New alignment-based sequence extraction software (ALiBaSeq) and its utility for deep level phylogenetics

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Abstract

Despite many bioinformatic solutions for analyzing sequencing data, few options exist for targeted sequence retrieval from whole genomic sequencing (WGS) data. Available tools especially struggle at deep phylogenetic levels and necessitate amino-acid space searches, increasing rates of false positive results. Many such tools also suffer from difficult installation processes and lack adequate user resources. Here, we describe a program using freely available similarity search tools to find homologs in assembled WGS data with unparalleled freedom to modify parameters. We evaluate its performance as well as that of other utilized bioinformatics tools on two divergent insect species (>200 My) for which annotated genomes exist, as well as on one large set each of highly conserved and more variable loci. Our software is capable of retrieving orthologs from well-curated, low and high depth shotgun, and target capture assemblies as well or better than other software as assessed by finding the most genes with maximal coverage and with a low rate of false positives throughout all datasets. The software (implemented in Python), tutorials, and manual are freely available at https://github.com/AlexKnyshov/alibaseq.

Introduction

Phylogenetic reconstructions use a fraction of the sequence data of an organism's genome though the quantity of data continues to increase with the widespread application of Next Generation Sequencing (NGS) to phylogenetics. Because of the relative ease to determine homology, orthologous genes have traditionally been the most widely used type of sequence data. Applications of NGS for the generation of phylogenetic markers have therefore heavily relied on a handful of reduced representation

approaches including transcriptome sequencing (RNASeq), DNA-based reduced representation techniques, and genome skimming. RNASeq was among the early, still fairly expensive, techniques to obtain large numbers of loci that are informative for deep phylogenetic divergences. Recently, the more cost-effective targeted sequencing of genomic DNA, enriched via hybrid capture, became popular and is at the core of widely used approaches including Ultra Conserved Element (UCE) (McCormack et al., 2012) and Anchored Hybrid Enrichment (AHE) (Lemmon, Emme & Lemmon, 2012) methods. As sequencing costs have dropped during the past decade, genome skimming has become a viable alternative to hybrid enrichment, at least for taxa with relatively small (1 Gbp) genomes. This technique is less challenging with respect to sample quality, involves less complicated lab protocols and does not require expensive probe synthesis. This last point is critical for sampling phylogenetically diverse taxa because the recovery of target sequences is not bound by limitations of the probe design.

However, data obtained from genome skimming can be difficult to parse or integrate into a phylogenetic dataset and can pose substantial problems for analysis. For example, they may differ from deep-sequenced model taxon genomes in that assemblies are much less contiguous, and annotations are unavailable. They also differ from RNASeq data, most notably by the presence of untranslated highly variable regions such as introns. As opposed to typical target capture data, genome skimming does not result in differential coverage between target and non-target loci. Also unlike hybrid capture, where probes are typically designed for a particular set of taxa based on a related reference taxon (e.g., Faircloth, 2017; Young et al., 2016), genome skimming can ideally be applied to taxa with or without available reference genomes or transcriptomes. This potential lack of a closely related reference taxon to guide locus extraction can be challenging for currently available software. Nevertheless, hybrid capture-based bioinformatic solutions are most commonly applied to the phylogenetic analysis of genome skimming data (Chen et al., 2018; Zhang et al., 2019).

Phylogenetically-oriented hybrid capture and genomic pipelines are subdivided into two main groups of approaches. Software in the first group identifies reads of interest and subsequently assembles this limited pool of reads (aTRAM [Allen et al., 2015, 2018], HybPiper [Johnson et al., 2016], Assexon [Yuan et al., 2019], Kollector [Kucuk et al., 2017], and HybPhyloMaker [Fér and Schmickl, 2018]). The search for reads that match target regions typically makes use of read aligners (HybPiper, Kollector, HybPhyloMaker) or local similarity search algorithms on both the nucleotide and protein levels (aTRAM, HybPiper, Assexon). After reads are gathered, they are fed to an assembler, and assembled contigs are further processed. A benefit of this group of approaches is that there is no need to assemble the entire read pool, making them potentially faster and less memory demanding. Some drawbacks are the need to perform new read searches and assemblies for each new set of baits and the inability to work with assembled data.

The second group of approaches uses all reads to produce an assembly. The assembly is queried for target sequences, which are then extracted and processed. Post-assembly dataset-specific target searches can be performed relatively quickly. However, especially for highly divergent taxa, assembly may be both a memory- and time-demanding procedure. Generating a set of contigs from transcriptomic assemblies can be relatively straightforward, because they mostly consist of spliced protein coding sequences. This approach is utilized in HaMStR (Ebersberger, Strauss & von Haeseler, 2009), Orthograph (Petersen et al., 2017), and FortyTwo (Simion et al., 2017), among other applications. However, genomic assemblies may have contigs comprised of multiple genes or untranslatable introns of varying size. Gene prediction and protein extraction may be complicated when a target gene is

fragmented into many small contigs. Recently, (Zhang et al., 2019) suggested using Phyluce (Faircloth, 2016) for UCE extraction and Benchmarking Using Single Copy Orthologs (BUSCO) (Simão et al., 2015; Waterhouse et al., 2017) for OrthoDB Single Copy Ortholog (SCO) extraction from genomes at shallow phylogenetic levels, i.e. from relatively closely related taxa. Between these two solutions, only BUSCO is specifically designed for genomic assemblies and has the capability to search for and predict genes de novo, but it is only feasible for a few predetermined sets of proteins. Phyluce was originally designed for short, conserved fragments and it is unclear how well it performs on longer multiexon genes. The recently published Assexon software (Yuan et al., 2019) is capable of searching for and retrieving sequences from genomic assemblies, but this module has not yet been extensively tested.

To address issues with commonly-used techniques for including genome-skimming data in phylogenies, we have developed a software, named ALiBaSeq (ALignment Based Sequence extraction), that is designed for sequence extraction based on a local alignment search and is applicable to all types of assembled data and a wide range of assembly qualities. The software is flexible with respect to both input and output, which will facilitate its incorporation into existing bioinformatics pipelines. Any read processing technique and assembler are supported to generate the input for the software, while the resulting sequences are output in FASTA format and can be grouped in several ways (per target locus, per sample, etc.) depending on what is required in downstream analyses. The software also allows for the integration of different types of datasets (e.g., transcriptomic and sequence capture data) that are increasingly becoming available and that will result in phylogenies with more complete taxon sampling (Kieran et al., 2019). Existing tools frequently rely on a particular type of sequence aligners [BLAST (Altschul et al., 1990) for aTRAM and FortyTwo, both BLAST and BWA (Li & Durbin, 2009) for HybPiper, Usearch for Assexon, LASTZ (Harris, 2007) for Phyluce]. Our software supports several commonly utilized similarity search programs and their outputs. While we provide utility scripts for some of the tools, no special software is required to run these similarity searches, thus giving the user full control over search program settings if needed. Finally, compared to other programs, we offer greater customization of parameters, including different alignment score cutoff criteria, specification of number of alternative matches, and sequence output structure. The software is available for download at https://github.com/AlexKnyshov/alibaseq.

We here describe the implementation of this software, assess its performance, and benchmark it against other commonly utilized algorithms. Tests are conducted on 1) both conserved and variable loci as determined by average pairwise sequence distance, on 2) contiguous whole genome assembly, short read assemblies of variable depth of coverage, and a hybrid capture sample. We focus testing on the insect samples (see below), but also perform a subset of tests on a plant system to verify the software's versatility, the details of which are available in the supplemental text S1. Overall, we find that our software matches or outperforms other techniques applied to genome skimming data in recovering the most orthologous genes with the lowest amount of error in low coverage genome assemblies. Furthermore, we determine that it works as well or better than other tools on high coverage genome assemblies and target capture assemblies especially at relatively deep phylogenetic levels (100-200 Mya). Thus, it is a potentially valuable tool for compilation of phylogenomic datasets across diverse taxa and diverse data types.

