datasets using GetOrganelle, but only 12 using NOVOPlasty. GetOrganelle assemblies are more accurate than published and/or NOVOPlasty-reassembled plastomes as assessed by mapping. We also assembled complete mitochondrial genomes using GetOrganelle. GetOrganelle is freely released under a GPL-3 license (https://github.com/Kinggerm/GetOrganelle).

Keywords: Assembler; Assembly graph; Plastome; Mitogenome; Organelle genome.

Background

The plastid genome (plastome, including the chloroplast and other plastid forms) and mitochondrial genome (mitogenome or chondriome) represent the portions of endosymbiotic organelle inheritance in eukaryotes that have remained in the organelle without being transferred to the nucleus or lost. The plastomes of photosynthetic eukaryotes are generally 120-150 kb in size and typically map as a highly conserved circular and quadripartite structure, with a pair of inverted repeat regions (IRs) that separate the large single copy (LSC) from the small single copy (SSC) regions [1, 2]. Mitogenomes exist in nearly all eukaryotic organisms and vary greatly in genome size and form. To date, six main types of mitogenome organization have been recognized [3]. Animal mitogenomes map as a single circle molecule, ranging from 11-28 kb in size and either lacking introns (i.e., type I), or including introns (types II - VI). Fungi and plants have single circular mitogenomes with introns from 19-1000 kb in size (type II), or a large and homogenous circular molecule from 20-1000 kb in size with small circular plasmid-like molecules (type III), or homogenous linear molecules from 1-200 kb in size (type V). Because of their near universal presence and high copy numbers in the cell (individual organelles contain numerous plastome and/or mitogenome copies), organelle DNA markers and genome sequences are easily obtained and have been extensively used for phylogenetic and evolutionary analyses [4-8], and DNA barcoding [9-12]. Since the rapid advances of high throughput sequencing technologies, sequencing costs have decreased tremendously in recent years. Due to the high copy numbers of organelle genome in a single cell, it is feasible to get enough reads from the low coverage whole genome sequencing (WGS) data to assemble complete organelle genomes [13, 14].

To date, there are 2937 plastomes and 9217 mitogenomes (including 8128 animals, 563 fungi, 60 plants, and 255 protists) available in GenBank (https://www.ncbi.nlm.nih.gov/genome/browse#!/organelles/, accessed on April 15, 2019). Many processes or pipelines for assembling organelle genomes have been described, but their assembly qualities vary widely. For example, SPAdes [15], SOAPdenovo2 [16], and CLC Genomics Workbench (https://www.qiagenbioinformatics.com/) have been widely used to assemble the WGS data, after which the organelle genomic scaffolds/contigs were selected or filtered out using a reference genome for further concatenation [17] or post-assembly gap-filling and closing [18, 19]. However, those approaches are computationally intensive and rarely assemble complete organelle genomes [20]. The IOGA (Iterative Organellar Genome Assembly) pipeline [21] incorporates Bowtie2 [22], SOAPdenovo2, SPAdes and other dependencies for recruiting plastid-associated reads and conducting de novo assembly, but the plastomic scaffolds/contigs need to be finalized by other programs. The Fast-Plast (https://github.com/mrmckain/Fast-Plast), NOVOPlasty [23], and ORG.asm (https://git.metabarcoding.org/org-asm/org-asm/org-asm) were proposed as fast tools to conduct *de novo* assembly of complete organelle genomes from WGS data. However, these tools have not been systematically evaluated until a recent preprint was posted at bioRxiv [24]. Freudenthal *et al.* [24] presented a benchmark comparison of seven chloroplast assembly pipelines/toolkits and found significant differences among those assemblers. In their tests, our toolkit, GetOrganelle

(https://github.com/Kinggerm/GetOrganelle), significantly outperformed all other assemblers in consistency, accuracy and success rate. Nevertheless, Freudenthal *et al.* [24] only tested the plastome assembly with a limited range of applications and parameters using GetOrganelle. Additionally, organelle genomes may produce flip-flop configurations or other assembly isomers mediated by repeats [25-27]; these outcomes are not addressed by any of the above-mentioned pipelines/toolkits, except GetOrganelle. This capability was not investigated by Freudenthal *et al.* [24].

The GetOrganelle toolkit includes a number of scripts and libraries for recruiting target organelle reads from the WGS data, manipulating and disentangling assembly graphs, and generating reliable organelle genomes, accompanied by labeled assembly graphs for user-friendly manual completion and correction (Figure 1). The from-reads-to-organelle process can be completed using the script "get_organelle_from_reads.py" with a single line command, which serves as the main workflow of GetOrganelle. This script exploits Bowtie2, BLAST [28], SPAdes, as well as the Python libraries Numpy, Scipy, and Sympy as dependencies. It starts with recruitment of initial target-associated reads by using Bowtie2 and taking target genome(s) or sequence(s) as the seed; the initial target-associated reads (seed-mapped reads) are treated as "baits" to get more target-associated reads through multiple extension iterations, which is similar in concept to those of the MITObim [29] and IOGA [21] pipelines. However, the core algorithm of GetOrganelle for read extension uses a hashing approach, which cuts the reads into substrings ("Words") with a uniform length ("Word size"), and adds them to a hash table ("Accepted Words", AW). During each extension iteration, the AW dynamically increases as new target-associated (overlapped) reads are cut and added as Words. Then, the total target-associated reads are *de novo* assembled into a FASTA assembly Graph ("FASTG") file using SPAdes. Non-target contigs in the FASTG assembly are further automatically identified and trimmed by their connections, coverages and BLAST hit information using a target-gene-based "label" database. The slimmed FASTG file is used to calculate all possible paths of the complete target organelle genome based on the graph characteristics and the coverages of the contigs (see Figure 2). In some cases, when the assemblies cannot be solved as a circular path or are too complicated to be solved, GetOrganelle will conservatively export the target contigs/scaffolds. Meanwhile, the slimmed assembly graph (FASTG) and selected target assembly graph (GFA) can also be visualized by Bandage [30] to assess the completeness of the final graph, or export selected contigs/scaffolds, or manually remove "noisy" and non-target contig/scaffold connections. The semi-manually-cleaned assembly graph can then be used to get the complete target genome or the target contigs/scaffolds using the script "get_organelle_from_assembly.py". In this study, we illustrated the mechanism and workflow of GetOrganelle. Moreover, we tested GetOrganelle with a wide range of parameters and samples of plants, animals and fungi in assembling plastomes and mitogenomes, and provide a detailed comparison of the assemblies of NOVOPlasty, the assemblies of GetOrganelle, and the published plastomes.

Results

Reassembling plastomes from 50 published plant datasets using GetOrganelle

GetOrganelle reassembled the complete circular plastome(s) from 47 out of the 50 plant datasets (Additional file 2: Table S1), including samples with typical IRs, contracted IRs, no IRs, and large directed repeats (DRs) (Figure 3). Of the remaining three species, two species (*Ginkgo biloba* L., ERR2206741 and *Salvinia cucullata* Roxb., SRR6478596) had two break points in the LSC/SSC region, and one species (*Musa balbisiana* Colla, SRR2057084) consisted of 14 plastome fragments (Additional file 2: Table S1; see details at https://github.com/Kinggerm/GetOrganelleComparison). The GetOrganelle-reassembled complete circular plastomes were identical to the published ones in 14 samples, and different from published plastomes by fewer than five site-differences and/or less than 100-bp differences in 30 samples (Additional file 2: Table S1, Table S2). Most of the differences were due to repeats or indels. Read mapping generally supported the GetOrganelle-reassembled plastomes more than the published plastomes (Figure 4; Additional file 2: Table S2). For example, the IR boundary regions of *Laurus nobilis* L. (SRA: SRR5602602; GBK: KY085912.1), which showed significant assembly differences between the published and GetOrganelle-reassembled plastomes, had a smooth mapping plot for the GetOrganelle plastome but a drastically fluctuating mapping plot for the published one (Additional file 1: Figure S1). Those differences can also be monitored from the summarized mapping quality (Additional file 2: Table S2), where the GetOrganelle-reassembled plastome had similar matched depth to the published plastome (221.60 vs 221.65), with a much smaller deviation (31.43 vs 37.88), as well as a smaller error rate (1.16% vs 1.17%) with a much smaller deviation (1.11% vs 1.56%).

Evaluations of assembling the 50 plant datasets showed that the mean memory, the mean duration, and the number of circularized genomes all decreased with the word size ratio (WSR, defined as the Word size over the effective mean read length) adjusted from 0.6 to 0.8 (Figure 5). Runs in an "auto" mode using an automatically estimated word size for read extension generated circular plastomes in 39 samples, ranked as the highest success ratio among the tested parameter sets, though had the most intense computational usage in both mean memory and duration (Figure 5). Eight more datasets were assembled into the complete circular plastome(s) by adjusting the Word size for extension, and/or adjusting the *k*-mer values for *de novo* assembly using SPAdes, and/or using other empirical options (see details at https://github.com/Kinggerm/GetOrganelleComparison).

GetOrganelle exported all possible configurations mediated by potential flip-flop recombination induced by IRs or other shorter repeats. When a complete circular plastome was obtained, GetOrganelle generally outputted consistent assemblies with the same raw reads using different parameters. In the 50 plant datasets, there were 33 samples that resulted in complete circular plastome reassembled from multiple runs using different parameters of GetOrganelle. Twenty-five out of 33 samples produced identical assemblies using different parameters. Five out of 33 samples (SRA: SRR5602586, SRR5602590, SRR5602594, SRR5602605, SRR6932851; we will use the SRA number to represent the relevant samples thereafter) had assemblies that diverged where there were 1 or 2 bp indels. For SRR5028199, the plastome of the GetOrganelle-W0.6 run is

longer than the plastome of any other three runs in a 19-bp repeat ("GAAAAGAAAGAATGAGAAA"). For SRR5602597, the plastome of the GetOrganelle-W0.8 run is shorter than the plastome of any other three runs in a 36-bp repeat and a 273-bp indel/repeats. For SRR5602577, the plastome of the GetOrganelle-W0.8 run is shorter than the customized run in a 6-bp poly-A/T indel.

Reassembling plastomes from 50 published datasets using NOVOPlasty

For reassembling plastomes from the same 50 plant datasets (see Additional file 2: Table S1) using NOVOPlasty, only 15 samples were "claimed circular" (i.e., eight for *k*-mer 23, ten for *k*-mer 31, eight for *k*-mer 39, and seven for *k*-mer 47) (Figure 4; Additional file 2: Table S2). Of the "claimed circular" samples, three samples had gaps or ambiguous sites in K23 runs, six samples had gaps or ambiguous sites in K31 runs, five samples had gaps or ambiguous sites in K39 runs, and five samples had gaps or ambiguous sites in K47 runs (see details at https://github.com/Kinggerm/GetOrganelleComparison). In addition, some claimed circular samples were not really circularized. For example, there are two contigs in both the K31 run of SRR5602581 and the K47 run of SRR5602590. Some claimed circularized runs (such as the K23/K31 runs of SRR2037123, and the K23 run of SRR5602581) were problematic because the lengths of assemblies were deviated strongly from the published plastomes and the reassembled plastomes using GetOrganelle. Of the remaining 12 samples for which NOVOPlasty obtained reasonable lengths, the read mapping qualities for NOVOPlasty assemblies were good though generally not better than those of GetOrganelle, especially in the error rate (Figure 4; Additional file 2: Table S2). Only three datasets (SRA: ERR1917165, ERR964904, and SRR5602589) were assembled by NOVOPlasty into plastomes identical to the published ones (Additional file 2: Table S2).

