



Best practices in insect genome sequencing: what works and what doesn't

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The last decade of decreasing DNA sequencing costs and proliferating sequencing services in core labs and companies has brought the de-novo genome sequencing and assembly of insect species within reach for many entomologists. However, sequence production alone is not enough to generate a high quality reference genome, and in many cases, poor planning can lead to extremely fragmented genome assemblies preventing high quality gene annotation and other desired analyses. Insect genomes can be problematic to assemble, due to combinations of high polymorphism, inability to breed for genome homozygosity, and small physical sizes limiting the quantity of DNA able to be isolated from a single individual. Recent advances in sequencing technology and assembly strategies are enabling a revolution for insect genome reference sequencing and assembly. Here we review historical and new genome sequencing and assembly strategies, with a particular focus on their application to arthropod genomes. We highlight both the need to design sequencing strategies for the requirements of the assembly software, and new long-read technologies that are enabling a return to traditional assembly approaches. Finally, we compare and contrast very cost effective short read draft genome strategies with the long read approaches that although entailing additional cost, bring a higher likelihood of success and the possibility of archival assembly qualities approaching that of finished genomes.

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Sanger beginnings: the first insect genome

The sequencing of the first arthropod genome — *Drosophila melanogaster* [1] — was planned to generate the ideal dataset for whole genome assembly [2•] and the principals employed then are still valid today. An isogenic strain

avoided DNA polymorphism assembly issues; milligrams of high quality DNA were isolated from embryonic nuclei avoiding gut and mitochondrial contamination; polytene, genetic and BAC based maps provided long range information for assembly validation; high genome coverage sequence information of different scales (2 kb and 10 kb inserts, and BAC end sequences) was generated enabling assembly of contigs, and determination of their order and orientation to produce scaffolds. The Celera Assembler [2•] was designed with exactly this dataset in mind, and has been improved over the years continuing to be a high quality assembly tool today.

Variations on this approach have been applied to many other species, but in many cases the required inputs — especially isogenic DNA and high long read sequence coverage — could not be provided. *Drosophila simulans* [3] provided an early warning of polymorphism problems. The initial sequencing plan attempted to collect polymorphism data in addition to a draft reference, generating 1× sequence coverage from multiple strains of *D. simulans* — a seductive goal. Unfortunately the dataset could not be assembled to high quality, and additional sequence had to be generated from a single inbred strain to rescue the assembly. This same dynamic has played out through successive sequence technologies and insect species, providing a cautionary tale for the designers of *de novo* reference genome-sequencing projects.

Shorter and shorter (but cheaper and cheaper) sequence reads

New technologies have given us cheaper, but shorter reads, enabling genome sequencing of many more species. *De novo* sequencing costs for genome assembly fell by a factor of 10 with the introduction of 454 sequencing [4] and another factor of 10 with Illumina short read assembly enabling sequence coverage decisions to be based on assembly strategy rather than cost. The downside has been the increased assembly difficulty leading to lower contig N50 lengths (more assembly false negatives) as short reads cannot straddle as many repeats or polymorphic regions. 454 assembly tools include Newbler ([5] but see [6]) and the CABOG variant of the Celera assembler [7] and have proven adept at assembling reasonable coverage (20× fragment, 30× clone coverage in 3 kb and 8 kb insert paired end) of inbred insects, but do not address sequence polymorphism. Results can be impressive for inbred *Drosophila* ([8] Table S1 shows N50's of 100–400 kb except for *D. rhopaloea* which could not be inbred resulting in a contig N50 of 19 kb). More

typical results using outbred species include the centipede ([9] 24.7 kb contig N50) and the somewhat inbred *Heliconius* butterfly ([10] 51 kb contig N50) that required manual partitioning of haplotypes and re-assembly to improve genome contiguity.