Materials & methods

Algorithm and Implementation

The workflow is shown in Fig. 1 and the terminology is outlined in Table 1. The software parses an alignment table (generated by a number of programs like BLAST and HMMER [Eddy, 2011]) and groups all HSPs for each contig (hit). For each query-hit pair, HSP hit regions are merged in case of overlaps and joined in case of no overlap, producing a pseudocontig. Contig regions derived from HSPs with overlap only in the bait sequence (different contig regions matching the same bait region) are separated into alternative "pseudocontigs". This accounts for a case of a contiguous assembly where several homologous genes occupy the same chromosomal contig and could be located nearby. Thus, no preliminary contig splitting (for example, as done in Phyluce) is needed for genomic data. For the translated searches, the hits derived from the opposite strands are kept as separate "pseudocontigs". By default, each particular region of every contig is allowed to match only one bait (to prevent the same sequence from being assigned to multiple baits).

An optional reciprocal best hit (RBH) check can ensure that each pseudocontig which matches a particular bait also matches the bait-matching contig in the reference assembly. This check removes out-paralogs and is also a part of Orthograph, FortyTwo, and Assexon, although is not implemented in most popular target-enrichment bioinformatics pipelines (e.g., aTRAM, HybPiper, and Phyluce). A strict RBH check however may represent a too conservative solution, especially when a DNA-based reciprocal search was performed on divergent taxa. The algorithm may result in too many false negatives, the truly homologous contigs that did not get any significant matches to a reference taxon. Thus, we added a relaxed option of retaining the contigs without matches to the reference.

After verifying that all pseudocontigs match best to the bait they were initially assigned, they are ranked by alignment scores. Optional contig stitching is then performed by attempting to iteratively fill all regions of the bait by non-overlapping pseudocontigs. Only pseudocontigs derived from different contigs are allowed to be stitched. Stitched supercontigs receive combined scores of the included contigs to prevent longer incorrect matches rank higher than the composite correct match. A similar operation is done by HMMER when combining the protein domain scores into an overall hit score. While potentially prone for chimeric sequence creation, contig stitching greatly improves coverage of the long loci mined out of discontiguous assemblies. Apart from ALiBaSeq, a contig stitcher is also implemented in Orthograph, while aTRAM and HybPiper are among the only read-based tools to perform the stitching of shorter assembled contigs.

The resulting supercontigs are then ranked by scores again, and the best supercontig or several high scoring supercontigs are then selected. We also implemented an option to select several competing supercontigs with similar scores matching the same bait in case the user wants to perform a more thorough tree-based orthology prediction. The procedure outlined thus far runs without access to an assembly FASTA file and is solely based on the alignment tables. We currently support BLAST, HMMER, and LASTZ searches, with a helper script enabling a tblastn-like search for HMMER.

After the list of matching sequences is prepared, the target assembly file is read, and sequences are extracted. Several extraction modes are offered: whole contigs (no contig stitching allowed), only single best matching region, all matching regions, and all matching regions plus the sequence in between (typically to get introns and variable regions as well). Additionally, extraction of flanking regions is permitted, although no similarity check is implemented for those regions. By default, the output sequences are grouped by locus and appended to existing alignments, but they may also be grouped by

the sample they are coming from, or can all be written into a single file. Output sequences can also be translated if a protein-based search is used and only matching regions are retrieved. Additionally, extensive logs and bait-sample correspondence tables with contig names, combined scores, and coordinates are saved.

Samples

In order to evaluate the software's performance at deep phylogenetic levels, we used the two species *Rhodnius prolixus* and *Cimex lectularius*. They belong to the two distantly related families Reduviidae and Cimicidae, respectively, within the heteropteran infraorder Cimicomorpha (Hemiptera). The divergence between the two species was recently dated to the mid-Triassic, roughly 225 million years ago (Johnson et al., 2018). Since genomes and annotations are available for both (Mesquita et al., 2015; Rosenfeld et al., 2016), we tested the retrieval of *Rhodnius* sequences using divergent *Cimex* bait sequences for regions known to be shared by both species.

We downloaded the original assemblies of the *Cimex* and *Rhodnius* genomes (ClecH1 and RproC3) and transcripts / gene sets (ClecH1.3 and RproC3.3) from VectorBase (Giraldo-Calderón et al., 2015). To test and compare both read-based and assembly-based software, we simulated Illumina reads derived from the *Rhodnius* assembly using Art (Huang et al., 2011). Six simulations at various depths (1x, 3x, 5x, 10x, 20x, 40x) were performed, with uniform coverage across target and off-target regions. We used recently published results (Kieran et al., 2019) of empirical enrichment of a closely related species, *Rhodnius robustus*, as a target capture sample for the UCE dataset (see below). To review assembly-based software equally, we processed all reads in a similar way, i.e., with Clumpify (Bushnell, 2014) and Trimmomatic (Bolger, Lohse & Usadel, 2014) and assembled them using SPAdes (Bankevich et al., 2012). Properties of all used assemblies were evaluated using QUAST (Gurevich et al., 2013) and are shown in Table 2. Whenever non-default settings were used, they were indicated in Table S2.

Baits

To ensure our software was applicable for two commonly-used data types, two sets of loci were used as baits, the Ultra Conserved Elements (UCE) Hemiptera 2.7K v1 set (Faircloth, 2017) and the OrthoDB single copy orthologs for Hemiptera (ODB SCO). UCE loci and ODB SCO orthogroups used are listed in Supplemental Text S3.

For UCEs, we extracted loci shared between *Cimex* and *Rhodnius* from the Hemiptera UCE 2.7K v1 set (Faircloth, 2017) and merged probes to obtain the overall bait regions (since typically two probes tile a 160 bp region). Since 99.9% of the kit was shown to target exons of protein coding genes in the taxon with the most well-annotated genome (Kieran et al., 2019), we prepared DNA and AA sequences for both nucleotide and protein modes of the programs we were assessing. A few particularities of the probe set caused us to perform additional filtering and processing: 1) some introns flanking UCE exons are part of the probes despite their relative high rate of mutation; 2) several UCE "loci" in *Cimex* and/or *Rhodnius* are overlapping for about a third of their length, with probes targeting opposite strands (likely due to intron drop out in either of the taxa compared to the other probe set taxa); 3) the bait region borders (probe start/stop coordinates) are not always homologous between *Cimex* and *Rhodnius*

(because of issue 1); 4) some UCE loci are too close to each other on the scaffold to exclude the possibility of capturing both loci with probes derived from only one (we did not address this issue, since it did not impede our evaluation analyses). Thus, to obtain homologous protein coding regions of the probe set, we first overlapped *Cimex* probes of the same region and aligned the resulting sequences using BLAST to *Cimex* genomic scaffolds. We randomly removed one of the UCE loci which were overlapping since these duplicate UCEs could be flagged by some of the software we were testing. We compared the mapping coordinates with the assembly annotation and truncated the probe regions to stay within the CDS. We then compared the CDS with the *Cimex* proteome to find the start and stop of the ORF and get the protein sequence of the probes. Using protein BLAST, we aligned *Cimex* AA probes to *Rhodnius* DNA probes and truncated the latter to make sure that homologous *Rhodnius* regions are used for evaluations. The resulting UCE dataset had 2,037 loci, with locus lengths ranging from 60-160bp, nucleotide pairwise sequence distances (n-psd) ranging from 1.59% to 50.69% (average 22.44%) and 0% to 60.42% (average 6.88%) on the protein level.

For ODB SCO, we retrieved orthologs that are single copy for Hemiptera from OrthoDB v9. We then extracted *Rhodnius* and *Cimex* protein sequences, aligned them with MAFFT (Katoh & Standley, 2013), and truncated alignments until both sequences were present. We subset the alignments to retain only those with >100 AA and less than 10% gaps between the two species, then used the resulting truncated selected protein sequences to obtain corresponding CDS from the VectorBase sourced transcriptomes of the two species. We obtained 1027 loci and initially used those for benchmarking. However, when investigating false positive results, we discovered that some results seemed to be correct based on the pairwise distance and were in fact correct according to the VectorBase orthology predictions. We thus compared orthology assignment of 1027 selected loci between OrthoDB and VectorBase. We found cases where one (primarily *Rhodnius*-sourced) gene was assigned to an incorrect orthogroup on OrthoDB, as well as cases where a gene was not single-copy (had paralogs in *Cimex* and/or *Rhodnius*) according to VectorBase, while being listed as single-copy on OrthoDB. A total of 43 such loci were found and discarded from the evaluations. We additionally inspected the *Rhodnius*-*Cimex* alignments and discarded 10 other loci that had poor alignment between the two species. The resulting ODB SCO dataset had a total of 974 loci, with locus lengths ranging from 216 to 20,862 bp, and nucleotide pairwise sequence distances ranging from 15.22% to 52.89% (average 29.84%) and 0% to 61.62% (average 20.15%) on the protein level.