The average duration of NOVOPlasty runs increased as the *k*-mer values increased from 23, through 31 and 39, to 47 (marked as K23, K31, K39, K47 runs separately), while the average memory cost was very close among the four *k*-mer values (Figure 5). The average duration of NOVOPlasty K23 and K31 runs was generally shorter than those of GetOrganelle runs. The average duration of K39 runs was similar to that of GetOrganelle-W0.8 runs. The average duration of K47 runs was longer than those of GetOrganelle runs except for GetOrganelle-auto runs. The average memory cost of the NOVOPlasty runs was lower than those of GetOrganelle-W0.60 and GetOrganelle-auto runs, but greater than those of GetOrganelle-W0.7 and GetOrganelle-W0.8.

Additionally, the assemblies of NOVOPlasty are largely inconsistent and varied greatly when using different *k*-mer values. In nine samples, NOVOPlasty obtained the complete circular plastome in multiple runs. However, NOVOPlasty generated consistent assemblies using four *k*-mer values in only three samples (SRA: ERR1917165, SRR5602589, and SRR5602602) out of the nine.

Assembling mitogenomes using GetOrganelle

For the animal mitogenome test, GetOrganelle successfully assembled the complete circularized mitogenome in 29 out of 56 datasets (Additional file 2: Table S3). Of the 56 datasets, GetOrganelle failed in calling any animal mitogenome contigs in four samples, which have been reported by Yang *et al.* [14]. The

average duration for those tests was 10,428 s, ranging from 843 s (SRA: SRR5803928, circularized) to 41,311 s (SRA: SRR5804145, circularized). The average memory cost for those tests was 12.1 G, ranging from 2.9 G (SRA: SRR5804137, circularized) to 40.1 G (SRA: SRR5804145, circularized).

For the fungal mitogenome test, GetOrganelle successfully assembled the complete circularized mitogenome in 24 out of 50 datasets (Additional file 2: Table S4). The average duration for those tests was 10,428 s, ranging from 843 s (SRA: SRR5803928, circularized) to 41,311 s (SRA: SRR5804145, circularized). The average memory cost for those tests was 12.1 G, ranging from 2.9 G (SRA: SRR5804137, circularized) to 40.1 G (SRA: SRR5804145, circularized). The sizes of circularized results ranged from 33,655 bp (SRA: SRR5803909) to 209,881 bp (SRA: SRR5804018).

Features of GetOrganelle

In exploring the influences of parameters and seed, we assembled reads of an angiosperm species, *Haberlea rhodopensis* Friz. [GenBank Sequence Reads Archive accession number (SRA): SRR4428742], using the complete plastome or a short plastomic fragment *rbcL* gene of a gymnosperm species, *Gnetum parvifolium* (Warb.) W.C. Cheng [GenBank accession (GBK): NC_011942.1] as the seed (Figure 6, Additional file 1: Figure S2, Additional file 2: Table S5). The runs for all tested WSR, except for 0.9, assembled an identical complete plastome, no matter whether the initial seed was a complete plastome or a short plastomic fragment *rbcL* gene, and regardless of whether pre-grouping (an *ad hoc* speeding-up algorithm, see more in the Methods) was enabled or not.

The run duration of GetOrganelle varied as the WSR set from 0.3 to 0.9 (Figure 6). The duration of the runs with unlimited number of rounds (lines with circle marks in Figure 2) increased drastically when the WSRs were 0.65 and 0.70. The duration of the runs with minimum number of rounds and pre-grouping disabled (grey line with cross marks) decreased when the WSR ranged from 0.3 to 0.65 and increased when the WSR ranged from 0.7 to 0.90. The time cost of the runs with the minimum number of rounds and pre-grouping enabled (green line with cross marks) decreased when the WSR ranged from 0.3 to 0.55, but it did not increase when using large WSRs. The maximum memory occupation generally decreased when the WSR was smaller than 0.65 and remained unchanged when the WSR reached 0.65. A too large WSR, such as 0.9 in our test, risked producing an incomplete result.

When testing with unlimited number of rounds, the runs with pre-grouping enabled (grey line with circle marks) and the runs with pre-grouping disabled (grey line with cross marks) recruited exactly the same number of the final accepted reads (Figure 6), thus they generated the same final assemblies, and similar computational durations and memory costs. However, when considering the runs with minimum rounds, the pre-grouping runs generally needed significantly fewer rounds of extension (< 6) to recruit most of the target-associated reads to achieve a complete plastome or to stabilize an incomplete result. Extension beyond the first five rounds is mostly to recruit non-target reads. By cutting off the latter unnecessary rounds of extension with minimum rounds (lines

with cross marks), the pre-grouping runs (green line with cross marks) can reduce both time and memory cost, sometimes significantly.

Discussion

Accuracy of the published/reassembled plastomes

GetOrganelle-reassembled plastomes contained identical sequences to the published plastomes in 14 of 50 plant samples, including a lycopod species, *Selaginella kraussiana* (Kunze) A. Braun (SRR2037123) that was reported to have large DRs [31]. Read mapping plots of GetOrganelle reassembled complete or near complete plastomes are generally smooth. In contrast, the NOVOPlasty reassembled plastomes (e.g. ERR964904, SRR2037123, SRR5602581, as mentioned above) had unreasonably shorter lengths, but were claimed to be circularized. In addition, read mapping plots (see https://github.com/Kinggerm/GetOrganelleComparison/tree/master/eval/Published) showed that some published plastomes (e.g. SRR1145775, SRR2057084, SRR5602601, SRR5602602, SRR6478596, SRR7630500) have incorrect IR boundaries or contig overlaps as revealed by the local coverages dramatically increasing or decreasing in the regions or sites.

Our evaluation using read mapping (Additional file 2: Table S2) showed that 37 of 50 assemblies generated by GetOrganelle had the best-ranked matched depths (smaller average and smaller deviation). For three samples (SRR2057084, SRR6478596, ERR2206741), GetOrganelle failed to generate a circular plastome. For SRR2037123, NOVOPlasty produced a problematic assembly with aberrantly high coverage and gain the best-ranked matched depths by cheating. Of the remaining ten samples that GetOrganelle did not have the best-ranked matched depths, all the GetOrganelle-reassembled plastomes have the best-ranked error rate (smaller average and smaller deviation) and the best-ranked or nearly-best-ranked number of mapped reads.

Our evaluation using read mapping (Additional file 2: Table S2) showed that 45 assemblies using GetOrganelle had the best-ranked error rate; the remaining five assemblies, of which three assemblies were not circular, had values very close to the best-ranked ones. However, many published plastomes (e.g. SRR5602575-SRR5602578, SRR5602581-SRR5602584, SRR5602587, SRR5602588, SRR5602592, SRR5602593, SRR5602595, SRR5602597, SRR5602598, SRR5602599, SRR5602600, SRR5602609, SRR5602610, SRR6932851, SRR7630500) have nucleotide sites that are incongruent with the consensus of most mapping reads, as revealed by extremely high mismatch/indel rates at some sites, identifiable as extremely outlier points in the mapping plot.

Consistent with Freudenthal *et al.* [24], our results also showed that GetOrganelle had outstanding consistency. Of the 33 samples reassembled to complete circular plastome by multiple runs, there are 30 samples had identical or nearly identical (<= 2 bp difference) results using different parameter values. There are three main reasons for the discrepancies in the remaining few samples. First, a large Word size recruits significantly fewer reads (e.g., -w 0.8 in SRR5602597), or may even cause an incomplete result (e.g., -w 0.8 in SRR5028199,

SRR5602590 and SRR5602594, and -w 0.9 in the performance test). Second, there were small repeats that could not be precisely identified by low local coverage, which might be the reason for inconsistency in SRR5028199. Third, there were polymorphisms in the data causing an incomplete result, such as in SRR5602577 recovered by the auto mode.

Our results also suggest that using read mapping quality is an easy and important approach of evaluating organelle genome assemblies. We tested 50 plant datasets here, not only to show that GetOrganelle has higher accuracy for assemblies than other currently available assembly toolkits, but also to argue for the necessity for all plastome providers to make raw data available to the public (e.g. SRA) so that reproducible assemblies that can be evaluated and amended will be available to benefit future comparative analyses of organelle genomes [32].

Large Inverted, Directed Repeats and "weird" plastomes

The entanglement of repeats is one of the challenges in assembling plastomes, though none of the reported plastome assemblers addressed it. The largest repeat pair in a canonical plastome is the IR. Given that flip-flop recombination mediated by the IRs is common [33, 34], a genuine *de novo* assembler such as GetOrganelle should export at least two configurations when IRs exist. Even if we assume that there was only one configuration *in vivo*, short sequencing reads theoretically cannot tell the difference between those two configurations when IRs exist. However, for example, NOVOPlasty produces, when successful, only one representative of the plastome structure, which risks misleading less experienced researchers to treat SSC orientations as an important inversion [34].

Another issue concerning IRs is the identification of the IR boundaries. A traditional assembly method which uses CLC or any other assemblers to make the contigs and complete the circular assembly based on a reference is prone to create a plastome with a similar IR length/boundary as the reference. That was why PCR verification was required for the four boundaries of a canonical quadripartite plastome in conventional assemblies. This obsolete concept still overshadows many empirical studies in the NGS age. However, our graph-based method for completing the circular plastome uses the original contig connections that supported by the nature overlap of reads. When sufficient read coverages (e.g. 100×) support the contig connections between the boundaries, there would be neither need for PCR verification nor reads-mapping. For some recalcitrant opponents who still insist the need for PCR, we may ask, why not use PCR to verify any other parts of the plastome since they are also assembled by short reads? Based on the nature of overlap of short reads, the credibility of the IR boundaries is actually the same to that of other parts of the plastome. For a genuine organelle genome *de novo* assembler, the IR boundaries can be constructed purely from read information rather than any references, the same as any other regions of the genome. As evidence, GetOrganelle correctly recovered the extreme contraction or loss of a large pair of IRs in *Juniperus cedrus* Webb & Berthel. (SRR1145775) and two *Picea* species (ERR268390, SRR5028199). Another interesting example is that GetOrganelle successfully assembled the plastome of *Selaginella kraussiana* (SRR2037123) with two large DRs, without being supplied

prior knowledge of the IRs or DRs. However, for the same sample, NOVOPlasty only achieved part of the complete plastome, lost the two large DRs, and claimed it to be circular. Our reassembled plastome is identical to the published one (MH549643.1), which was assembled using Velvet and SPAdes, and then verified using PCR [31]. For an IR lacking species, such as a species in the IR-lacking clade of Fabaceae, GetOrganelle could correctly generate a plastome without IRs, with a single circle assembly graph as visualized by Bandage (e.g. R. Zhang et al., unpublished data).

It is a challenge to assemble some "weird" plastomes (e.g., reduced plastomes, plastomes with gene translocations, IR expansions, contractions or losses) using some traditional methodologies [20]. For example, non-photosynthetic plants typically have reduced plastomes and many gene translocations [20, 35, 36]. We successfully assembled a complete and yet highly reduced plastome without IRs for the holoparasite *Cytinus hypocistis* (L.) L. (ERR964904) using a customized strategy. We also were able to assemble circular plastomes for many holoparasites from Balanophoraceae, Convolvulaceae (*Cuscuta* spp.), Lennoaceae, and Orobanchaceae, as well as hundreds of hemiparasites (W.-B. Yu et al., unpublished data).

