Manual for Sondovač 0.9 beta

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Sondovač[1] is a script to create orthologous low-copy nuclear probes from transcriptome and genome skim data for target enrichment.

Abstract

Phylogenetics benefits from using a large number of putatively independent nuclear loci and the combination with other sources of information, such as the plastid and mitochondrial genome. Selecting such orthologous low-copy nuclear (LCN) loci is still a challenge for non-model organisms. In recently published phylogenies based on target enrichment of several hundred LCN genes, these loci were selected from transcriptomes, genomes, gene expression studies, the literature, or a combination of these sources. Automated bioinformatic pipelines for the selection of LCN genes are, however, largely absent. We created a user-friendly, automated and interactive script named Sondovač to design LCN loci by a comparison between transcriptome and genome skim data. The script is licensed under open-source license GPL v.3 allowing further modifications. It runs on major Linux distributions and Mac OS X. Strong bioinformatics skills and access to high-performance computer clusters are not required; Sondovač runs on a standard desktop computer equipped with modern CPU like Intel i5 or i7.

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[1] English pronunciation is "Sondovach".

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Sondovač is a script to create orthologous low-copy nuclear probes from transcriptome and genome skim data for target enrichment. For information and download see https://github.com/V-Z/sondovac/wiki.

1 Introduction

High-throughput sequencing (HTS) has the potential to greatly increase the amount of phylogenetically informative signal in molecular datasets (Parks et al., 2009, 2012) and overcome difficulties in phylogenetic reconstructions, such as polytomies and low support values, that are often the result of using only a small fraction of the genome. However, HTS also "opens the era of real incongruence" (Jeffroy et al., 2006), and even massive amounts of sequence data do not always result in strongly resolved phylogenies (Pyron, 2015).

Currently, target enrichment (sequence capture) of hundreds of loci is becoming increasingly popular in phylogenetics. In animal phylogenomics non-exonic or partly exonic ultraconserved elements and their quite variable flanking regions are often utilized (e.g. Faircloth et al., 2012; Hedtke et al., 2013; Smith et al., 2014). For plant phylogenetics, low-copy nuclear (LCN) genes are targeted (Mandel et al., 2014; Weitemier et al., 2014; Grover et al., 2015; Heyduk et al., 2015; Mandel et al., 2015; Nicholls et al., 2015; Stephens et al., 2015a,b) due to the paucity of ultraconserved nuclear sequences (Reneker et al., 2012). Target sequencing strategies for plant nuclear genomes are largely lineage-specific, requiring the de novo design of target

enrichment probes. Chamala et al. (2015) recently introduced a pipeline for phylogenetic marker development in angiosperms using transcriptomes, and they obtained several hundred putative LCN genes that can be utilized at three phylogenetic levels (genus, family, order); however empirical evidence for the phylogenetic utility of these loci was not demonstrated. Alternative phylogenetic marker developments, also utilizing transcriptomes (Pillon et al., 2014; Rothfels et al., 2013; Tonnabel et al., 2014), resulted in a much smaller number (up to 20) of mainly LCN loci, but these loci were evaluated with PCR in the empirical datasets, not target enrichment. In recently published phylogenies based on target enrichment of several hundred LCN genes these loci were selected from transcriptomes, gene expression studies, the literature, or a combination of these sources (Mandel et al., 2014; Grover et al., 2015; Heyduk et al., 2015; Mandel et al., 2015; Nicholls et al., 2015; Stephens et al., 2015a,b). Weitemier et al. (2014) designed LCN probes for target enrichment based on a combination of transcriptome and genome data. The limitation of this probe design pipeline is that (draft) genomes are still infrequent, especially for non-model species, and are costly to generate. This limitation also applies to the approach of de Sousa et al. (2014), who selected 50 LCN loci from a genomic source and amplified them using target enrichment. Except for Chamala et al. (2015), who offer a user-friendly but empirically untested probe design pipeline, and Weitemier et al. (2014), whose Hyb-Seq pipeline is designed for more advanced users, no automated probe design pipeline for LCN genes is currently available.

In this study we developed a novel probe design pipeline for targeting orthologous LCN loci for phylogenetic reconstruction by using genome skim and transcriptome data. In particular, genome skim data of one accession of the studied plant group were combined with a congeneric transcriptome from the 1000 Plants (1KP) initiative (http://www.onekp.com/). We implemented our software workflow in the user-friendly, automated and interactive BASH script Sondovač, which allows a straightforward design of LCN probes also for users with limited bioinformatics skills.

Sondovač workflow is divided into three parts (see details at page 5 and Figure 1):

1. Raw input data are analyzed by `sondovac_part_a.sh`.
2. Obtained sequences are assembled by Geneious manually by user.
3. Final probes are produced by `sondovac_part_b.sh`.