Software and settings

For read-based approaches, we tested four software packages, Assexon, aTRAM, HybPiper, and Kollector. In order to make the comparison equal, we used processed reads for the SPAdes assemblies (see above) as an input for the read-based software. The default settings of the evaluated software were drastically different, and in some cases inappropriate for the deep level divergence we were looking at, causing suboptimal performance. We thus attempted to standardize them and set parameters of the programs to a very generous minimal level (Table S2). Due to specific requirements of Assexon on NGS read format, we could not run it on our simulated reads. aTRAM's performance with the SPAdes assembler was poor due to coverage cut-off values and timeout problems; when the time parameter was increased, the analyses took days to complete; thus we ran the protein-based analyses with Velvet (Zerbino & Birney, 2008) for evaluation purposes. HybPiper's protein-based search speed on the ODB

dataset, particularly when using 20x and 40x reads, was extremely slow (did not complete in eight days), thus we set the filtering settings to default for these trials to maximize locus recovery. We note that changing the HybPiper settings to default did not change the results of the low depth trials (1x-5x), while improving the results on the 10x dataset.

For assembly-based approaches, we tested Assexon, FortyTwo, Phyluce, and our software ALiBaSeq. We note that Orthograph is very close in capabilities to FortyTwo, with both programs being primarily designed to work with transcripts. However, Orthograph has a substantial list of dependencies, and requires a relational database, thus, we have not included it in our evaluation. Despite the program BUSCO's capability to process genomic data and retrieve target sequences with high precision, this software is hard to customize. The lowest level relevant gene set available are orthologs at the level of Insecta. The presence of *Rhodnius* in the profiles would not allow us to fully test the performance with a divergent reference. We thus did not include BUSCO in our comparison. Settings used are listed in the Table S2. For ALiBaSeq we ran the software both with strict and a relaxed RBH check (see implementation). Figures 2 and 4 represent the best performing combination of such analyses (strict for 1-3x assemblies, relaxed for others), for details see Supplementary Tables S3 and S4. For Phyluce, we generally used guidelines in tutorial 3 (https://phyluce.readthedocs.io/en/latest/tutorial-three.html). We had to use the 0 bp flank option to maximize the locus recovery since many loci were being discarded due to co-location on the same contig. The capture sample was processed directly starting with the SPAdes assembly, bypassing read processing and Trinity assembly for comparison purposes. The phyluce_assembly_match_contigs_to_probes command for the capture sample was run with the same settings as for genomic samples.

We additionally capped all read- and assembly-based programs to run maximally with 32 threads to make the performance comparison fair. To assess accuracy of the recovery, obtained DNA (or protein, in the case of FortyTwo) sequences were aligned back to the *Rhodnius* sequences that were homologous to the *Cimex* bait sequences. The alignment was done with MAFFT G-INS-i or E-INS-i (Katoh & Standley, 2013); and the --adjustdirectionaccurately option was used for outputs of Kollector, since Kollector does not maintain hit sequence direction with respect to the query sequence. Since only ALiBaSeq has the capability to report the source contig names and coordinates for the retrieved sequences, we had to rely on sequence similarity to determine whether an ortholog or a paralog was recovered. We observed that sequence similarity of less than 100% was too strict a criterion for assessing false positive results. Small alterations in simulated reads, assembly of such reads, incorrect flanking bases of the exon-intron junctions, and the alignment errors lower the apparent sequence similarity when the retrieved sequence is compared to the original sequence. Thus, we used a threshold of 10% difference to track strongly false positive results. Since our empirical UCE capture sample belonged to a different species (*R. robustus*), we used a larger threshold of 20% to account for additional interspecific variation.

We also evaluated software performance for difficult loci, which either have multiple introns, or are represented by multiple contigs in the assembly (for assembly-based tools only). The evaluation of the performance on multi-intron and multi-contig loci was evaluated on the 40x ODB dataset. To assess the impact of introns on the software performance, we queried the annotation file for *Rhodnius* to obtain the number of introns per SCO, and then regressed the SCO coverage produced by each software on the number of introns using a linear model. To evaluate the impact of SCO fragmentation in the assembly, we performed blastn search for *Rhodnius* bait homologs in the *Rhodnius* 40x read assembly, processed

the results with ALiBaSeq to estimate the number of contigs representing each SCO locus, and then regressed the SCO coverage produced by each software on the number of contigs.

Additional evaluation methods

Methods of the software evaluation using the plant dataset are available in the supplemental text S1.

Results

The UCE dataset

Number of loci recovered (Fig. 2, correct loci panel). On the original assembly, ALiBaSeq recovered up to 2,032 out of 2,037 UCE loci. Protein based approaches (tblastn, tblastx, and phmmer) performed best, with relaxed nucleotide-based searches (blastn with wc9 and discontinuous megablast) performing almost as well. Both Assexon and Phyluce recovered around 1,500 loci, while FortyTwo only found ~300 loci. On the assemblies of simulated reads, ALiBaSeq generally performed best, or as well as other assembly-based software. ALiBaSeq, Phyluce, and FortyTwo reached their maximum performance at assemblies of 10x and deeper and also performed well on the target capture data. Read-based methods using nucleotide space searches performed very poorly, with the exception of Assexon (~1,500 out of 2,037 loci), which we could only test on the capture sample. Protein-based aTRAM performed the best of these methods in finding the maximum number of loci on the simulated genomic reads. Protein-based HybPiper only performed well on 40x and target capture data, with only ~1,300 out of 2,037 loci on the 20x data, and much less on the shallower data. Interestingly, the genomic pipeline of Phyluce performed better (1,939 loci) on the target capture data of Kieran et al. (2019) than the standard Phyluce capture pipeline (1,471 loci), a result closer to that reported in the original study (Kieran et al., 2019) for this sample (1,508 loci).

False positives (Fig. 2, incorrect loci panel). All tools performed well on the original assembly (0.0-0.25% false positives) with the exception of FortyTwo (1.4%). The DNA-based ALiBaSeq search on simulated read assemblies had less than 1% false positives. Protein-based ALiBaSeq had a higher proportion of false positives (1-2%) on the low-coverage assemblies (1x and 3x respectively), with a much lower proportion (0.25%) for 10x-40x assemblies. This is comparable to other assembly-based software false positive rates, and much lower than that of read-based programs: up to 9% for protein-based aTRAM and up to 12% for protein-based HybPiper. Lower-depth (1-3x) reads and read-based assemblies were more challenging for accurate orthology prediction. The conservative approach of exclusion of contigs that lacked any hits in the reciprocal table improved orthology assignment for 1-5x data however worsened the results of 10-40x data analysis (true orthologs were incorrectly discarded, see Table S3).

Bait region coverage (Fig. 2, avg bait coverage panel). As UCE bait regions are short, generally ALiBaSeq and other tools recovered almost complete bait regions (~80-100% of the length), with a minimum of 78% recovered on the lowest coverage assembly.

Time (Fig. 3). Depending on the search type, the ALiBaSeq pipeline on the original assembly completed a forward BLAST search in between 2 sec (dc-megablast) and 4 minutes (tblastx) and a reciprocal search in between 8 and 90 minutes. HMMER searches ran much longer (up to 7 hours). The main script ran for 15 sec to 2 minutes. The total processing time for ALiBaSeq thus was between 8 min and 1.5 hours with BLAST searches, and up to 8 hours with HMMER searches. Assexon and Phyluce worked faster than ALiBaSeq searches (30 sec to 2 min total time), while FortyTwo was slower (several days). The same relationship was generally true for the searches on simulated read assemblies. On smaller datasets (1-5x) ALiBaSeq with nucleotide blast was as fast as Phyluce and about as fast as Assexon with protein blast. DNA-based read-based tools worked very quickly, but that can be explained partially by much lower locus recovery. Adding the assembly time to the analysis time, assembly-based tools' speed (1 to 1.5 days) is higher on the larger datasets than that of protein-based aTRAM (20x and 40x with 4 to 10 days). Processing time of protein-based HybPiper on the UCE dataset (9 min to 5.5 hours) is smaller than the total time of assembly-based tools (2.25 hours minimum). Interestingly, the SPAdes assembly time of the capture sample was much lower (~7 min) despite the comparable read number with 1x whole genome data, likely due to lower K-mer diversity. This gave the assembly-based tools a clear advantage in speed (7 to 17 min total time) over the read-based tools that found a considerable amount of loci (Assexon, protein-based HybPiper, and aTRAM with 19 min, 47 min, and several days respectively).