Illumina 100 bp reads required higher coverage (as a high proportion of read information is used for overlap determination instead of contig extension) and new de Bruijn kmer graph based assembly tools to efficiently deal with the large numbers of sequence reads [11–14]. Note that storing kmer graph structures in memory for assembly requires large amounts of RAM — often 1 Terabyte RAM, 32 core servers are used. Table 1 shows a typical ALLPATHS-LG [12] sequencing strategy of ~150× genome coverage, 100 bp paired end (pe) Illumina reads. Short read assembly of arthropod genomes works well (20–50 kb contig N50s, 1 Mb Scaffold N50) for inbred material (or haploid male hymenoptera), generally requiring ~20 ug DNA (1 ug of DNA for the 180 bp and 500 bp libraries, 5 ug for the 3 kb insert library, and 10 ug for the 8 kb). However, this and other assemblers are not tuned for polymorphic material, and even mediocre quality assembly for publication (say >10 kb contig N50 enabling annotation of gene models without excessive fragmentation) is not guaranteed. Additionally, despite routine success assembling 3 Gb mammalian genomes, it is extremely difficult to assemble polymorphic genomes larger than 2 Gb.

The problem with insects (and many other species)

The major goal of high quality genome references is high quality gene model annotation. As average gene loci range in size from 12 kb in *Drosophila* to 25 kb and more in larger insect and mammalian genomes, contig N50s of at least 10 kb, and scaffold N50s >300 kb are the minimum for high quality gene annotation.

Unfortunately, insects and other invertebrates have a particularly bad list of attributes for genome assembly that compromise contig N50 sizes:

1. Often they cannot be reared in the lab — which precludes any breeding for genome homozygosity — and instead must be collected on field trips necessitating the use of some material for species identification. Even if research colonies are available, annual and longer lifecycles can make inbreeding unrealistic.
2. Insects are often physically small, such that very little DNA (nanograms) can be obtained from a single individual, necessitating pooled polymorphic individuals to make libraries. In cases with intermediate sized individuals, we prioritize a single individual for the majority of sequence, and pooled individuals for larger

insert libraries, where significant material is lost in agarose gel size selection.

3. Due to the large species diversity within the arthropods, there are generally no high quality genome assemblies of phylogenetically close species to aid in assembly (with the possible exception of the Lepidoptera [15])
4. DNA preps often have to be optimized for a new insect species, as entomologists are not trained in molecular methods and standard protocols have not been determined. From our experience, Qiagen spin columns do not produce DNA of appropriate quality, but Qiagen midi sized drip column kits often give good results.
5. Although holometabola often have small (~500 Mb) genomes, outside the holometabola, arthropods can have large genomes (1.5 Gb spiders, 3 Gb cockroaches, 5 Gb mantis, and bristletails, 7 Gb grasshoppers [16]), thus costs are variable (compared to the relative stability of the 3 Gb mammals) and larger than the 175Mb *Drosophila* experience would indicate.

New technologies are revolutionizing genome assembly

The above describes the reality that high quality insect genome assembly is not guaranteed.

Optimization of assembly parameters by empirical testing is critical, and can perhaps improve a current genome assembly 2× or more for a specific dataset and software combination, but the limitations of current data and techniques remain. New sequencing technologies and assembly strategies are overcoming the traditional assembly problems of data polymorphism and repetitiveness with the goal of creating near ‘finished’ archival quality genome assemblies.

New assembly software

New assembly software attempts to account for polymorphism and take advantage of longer 250 bp Illumina reads. DISCOVAR [17*] from David Jaffe’s group who wrote the excellent ALLPATHS-LG is designed around 250 bp paired end reads from a single PCR free library — an extremely low cost strategy. However, as it was designed with relatively low human polymorphism in mind, additional testing will be required to assess the extent of polymorphism it can handle, despite excellent results in mammalian assemblies. Platanus [18*] has been used extensively for assemblies by the BGI, and is designed with multiple approaches to dealing with polymorphism. Its use of multiple kmer lengths in the de Bruijn graph, enables effective use of Illumina reads from 100 bp to 250 bp.

Long reads and their assembly

Long reads offer inherent advantages for genome assembly: they span polymorphic regions, repeats and transposable elements, and provide long-range information for contig scaffolding intrinsically avoiding many assembly problems.