1.1 Pipeline – how the data are processed

A transcriptome assembly and paired-end genome skim raw data are combined to get hundreds of orthologous LCN loci. Enrichment of multi-copy loci is minimized by using unique transcripts only, which are obtained by comparing all transcripts and removing those sharing ≥90% sequence similarity using BLAT. Before matching the genome skim data against those unique transcripts, reads of plastid (and mitochondrial) origin are removed with Bowtie 2, SAMtools and bam2fastq utilizing reference sequences. Paired-end reads are subsequently combined with FLASH. These processed reads are matched against the unique transcripts sharing ≥85% sequence similarity with BLAT. Transcripts with >1000 BLAT hits, indicating repetitive elements, and BLAT hits containing masked nucleotides are removed before de novo assembly of the BLAT hits to larger contigs with Geneious, using the medium sensitivity / fast setting. After assembly, only those contigs that comprise exons of a minimum bait length (usually ≥120 bp in case of probe design for phylogenies) and have a certain minimum total locus length (multiple of the bait length, should not be too short in order to obtain sufficient phylogenetically informative signal; we recommend at least ≥600 bp) are retained. To ensure that probes do not target multiple similar loci, any probe sequences sharing ≥90% sequence similarity are removed using cd-hit-est, followed by a second filtering step for contigs containing exons of a minimum bait length and totaling minimum loci length (see comments above). To ensure that plastid sequences are absent from the probes, the probe sequences are matched against the plastome reference sharing

≥90% sequence similarity with BLAT and the hits removed from the probe set. The workflow of Sondovač is summarized in the Figure 1. The direction of the workflow is indicated by arrows. Optional removal of reads of mitochondrial origin from the genome skim data is marked by light background. The required input files of Sondovač are highlighted in bold.

For comprehension improvement the steps of Sondovač are consecutively numbered. Sondovač has three parts: two script parts and an intermediate part using Geneious. The workflow is as follows:

A. `sondovac_part_a.sh`: Covers steps 1 to 6.

1. Removal of transcripts sharing ≥90% sequence similarity.
2. Removal of reads of plastid origin.
3. Removal of reads of mitochondrial origin (optional).
4. Combination of paired-end reads.
5. Matching of the unique transcripts and the filtered, combined genome skim reads sharing ≥85% sequence similarity.
6. Filtering of BLAT output:
6.1. Choice of transcript or genome skim sequences for further processing.
6.2. Removal of transcripts with >1000 BLAT hits.
6.3. Removal of transcript or genome skim BLAT hits [depending on the selection in (6.1)] containing masked nucleotides.

Input files for `sondovac_part_a.sh` are FASTA transcriptome data, FASTQ paired-end genome skim reads and a plastome (and possible as well mitochondriome) reference. The input file for Geneious is the output of `sondovac_part_a.sh`.

B. Geneious: Covers step 7 (see page 10).

7. De novo assembly of the transcript or genome skim BLAT hits [depending on the selection in (6.1)] to larger contigs. Note you need a copy of Geneious for this step.

The output files of Geneious are input files for `sondovac_part_b.sh`.

C. `sondovac_part_b.sh`: Covers steps 8 to 11.

8. Retention of those contigs that comprise exons ≥ bait length and have a certain total locus length.
9. Removal of probe sequences sharing ≥90% sequence similarity.
10. Retention of those contigs that comprise exons ≥ bait length and have a certain total locus length.
11. Removal of probe sequences sharing ≥90% sequence similarity with the plastome reference.

The output file of `sondovac_part_b.sh` is the final list of probes.

When Sondovač starts, directory `bin` is created in current working directory. Sondovač saves here binaries of required software packages (if they are not available). User can then add this directory to PATH, move or delete it afterward.

2 Installation of Sondovač

Sondovač is simple BASH script, but it requires some software. The script will check for presence of all required software and if needed, it will offer installation. The most easy way is just to launch the script (see chapter 2.4 at page 8) and let yourselves to be guided through whole needed process.