The ODB SCO dataset

Number of loci (Fig. 4, correct loci panel). ALiBaSeq found the highest number of loci (up to 933 out of 974) on the original assembly by a very large margin, twice as many as the best alternative (404 out of 974 for Phyluce). On the assemblies of simulated reads, FortyTwo found most loci in the low coverage assemblies (1-3x). ALiBaSeq performed well on low coverage assemblies and the best of all assembly-based tools on assemblies of 10x and deeper. As in the UCE dataset, only protein-based versions of the read-based tools found any significant number of loci. The software aTRAM outperformed HybPiper on low coverage data (1-10x) but recovered fewer loci than HybPiper when analyzing 20x and 40x reads.

False positives (Fig. 4, incorrect loci panel). The highest levels of false positives were found in the results of the read-based tools: protein-based aTRAM with 11-23% and protein-based HybPiper with 3-100%. Among assembly-based tools, ALiBaSeq had a rate of 1-3% on nucleotide and protein levels on complete assemblies, but up to 6.4% on protein-level on 1x data. This rate was comparable or lower among the programs finding >100 loci, with Phyluce having 8% and FortyTwo 12% false positives. Most of ALiBaSeq false-positives are due to non-orthologous reciprocal best hit results (since we conducted the reciprocal searches on the nucleotide level) as well as paralogous longer hits having a much larger score than the sum of small orthologous hits (i.e., paralogs with less introns were preferred over orthologs with more introns).

Bait region coverage (Fig. 4, avg bait coverage panel). Protein-based aTRAM and Kollector recovered the largest regions (>85%), followed by ALiBaSeq and Phyluce. Since ODB SCO baits were much larger than UCE baits, much smaller portions of the bait regions (up to 29%) were recovered from the lower coverage assemblies (1x-5x), with recovery reaching maximum values on 10x and deeper data.

Time (Fig. 5). Processing time on this dataset was longer compared to the UCE dataset, likely due to the larger size of the loci. However, relative performance of the tools was generally similar between the two

datasets. The notable exception was HybPiper, with its exonerate steps taking much longer on 20x and 40x data leading to a slower performance compared to aTRAM on the same data.

Target-adjacent sequences. Since the majority of software did not output non-target sequences with the settings used in our analyses, we did not perform a formal evaluation. Below we outline detailed features of the programs pertinent to target-adjacent sequence extraction.

External non-target sequences (flanks). Flanks are retrieved by HybPiper and Kollector and bound by the read size. Although ALiBaSeq has the capability to extract flanks, we have not used it for the evaluation analyses. Phyluce is capable of extracting flanks, however it discards loci which overlap in flanking regions, thus we set flanks to 0bp during the evaluations to maximize locus recovery. The output of the aTRAM main script has flanks bound by the read size, however in order to maximize locus coverage we had to run the exon stitching pipeline, which outputs a spliced sequence devoid of flanks. Similarly, Assexon and FortyTwo output spliced sequences without flanks.

Internal non-target sequences (introns). HybPiper, Kollector and Phyluce extracted introns as part of their output in our analyses, with introns of the two former programs often being partial and bound by the read size. ALiBaSeq has a capability to extract internal non-target regions, while Assexon, aTRAM, and FortyTwo output a spliced sequence without introns.

Number of introns vs coverage (Fig. 6). ALiBaSeq in conjunction with HMMER and protein-based BLAST searches retrieved the largest regions of the multi-intron genes among all other assembly-based software. Phyluce and Assexon generally failed to recover genes with over 10 introns, while FortyTwo retrieved less than 25% of the sequence of the genes with over 10 introns. Among the read-based software, protein-based aTRAM and HybPiper recovered the largest regions of the multi-intron genes, slightly outperforming ALiBaSeq.

Number of contigs vs coverage (Fig. 7). Among the assembly-based software only ALiBaSeq performed well in finding and stitching contigs comprising multi-contig genes. Assexon barely reported any multi-contig genes, while FortyTwo and Phyluce output highly incomplete sequences for loci represented by three or more contigs in the assembly.

Additional results

Results of the software evaluation using the plant dataset are available in the supplemental text S1.

Discussion

We show that ALiBaSeq is a versatile tool, capable of retrieving orthologs from curated contiguous assemblies, low and high depth shotgun assemblies, and target capture data (Table 3). While utilizing a divergent reference taxon, separated from the sample by over 200 My of evolution, ALiBaSeq performed as well or better than other software in finding most genes with maximal coverage breadth and has a comparable rate of false positives throughout all datasets. With respect to the most similar programs, Assexon and FortyTwo, our program recovered more and longer sequences compared to the former, and was much faster in recovering longer sequences with a lower false positive rate compared to the

latter. We highlight that this outcome applies to deep level divergences between the bait taxon and the sample taxon. This conclusion will likely not be valid for shallow-level datasets, since all tested programs perform well in retrieving highly similar sequences in our evaluations, and some can do it even faster than ALiBaSeq (e.g., Assexon and Phyluce). With respect to accuracy at the protein level, additional improvements may be achieved by running the protein-based reciprocal search instead of the nucleotide-based as we did in our analyses, as well as removing outliers using alignment and gene tree screening software (de Vienne, Ollier & Aguileta, 2012; Kocot et al., 2013; Borowiec, 2019).

Performance tests demonstrated (Fig. 3) that for deeply sequenced samples (10x and above), whole read pool assembly followed by target searches can be completed much faster than analyses using read-based tools. At the depths where read-based tools run faster, they are unable to retrieve the same number of loci. We emphasize that this outcome is a consequence of protein-based searches for deep level analyses, since much faster DNA-based search and read aligners can be successfully used in shallow-level applications. In addition, read-based tools have a higher proportion of false-positives compared to assembly-based methods, most likely due to the lack of RBH checks. With regard to ALiBaSeq's RBH check, the relaxed option of not discarding the contigs that did not have any hits in the reciprocal tables proved beneficial for the deep coverage assemblies, resulting in recovering many more loci at the expense of few additional false positives. However, for incomplete low-coverage assemblies the relaxed option results in too many false positives, and we recommend a safer strict option for such situations.

Assessing the performance of different search engines with ALiBaSeq, we highlight the outstanding performance of discontinuous megablast. Its forward search time on our datasets was only a few seconds, while the number of loci found was comparable with the protein-based searches and the number of false positives was smaller. We speculate that this outcome is due to the gapped initial match, which allows for variable third codon positions in between more conserved positions. Even with a hit reduced to a 9 bp initial match (default at 11 bp), blastn did not achieve the same result, as it requires an exact initial match. Due to its sensitivity and computational efficiency, dc-megablast may thus be a viable alternative to costly protein-based searches. Nucleotide-based HMMER search (nhmmer) was generally as effective in finding loci as protein-based BLAST searches, and had less false-positives, but took several times longer to run. Protein-based HMMER search (phmmer) was about as effective as the nucleotide version but had much higher number of false-positives.

Finally, we want to emphasize that our software has a small list of dependencies and is easy to install locally or on a bioinformatics cluster. Although a software of choice is needed to generate alignment tables, it is not required to be installed, and the actual alignment search can be done on a different machine or environment. Since the alignment software is external to the script, it can be run with a particular setting independent of our script and thus users are not constrained with default settings. Simple input and output formats allow for easy incorporation of this software as a step into existing pipelines. In addition to phylogenetic applications, the software can be used as a general similarity search results parser and sequence extractor. Even when only working with search results tables and without access to FASTA files, ALiBaSeq outputs extensive logs and query-hit correspondence tables, which include a list of contigs found for each bait, their combined score and coordinates, as well as a list of baits located on the same contigs. We provide simple easy to follow tutorials for several examples on how to use the program as well as a test dataset.