Mitogenomes of fungi and animals

In our tests, assembling animal mitogenomes, generally required more computational resources, in terms of both duration and memory, than assembling plastomes and fungal mitogenomes, even though animal mitogenomes are much smaller. One reason for the increased computational burden could be the increase of dataset size, which is the number of total bases. Another reason may be the inaccurate target organelle coverage estimation, therefore improper Word size estimation. Most of the time, GetOrganelle underestimated the target organelle coverage, which generated extremely small Word size values and extended too many non-target reads. The underestimation of target organelle coverage may be due to the limitation of the default seed database (see more discussion below). It is also worth mentioning that animal mitogenome representations in tested WGS data sets are relatively low (ca. 0.5% on average), compared to plastome (ca. 3% on average) and fungal mitogenomes (ca. 3% on average) represented in the tested low coverage WGS datasets, which at least leads to lower efficiency in recruiting target reads.

The mitogenomes of fungi and animals usually have a much higher nucleotide substitution rates than plastomes [37, 38]. Therefore, unlike assembling plastomes, a relatively closely related seed or label database would be indispensable for assembling and identifying mitogenomes using the extension and assembly strategy of GetOrganelle. If GetOrganelle fails using the default database for animals or fungi mitogenome assembly, we suggested that users rerun the same sample using their own seed and label databases. Limitations of the default seed database or the default label database of the current GetOrganelle version may be responsible for some failures. For example, SRR1946581 (*Cephus cinctus* Norton, a wheat stem sawfly) has 1,796,709 reads (ca. 225 Mbp) mapped to the default seed database, but none of its assembled contigs hit the label database, because the default label database is currently still a small, near-random subset of the default seed database.

Other small repeats, broken graph and manual completion

When attempting to solve the organelle genome structure, other small repeats can cause awkward tangles in the assembly graph. Plastomes of some plant clades have been reported to have multiple short repeats, such as some clades in Ericaceae [39, 40], Geraniaceae [41], and Pinaceae [26, 42]. Most plant mitogenomes contain a high number of short repeats [43-45]. In contrast, mitogenomes in animals and most fungi are contracted and generally lack repeats [46]. However, in our trial analyses we did discover a few samples of fungi (e.g. SRR5801935, *Macrolepiota dolichaula* (Berk. & Broome) Pegler & R.W. Rayner, SRR5802125, *Collybia* sp., and SRR5804018 *Grifola frondosa* (Dicks.) Gray), and animals (e.g. SRR136494, *Mayetiola destructor* Say, SRR4340274, *Daphnia magna* Straus, SRR1298377, *Halyomorpha halys* Stål) that have multiple repeats, which may result in isomeric assemblies.

One common feature of organelle genomes is tandem repeats, which can be visualized as whirls in the assembly graph. The main challenge with disentangling tandem repeats in an assembly graph is determining the multiplicity (copy number) of the contigs. Many bacterial genome assemblers use the minimum or the greedy algorithm for detecting multiplicity, which is simply assuming every tandem repeat contig has multiplicity two and constructing the assembly as a path that traverses the repeat twice [47-49]. GetOrganelle disentangles the tandem repeats based on both the connection information and the contig coverage, a more reality-based approach, which has a better chance of success if the target organelle genome has some tandem repeats of more than two copies and the reads are of sufficiently high coverage to overcome the error (Figure 2). Other repeats, such as short inverted repeats other than the large IRs, cause not only assembly graph tangles, but could also mediate recombination, potentially producing isomers in the same individual [25-27, 50]. For researchers studying plastome rearrangement and recombination dynamics, GetOrganelle is the right tool to exhaust the limits of the assembly graph and present all possible configurations, such as plastomes of *Juniperus cedrus* (SRA: SRR1145775) and *Picea* species (ERR268390, SRR5028199), and mitogenomes of some fungal (SRR5801935, SRR5804018) and animals species (SRR4340274, SRR136494). Currently, the potential configurations mediated by short repeats need to be confirmed by read pair mapping or PCR verification. With the development of long insert size libraries and long-read sequencing platforms (e.g. PacBio), these data can be added for improving repeat resolution and configuration confirmation. A function that incorporates long library reads or long-read sequencing data in estimating the proportion of all candidate isomers is expected to be added in future versions of GetOrganelle.

Some assembly graphs may not be organelle-sufficient (broken graph) or are too complicated to estimate multiplicity. In such cases, users are recommended to perform manual completion or manual multiplicity estimation with the simplified assembly graph using Bandage, along with the target-hit-contig table from the concomitant, cognominal TAB-formatted file. An advantage of manual completion is that it is possible to detect sequence interchange between the nuclear genome, mitogenome and plastome. This is especially the case when multiple organelle genome mode is selected for plant datasets. The result could be a tangly assembly graph mixed with plastome, mitogenome, and plastome-mitogenome-shared contigs. If *de novo* assembly and manual

completion cannot yield a complete sequence of the plastome, gap filling and PCR verification are needed [20, 39].

Seed and label database in GetOrganelle

It is feasible to assemble the plastome from the reads of an angiosperm species, *Haberlea rhodopensis,* with high and homogeneously-distributed plastid coverage, using the plastome of a gymnosperm species, *Gnetum parvifolium*, as the initial seed. For the same set of reads, using a short, conserved DNA fragment (*rbcL)* as the seed is sufficient to recruit almost all target-associated reads to assemble a complete plastome, but less efficient. The reason for this is that the seed is only used for reads mapping to collect the initial target-associated reads, after which the seed will no longer be used (Figure 1). GetOrganelle then uses the initial target-associated reads to recruit more target-associated reads using multiple rounds of extension, which is mainly based on the nature of the read overlaps with the previously-recruited target-associated reads, rather than using the seed as a reference in assembly process. Nevertheless, using the organelle genome of a non-related species as the seed can only be applied to plastome seed recruitment, but not to animal and fungal mitogenome seed recruitment dues to their highly divergent rates of evolution (See section on "mitogenomes of fungi and animals").

Since the label database (see Figure 1) in GetOrganelle is used to provide a supervision signal for labeling contigs prior to semi-supervised learning, the label database should come from conserved regions, such as coding regions, but not necessarily a complete set of genes. After labeling, GetOrganelle uses an integrated strategy (see Methods section) to identify target contigs, which largely relies on the contig coverages and assembly graph characteristics. Thus, the label database of gene fragments has limited influence on the final organelle-complete graph output and would have only a minor effect on the scaffolding of organelle contigs, except for cases where the assemblies have no hits against the database (e.g., SRR1946581).

Word size in GetOrganelle

Word size, like the *k*-mer in De Bruijn graph-based assembly, is important to the feasibility and efficiency of read extension, but it differs from *k*-mer in providing the overlap threshold for data filtering. The smaller the Word size is the lower the threshold is. The use of Word size does not only examine the nature of connection between any two target reads, but also in a way assesses the connection strength by the coverage. Even if the plastome shares similar sequence with mitogenome or nuclear genome, the reads of those non-target genomes would not be largely recruited due to the low coverage, meaning that any two reads of those non-target reads are less likely to be connected in the shallow coverage WGS data.

The Word size is generally negatively correlated with memory usage (Figure 6), while *k*-mer is generally positive correlated with memory usage. Memory cost decreases when the Word size increases because the larger the overlap threshold (causing fewer reads to be connected and recruited based on the overlap), the smaller the word space during extension. Thus, *k*-mer only influences the assembly process, while the Word size influences the extension process, the numbers of reads to be recruited and therefore the assembly process. For many real

datasets, because the plastome might share homologous regions with non-target genomes, a small Word size means that a relaxed threshold would not promptly stop the extension when the extension was walking through the non-target genomes. In the case shown in Figure 6, using a WSR < 0.65 wastes computational resources.

The duration of a whole assembly run is affected by the Word size in various ways (Figure 6). Generally, with unlimited number of rounds, there are two significant but antagonistic effects shaping the curve of the duration against the Word size (or in the form of WSR). The first one is the "slowing-speed effect", meaning that a larger Word size means smaller steps in extension and consumes more rounds and more time to recruit the same set of reads. The second one is the "reducing-data effect", meaning that a larger Word size also results in a briefer extension period with fewer non-target reads, reducing the run time in *de novo* assembly and other downstream calculation. In the performance test (Figure 6), a typical manifestation of both effects was where the time cost remarkably increased at WSRs in 0.65 and 0.70, which was caused by the relatively slow speed in extension and a relatively high amount of non-target reads to recruit. In this test, using the minimum number of rounds could cut off most non-target reads and eliminate the "reducing-data effect" in a slow-paced extension (a relatively large WSR, i.e. ≥ 0.65 in the pre-grouping disabled case and ≥ 0.55 in the pre-grouping enabled case), but cut off less non-target reads as the WSR become smaller (Figure 6). Therefore, when pre-grouping was disabled and the minimum rounds were run, the plot of duration against WSR was not a monotonic rising curve ("slowing-speed effect") but suffered the "reducing-data effect" when the WSR < 0.65 (Figure 6). Using the pre-grouping algorithm could generally eliminate the "slowing-speed effect". Consequently, in this test, the time cost is nearly constant when the WSR ≥ 0.55, but still suffers the "reducing-data effect" when the WSR < 0.55 (Figure 6). In empirical studies, the minimum number of rounds required is unknown. Using the pre-grouping algorithm with a sufficient value (i.e. empirically 2E5 for normal plastome percent), five rounds of extension typically recruited target reads covering the whole plastome (although 15 rounds were required in a very few cases with extremely low plastome coverage (data not showed)).

The optimal Word size is dictated by read length, read quality, total number of reads, percentage of organelle DNA content, heterogeneity of organelle base coverage, and other factors. If there is no user-assigned Word size value, the "get_organelle_from_reads.py" will automatically estimate an optimal Word size value using a set of customized empirical functions based on the characteristics of the dataset. The automatically estimated Word size tends to be small to enhance the success rate, but possibly at the cost of increasing the computational burden (Figure 5). Importantly, though, the automatically estimated Word size does not guarantee the best performance in assembly results (see eight plastome samples with customized parameters in our tests) nor in computational costs.

K-mer in GetOrganelle

GetOrganelle uses SPAdes as the core *de novo* assembler, which allows the user to use a *k*-mer gradient for assembly. One advantage of this is that SPAdes combines the assemblies from multiple *k*-mers. In our tested 50 plant datasets, most successful assemblies were the results of the largest *k*-mers that the data permitted, which is

127 for read length ≥ 150 bp or 91 for read length up to 100 bp. Someone might draw a specious conclusion that the base coverage is usually high enough to use the largest *k*-mer for assembling the complete plastome or mitogenome from WGS data. However, except for those with low base coverage for the plastome (e.g. SRR5602610, ca. 14×), plastome assembly still suffered from the base coverage heterogeneity or read error, which create regions where large *k*-mers do not overlap. For example, if only one large *k*-mer value was used for each run (i.e. 91 for read length ca. 100 bp and 127 for read length ≥ 150 bp), some samples (tests not shown) would not assemble a complete circular plastome, while by using a *k*-mer gradient, those samples achieved a complete plastome at the same large *k*-mer.

Another advantage of using a *k*-mer gradient is that GetOrganelle could iteratively attempt to disentangle the assembly graph of each *k*-mer from the largest to the smallest, then find a larger one with the organelle-sufficient graph. A larger *k*-mer value is preferable when there are longer repeats and coverage is sufficiently high. However, when the largest *k*-mer used in the analysis does not obtain the complete circular plastome/mitogenome, the assembly graph of a smaller *k*-mer is checked automatically. The "GetOrganelle-auto" runs of SRR5602587 and SRR5028199 benefitted from this design.