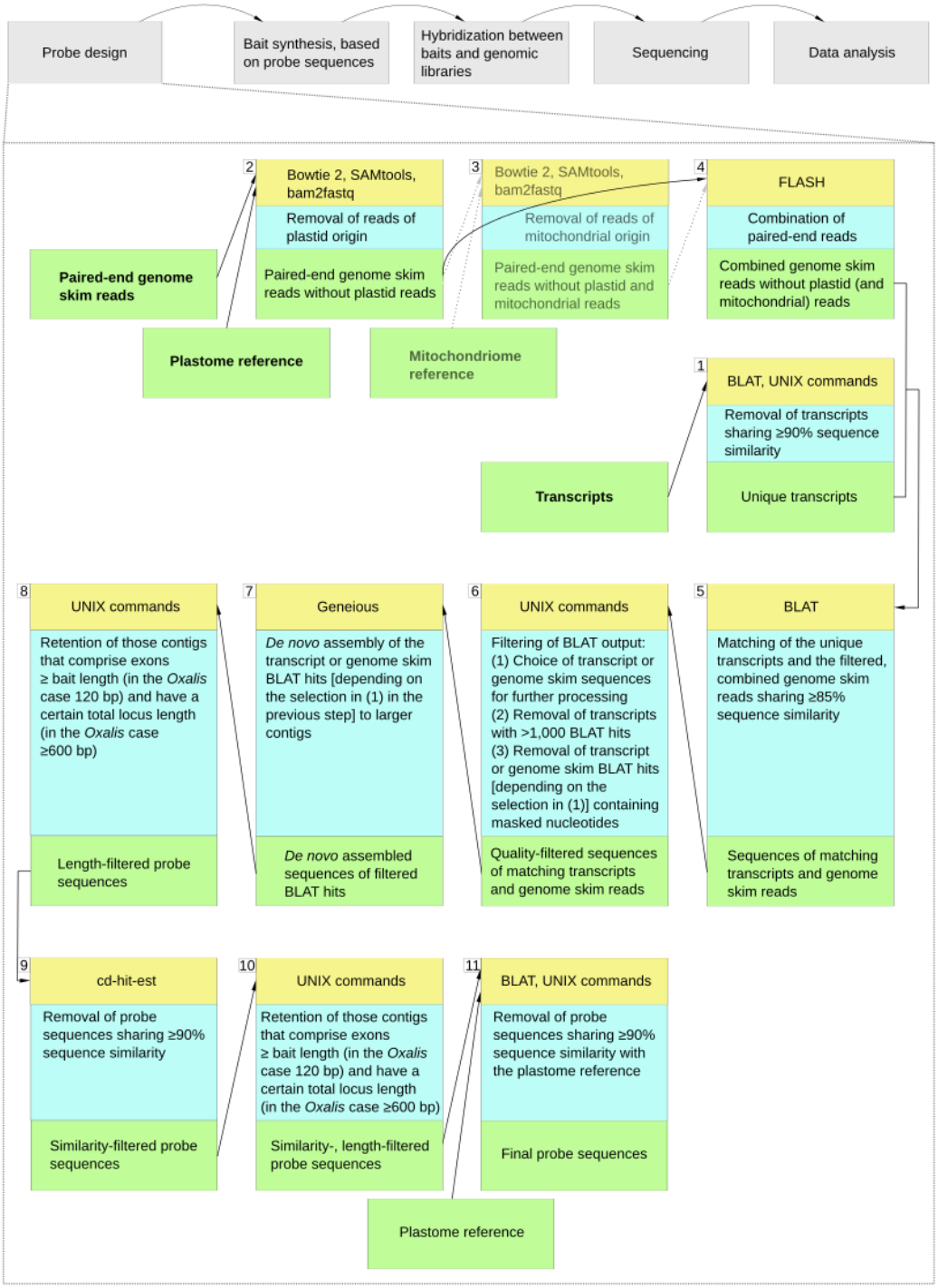

Figure 1: Workflow of the probe design script Sondovač. An overview on the main steps of Hyb-Seq are given in the top part of the figure; probe design is the first one. Each step of Sondovač is numbered and illustrated by three boxes each: Software is highlighted in yellow, a summary of each step is given in light blue, and input/output of each step is depicted in light green. Optional removal of reads of mitochondrial origin from the genome skim data is marked by decoloration of the text. The required input files of Sondovač are highlighted in bold. The direction of the workflow is indicated by arrows.

2.1 Requirements to run Sondovač

Sondovač is currently tested on the Linux distributions Ubuntu and openSUSE. Testing on Mac OS X and other major Linux distributions is on the way and full support will be added soon. Thank you for patience and reporting[2] any problems and wishes.

In order to run Sondovač you need a UNIX-based operating system (preferably Linux or Mac OS X) equipped with BASH or compatible shell interpreter (this should be by default available for any Linux distribution, Mac OS X and any other UNIX-based operating system like Solaris, BSD and its variants etc). You should use the current operating system version supported by upstream. Otherwise we will not be able to help you in case of problems. Older operating systems can have different versions of shell and system libraries, which can cause various problems and incompatibilities.

Sondovač is using several scientific software packages (namely bam2fastq, BLAT, Bowtie2, CD-HIT, FASTX toolkit, FLASH, Geneious, htsjdk, libgtextutils, Picard and SAMtools – see required versions and links, Table 1), and basic UNIX tools. Sondovač will check if those programs are installed – available in the PATH (i.e. if the shell application can locate and launch respective binaries). If you have those packages installed (in current versions), ensure their binaries are in PATH. This should not be a problem for basic tools available in any UNIX-based operating system, as basic installation usually contains all needed tools. If you are lacking some of the required tools, the script will notify you, and you will have to install them manually. If this will be needed, check the documentation for your operating system.

If required scientific programs are not installed, Sondovač will offer you installation. You can use precompiled binaries available together with the script or (sometimes) from the web. This is the recommended way. In case you would like to compile required software yourselves, the script will guide you through this process. Anyway, it is recommended only for advanced users, as compilation might sometimes be very tricky. It is currently fully implemented only for the Linux distributions Ubuntu and openSUSE. Support for other Linux distributions and Mac OS X will be added soon. For compilation you need Apache Ant, GNU G++, GNU GCC, GIT, Java, libpng developmental files and zlib developmental files. Ensure you have those tools available – they should be readily available for any UNIX-based operating system.

Following UNIX tools are required to run Sondovač. They are usually readily available in Mac OS X, any Linux distribution or another UNIX system, so there is usually no need to install them manually. The tools are `awk`, `bc`, `bunzip2`, `cat`, `cp`, `curl`, `cut`, `dirname`, `echo`, `egrep`, `cd`, `g++`, `gcc`, `grep`, `gunzip`, `join`, `less`, `lsb_release`, `make`, `mkdir`, `perl`, `pkg-config`, `pwd`, `sed`, `sort`, `tar`, `tr`, `uname`, `uniq`, `unzip`, `wc`. Not all tools are required every time – some are used only during particular actions (e.g. when user decide to compile required software manually). And user usually do not need to bother about them. See also details in following subchapters for some common Linux distributions and Mac OS X.

2.2 Installation of required software in Linux

XXX

2.2.1 openSUSE and SUSE Linux Enterprise

XXX

2.2.2 Debian and derivatives

XXX

[2] You can report any problems at https://github.com/V-Z/sondovac/issues. We will try to help you.

2.2.3 Ubuntu and derivatives

XXX

2.2.4 Linux Mint

XXX

2.2.5 Fedora, RedHat, Centos and Scientific Linux

XXX

2.3 Installation of required software in Mac OS X

XXX

2.4 First launch of Sondovač

Download the latest version from https://github.com/V-Z/sondovac/releases/ and unpack the archive. You can run Sondovač from any directory. In command line navigate to the directory with the unpacked Sondovač files (see Figure 2):

```
cd /path/to/directory_with_sondovac
```

and start it by

```
./sondovac_part_a.sh -h
```

to see basic usage instructions. See chapter 3 at page 14 for more information.