Table 1

De novo genome assembly strategies. Assembly software is designed for a specific sequencing and assembly strategy. Thus sequence must be generated with the assembly software and algorithm in mind, choosing a sequence strategy designed for a different assembly algorithm, or sequencing without thinking about assembly is usually a recipe for poor un-publishable assemblies [38]. Here we survey different assembly strategies, with different sequence and library construction requirements. A typical genome project starts with high quality DNA of as low polymorphism as available, and extends beyond genome assembly to include gene annotation. Relatively inexpensive RNAseq from multiple tissues/or life stages (the authors often chooses adult male, adult female and mixed other life stages) provides transcript data for final genome annotation. For the definition of sex chromosomes, it is often useful to re-sequence at 30× coverage one individual of each sex. Additionally, re-sequencing of individuals at 30× genome coverage followed by alignment to the final reference using standard human analysis tools is the best way to characterize sequence variation within a species.

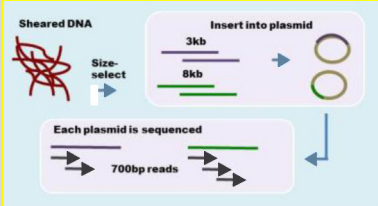
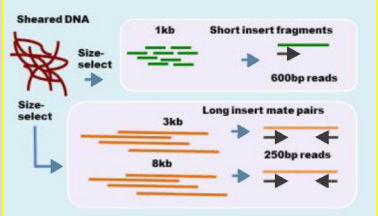
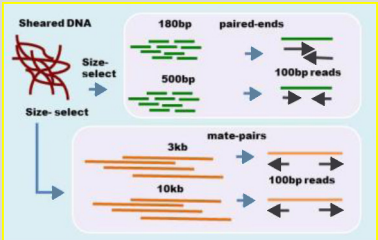

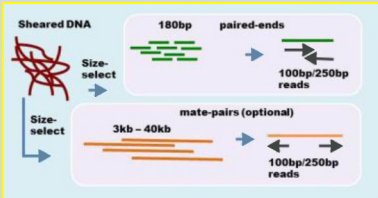
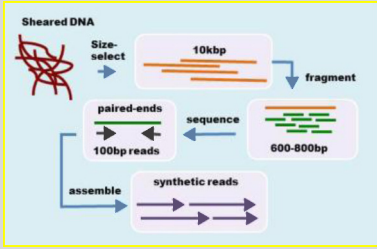
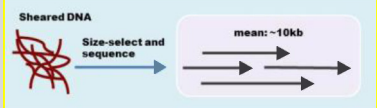

Technology	Strategy	Software	DNA	Cost	Notes
Sanger Paired-end	Overlap-layout-consensus Read length: 700 bp Insert sizes, coverage 3 kb, 15× 8 kbp, 5× 	Celera assembler	++++	++++	The original sequencing technology is now obsolete for genome assembly due to cost.
454 Fragment + Paired-end	Overlap-layout-consensus Read length: 600 bp Insert sizes, coverage 600 bp (fragment), 15× sequence coverage 3 kb (pe), 15× clone coverage 8 kb (pe), 15× clone coverage 	Celera assembler Newbler	+++	++++	Later revisions of the chemistry gave almost Sanger length sequence reads. Systemic homopolymer errors in assemblies can easily be corrected with Illumina sequence. Roche has announced a 2016 end of life for 454 sequencing support.
Illumina Paired-ends + mate pairs	de Bruijn graph based assembly Read length: 100 bp Insert sizes, coverage 180 bp, 40× 500 bp, 40× 3 kb, 40× 8 kb, 20× 	All Paths-LG SOAP <i>de novo</i> SGA Platanus	+++	+	Needs large memory machine for assembly. Can assemble large eukaryotic genomes. Not designed for polymorphic genomes (except Platanus)
Illumina PCR-free Single library paired-end	de Bruijn graph based assembly Read length: 250 bp Insert sizes, coverage 450 bp, 60× 	DISCOVER <i>de novo</i>	+	+	Simplified library production. Designed for mammalian levels of sequence polymorphism. DISCOVER is designed for PCR-free library construction, Platanus is more flexible.
	Multiple k-mer de Bruijn graph Read length: 100 bp, 250 bp Insert sizes, coverage 450 bp, 60× 3–40 kb (optional) 	Platanus			Platanus documentation gives no indication of desired sequence coverage or insert size inputs. We would recommend a longer insert size for scaffolding, in addition to shorter insert sizes for the primary sequence data.