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Tables

Table 1. Terminology used in the paper.

Term	Definition
Bait	Sequence in reference organism for which homologs are to be found in the sample
Forward search	Operation to match bait sequences to assembly contig sequences
Genome skimming	Low-depth whole genome sequencing
High-scoring Segment Pair (HSP)	A local alignment between the query region and the hit region.
Hit	Sequence with a match to query found by the local alignment search tool, a contig in a typical ALiBaSeq's application
Pseudocontig	Part of the contig, comprised of one or several HSPs, produced by the HSP stitcher; for the small contigs, i.e. transcriptomic or hybrid capture, a pseudocontig may incorporate most of the original contig, while for chromosome-sized contigs, a pseudocontig comprises a small region of the original contig
Query	Sequence used for search, a bait sequence in case of the forward search, and a contig sequence in case of the reciprocal search
Reciprocal search	Operation to match a sample assembly contig sequences to the reference assembly contig sequences
Supercontig	Several pseudocontigs stitched together if contig stitching is enabled; only pseudocontigs derived from different targets (original contigs) are allowed to be stitched
Sequence extraction / retrieval	For the purposes of this manuscript, a procedure of searching and outputting sequence regions homologous to baits from a collection of sequences (e.g., an assembly file)
Target	Sequence in a sample that is homologous to the bait

Table 2. Samples used for software evaluation in conjunction with UCE and ODB SCO datasets.

Sample	Source and version / accession	Number of PE clusters	Assembly Size (bp)	Assembly N contigs	Assembly N50	Assembly L50
Original assembly	*R. prolixus* RproC3.3	NA	706,824,083	16,537	1,088,772	170
Art 1x	*R. prolixus* RproC3.3	1,853,060	55,221,538	103,921	524	35,823
Art 3x	*R. prolixus* RproC3.3	5,548,045	242,206,429	311,385	923	84,342
Art 5x	*R. prolixus* RproC3.3	9,244,580	367,224,880	287,779	1,736	66,353
Art 10x	*R. prolixus* RproC3.3	18,478,995	580,423,518	1,305,726	3,209	41,579
Art 20x	*R. prolixus* RproC3.3	36,965,240	539,547,878	740,413	6,029	20,520
Art 40x	*R. prolixus* RproC3.3	73,929,196	539,002,827	698,488	6,826	18,378

Capture	*R. robustus* SRR7819296	1,776,377	2,357,426	2,384	1,107	851

Table 3. Software used for evaluation

Software	Application	Sequence type (>> - in, << - out)	Search engine	Dependencies	Reciprocal best hit check	Reference
ALiBaSeq	Assembly-based	>> DNA/AA < DNA/AA	External	Python 2 or Python 3, Biopython, search tool (BLAST, HMMER, LASTZ)	Optional	This study
Phyluce	Assembly-based	>> DNA < DNA	LASTZ	Python2, several python packages, LASTZ	No	(Faircloth, 2016)
FortyTwo	Assembly-based	>> DNA < AA	BLAST	Perl, Perl Bio::MUST, BLAST, exonerate	Optional	(Simion et al., 2017)
Assexon	Read- and assembly-based	>> DNA + AA < DNA/AA	Usearch	Perl, several perl modules, Usearch, exonerate, SGA	Mandatory	(Yuan et al., 2019)
aTRAM	Read-based	>> DNA/AA < DNA	BLAST	Python3, BLAST, SPAdes, exonerate	No	(Allen et al., 2018)
HybPiper	Read-based	>> DNA/AA < DNA/AA	BWA, BLAST	Python2, BLAST / BWA, SPAdes, parallel, BWA, samtools	No	(Johnson et al., 2016)
Kollector	Read-based	>> DNA < DNA	BWA	ABYSS, gmap, BWA, samtools	No	(Kucuk et al., 2017)

Table 4. Performance assessment summary

	ALiBaSeq (tblastx)	Assexon	FortyTwo	Phyluce	aTRAM (protein)	HybPiper (protein)	Kollector
Intended input sequence / data type	Assemblies, any type	Reads and assemblies, any type	Assemblies, transcriptomic	Reads and assemblies, any type	Reads, genomic	Reads, hybrid capture	Reads, genomic
Possible bait to target divergence	Low to high	Low	Low to high	Low	Low to high	Low to high	Low
Amount of data obtained on UCE	**14-99% (avg 83%)**	9-71% (avg 58%)	14-98% (avg 65%)	14-95% (avg 77%)	19-100% (avg 83%)	0-100% (avg 37%)	0-7% (avg 5%)
Amount of data obtained on ODB SCO	**37-98% (avg 86%)**	3-4% (avg 4%)	12-90% (avg 63%)	13-42% (avg 36%)	53-89% (avg 80%)	0-96% (avg 32%)	0-1% (avg 0%)
Amount of false positives on UCE	0.2-2.1% (avg 0.6%)	**0-0.1% (avg 0.1%)**	0.1-5.3% (avg 1.4%)	0.1-4.2% (avg 0.8%)	1.6-8.4% (avg 4.6%)	0-12% (avg 3.8%)	0-19.9% (avg 3.3%)
Amount of false positives on ODB SCO	0.7-5.0% (avg 1.7%)	**0% (avg 0%)**	8.5-11.9% (avg 9.7%)	2.4-7.5% (avg 3.8%)	11.2-23.1% (avg 15.1%)	2.5-100% (avg 47.5%)	0-12.5% (avg 4.7%)
UCE locus completeness	90-99% (avg 97%)	87-93% (avg 92%)	**90-100% (avg 98%)**	92-99% (avg 97%)	87-100% (avg 96%)	0-100% (avg 71%)	0-100% (avg 86%)
ODB SCO locus completeness	23-87% (avg 72%)	20-56% (avg 46%)	20-64% (avg 41%)	31-77% (avg 64%)	29-91% (avg 74%)	0-81% (38%)	0-100% (avg 83%)
Speed (assembly-based only)	2 min – 2 h	1 min – 2.5 min	10 min – 2 days	**30 sec – 1 min**	NA	NA	NA
Splicing	Optional	Yes	Yes	No	Optional but coupled with exon stitching	Optional	No
Performance on multi-intron genes (>20 introns)	Good	Poor	Poor	Poor	Good	Good	Poor
Performance on multi-contig genes (>= 2 contigs)	Good	Poor	Average	Average	NA	NA	NA

Figure legends

Figure 1. Software workflow, illustrated by a hypothetical example of a bait for which both an ortholog and several paralogs are present in the sample. For terminology see Table 1. White boxes show contig boundaries, colored boxes represent similarity regions (shown both for the bait and the sample), shading denotes different paralogous genes. Abbreviations are as follows: B1 – bait sequence for the locus 1, C1-3 – assembly contigs 1-3, PC1-2 – pseudocontigs 1-2, SC1-3 – supercontigs 1-3, HSP - high-scoring segment pair.