2.4.1 Examples

See page 14 for explanation of command line parameters. Basic and the most simple usage (running in interactive mode, see chapter 3 at page 14):

```
./sondovac_part_a.sh -i
```

Specify some of required input files, otherwise run interactively:

```
./sondovac_part_a.sh -i -f input.fa -t reads1.fastq -q reads2.fastq
```

Running in non-interactive automated way (parameter "`-n`", see chapter 3 at page 14) with example data downloaded from https://github.com/V-Z/sondovac/wiki/Sample-data:

```
./sondovac_part_a.sh -f input1_JHCN_Oxalis_corniculata_transcriptome_data.fa \
 -c input2_Ricinus_communis_reference_plastid_genome.fsa -m \
 input5_Ricinus_communis_reference_mitochondrial_genome.fasta -t \
 input3_J12_Oxalis_obtusa_genome_skim_data_R1.fastq -q \
 input4_J12_Oxalis_obtusa_genome_skim_data_R2.fastq -n
```

Modify parameter "`-a`", otherwise run interactively:

```
./sondovac_part_a.sh -i -a 300
```

Run in non-interactive mode (parameter "`-n`", see chapter 3 at page 14) – in such case user must specify all required input files (parameters "`-f`", "`-c`", "`-m`", "`-t`" and "`-q`"). Moreover, parameter "`-y`" is modified:

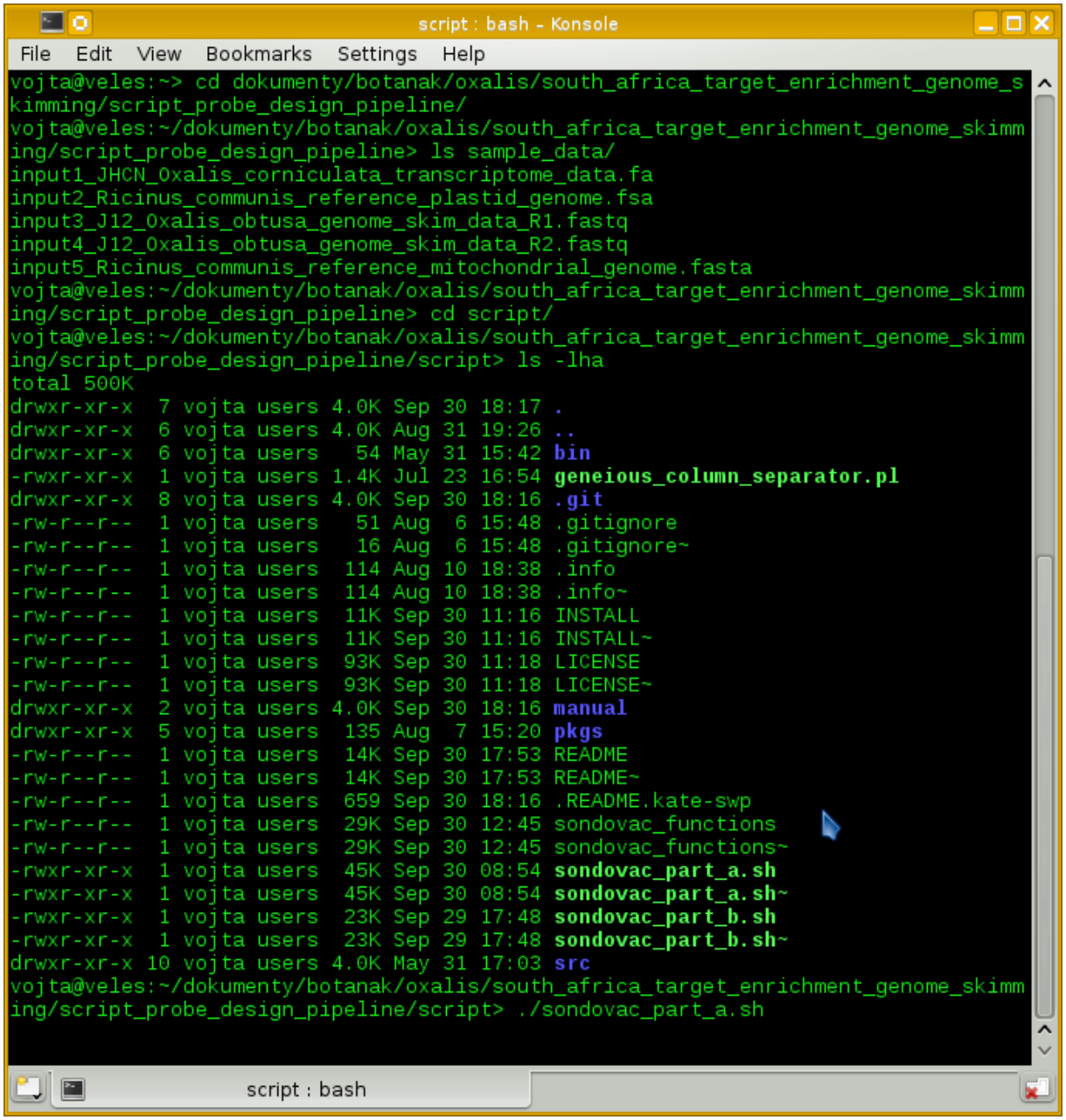

Figure 2: Starting terminal and navigating to Sondovač. First look at the terminal (command-line, shell) window, navigation to directory with Sondovač (using command `cd`), listing directory content (command `ls`) and preparing to launch Sondovač (`./sondovac_part_a.sh`).

```
./sondovac_part_a.sh -n -f input.fa -c referencecp.fasta \
 -m referencemt.fsa -t reads1.fastq -q reads2.fastq -y 90
```