Computational consumption of GetOrganelle

Freudenthal *et al.* [24] showed that GetOrganelle has moderate efficiency in both time and memory usage among tested assemblers. However, they only used the default options for evaluation which are inclined to have the highest chloroplast genome completion rate, rather than high computational efficiency. If the aim of studies is not to assemble complete organelle genomes, researchers could easily adjust the options, such as increasing the Word size or turning on "--fast" to significantly speed up assembly and reduce memory usage, and still keep both the success rate and assembly quality at a higher level than those of other assemblers. However, we do not recommend these options. Firstly, in the current version of GetOrganelle, the complete circular assemblies are better than incomplete results. Secondly, extremely high Word size may generate higher error rate in assemblies with two cases in the 50 plant datasets (i.e., GetOrganelle-W0.8 runs of SRR5602577 and SRR5602597).

Conclusions

GetOrganelle is a fast and versatile toolkit for *de novo* assembly of complete and accurate organelle genomes using low coverage WGS data. Our evaluations show that the GetOrganelle toolkit can efficiently and accurately assemble different types of organelle genomes from a broad range of organisms. In general, compared with NOVOPlasty, GetOrganelle has far better success rates for assembling plastomes while consuming similar or even less computational resources. Additionally, GetOrganelle-reassembled plastomes generally have much higher accuracy than plastomes reassembled by NOVOPlasty or published plastomes that were assembled by various tools in accordance with the read mapping evaluation. GetOrganelle can also generate all possible

configurations when plastomes or mitogenomes have flip-flop configurations or other isomers mediated by repeats.

Potential applications of GetOrganelle include quickly extracting organelle genomes from whole genome assemblies and evaluating organelle genome quality. Assembling organelle genomes from metagenomic data would also be possible by using a customized database and scheme. The maximum extending length option enables rough control of the length of the target assembly, which could be used to quickly assemble interesting loci or genes from the metagenomic and transcriptomic data. Additionally, the Python Classes and Functions defined in GetOrganelleLib could be used to manipulate and disentangle non-organelle assembly graphs.

Currently GetOrganelle exports all possible configurations without using library information of the paired-end reads. However, the long insert size library or long-read sequencing data can be used for repeat resolution and configuration verification. A function that could use this information and estimate the proportion of all the potential isomers (configurations) is expected in a future version of GetOrganelle. Improvements in the seed databases and the label databases are also expected, which should result in better parameter estimation and higher success rates in assembling mitogenomes.

Methods

Workflow of organelle genome assembly using GetOrganelle

GetOrganelle v1.6.2 consists of two major scripts ("get_organelle_from_reads.py" and "get_organelle_from_assembly.py") and 17 minor scripts (under the directory "Utilities"; for processing or evaluating organelle assemblies) (Figure 1), a series of libraries (under the directory "GetOrganelleLib"; including default seed database, default label database, and Python Classes/Functions), and dependencies (under the directory "GetOrganelleDep"; including Bowtie2, SPAdes, BLAST+). The major script "get_organelle_from_reads.py" could pipe all 5 steps described below (also see green solid arrows in Figure 1) together using a single line command to assemble organelle genome(s) from raw reads. The major script "get_organelle_from_assembly.py" could extract organelle genome(s) from assembly graphs generated using SPAdes [15] or Velvet [51] (steps 4-5; also see blue solid arrows in Figure 1) .

Step 1. Mapping reads to seed and assembling seed-mapped reads for parameter estimation

The initial step of assembling target organelle genome(s) from reads via GetOrganelle ("get_organelle_from_reads.py") is using Bowtie2 to map reads to seed sequence(s) (i.e. the default seed database), which may include complete reference organelle genome(s) or organelle fragment(s) (Figure 1, green solid arrow 1). Currently, the default seed of GetOrganelle (under the directory "GetOrganelleLib/SeedDatabase") covers embryophyte plastomes, non-embryophyte plastomes, embryophyte mitogenomes, embryophyte nuclear ribosomal DNA, animal mitogenomes, and fungal mitogenomes (in the

option referred as “embplant_pt”, “other_pt”, “embplant_mt”, “embplant_nr”, “animal_mt”, “fungus_mt”, respectively). These mapped reads are called seed-mapped reads here (stored at *.initial.fq).

The seed-mapped reads will be treated as initial “baits” to recruit more target-associated reads in the next step. In the “auto” mode (see the last paragraph of Step 2 below), the seed-mapped reads will be also coarsely assembled into seed contigs, which will be used for parameter estimation in Step 2.

Step 2. Recruiting more target-associated reads through extending iterations

After creating “baits”, GetOrganelle (“get_organelle_from_reads.py”) recruits new target-associated reads by comparing candidate reads to “baits” and updating the “baits” with overlapped new reads (Figure 1, green solid arrow 2). In this extension process, the key comparison method for determining overlaps is the classic substring hashing. Substring(s) are referred as Word(s) here to distinguish them from *k*-mers, a similar concept in the assembly process. The uniform length of the Words is thus named as Word size.

Before the core extension iterations, the “get_organelle_from_reads.py” creates an index and assign unique ids for each set of duplicated reads to avoid repeatedly calculating (information stored at file “temp.indices.1”). Those reads with duplicates can also be used for downstream “pre-grouping”. The pre-grouping is an algorithm for speeding up target-reads recruitment. This algorithm is based on the idea that it would be more efficient to firstly compare reads that are more likely to be target-associated. Given that the organelle genomes usually have more copies, and hence higher base coverage than most non-organelle chromosomes, the duplicated reads are more likely to be organelle-associated than non-duplicated reads. The “get_organelle_from_reads.py” will group a certain number of duplicated reads (after option “-P”) into groups based on read overlap using the same substring hashing method mentioned above. Any groups, including those with only a single read, will have a hash table storing Words chopped from all reads of this group. Any two groups sharing at least a single Word in their hash table will be merged. After pre-grouping, a group resembles a set of connected pseudo-contigs (information stored at file “temp.indices.2”). During the following extension iterations, once a read is accepted as a target-associated read, all other reads (ids) in the same group will be marked as acceptable.

The “get_organelle_from_reads.py” begins the core extension iterations with constructing a hash table, by cutting the initial reads (“baits”) into Words and adding those initial Words to the hash table (AW). If a Word of a candidate read hits the AW, all Words of this read are also added to AW, and the index of this read will be marked as accepted (Accepted Index, AI). As mentioned above, all other read ids in the same group will be treated as accepted. During each iteration (round), the “get_organelle_from_reads.py” goes through all candidate reads one by one to check whether a read is acceptable. After a user-specified number of rounds or when no new read been accepted in a complete round, the “get_organelle_from_reads.py” will stop the extension process and output all accepted reads (stored at file “filtered_*.fq”). For low memory machines or testing purposes, accepted reads per round can be outputted separately after each round (followed with flag “--out-per-round”) along with AW emptied after each round.

Word size (followed with flag "-w"), like *k*-mer length in assembly, is crucial to the feasibility and efficiency of this process. The best Word size for extension is affected by read length, read quality, total number of reads, percentage of organelle genome reads, heterogeneity of organelle base coverage, and other factors. In the "auto" mode when there is no user-assigned Word size value, the "get_organelle_from_reads.py" will automatically estimate a proper Word size with a set of empirical-customized functions, based on the data characteristics.

Step 3. Conducting *de novo* assembly

The recruited target-associated reads will be then automatically assembled using SPAdes (Figure 1, green solid arrow 3). Both paired and unpaired reads will be used. The outputs of each *k*-mer of SPAdes include an assembly graph (FASTG format), which records the connections of contigs as a graph with some allelic polymorphism and assembly uncertainty. Other assemblers that are able to generate the assembly graph, such as Velvet, may be used for completing this step, but are not implemented in GetOrganelle yet. The intermediate results are stored in the subfolder "filtered_spades".

Step 4. Roughly filtering for target-like contigs

Because sequences are often shared among plastomes, mitogenomes, and nuclear genomes, the accepted reads from Step 2 sometimes unavoidably include non-target reads. As a consequence, the output assembly graph might also include non-target contigs. However, previously reported tools did not account for or adequately addressed this concern.

GetOrganelle searches for the target-like contigs from the original assembly graph file by jointly using the contig label table, contig connections and contig coverages (Figure 1, green solid arrow 4 and blue solid arrow 1). To create the contig label table, GetOrganelle takes the contigs in the assembly graph as the queries, conducts the BLAST search against a local label database (see next paragraph), generates the BLAST hit table, and converts the generated BLAST hit table into the contig label table, which records the gene identities and organelle types of those BLAST matches. By conservatively deleting non-target contigs, GetOrganelle outputs a simplified assembly graph file, along with a concomitant cognominal TAB-formatted file recording the contig label table (with the extension ".csv" to be in conformity with Bandage). This step is completed automatically by the two major scripts or can be independently executed using the script "slim_fastg.py".

For GetOrganelle, the default label database of a certain organelle is made from the coding regions of that organelle genome. We created six default label databases correspond to the six types of organelle genome in the seed databases. A contig that hit the target organelle database will be labeled with gene identity in the ".csv" file, and called target-hit-contig here. Any contig that is directly or indirectly connecting to that target-hit-contig, is called a target-associated-contig. Here, we define a group of interconnected contigs as a connected component of the assembly graph. GetOrganelle by default retains all connected components with target-hit-contig(s). Additionally, in the embplant_pt or embplant_mt mode, GetOrganelle by default retains both plastome and

mitogenome connected components for downstream clustering contigs by coverage. Generally, this roughly filtering step is designed to be conserved and avoiding removing true target contigs.

Step 5. Identifying target contigs and exporting all configurations

GetOrganelle then uses the simplified assembly graph file and the contig label table to: 5a) further accurately identify (narrow down to) target organelle contigs, 5b) estimate multiplicities (copy number) of contigs in an organelle-only graph, and 5c) export all possible distinctive path(s) [stored as FASTA file(s)] from the organelle assembly graph (stored as a cognominal GFA format file) (Figure 1, green solid arrow 5 and blue solid arrow 2). Each path represents a possible configuration of the target organelle genome. This step is fulfilled by the two major scripts or can be separately executed using the script "disentangle_organelle_assembly.py". In case of organelle genome with a large number of repeats, GetOrganelle sets up an option for limiting the calculation time of disentangling to avoid generating inexhaustible combinations. When the major scripts failed to export circular sequence(s) from the assembly graph for the reasons enumerated below in Step 5' or because of the time limit, they will execute a second run to export the contigs, which would be mainly the target-hit-contigs.

Three concepts need to be clarified:

1. An "organelle-sufficient graph" is an assembly graph with contigs completely covers one complete organelle genome.

2. An "organelle-only graph" is an assembly graph only with true contigs of one organelle genome.

3. An "organelle-equivalent graph" is both an organelle-sufficient graph and an organelle-only graph.

GetOrganelle requires three assumptions to disentangle the assembly and declare the result as a complete circular organelle:

Assumption 1: All configurations, if there are more than two, of the target organelle genome are compositionally identical. This assumption limits the multiplicities of contigs to be the same among different configurations. In other words, polymers are found in real plastid DNA molecules [52], whereas GetOrganelle can only export the monomer form; potential sub-genomic configurations are currently not implemented in the current version. If there are parallel contigs caused by nucleotide polymorphism, all subgraphs composed of any of those polymorphisms will be disentangled independently. Therefore, all configurations of each subgraph will be compositionally identical.

Assumption 2: The topology of each organelle genome will be represented as a single circular molecule. This assumption holds when the real organelle genome is a circular molecule or organized in polymers (most plastomes, and type I and type II mitogenomes) and the assembly graph is an organelle-sufficient graph. If this assumption is violated, GetOrganelle only exports the target contigs.