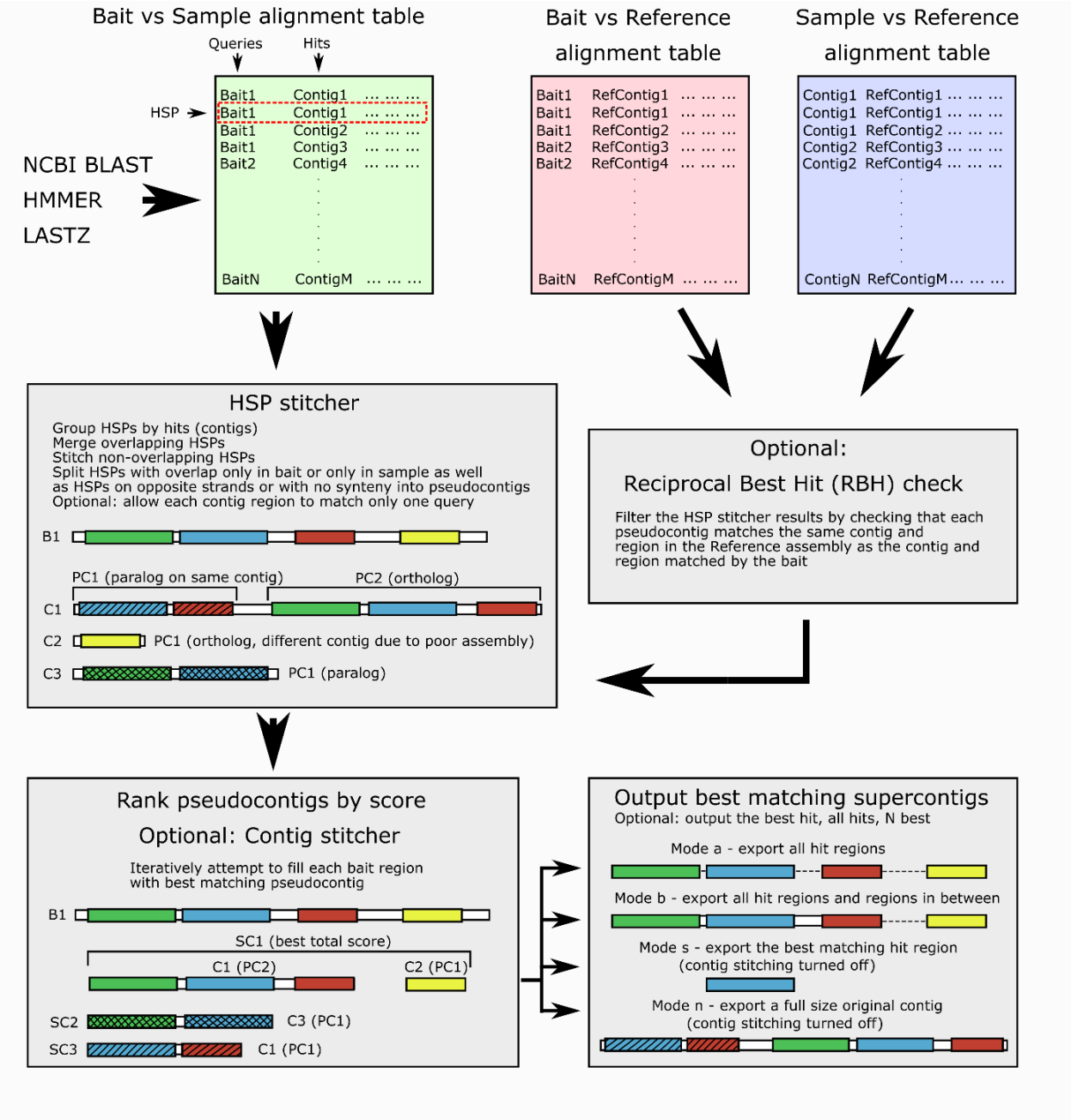


Figure 2. Performance on the UCE dataset. The correct loci panel shows amount of loci with a high sequence identity to the *R. prolixus* bait region sequence, the incorrect loci panel shows amount of loci with a low sequence identity to the *R. prolixus* bait region sequence, the avg bait coverage panel shows the average percentage of the bait region recovered. Vertical panels refer to different datasets (see text for details). Abbreviations are as follows: bln – blastn, blnWC9 – blastn with word length 9bp, dcmgbl – discontinuous megablast, tbln – tblastn, tblx – tblastx, nhmm – nhmmer, phmm – phmmer, phyluceC – capture pipeline of phyluce, assexonR – read-based version of Assexon.

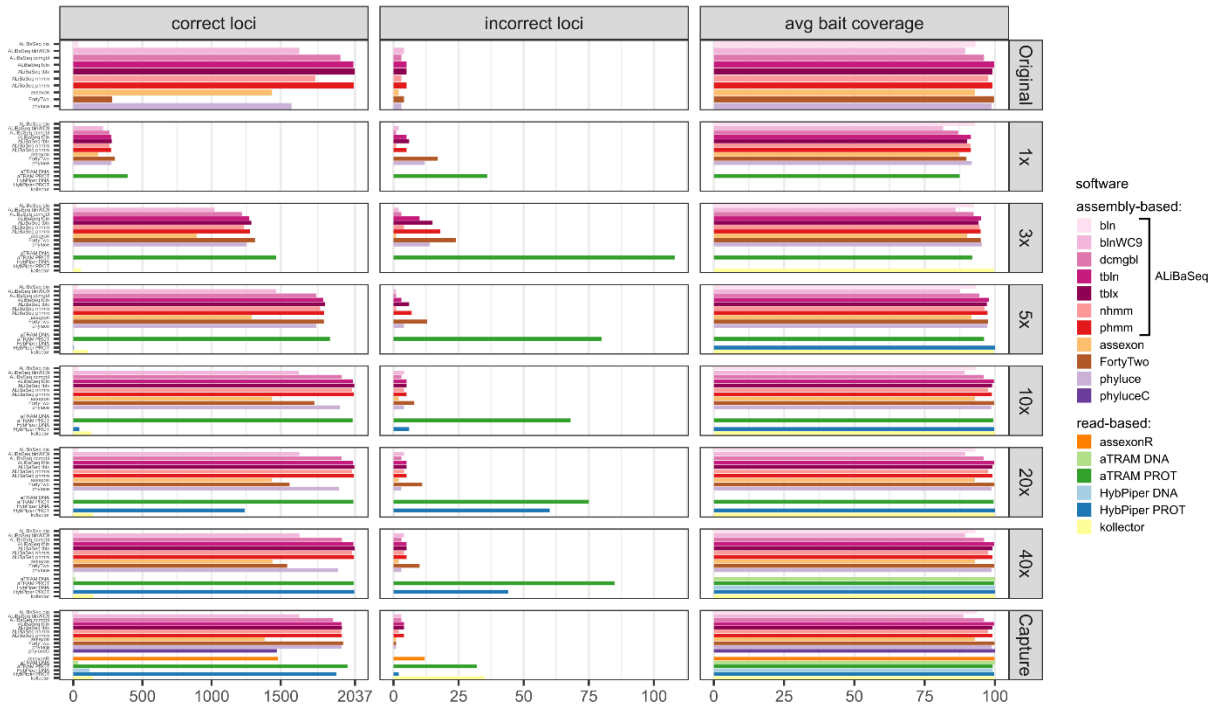


Figure 3. Speed of the processing of the UCE dataset, x axis scale is log-transformed. The analysis panel shows the search and sequence retrieval time (including assembly in case of the read-based tools), the assembly panel shows the assembly stage time for the read-based tools or the database creation time for aTRAM, the total panel shows the total time elapsed.