Modifying parameter "`-s`". Note interactive mode "`-i`" is implicit and does not need to be specified explicitly:

```
./sondovac_part_a.sh -s 950
```

2.5 Help for usage of terminal

If you are not familiar with the usage of command line, see some basic tutorial first. You can try some of those:

- https://activedoc.opensuse.org/book/opensuse-start-up/chapter-17-shell-basics
- https://help.ubuntu.com/community/UsingTheTerminal
- https://www.gnu.org/software/bash/manual/ (advanced – full reference manual)
- http://ubuntuguide.org/
- https://www.debian.org/doc/manuals/debian-reference/ch01.en.html
- https://trapa.cz/en/course-linux-command-line-2015
- https://docs.fedoraproject.org/en-US/Fedora/22/html/System_Administrators_Guide

2.6 Geneious

For the part (2) user must have Geneious (Kearse et al., 2012). Geneious is a DNA alignment, assembly, and analysis software and one of the most common software platforms used in genomics. It is utilized for de novo assembly in Sondovač. We plan to replace it by some free open-source command line tool in some future release of Sondovač. Visit http://www.geneious.com/ for download, purchase, installation and usage of Geneious. It is very feature-rich application. The input data are processed (interactively or not) by `sondovac_part_a.sh` and then user must process its output manually by Geneious according instructions below. Output of Geneious is then processed by `sondovac_part_b.sh`, which produce final probe set. Geneious was tested with versions 6, 7 and 8.

Import output file of part A of the script (`sondovac_part_a.sh`): go to menu **File | Import | From File...** This file is named as: `YourInputFile_blat_unique_transcripts_versus_genome_skim_data-no_missing_fin.fsa`

Select the file and go to menu **Tools | Align / Assemble | De Novo Assemble**. In **Data** frame select **Assembe by 1st (...) Underscore**. In **Method** frame select **Geneious Assembler** (if you don't have other assemblers, this option might be missing) and **Medium Sensitivity / Fast Sensitivity**

In **Results** frame check **Save assembly report**, **Save list of unused reads**, **Save in sub-folder**, **Save contigs** (do not check **Maximum**) and **Save consensus sequences**. Do not trim. Otherwise keep defaults. Run it. Geneious may warn about possible hanging because of big file. Do not use Geneious for other tasks during the assembly. Running may take long time.

Select all resulting contigs (typically named *** Contig #**) and export them (go to menu **File | Export | Selected Documents...**) as **Tab-separated table values (*.tsv)**. Save following columns (Hold `Ctrl` key to mark more fields): **# Sequences**, **% Pairwise Identity**, **Description**, **Mean Coverage**, **Name** and **Sequence Length**. If this option would be inaccessible for you, export all columns. Warning! Do not select and export *** Consensus Sequences**, *** Unused Reads** or *** Report** – only the individual *** contig #** files.

Select items **Consensus Sequences** and **Unused Reads** and export them as one **FASTA**. Go to menu **File | Export | Selected Documents...** and choose **FASTA file type**.

Use exported files from Geneious as input for part B of the script (`sondovac_part_b.sh`).

2.7 Software used by Sondovač

Table 1 lists all software used by Sondovač, including minimal required versions and homepages. As soon as you have recent version of your operating system and you are using automated way of installation of additional software offered by Sondovač, you do not have to worry about this. In case you installed some of required scientific packages manually, ensure you have required minimal version. Following list refers papers and web resources describing methods used by software used by Sondovač:

Table 1: Required software, its versions and homepages. "X" denotes any subversion of particular lineage and "v. >" denotes any version higher then noted. Generally, any current version should usually be fine.

Software	**Version**	**Homepage**
Apache Ant	1.9.X	https://ant.apache.org/
bam2fastq	1.1.0	http://gsl.hudsonalpha.org/information/software/bam2fastq
BASH	v. > 3	https://gnu.org/software/bash/bash.html
BLAT	v.36	http://genome.ucsc.edu/FAQ/FAQblat.html
Bowtie2	2.2.6	http://bowtie-bio.sourceforge.net/bowtie2/index.shtml
CD-HIT	4.6	http://weizhongli-lab.org/cd-hit/
FASTX, libgtextutils	0.0.13	http://hannonlab.cshl.edu/fastx_toolkit/
FLASH	1.2.11	http://sourceforge.net/projects/flashpage/
G++, GCC	v. > 4.2	http://gcc.gnu.org/
Geneious	v. > 6.1	http://www.geneious.com/
GIT	v. > 2.0	http://git-scm.com/
GNU core utils	8.X	https://gnu.org/software/coreutils/coreutils.html
Java/OpenJDK	v. > 7	https://www.java.com//http://openjdk.java.net/
libpng	1.6.X	http://www.libpng.org/
Picard	v. > 1.137	https://broadinstitute.github.io/picard/
SAMtools, htsjdk	1.2	http://www.htslib.org/
Sondovač	0.9	https://github.com/V-Z/sondovac/wiki
zlib	1.2.8	http://zlib.net/

`sondovac_part_a.sh` requires (and will install) following software packages:

- BLAT
- Bowtie2
- SAMtools
- bam2fastq (will be replaced by Picard in future release)
- FLASH
- FASTX-toolkit

`sondovac_part_b.sh` requires (and will install) following software packages:

- CD-HIT
- BLAT

Papers describing the software used by Sondovač:

BLAT Kent (2002): BLAT – the BLAST-like alignment tool.