Assumption 3: The coverage values of contigs of the same organelle genome are generally proportional to their multiplicities (copy numbers). Therefore, coverage values of contigs with the same multiplicity of the same organelle genome generally are consistent.

5a) *Further identifying target organelle contigs*

Using the BLAST hit information alone to identify target organelle contigs is risky. Some contigs, including mitochondrial contigs that have short sequence of plastome origin or target-like shallow-depth contaminant contigs, would be labeled incorrectly as target-hit-contigs (false positive). On the other hand, some sequences might be true target contigs but are too short or divergent from sequences in the label database to be labeled as target-hit-contigs (false negative). Therefore, we used additional information to improve the identification of target contigs, such as the assembly graph characters (Assumption 1 and 2) and contig coverage values (Assumption 3). GetOrganelle uses an integrated strategy that iteratively uses all or part of following modules to approach this task until no more changes is going to be made to the assembly graph.

1) Using the BLAST hit information to roughly cluster contigs with target labels. Specifically, GetOrganelle first calculates a customized hit weight value (HW) for each BLAST hit record (gene identity label restored from the contig label table). For records representing the same gene in the local BLAST database, only the record with the best HW is kept as the only valid hitting record for that gene. The HW of a hit record is simply defined as the product of the hitting length of the query (HL) and query contig coverage (QC) (HW = HL * QC). Given our experience that the false positive hits generally correspond to shorter length and shallower depth contigs, HW can be a criterion for excluding the false positive hit records. Each gene in the BLAST database is thus aligned to no more than one contig in the assembly graph. Then, GetOrganelle calculates a customized contig weight value (CW) of a target organelle genome for each contig as $\mathrm{CW}_{\mathrm{target}} = \sum \mathrm{HW}_{\mathrm{target}}$. For example, the plastid CW for a contig is defined as the sum of the HWs of all plastid gene hit records of that contig, while the mitochondrial CW for the same contig is defined as the sum of the HWs of all mitochondrial gene hit records of the same contig. For a contig, if the target CW is much larger (default factor: 3 times) than the non-target organelle CW, this contig would be labeled as a target-anchor contig (very likely to be a true target contig), and vice versa. Using the HW and CW, GetOrganelle roughly eliminates most non-target contigs with false positive BLAST hits.

2) Adding more target labels to some target contigs that do not hit the label database according to assembly graph characters. Based on Assumption 2, any configuration of the target organelle genome is a single circular molecule. As a result, in an organelle-sufficient graph, both ends of any true target contig should be connected to at least one true target contig. If the tail end of a true target contig, Contig A (marked as A_{tail}) has only one edge that connects A_{tail} and the head end of another unknown contig, Contig B (B_{head}), then Contig B should be a true target contig. However, in a real assembly graph with missing contigs (incomplete organelle genome), Contig B may be missing and the unknown contig connected to A_{tail} may be a non-target contig. In consideration of complicated situations like this, only when Contig B is between two target-anchor contigs (Contig A and Contig C) with the sequence (A_{tail}-B_{head}-B_{tail}-C_{head}), and when A_{tail} only connects to B_{head} and C_{head} only connects to B_{tail}, GetOrganelle labels Contig B as a target-anchor contig with CW=0.

3) Using coverage values of contigs to remove contigs with coverage value that significantly deviates from the target-anchor contigs. Based on Assumption 3, GetOrganelle uses the Gaussian mixture distribution to

approximate the coverage values of all contigs in the simplified assembly graph, which is a mixture of different organelle contigs and nuclear contigs. In most cases of empirical plant genome skimming data, the plastome has significantly higher coverage than the mitogenome, the coverage of which in turn is higher than the nuclear genome except for highly repeated regions. Therefore, in a plant WGS dataset, the coverage values of plastid, mitochondrial and nuclear contigs are expected to be classified into different Gaussian components of the Gaussian mixture distribution. GetOrganelle could thus delete the contigs with coverage value far from the target coverage distribution. Specifically, GetOrganelle applies an EM (Expectation-Maximization) algorithm with the semi-supervised learning and the weighted Gaussian mixture model to cluster the coverage values of all candidate contigs. Here, the semi-supervised learning means that the coverage values of the target-anchor contigs (the labeled data) are not updated during EM iterations. The coverage value of a contig in the Gaussian mixture model is weighted by the length of the contig.

4) Removing contigs isolated from the main target connected component that includes the target-anchor contigs. Based on Assumption 2, true target contigs in an organelle-sufficient assembly graph should occur in one connected component. Thus, for a real organelle-sufficient assembly graph, GetOrganelle retains the connected component with the most target-anchor contigs and deletes other such connected components. Specifically, GetOrganelle calculates a customized target weight value (TW) for each connected component of the assembly graph. The TW of a connected component is defined as the sum of the target CWs of all contigs in that connected component. Assuming organelle-sufficiency, GetOrganelle sorts connected components by their TWs, finds the connected component with the significantly largest TW (100 times larger than the second largest TW by default) and removes the contigs of other connected components from the assembly graph. If GetOrganelle fails to find such a connected component, disentangling the assembly graph as a circular organelle genome fails and GetOrganelle reverts to "linear mode". In a "linear mode", when GetOrganelle tries to disentangle the assembly graph as contigs or scaffolds, several connected components with large TW are retained, and only the connected components with TWs 10000 times (by default) less than the largest TW will be removed.

5) Removing tip contigs. A tip contig is a contig with one or both ends that do not connect to any other contigs in the assembly graph nor to itself as circular. Based on Assumption 2, an organelle-equivalent graph will not contain any tip contig, because any partial sequence of a circular DNA molecule will have both upstream and downstream sequences. GetOrganelle will check whether a tip contig is a target-anchor contig before removing it. If the tip contig is a target-anchor contig, it is likely that Assumption 2 is violated, and in most cases, the assembly graph is not an organelle-sufficient graph but rather "a broken organelle graph".

5b) *Estimating the multiplicities of contigs in an organelle-only graph*

There are sources of information resources for estimating multiplicities for contigs in GetOrganelle. One is contig coverage value (*k*-mer coverage in practice but referred to as coverage in this paragraph). According to Assumption 3, we could roughly estimate the multiplicity of each contig based on its coverage. Given that any contig in an organelle-only graph would have at least one copy, GetOrganelle first assumes all contigs have the

multiplicity of one and estimates a primary value of the average coverage of the target genome. Then, GetOrganelle optimizes the multiplicities of all contigs by dividing the coverage value of a contig by the average coverage and rounding the result to the nearest integer. GetOrganelle iteratively optimizes the average coverage of the target genome and the multiplicities of the contigs, with the constraint that the genome average coverage should not below the minimum coverage of all contigs according to Assumption 1. Here, the stabilized multiplicities estimated on the base of the coverage values of contigs are called observed multiplicities (O_{Ci}, with $_{Ci}$ denoting the contig name) (Figure 2).

Another type of information comes from the graph characteristics, which offers a set of hard constraints for the multiplicities of contigs (M_{Ci}, with $_{Ci}$ denoting the contig name). Using the Python library Sympy, GetOrganelle creates a set of linear equations to characterize the multiplicity relationship among connected contigs. In detail, there are mainly four constraints to build this set of equations. First, the multiplicity of a self-loop contig has no constraints. Second, if Contig A is not a tip contig nor a self-loop contig, M_{CA} is equal to sum of the multiplicities of the contigs connected to A_{head}, and equal to sum of the multiplicities of the contigs connected to A_{tail}. Third, the multiplicity of a tip contig is arbitrarily set to 1 to avoid over-estimation, although this risks failure in solving the equations. Last, in considering of symmetry of large inverted repeats (such as IRs in plastome), the multiplicity of a sequential repeat contig is constrained to be an integer multiple of the multiplicity of one of its nearby contigs, e.g. the multiplicity of a sequential repeat inside the IR region of a plastome assembly graph must be an even number and no smaller than 4. This set of equations would be then simplified using Sympy.solve. The multiplicity values of all contigs would be represented as linear expression of several free variables.

By minimizing the difference of the multiplicities based on above two types of information using least square, meaning minimizing $\sum_{i=0}^{n}(M_{Ci} - O_{Ci})^2$, GetOrganelle achieved the values of those freedom variables, therefore all the M_{Ci} (Figure 2).

5c) *Exporting all possible distinctive path(s)*

GetOrganelle then exhaustively searches for all possible paths from this organelle-only graph with contig multiplicities. Each configuration combination would be saved as an independent FASTA file, with the same sequence-name style to manual completion using Bandage [30] (see below). A circular sequence would also be marked "(circular)" in the sequence name. For plastomes with repeats inside the large IRs, there would be 6 paths, meaning 6 potential isomers (see Figure 2; another similar but more complicated example is SRR5602601 with 12 paths). However, in considering symmetry, only those isomers with identical large IRs (path1 & 2 in Figure 2) are biologically possible paths. In these cases, GetOrganelle would mark these results with identical IRs as the first repeat pattern in the file name.

Step 5'. Manual Completion

If GetOrganelle fails to export a complete circular organelle genome, because of insufficient target assembly

graph, too short a disentangling run-time, too many possible configuration sequences, or misidentification of target contigs, manual completion is needed to clean "noisy" and non-target contig/scaffold connections (Figure 1, grey solid arrow 4 and 5). The simplified assembly graph can be visualized using Bandage [30]. Meanwhile, the concomitant annotation "csv" file can be imported into Bandage and added to the graph as labels, which helps to manually identify target-like contigs/scaffolds. The semi-manually-cleaned assembly graph can be then finalized using another main script "get_organelle_from_assembly.py" (Figure 1) or be manually exported from the cleaned assembly graph using Bandage.

Assessing the performance characteristics of GetOrganelle

To investigate the performance characteristics of GetOrganelle, we varied relevant parameters of the program: word size values (as the form of WSR, i.e. 0.3, 0.35, 0.4, 0.45, 0.5, 0.55, 0.6, 0.65, 0.7, 0.75, 0.8, 0.85, 0.9); the number of rounds (unlimited vs. minimum), pre-grouping either activated (-P 2E5, i.e. using the top 2E5 duplicated reads to conduct the pre-grouping) or disabled (-P 0). The minimum number of rounds mode is defined as fewest rounds of extension iterations required to obtain a complete plastome or stabilizing an incomplete plastome.

We used these various settings to assemble the plastome of the angiosperm species *Haberlea rhodopensis* Friv. (Gesneriaceae) from a reduced published dataset (500Mb, SRA: SRR4428742), with the complete plastome of a gymnosperm species, *Gnetum parvifolium* (Warb.) W.C.Cheng (GBK: NC_011942.1) as the seed. We additionally use only the *rbcL* gene sequence of *Gnetum parvifolium* (GBK: NC_011942.1) as the seed and the WSR of 0.75 to assemble the same dataset.

To assess the characteristics of recruited reads per round, the option "--out-per-round" was specified for the script "get_organelle_from_reads.py". The script "round_statistics.py" was used to assess the increasing cover percent of the organelle genome using a reads mapping approach. Using Bowtie2, this script maps reads of each round to the final assembled plastomes and calculates the percentage of bases in the plastome that are covered by mapped reads over a certain coverage threshold (the defaults were 0 and 10). The minimum number of rounds occurs when the percentage of covered bases reaches 100% or stays unchanged and when the final assembly is stabilized. Besides, if Python library Matplotlib is available, the script "round_statistics.py" could generate the base coverage across the organelle genome plot for each extension round to visualize the extension process.