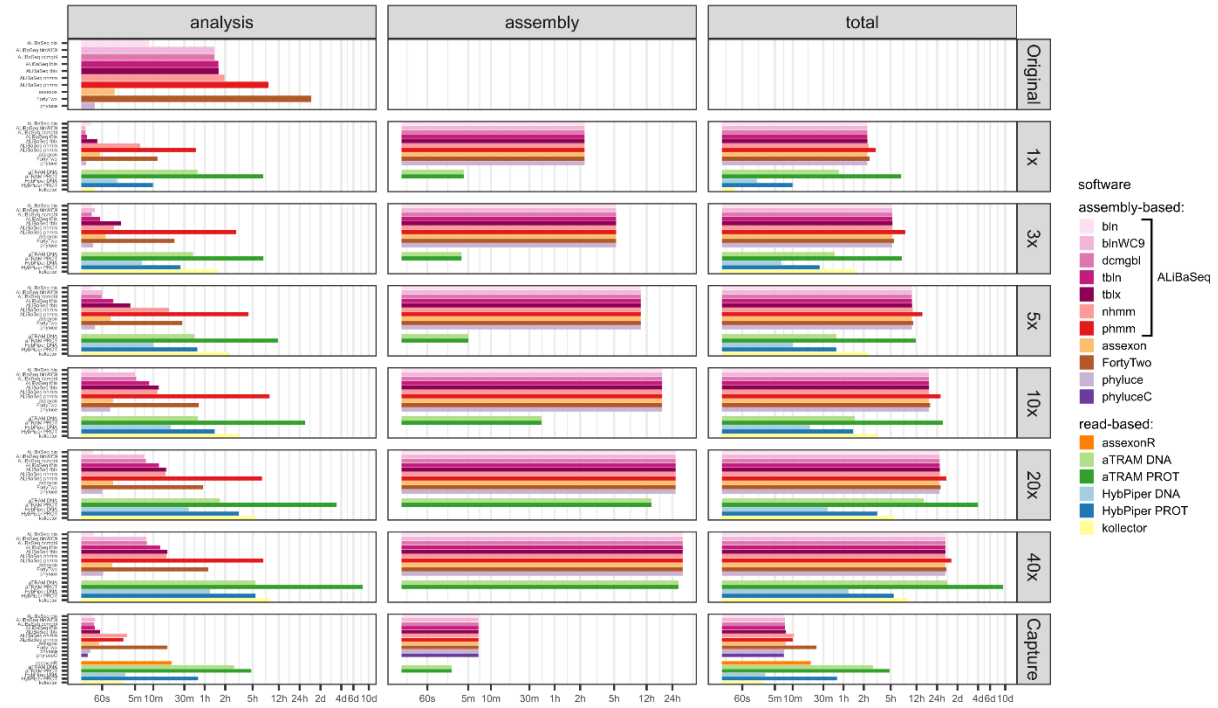


Figure 4. Performance on the ODB SCO dataset. The correct loci panel shows amount of loci with a high sequence identity to the *R. prolixus* bait region sequence, the incorrect loci panel shows amount of loci with a low sequence identity to the *R. prolixus* bait region sequence, the avg bait coverage panel shows the average percentage of the bait region recovered. Vertical panels refer to different datasets (see text for details). Abbreviations are as follows: bln – blastn, blnWC9 – blastn with word length 9bp, dcmgbl – discontinuous megablast, tbln – tblastn, tblx – tblastx, nhmm – nhmmer, phmm – phmmer.

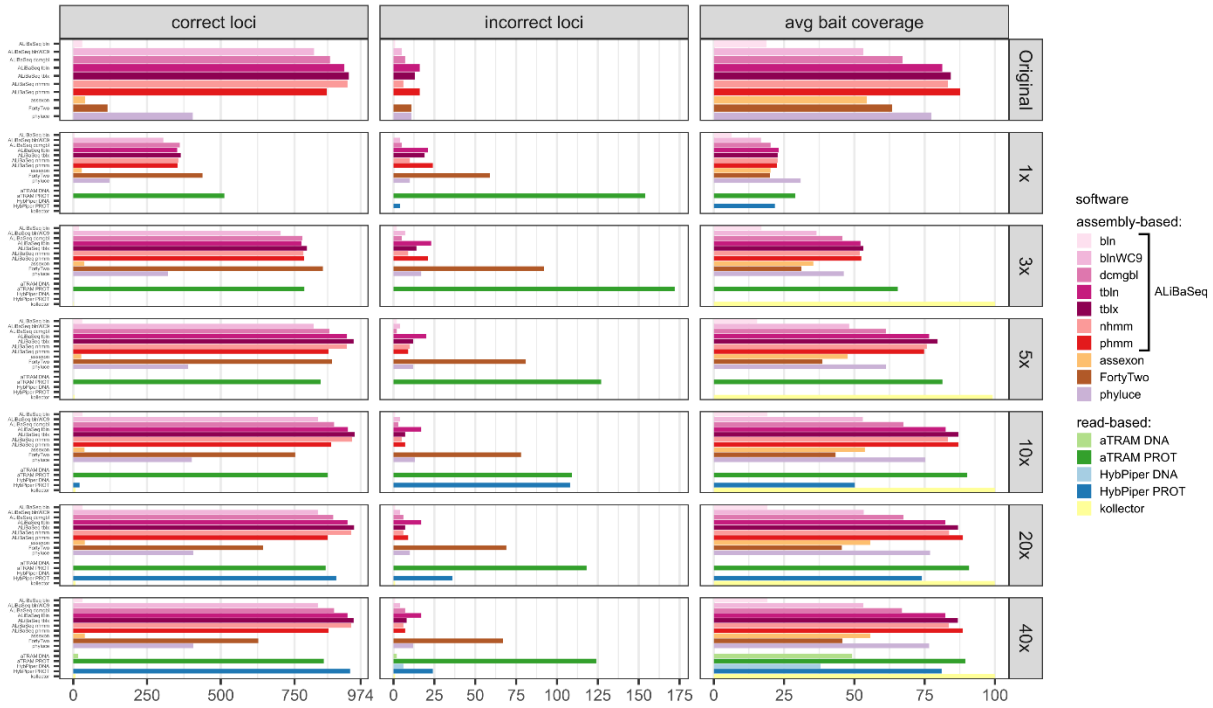


Figure 5. Speed of the processing of the ODB SCO dataset, x axis scale is log-transformed. The analysis panel shows the search and sequence retrieval time (including assembly in case of the read-based tools), the assembly panel shows the assembly stage time for the read-based tools or the database creation time for aTRAM, the total panel shows the total time elapsed.

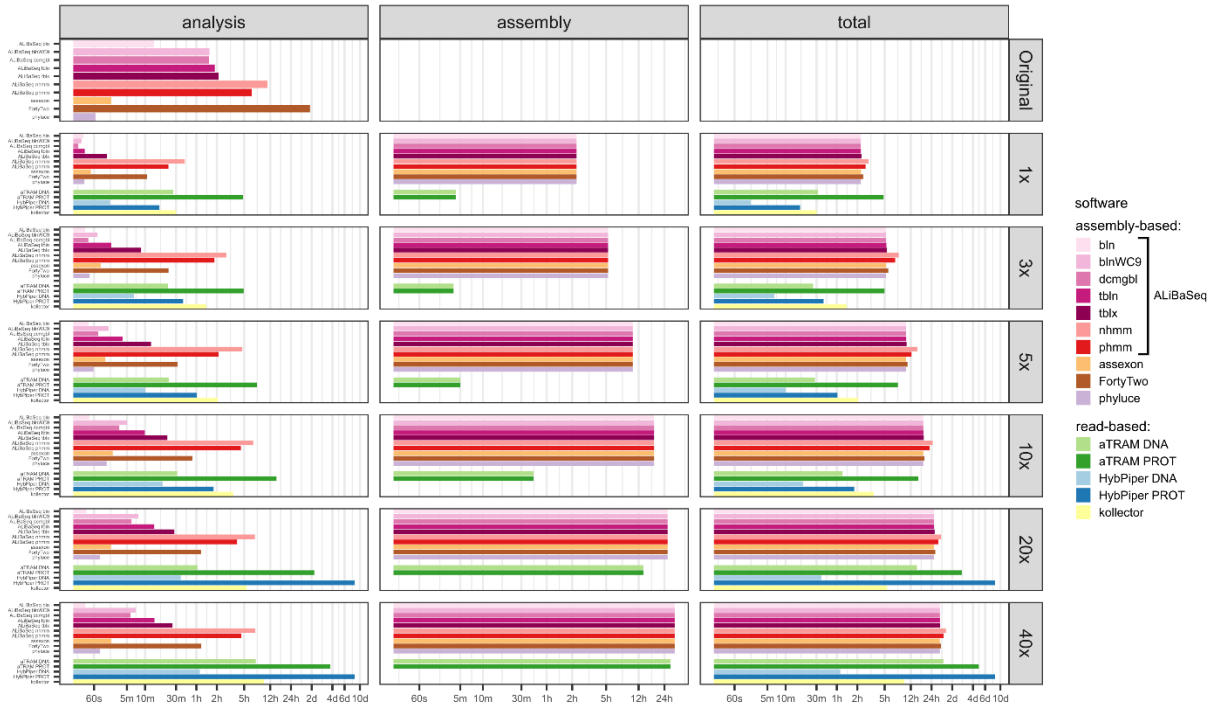


Figure 6. Relationship between the number of introns in a locus and its coverage in the software output. Points represent correct loci found by each program using the 40x ODB SCO dataset.