Bowtie2 Langmead and Salzberg (2012): Fast gapped-read alignment with Bowtie 2.

CD-HIT There are several papers describing CD-HIT:

- Li et al. (2001): Clustering of highly homologous sequences to reduce the size of large protein databases.
- Li et al. (2002): Tolerating some redundancy significantly speeds up clustering of large protein databases.
- Li and Godzik (2006): Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences.
- Fu et al. (2012): CD-HIT: accelerated for clustering the next generation sequencing data.
- Huang et al. (2010): CD-HIT Suite: a web server for clustering and comparing biological sequences.
- Niu et al. (2010): Artificial and natural duplicates in pyrosequencing reads of metagenomic data.
- Li et al. (2012): Ultrafast clustering algorithms for metagenomic sequence analysis.

FASTX toolkit Gordon and Hannon (2010): FASTX-Toolkit. FASTQ/A short-reads preprocessing tools.

FLASH Magoč and Salzberg (2011): FLASH: fast length adjustment of short reads to improve genome assemblies.

Geneious Kearse et al. (2012): Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data.

SAMtools There are several papers describing SAMtools:

- Li et al. (2009): The Sequence alignment/map (SAM) format and SAMtools.
- Li (2011a): A statistical framework for SNP calling, mutation discovery, association mapping and population genetical parameter estimation from sequencing data.
- Li (2011b): Improving SNP discovery by base alignment quality.

Sondovač XXX

2.8 Vocabulary

Binary An application in form understandable by the computer, but usually not transferable among operating systems and/or hardware platforms. Binaries in Windows usually have extension *.exe, in UNIX there use to be no extension.

BASH "The command line" – fully featured programming scripting language accessible through terminal of any UNIX-based operating system (any Linux, Mac OS X, Solaris, any variant of BSD and more). BASH scripts usually have extension *.sh.

BSD Group of popular UNIX-based operating systems. See https://en.wikipedia.org/wiki/Berkeley_Software_Distribution.

Compilation "Translation" of software application from the source code (text readable by human programmer) into binary form launchable by the computer. It requires special tools, and it usually must be done for every operating system and hardware platform.

Console See "Shell".

GNU Major project providing free software widely used in many operating systems, see https://gnu.org/.

Library Pack of software tools and functions used by another applications.

Linux One of the most common variants of UNIX-based operating systems. Linux kernel is used by many developers, so that there are plenty of Linux distributions ("flavors") from various sources (e.g. Ubuntu and derivatives, openSUSE, SLE, Debian, Linux Mint, Fedora, Centos, RedHat etc.). They share many features although on the first look they can look differently. See https://en.wikipedia.org/wiki/Linux.

Mac OS X Popular operating system produced by Apple. The system kernel is based on UNIX, see https://www.apple.com/osx/.

openSUSE Popular Linux distribution, see https://www.opensuse.org/.

Operating system Basic system running on your computer – typically MS Windows (not supported by Sondovač, although it might be working), Mac OS X or some Linux distribution (Ubuntu and derivatives, openSUSE, SLE, Debian, Linux Mint, Fedora, Centos, RedHat etc.).

Parameter(s) Option(s) passed to any function/command line application to modify its usage. Some can be required, some are optional and some can be used only in particular cases. In case of shell applications, parameters are usually given in way like "application -X", "application -parameter", "application -Param SomeValue" and so on. See manual for particular application (e.g. "man application"), in case of Sondovač see page 14.

PATH Directories in the computer where the system is looking for installed software (in a UNIX-based system you can view it by the command "`echo $PATH`"). If you need to modify it manually, see the documentation for your operating system.

Script Software application. It requires an interpreter (application installed on the computer that is able to launch scripts written in a particular language), but the application itself is portable among operating systems and hardware architectures, and it is written in plain text, so that developers can easily modify it. Common examples are Python, Perl or BASH.

Shell "The command line" – the interface to interact with software using commands typed into the terminal window (See Figure 2).

Solaris Popular (mainly on servers) UNIX-based operating system, now developed by Oracle and including several independent clones. See http://distrowatch.com/table.php?distribution=solaris.

Terminal see "Shell".

Ubuntu Popular Linux distribution, see http://www.ubuntu.com/.

Upstream Developers usually support (e.g. by fixing of bugs) only newer versions of an application. If you use an older version and you encounter problems, no one will probably help you. Moreover, using old versions of software can be a security risk because of security issues fixed in newer versions.

UNIX (UNIX-like, UN*X, *nix, ...) Family of operating systems sharing the same logic, software architecture and plenty of tools. See https://en.wikipedia.org/wiki/Unix-like for details.

3 Usage of Sondovač

3.1 Command line parameters

Sondovač has some parameters useful especially for advanced users, on remote servers, for repeated analysis and so on. We recommend to start with basic interactive usage – script will ask for input files and when needed also for installation of additional software.

```
# Go to directory with unpacked Sondovač (in terminal):
cd /path/to/directory_with_sondovac
# Run sondovac_part_a.sh in basic interactive mode:
./sondovac_part_a.sh -i
# Then run Geneious and continue with sondovac_part_b.sh:
./sondovac_part_b.sh -i
```

3.1.1 General parameters

Shared by `sondovac_part_a.sh` as well as `sondovac_part_b.sh`.