Table 1 (Continued)

Technology	Strategy	Software	DNA	Cost	Notes
Illumina synthetic long reads (previously Moleculo)	Overlap-layout-consensus Reads: 10 kbp sheared to 500–800 bp and assembled into 1–18.5 kb synthetic reads, 20×	 Celera assembler	+	+++	Currently relatively expensive, but has continued potential for cost reduction. Synthetic long reads are very accurate. Possible uneven coverage.
PacBio Self-correction	Overlap-layout-consensus Read sizes, coverage 6–15 kb reads at 60×	 HBAR/Falcon & Celera assembler	++++	+++	All-against-all read alignment for error correction is processing intensive
PacBio Circular Consensus Sequencing	Overlap-layout-consensus Read sizes, coverage 3 kb CCS reads, 20×	 Celera assembler	++	+++	Trivial error correction to Sanger quality long reads. No possibility of sequence reads error corrected from disparate genomic loci. Assembly of 3 kb reads may not be as good as longer reads

Additionally, fewer reads for the same coverage reduces computational demands. Read lengths are often longer than contigs from mediocre short read assemblies.

The TruSeq synthetic long read technology from Illumina [19**] is a library construction kit and cloud software solution producing high-quality sub assemblies of barcoded 10 kb fragments (synthetic long reads) from shorter Illumina reads. Resulting synthetic long reads are then easily assembled into the full genome using the Celera Assembler or other overlap layout consensus assembler. This strategy was designed and successfully used for a highly polymorphic colonial tunicate genome [20**]. There can be some issues from un-even genome representation [19**], so a combination of this technique and a less biased short read assembly might be ideal. Synthetic long reads are especially exciting due to the continued potential for cost reduction from this ever more massively parallel sequencing technology.

Pacific Biosciences (PacBio) RSII reads have matured, and now routinely generate read lengths >20 kb with averages in the 10 kb range. Unfortunately, techniques for Illumina error correction of PacBio reads such as the pacbioToCA utility [21] within the Celera Assembler, and PBcR [21] have proved computationally inefficient in our hands due to the difficulties of aligning high coverage 100 bp Illumina data to high coverage 15% error rate long reads (although a promising new algorithmic approach was recently released [22]). A different approach, PBJelly [23] enables gap filling in draft assemblies by alignment to an existing draft genome, avoiding the error correction

step except when generating consensus sequence in gap regions. However, pure PacBio error correction strategies appear to give the best results. HGAP/HBAR/Falcon (Here called HGAP) [24–26] assembly trades high sequence coverage (50–70×) to overcome the 15% error rate, by using shorter reads to correct the longest 15–20× of reads enabling traditional assembly using the Celera assembler. This has produced effectively finished ‘archival’ quality genome assemblies with contig N50s 5 Mb and above for isogenic *melanogaster* and *Arabidopsis* [27**,28] genomes, although haploid human assembly, whilst equally successful, was computationally demanding for the error correction step [29]. The effectiveness of HGAP on polymorphic genomes has yet to be tested, but the length of the error corrected reads suggests a significant improvement over current methods. The megabase sized contigs of these assemblies simplifies and almost obviates finishing, and prevents future re-visiting of sequencing to make these assemblies truly archival — of high enough quality to be placed in a database and used for many decades to come.

Pacific Biosciences Circular Consensus Sequencing (CCS) [30] is an alternate, often neglected method of error correcting PacBio sequence reads. Circularized shorter templates are sequenced multiple times by a single long read enabling error correction on a single molecule. This avoids the possibility of independent, but similar, genomic loci erroneously error correcting each other. The advantage is computationally trivial error correction, as no all-against-all read comparison is required, but the disadvantage of shorter error corrected read lengths (current read lengths

enable ~3 kb CCS reads with at least three sequence passes of the molecule), and slightly less efficient production of error corrected data. This can be an effective approach for genomes with significant highly repetitive content and larger genomes to avoid the computational difficulties of all against all alignment for error correction.