***De novo* assembly of the plastomes of 50 plant datasets using GetOrganelle and NOVOPlasty**

To evaluate the working efficiency and assembly success, we selected 50 datasets of vascular plants with raw reads from the GenBank Sequence Reads Archive (SRA) (Additional file 2: Table S1). The 50 vascular plants represented 42 species of angiosperms (from eight major clades, 21 orders and 29 families), four species of gymnosperms, three species of ferns, and one species of lycophytes. Notably, the raw reads of these 50 samples are associated with published plastomes [53-56], allowing comparison with newly reassembled plastome using GetOrganelle. Besides, since 2018, NOVOPlasty has received more than 400 citations for assembly chloroplast

genome in Google Scholar (accessed 31 Dec 2019) and became one of the most widely used tools for plastome assembly. We thus reassembled 50 samples using NOVOPlasty for comparisons.

The data resources are paired-end reads. The read length varied from 100 bp to 300 bp (Additional file 2: Table S1). In all tests, if the tested data included fewer than 10,000,000 reads for each end, we used all the reads; if the data included more than 10,000,000 reads of each end, we only select the first 10,000,000 reads for each end. We set up four testing groups, i.e., three groups with different word size values (w = 0.6, 0.7, 0.8) (i.e. GetOrganelle-W0.6, GetOrganelle-W0.7, GetOrganelle-W0.8), and an auto-estimated word size group (i.e. GetOrganelle-auto). The extension rounds of all tests were set to 10. All other options including the seed were set to default. Because incomplete assemblies are unsuitable for comparing mapping qualities in the next part, we additionally added extra runs for eight samples, in which GetOrganelle-auto could not achieve complete plastomes, with customized options (GetOrganelle-customized) for mapping quality comparison. A detailed record of commands, as well as the final results and log files recording the memory usage and time cost of all the tests are available at https://github.com/Kinggerm/GetOrganelleComparison.

Plastomes from the same 50 datasets were also reassembled by NOVOPlasty using four *k*-mer values, i.e., 23, 31, 39 and 47. The config file of NOVOPlasty was downloaded from the NOVOPlasty GitHub repository (https://github.com/ndierckx/NOVOPlasty/blob/master/config.txt), with "Type" as "chloro", "Genome Range" as 15000-180000, "Save assembled reads" as "yes", "Seed Input" as the same seed as running GetOrganelle, "Read Length" as the mean read length of each sample (seed Additional file 2), with all other parameters unchanged.

Reads mapping to evaluate plastome assemblies

The script "evaluate_assembly_using_mapping.py" was used to evaluate circular/non-circular assemblies (Figure 1, grey solid arrow 8). It uses Bowtie2 to map reads to circular/non-circular assemblies, parses the SAM file, counts the number of mapped paired and unpaired reads, counts matched bases for each site (M), mismatched bases for each site (X), insertions between any two sites (I) and deletions for each site (D), and calculates the average and deviation of matched depth, average and deviation of mismatched depth, average and deviation of insertions between any two reference sites, and average and deviation of deletions per reference site for each contig and the whole assembly. If $\Sigma M > 0$, a customized error rate would be also calculated as $(\Sigma X + \Sigma I + \Sigma D)/\Sigma M$ with deviation $var(X + I + D)/\Sigma M$. If "--draw" is chosen, the script "evaluate_assembly_using_mapping.py" generates the plot of M/X/I/D at each site/site-interval across the whole assembly using Python library Matplotlib. For reproducibility when using the same random seed, a circular assembled sequence was relinearized to make sure that biologically the same circular plastome would have identical start and end since identical linear sequence. By default Bowtie2 does not support mapping reads to a circular sequence, the script "evaluate_assembly_using_mapping.py" gets around this problem by adding an

extra fragment of the head of the original sequence to the tail, and counting the mapping statistics (M/X/I/D) of the sites in the extra fragment back to the statistics of head part of the original sequence.

The script "evaluate_assembly_using_mapping.py" was used to assess all the assembled plastomes. The abnormal characters, such as "*" and "-" in the FASTA-format assemblies of NOVOPlasty were replaced with "N". NOVOPlasty would also produce multiple completely different sequences with the same sequence name in the assemblies, which would be also modified with different names before assessment. For NOVOPlasty, the evaluation statistics of each sample were based on the best result among those using different *k*-mer values. Here the best result is determined in turn by being true circularized, the largest number of mapped paired reads, the largest number of mapped unpaired reads, the largest matched depth, the smallest error rate, the smallest deviation of matched depth, and the smallest deviation of error rate. For GetOrganelle, except for 8 samples that were generated using the customized parameters, other evaluation statistics were based on the assemblies from the "GetOrganelle-auto" runs.

For each sample, when comparing the evaluation statistics of different assemblies, the "best" mapped reads (the hat mark in Table S3) is defined as the largest number of mapped paired reads or equal-largest number of mapped paired reads with largest number of mapped unpaired reads; the "best" mapped depth is defined as the largest matched depth or equal-largest matched depth with smallest deviation of matched depth; the "best" error rate is defined as the smallest error rate or equal-smallest error rate with smallest deviation of error rate.

De novo assembly of the mitogenomes using GetOrganelle

In total, 56 animal (the test samples of MitoZ [14], see Additional file 2: Table S3) and 50 fungal samples (Additional file 2: Table S4) were used to test the capability of GetOrganelle to assemble mitogenomes. The mitogenomes of plants have a relative slow nucleotide substitution rate [9, 57]; therefore it is feasible for GetOrganelle to achieve sufficient reads to construct the mitogenome-sufficient assembly graph even with a remotely related seed, provided that there is enough coverage. However, there are two main challenges for short-read sequencing data to confirm the mitogenome architecture. Firstly, lots of repeats in the plant mitogenome cause the awkward tangles in the assembly graph [44, 58, 59]. Secondly, most of plant mitogenomes are not one single circular structure; rather they often consist of one large circular molecule and small circular plasmid-like molecules (type III) , or homogenous linear molecules (type V) [60]. There are also frequent horizontal transfers from plastome to mitogenome. In this case, when the coverage of the plastome is much higher than that of mitogenome, the multiplicity of shared contigs would be hard to estimate. When the coverage of the plastome is similar to that of the mitogenome, the parallel contigs in between the shared contigs would be difficult to distinguish. Thus, we did not include plant mitogenome testing due to the general infeasibility of assembling complete circularized plant mitogenome from WGS data.

The data resources are paired-end reads. The read length varied from 92 bp to 301 bp (see Additional file 2: Table S3, Table S4). In animal tests, if the tested data included fewer than 75,000,000 reads for each end, we used all the reads; if the data was more than 75,000,000 reads of each end, we only used the first 75,000,000

reads for each end. The same maximum number of reads for fungi was 15,000,000. All tests were performed with default settings with the *k*-mer values set to 21,43,65,87,127. Commands, results and log files of all the tests are available at https://github.com/Kinggerm/GetOrganelleComparison.

Computer resources for testing

All assemblies were executed on an Intel Xeon CPU machine containing 144 cores of 2.40 GHz, a total of 3TB of RAM, and set up with Linux (Linux version 3.10.0-514.el7.x86 (Red Hat 4.8.5-11)). The testing environment also includes Python v3.6.5, Perl v5.16.3, GetOrganelle v1.6.2, Bowtie2 v2.3.5.1, SPAdes v3.13.0, BLAST 2.2.30+, Numpy v1.13.3, Scipy v0.19.1, Sympy v1.1.1, Matplotlib v3.0.2, Psutil v5.4.7, and NOVOPlasty 2.7.2 [23]. GetOrganelle is capable of multi-processing in mapping and assembly process, which was disabled in this test for comparable with NOVOPlasty. All tests can be reproduced by accessing https://github.org/Kinggerm/GetOrganelleComparison.

Additional files

Additional file 1.docx: Figure S1 - S2

Additional file 2.xlsx: Table S1- S5

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

No sequencing data were newly generated. See Additional file 1: Table S1, S3, & S4 for publicly archived datasets analysed in this study.

Assembly and other results are available via https://github.org/Kinggerm/GetOrganelleComparison.

Competing interests

The authors declare that they have no competing interests.

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Author contributions

J-JJ, W-BY, T-SY, D-ZL conceived the research; J-JJ wrote the toolkit code; J-JJ, W-BY analyzed the data; J-JJ, W-BY, T-SY wrote the draft manuscript. All authors revised and approved the manuscript.

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Legend of figures

Figure 1. The workflow of GetOrganelle toolkit. The thumbnails inside data objects show an example of plastome assembly. The solid arrows denote the data processing flows and associated directions, with their width proportional to general computational burden. All green solid arrows together describe a complete run from reads to organelle genome(s), which is encapsulated in a single command using the script "get_organelle_from_reads.py".

Figure 2. An example of exporting all configurations from a target-complete assembly graph in GetOrganelle (step 5b & 5c of the script "get_organelle_from_reads.py").

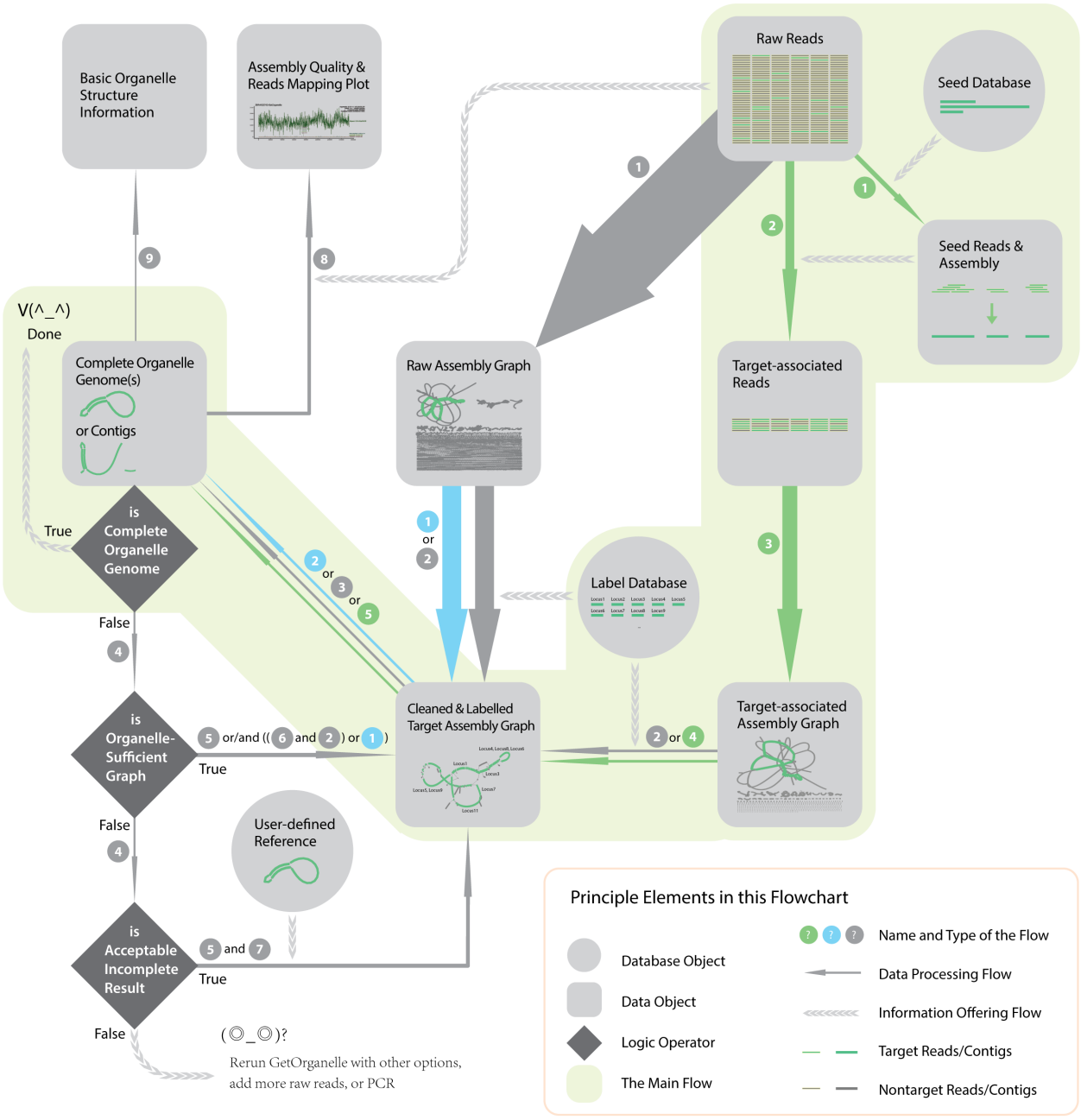
Figure 3. Selected assembly graphs of plastome show quadripartite structures with typical IRs, contracted IRs and large DRs, and no large repeats.