`-h, -v` Print help message and exit.

`-u` Check for updates. If there is newer version of Sondovač available on https://github.com/V-Z/sondovac/releases/ download of newer version will be offered to the user.

`-l` Display LICENSE for license information (this script is licensed under GNU GPL v.3, other software under variable licenses). Exit viewing by pressing the `Q` key.

`-r` Display README for detailed usage instructions. Exit viewing by pressing the `Q` key.

`-p` Display INSTALL for detailed installation instructions. Exit viewing by pressing the `Q` key. See also page 5.

`-e` Display detailed citation information and exit.

`-o` Set name of output files. Output files will start with that name. Do not use spaces or special characters - some software can not handle them correctly. Default value (if user does not provide) another name is ”output”. See below for list of produced output files.

`-i` Running in interactive mode – script will on-demand ask for required input files, installation of missing software etc. This is recommended default value (the script runs interactively without explicit using option `-n`).

`-n` Running in non-interactive mode. User must provide at least required input files (see below). You can use only one of parameters `-i` or `-n` (not both of them). If script fails to find some of required software packages, it will exit. This is recommended for batch or repeated analysis, on remote servers and for more advanced users. User must be sure that all required software is installed (see page 5).

3.1.2 Input files

Those parameters are required when running in non-interactive mode. The parameters are optional in default interactive mode. Please, use file names without spaces and without special characters.

`-f FILE` Transcriptome input file in FASTA format.

- `sondovac_part_a.sh`

`-c FILE` Plastome reference sequence input file in FASTA format.

- `sondovac_part_a.sh`, `sondovac_part_b.sh`
- Plastome reference sequences from taxa up to the same order of the studied plant group are suitable. See Straub et al. (2012).

`-m FILE` Mitochondriome reference sequence input file in FASTA format (optional).

- `sondovac_part_a.sh`
- This step is facultative, as plant mitochondrial genomes have largely variable sizes and high rearrangement rates.

`-t FILE` Paired-end genome skim input file in FASTQ format (first file).

- `sondovac_part_a.sh`

`-q FILE` Paired-end genome skim input file in FASTQ format (second file).

- `sondovac_part_a.sh`

`-x FILE` Input file in TSV format (output of Geneious assembly).

- `sondovac_part_b.sh`

`-z FILE` Input file in FASTA format (output of Geneious assembly).

- `sondovac_part_b.sh`

3.1.3 Optional parameters

See page 5 and Figure 1 for steps referred here. If those parameters are not provided, default values are used and it is not possible to change them any time later (not even in interactive mode).

`-a ###` Read length of paired-end genome skim reads (parameter -M of FLASH, see its manual for details).

- Step 4 of Sondovač, `sondovac_part_a.sh`.
- Ensure to use a certain insert size of the genome skim genomic library in combination with an appropriate read length for sequencing in order to enable merging of the paired-end genome skim reads.
- DEFAULT: 250
- OPTIONS: 125, 150, 250, 300

`-y ##` Sequence similiarity between unique transcripts and the filtered, combined genome skim reads (parameter -minIdentity of BLAT, see its manual for details).

- Step 5 of Sondovač, `sondovac_part_a.sh`.
- Consider the trade-off between probe specificity and number of remaining matching sequences for probe design. Sequence similarity is in percent.
- DEFAULT: 85
- OPTIONS: Integer ranging from 70 to 100

`-s ####` Number of BLAT hits per transcript when matching unique transcripts and the filtered, combined genome skim reads.

- Step 6.2 of Sondovač, `sondovac_part_a.sh`.
- Transcripts with a high number of BLAT hits, indicating repetitive elements, need to be removed from the putative probe sequences.
- DEFAULT: 1000
- OPTIONS: Integer ranging from 100 to 10000

`-b ###` Minimum exon (bait) length.

- Steps 8 and 10 of Sondovač, `sondovac_part_b.sh`.
- The minimum exon length should not fall below the bait length in order to facilitate specific binding between genomic libraries and baits during hybridization.
- DEFAULT: 120 (optimal length for phylogeny).
- OPTIONS: Integer ranging from 120 to 200

`-d 0.##` Sequence similarity between probe sequences (parameter -c of cd-hit-est, see its manual for details).

- Step 9 of Sondovač, `sondovac_part_b.sh`.
- Too similar probe sequences will interact with each other during hybridization and thereby reduce enrichment efficiency.
- DEFAULT: 0.9 (highly recommended).
- OPTIONS: Decimal ranging from 0.85 to 0.95

3.2 Input and output files

All names of input files and paths to them must be without spaces and without special characters (some software has difficulties to handle them in such case).

Script `sondovac_part_a.sh` requires as input files:

1. Transcriptome input file in FASTA format.
2. Plastome reference sequence input file in FASTA format.
3. Paired-end genome skim input file in FASTQ format (two files).
4. OPTIONAL: Mitochondriome reference sequence input file in FASTA format. This file is not required.

Script `sondovac_part_a.sh` creates the following files:

1. `*_blat_unique_transcripts.psl` – Output of BLAT (removal of transcripts sharing ≥90% sequence similarity).
2. `*_unique_transcripts.fasta` – Unique transcripts in FASTA format.
3. `*_genome_skim_data_no_cp_reads.bam` – SAM converted to BAM (removal of reads of plastid origin).
4. `*_genome_skim_data_no_cp_reads` – Genome skim data without cpDNA reads.
5. `*_genome_skim_data_no_cp_no_mt_reads.bam` – SAM converted to BAM (removal of reads of mitochondrial origin) – only if mitochondriome reference sequence was used.
6. `*_genome_skim_data_no_cp_no_mt_reads` – Genome skim data without mtDNA reads – only if mitochondriome reference sequence was used.