Finally, Oxford Nanopore's infamous single molecule sequencing is in beta testing at the time of writing. Anecdotal information suggests the technology enables extremely long reads up to 100 kb in length, but quality issues remain significant. Despite these high error rates, an initial assembly of a eukaryote has been reported [31]. Given the revolutionary assembly potential of such read lengths, we are watching this technology with interest.

New assembly validation and scaffolding technologies

Genome assembly validation has fallen out of favor, as the costs associated are often greater than that of the Illumina assembly itself. Genetic maps, physical maps and other long-range independent data are expensive to generate, and taxonomically close insect species with high quality genome sequences are rare. Minimally, BUSCO analysis should be performed (see Waterhouse's review in this issue [32]), indicating the completeness of the assembled gene set — an item of critical importance for most users. For comprehensive analysis of assembly quality, additional data are required. Two new technologies are described here, but it should be noted that long-range sequence information requires long pieces of DNA, either from intact nuclei or extremely long DNA molecules, which may require additional material.

Optical mapping (commercially available from OpGen) developed in 1990s [33] directly images restriction digests of confined long linear DNA molecules, generating large scale restriction maps. A similar, but more scalable technology from BioNano Genomics [34**] uses semiconductor fabricated nanogrooves, and sequence motif fluorescent labeling to enable high throughput data collection of similar motif maps. The high throughput data collection method has reduced costs to enable routine validation of genome assemblies, and has been used for assembly super scaffolding to increase scaffold N50 lengths.

An orthogonal data source for genome assembly validation is chromatin interaction sequencing (Hi-C) [35**]. This sequencing protocol captures pairs of DNA fragments physically close to each other in three dimensional chromatin structures. The proximity of the paired sequences is inversely related to the distance between them, and this fact is used to produce 'ultra-long-range' genome assembly scaffolding for entire chromosome arms [35**]. To date, this has been used on high quality genomes including *Drosophila*, but the technique is rapidly gaining acceptance.

Low input DNA and large genome sizes are still problematic

As described above, in many cases input DNA quantity from a single individual can be very small, and pooled individuals increase the polymorphism and chances of a poor assembly product. But some of the above strategies require relatively small amounts of DNA (although the DNA must still not be degraded). Illumina Synthetic reads require only 500 ng of DNA, depending on the amount of synthetic long-read sequence desired. The DISCOVAR or Platanus assembly of a 250 bp read length Illumina data can be made from as little as 10 ng of DNA using low input DNA library protocols. Unfortunately this is not a PCR free library technique, but ignoring this requirement may be a lessor of evils. In our hands Platanus assembly of an outbred 400 Mb diplura genome from 250 bp paired reads with a 400 bp insert generated 11 kb contig N50 from approximately 50 ng of DNA, although the scaffolds are small due to an absence of long-range sequence information.

Very large genomes are also problematic, with the definition of very large decreasing with increased polymorphism in the dataset. With ALLPATHS-LG, many groups have routinely assembled 3 Gb mammalian genomes to high quality, but more polymorphic insect genome assemblies often fail with genomes greater than 2 Gb. The ALLPATHS-LG software is not designed for genomes larger than 4 Gb, and run times grow into multiple months. MaSuRCA [36] is the only assembler designed for very large genomes having been successfully applied to the 22 Gb loblolly pine genome [37*], however, we have had difficulty applying this software to polymorphic insect genomes from 2.5 to 5 Gb. Additional research is required to address this problem.

Summary

New long read based assembly strategies, such as Pacific Biosciences and Illumina synthetic long-reads, are highly recommended due to a higher probability of assembly completion, longer contiguous sequences and potentially archival 'finished' quality assembly products. Cost effective draft assembly of longer 250 bp Illumina reads is also improved due to new software, although the success rate in significantly polymorphic datasets is unknown and expectations of archival quality genome references for little investment are unrealistic. New validation technologies enable quality assurance and improved super-scaffolding of genome assemblies. Genome assembly from low DNA quantity and of large and polymorphic genomes is still a significant challenge.

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have been highlighted as:

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A description of the assembly of the 22 Gb loblolly pine genome the largest genome assembled to date.

38. Richards S. Please note that this advice, often ignored by otherwise brilliant researchers, is the main reason we agreed to write this review.