Figure 4. Comparisons of four sets of runs using GetOrganelle and four sets of runs using NOVOPlasty when assembling 50 public plant datasets. The labels along the bottom denote the program and key settings for each set of 50 runs. The dots in the upper boxplot show the time cost in seconds for finishing each run. The dots in the middle boxplot show the maximum memory usage in gigabytes for finishing each run. The bottom histogram shows the number of circularized genomes for each set.

Figure 5. Evaluating assembly qualities of GetOrganelle plastomes, NOVOPlasty plastomes, and published plastomes using reads mapping.

Figure 6. Assessing the plastome assembly performance characteristics of GetOrganelle using online reads of an angiosperm species, *Haberlea rhodopensis* (SRA: SRR4428742) as the dataset, using the complete plastome of a gymnosperm species, *Gnetum parvifolium* (GBK: NC_011942.1) as the seed. The assessment was conducted across a range of Word sizes (in the form of WSR, after the flag "-w" along the x axis). The green marks denote using the pre-grouping value 200,000 (after the flag "-P"), while the grey marks denote that the pre-grouping was disabled. The cross marks denote using the minimum number of rounds of extension iterations for achieving a complete plastome or stabilizing the incomplete plastome result, while the solid circle marks denote using unlimited number of rounds ("-R 1000" in practise). From top to bottom, for assembling raw reads into the same complete plastome (or the same incomplete result in all runs of "-w 0.90"), the five subgraphs present the total computational time cost in seconds, the maximum memory cost in gigabytes, the actual number of rounds GetOrganelle took, the number of target plastid reads GetOrganelle recruited, and the number of non-target reads GetOrganelle recruited, respectively.

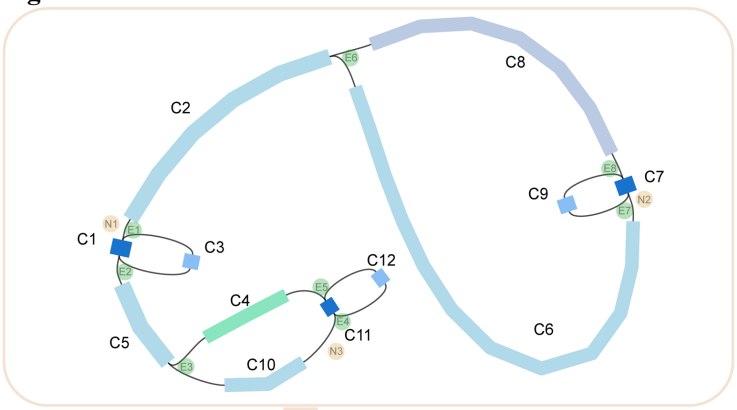
Figure 1



Brief Descriptions of the Processing Flow

- x The x-th step of get_organelle_from_reads.py in a single run
- 1 Mapping reads to seed and assembling seed reads for parameter estimation
- 2 Recruiting more target-associated reads through extending iterations
- 3 Conducting de novo assembly
- 4 Roughly filtering for target-like contigs
- 5 Identifying target contigs and exporting all configurations
- x The x-th step of get_organelle_from_assembly.py in a single run
- 1 Filtering for target-like contigs
- 2 Exporting all configurations of the target organelle genome
- 1 Conducting de novo assembly using SPAdes
- 2 Roughly filtering target-like contigs using Utilities/slim_fastg.py
- 3 Identifying target contigs and exporting all configurations using Utilities/disentangle_organelle_assembly.py
- 4 Visualizing, labeling and checking the assembly graph using Bandage
- 5 Manually cleaning assembly graph using Bandage
- 6 Converting assembly graph format using gfa_to_fastg.py
- 7 Adding gap contigs according to reference using Utilities/join_spades_fastg_by_blast.py
- 8 Conducting evaluate_assembly_using_mapping.py
- 9 Conducting plastome_arch_info.py

Figure 2.



Contig name	*k*-mer coverage (*k*C)	Contig length
C1	329.0×	128 bp
C2	162.7×	16,795 bp
C3	162.9×	170 bp
C4	81.7×	7,758 bp
C5	191.5×	6,294 bp
C6	88.0×	41,103 bp
C7	276.0×	448 bp
C8	90.4×	21,210 bp
C9	201.7×	374 bp
C10	92.4×	6,319 bp
C11	101.0×	370 bp
C12	85.5×	390 bp

Formulating equations for multiplicities (M_{Ci}) based on graph character

Estimating observed multiplicities (O_{Ci}) based on *k*C

$M_{C1} - M_{C2} - M_{C3} = 0$ E1
$M_{C1} - M_{C3} - M_{C5} = 0$ E2
$M_{C5} - M_{C10} - M_{C4} = 0$ E3
$M_{C11} - M_{C4} - M_{C12} = 0$ E4
$M_{C11} - M_{C10} - M_{C12} = 0$ E5
$M_{C2} - M_{C6} - M_{C8} = 0$ E6
$M_{C7} - M_{C6} - M_{C9} = 0$ E7
$M_{C7} - M_{C8} - M_{C9} = 0$ E8
$R_1 \cdot M_{C2} - M_{C1} = 0$ N1
$R_7 \cdot M_{C6} - M_{C7} = 0$ N2
$R_{11} \cdot M_{C10} - M_{C11} = 0$ N3

M_{Ci} is a non-negative integer, for any *i* in 1~12
R_j* is a non-negative integer and $R_j > 1$, for any *j* in 1,7,11

Average *k*-mer coverage: 87.5×

Contig name	O_{Ci} based on *k*C
C1	4
C2	2
C3	2
C4	1
C5	2
C6	1
C7	3
C8	1
C9	2
C10	1
C11	1
C12	1

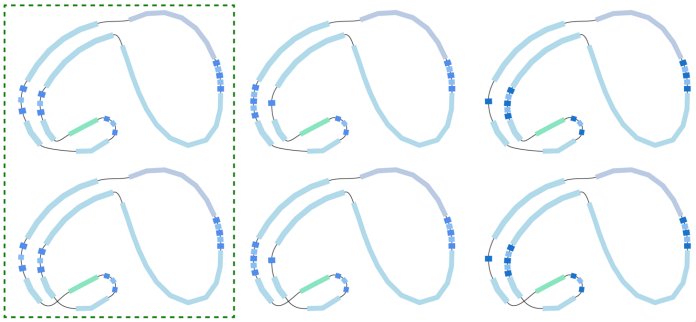
Simplifying equations and expressing M_{Ci} with four free integer variables a, x, y, z with a > 0 and x, y, z > 1

Optimizing free variables M_{C9}, R_1, R_7, R_{11} by minimizing the difference between M_{Ci} and O_{Ci}, $\sum_{i=1}^{12}(M_{Ci} - O_{Ci})^2$

M_{C1} =	$2 \cdot a \cdot x / (y - 1)$
$M_{C2} = M_{C5}$ =	$2 \cdot a / (y - 1)$
M_{C3} =	$2 \cdot a \cdot (x - 1) / (y - 1)$
$M_{C4} = M_{C6} = M_{C8} = M_{C10}$ =	$a / (y - 1)$
M_{C7} =	$a \cdot y / (y - 1)$
M_{C9} =	a
M_{C11} =	$a \cdot z / (y - 1)$
M_{C12} =	$a \cdot (z - 1) / (y - 1)$
R_1 =	x
R_7 =	y
R_{11} =	z

a = 2, x = 2, y = 3, z = 2

Contig name	O_{Ci} based on kC
C1	4
C2	2
C3	2
C4	1
C5	2
C6	1
C7	3
C8	1
C9	2
C10	1
C11	2**
C12	1



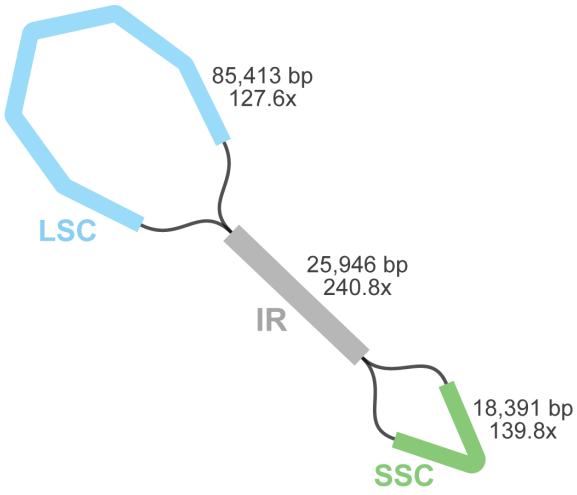
Exporting all possible configurations (potential isomers)

* Repeat Calues (R_j) are nuisance parameters for constraining the multiplicities of sequential repeats to be integer times of the multiplicities of their nearby contigs.
** The final multiplicity of MC11 is different from OC11.
⬚ Configuration(s) with symmetrical IRs in a plastome.

Figure 3.