7. `*_combined_reads_co_cp_no_mt_reads` – Combined paired-end genome skim reads.
8. `*_blat_unique_transcripts_versus_genome_skim_data.pslx` – Output of BLAT (matching of the unique transcripts and the filtered, combined genome skim reads sharing ≥85% sequence similarity).
9. `*_blat_unique_transcripts_versus_genome_skim_data.fasta` – Matching sequences in FASTA.
10. **`*_blat_unique_transcripts_versus_genome_skim_data-no_missing_fin.fsa` – Final FASTA sequences for usage in Geneious.**

Files 1-9 are not necessary for further processing by this pipeline, but may be useful for the user. The last file (10) is used as input file for Geneious in the next step. Asterisk (*) denotes beginning of the output files names specified by the user with parameter `-o`. If user does not select custom name, default value (`output`) will be used.

Geneious requires as input the last output file of `sondovac_part_a.sh` (file 10: `*_blat_unique_transcripts_versus_genome_skim_data-no_missing_fin.fsa`). Output of Geneious are two exported files (see page 10):

1. Final assembled sequences exported as TSV.
2. Final assembled sequences exported as FASTA.

Script `sondovac_part_b.sh` requires as input files:

1. Plastome reference sequence input file in FASTA format.
2. Assembled sequences exported from Geneious as TSV.
3. Assembled sequences exported from Geneious as FASTA.

Script `sondovac_part_b.sh` creates following files:

1. `*_prelim_probe_seq.fasta` – Preliminary probe sequences.
2. `*_similarity_test.fasta` – Contigs that comprise exons ≥ bait length and have a certain total locus length.
3. `*_target_enrichment_probe_sequences.fasta` – Probes in FASTA.
4. **`*_target_enrichment_probe_sequences_final.pslx` – Final probes ready for bait synthesis.**

Asterisk (*) denotes beginning of the output files names specified by the user with parameter `-o`. If user does not select custom name, default value (`output`) will be used. By default, output files are created in same directory from which Sondovač was launched. Output files can be saved in custom directory by specifying output directory together with parameter `-o`:

```
# Find current directory (e.g. /home/user):
pwd
# Launching Sondovač located in directory /home/user/sondovac
# and save output to e.g. desktop (/home/user/Desktop):
./sondovac/sondovac_part_a.sh -o Desktop/MyFile
# Sondovač will save software (if needed) in "bin" directory
# located in directory from which it was launched, see it:
ls bin/*
# Output files are in desired directory, see them e.g. by:
ls -lh Desktop/MyFile*
```

3.3 Record output of Sondovač

To record whole output of Sondovač script (regardless parameters used) use utility `tee`. It will produce plain text output with everything printed to the screen. It can be useful for reference or explorations is something went wrong. Use it as follows:

```
./sondovac_part_a.sh | tee records.log
man tee # See more options how tee can record script's output
# "|" is a pipe passing output of 1st command as input for 2nd command
less records.log # See the record. Quit viewing by "Q"
rm records.log # Delete the log file
```

You can use any command line arguments, script will behave as usually. Plain text file `records.log` will then contain all its output.

4 Sample data

Together with the script, we provide the ZIP archive (1.8 GB) that contains exemplary input files for running the script: *Oxalis* genome skim data as well as the *Ricinus* cpDNA and mtDNA reference sequences. See https://github.com/V-Z/sondovac/wiki/Sample-data for download of sample data.

The package contains:

1. `input1_JHCN_Oxalis_corniculata_transcriptome_data-unique_transcripts.fa` – reduced data of file `JHCN_Oxalis_corniculata_transcriptome_data.fa` (only unique transcripts) kindly provided by Prof. Soltis (parameter `-f`). See below for information how to get full dataset.
2. `input2_Ricinus_communis_reference_plastid_genome.fsa` – cpDNA reference (parameter `-c`), GenBank reference number NC_016736.
3. `input3_J12_Oxalis_obtusa_J12_genome_skim_data_R1.fastq` – paired-end genome skim data, file 1 (parameter `-t`).
4. `input4_J12_Oxalis_obtusa_J12_genome_skim_data_R2.fastq` – paired-end genome skim data, file 2 (parameter `-q`).
5. `input5_Ricinus_communis_reference_mitochondrial_genome.fasta` – mtDNA reference (parameter `-m`), GenBank reference number NC_015141.

Transcriptome input file is unpublished data from G. K.-S. Wong et al. (hopefully it will be published soon). When the data became public, we will post them here. Data can be now found under

- http://www.onekp.com/
- http://www.onekp.com/samples/list.php
- http://www.onekp.com/samples/single.php?id=JHCN

The transcriptome FASTA file used for the probe design is named JHCN-SOAPdenovo-Trans-assembly.dnas.out and can be found under JHCN/Assembly/JHCN-SOAPdenovo-Trans-translated/. Information about how to get access to data download is given in Matasci et al. (2014).

Explanation of command line parameters is at page 14.

5 Questions not covered here, reporting bugs and wishes

If you have any question or you encounter some problem, please, see https://github.com/V-Z/sondovac/issues and feel free to ask any question and/or express any wish. The authors will do their best to help you.

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