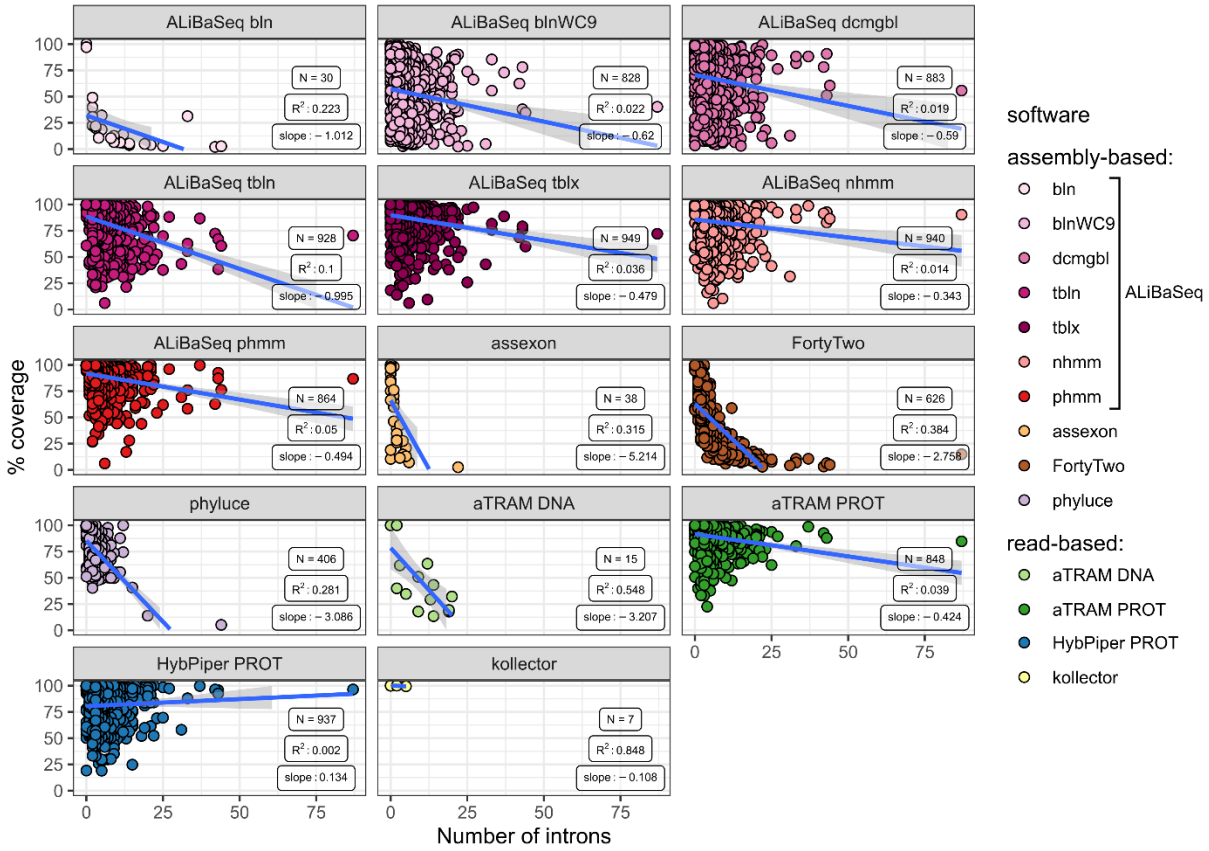
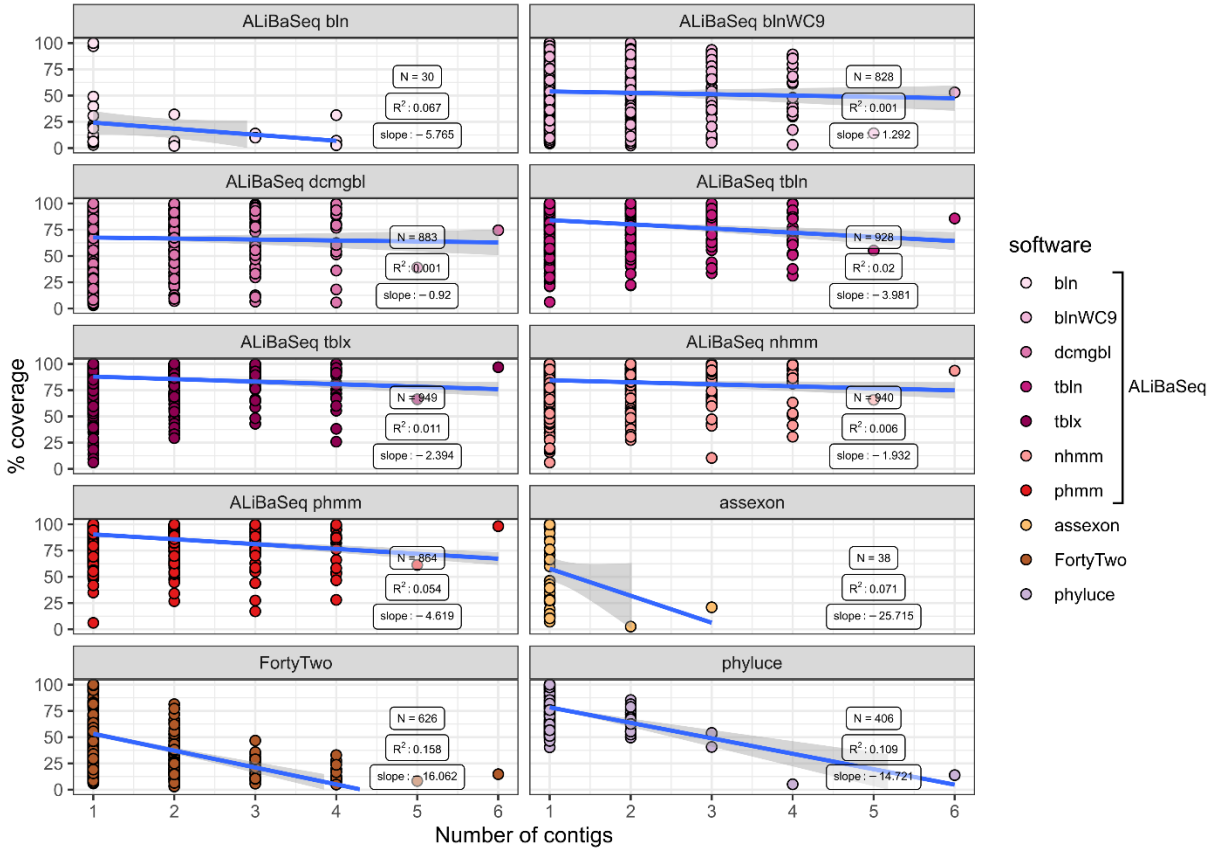


Figure 7. Relationship between the number of contigs that a locus is represented by in the assembly and its coverage in the software output. Only assembly-based programs are shown. Points represent correct loci found by each program using the 40x ODB SCO dataset.



Supplementary data.

Supplemental text S1. Details of the plant ODB SCO dataset preparation, assessment methods, and results.

Table S1. List of UCE, insect ODB SCO, and plant ODB SCO loci used for the evaluations.

Table S2. Program versions and settings used for evaluations.

Table S3. Performance data on the UCE dataset. Abbreviations are as follows: bln – blastn, blnWC9 – blastn with word length 9bp, dcmgbl – discontinuous megablast, tbln – tblastn, tblx – tblastx, nhmm – nhmmer, phmm – phmmer, phyluceC – capture pipeline of phyluce, assexonR – read-based version of Assexon, ST – strict RBH check mode, RX – relaxed RBH check mode.

Table S4. Performance data on the ODB SCO dataset. Abbreviations are as follows: bln – blastn, blnWC9 – blastn with word length 9bp, dcmgbl – discontinuous megablast, tbln – tblastn, tblx – tblastx, nhmm – nhmmer, phmm – phmmer, ST – strict RBH check mode, RX – relaxed RBH check mode.

Figure S1. Performance on the plant ODB SCO dataset. The correct loci panel shows amount of loci with a high sequence identity to the *A. trichopoda* bait region sequence, the incorrect loci panel shows amount of loci with a low sequence identity to the *A. trichopoda* bait region sequence, the avg bait coverage panel shows the average percentage of the bait region recovered. Vertical panels refer to different datasets and RBH check modes: original – original assembly with relaxed RBH check (ALiBaSeq only), original strict – original assembly with strict RBH check, 40x – simulated reads with 40x depth and relaxed RBH check (ALiBaSeq only), 40x strict – simulated reads with 40x depth and strict RBH check. Abbreviations are as follows: dcmgbl – discontinuous megablast, tblx – tblastx.