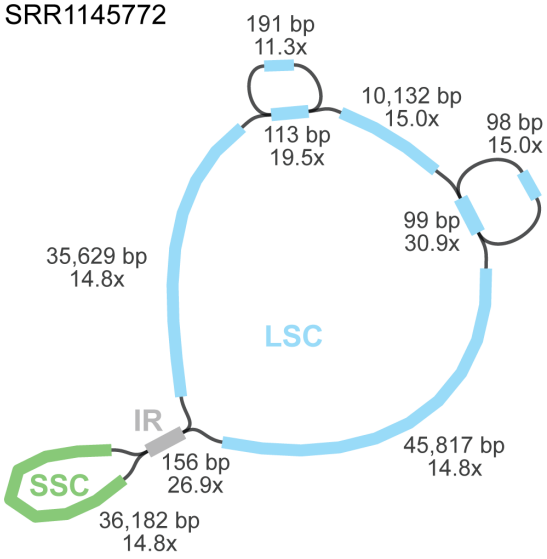
A) Typical quadripartite structure

SRR5602575



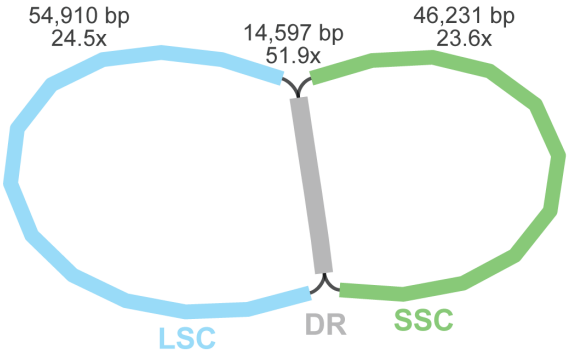
B) Quadripartite structure with contracted IRs

SRR1145772



C) Quadripartite structure with large DRs

SRR2037123



D) No large repeats

ERR964904

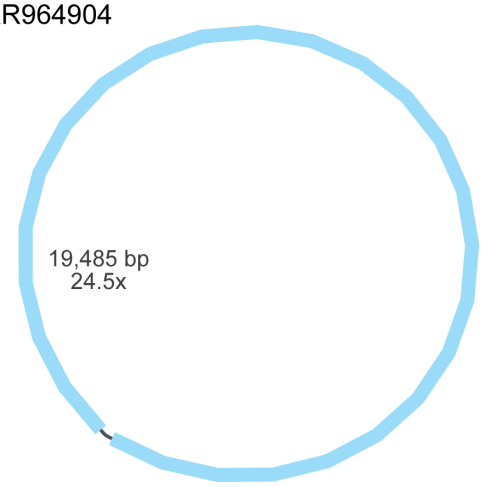


Figure 4

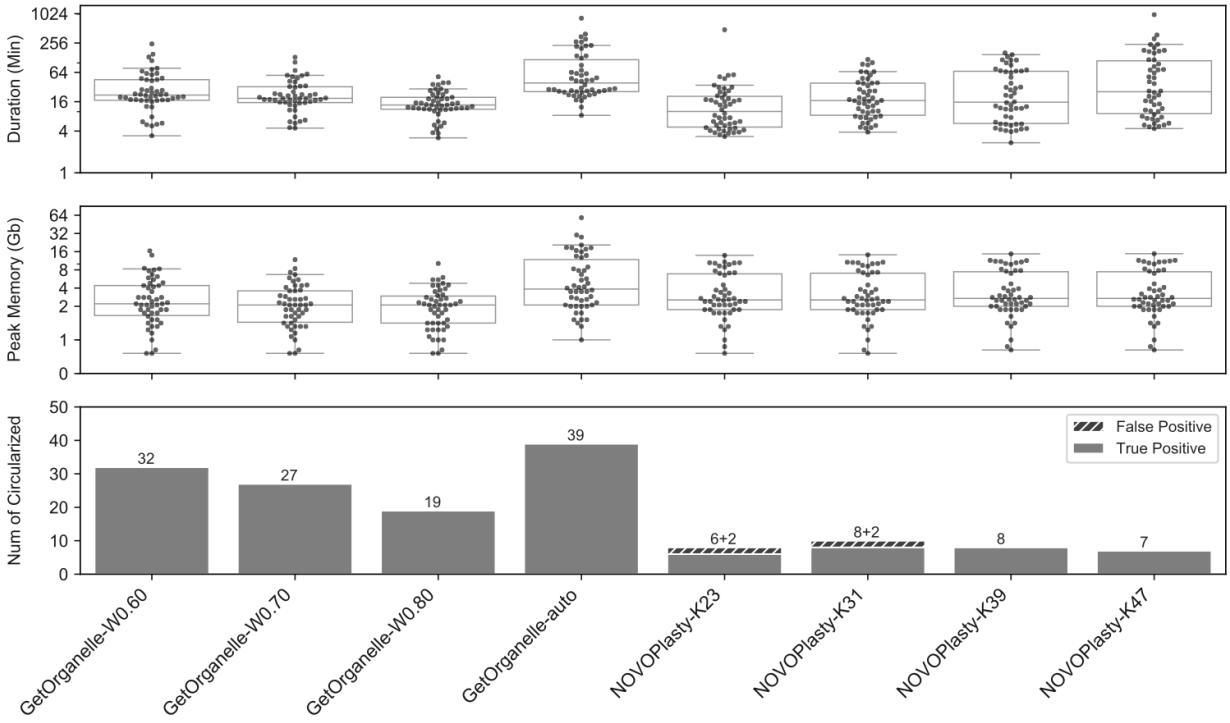

Duration (Min)
1024
256
64
16
4
1
Peak Memory (Gb)
64
32
16
8
4
2
1
0
Num of Circularized
50
40
30
20
10
0
False Positive
True Positive
32
27
19
39
6+2
8+2
8
7
GetOrganelle-W0.60
GetOrganelle-W0.70
GetOrganelle-W0.80
GetOrganelle-auto
NOVOPlasty-K23
NOVOPlasty-K31
NOVOPlasty-K39
NOVOPlasty-K47

Figure 5

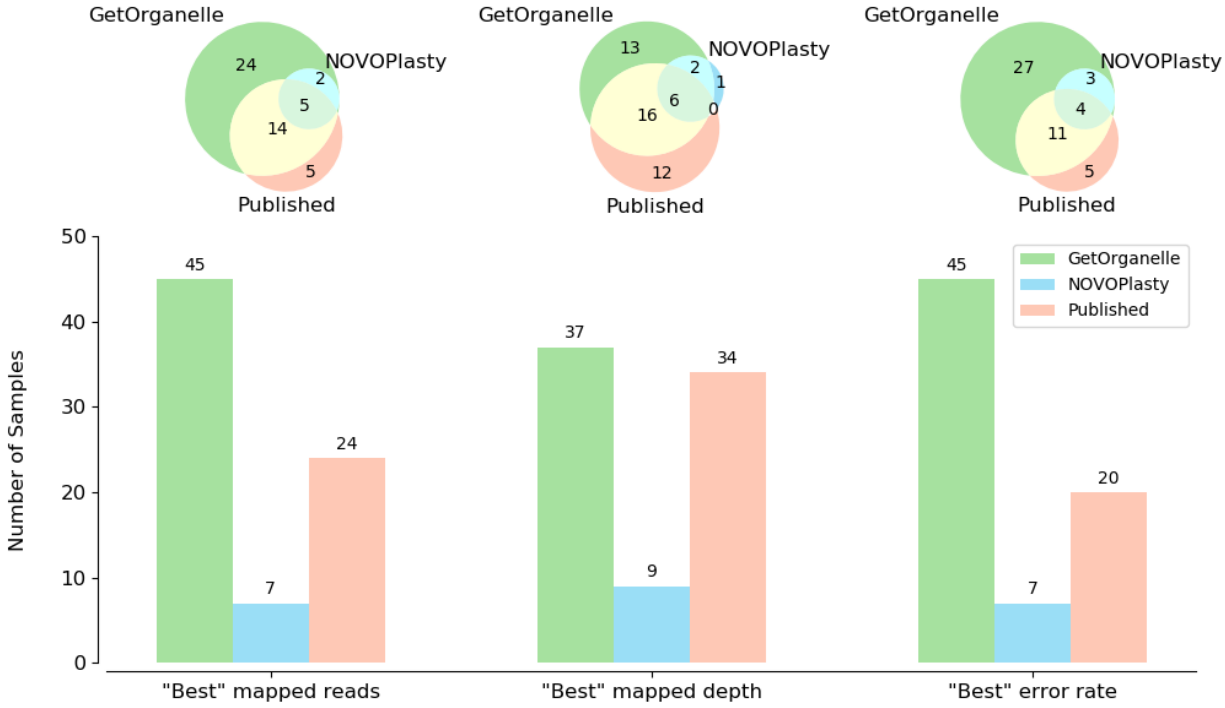
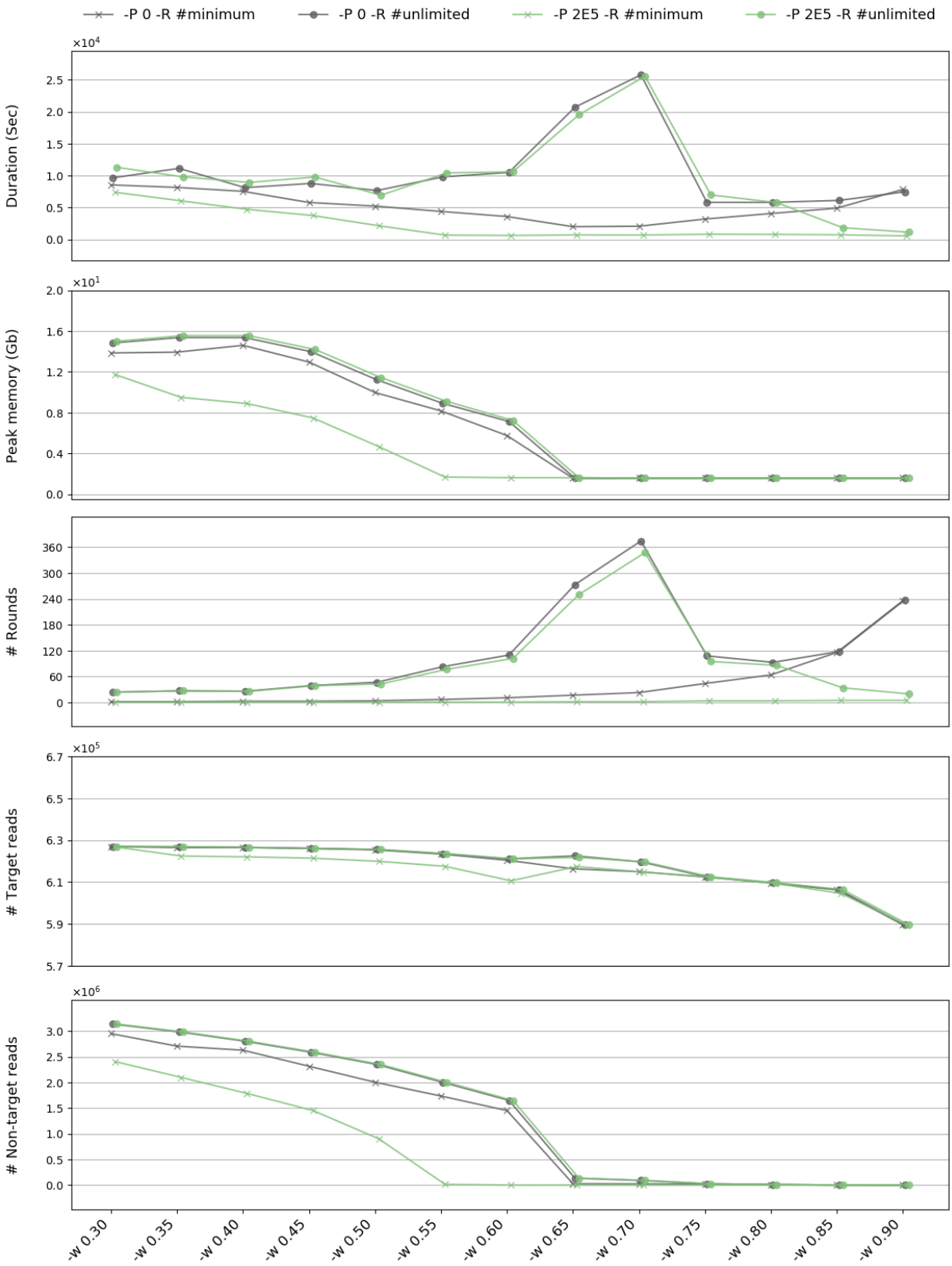

GetOrganelle
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16
12
GetOrganelle
NOVOPlasty
Published
27
3
4
11
5
Number of Samples
50
40
30
20
10
0
45
7
24
37
9
34
45
7
20
"Best" mapped reads
"Best" mapped depth
"Best" error rate
GetOrganelle
NOVOPlasty
Published

Figure 6



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