Vignette: hoardeR

Guided Cross-Species Identification of Novel Gene Candidates

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Contents

1 Installation

The installation of hoardeR is straight forward, it is located on Cran and can be installed with the command

```
> install.packages("hoardeR")
```

There are no special system requirements for the installation. However there are a few package dependencies that have to be met. Normally, these dependencies are installed automatically, too. If not, missing Cran packages be installed prior to the installation as above, with

```
> install.packages("packageName")
```

Missing Bioconductor packages (e.g. the package *Biostrings*) with

```
> source("https://bioconductor.org/biocLite.R")
> biocLite("Biostrings")
```

The latest developer version, of hoardeR is located on GitHub, here

https://github.com/fischuu/hoardeR

and the address on Cran with the latest stable release version is

https://cran.r-project.org/web/packages/hoardeR/

Packages from GitHub can be installed directly in R, using the *devtools* package. To install hoardeR from GitHub, the following commands are required

```
> install.packages("devtools")
> library("devtools")
> install_github("fischuu/hoardeR")
```

Once the package is installed, it can be loaded into the namespace

```
> library(hoardeR)
```

2 Using hoardeR for identifying cross-species orthologs of novel candidate genes

2.1 Typical workflow prior the use of hoardeR

The common application of hoardeR is to search cross-species orthologs of unannotated, but active regions in a present organism. For that, typically an RNA-seq experiment has been conducted, the reads have been mapped to a reference genome and gene expressions have been estimated using some annotation.

Further, reads from non-annotated regions have been analysed and a set of novel gene candidate regions has been identified. This can be done either across the whole data set, or individually for each sample.

From this analysis, the user has either a gtf file with novel loci (e.g. Cufflinks provides this), or then a bed file with the new loci.

The first rows of a typical gtf file would like this:

```
1       Cufflinks       exon    242203  242862  .       +       .       gene_id "XLOC_000002"; transcript_id "TCONS_00000002"; exon_number "1"; oId "CUFF.2.1"; class_code "u"; tss_id "TSS2";
1       Cufflinks       exon    242203  242646  .       +       .       gene_id "XLOC_000002"; transcript_id "TCONS_00000003"; exon_number "1"; oId "CUFF.1.1"; class_code "u"; tss_id "TSS2";
1       Cufflinks       exon    254559  256717  .       +       .       gene_id "XLOC_000002"; transcript_id "TCONS_00000003"; exon_number "2"; oId "CUFF.1.1"; class_code "u"; tss_id "TSS2";
1       Cufflinks       exon    254240  256717  .       +       .       gene_id "XLOC_000002"; transcript_id "TCONS_00000004"; exon_number "1"; oId "CUFF.3.1"; class_code "u"; tss_id "TSS3";
1       Cufflinks       exon    341982  343630  .       +       .       gene_id "XLOC_000003"; transcript_id "TCONS_00000005"; exon_number "1"; oId "CUFF.10.1"; class_code "u"; tss_id "TSS4";
1       Cufflinks       exon    342113  342607  .       +       .       gene_id "XLOC_000003"; transcript_id "TCONS_00000006"; exon_number "1"; oId "CUFF.11.1"; class_code "u"; tss_id "TSS5";
1       Cufflinks       exon    342961  343494  .       +       .       gene_id "XLOC_000003"; transcript_id "TCONS_00000006"; exon_number "2"; oId "CUFF.11.1"; class_code "u"; tss_id "TSS5";
1       Cufflinks       exon    3312599 3313720 .       +       .       gene_id "XLOC_000024"; transcript_id "TCONS_00000073"; exon_number "1"; oId "CUFF.75.1"; class_code "u"; tss_id "TSS37";
1       Cufflinks       exon    3446776 3447142 .       +       .       gene_id "XLOC_000024"; transcript_id "TCONS_00000073"; exon_number "2"; oId "CUFF.75.1"; class_code "u"; tss_id "TSS37";
1       Cufflinks       exon    9347375 9347527 .       +       .       gene_id "XLOC_000047"; transcript_id "TCONS_00000118"; exon_number "1"; oId "CUFF.115.1"; class_code "u"; tss_id "TSS63";
```

The first lines of a bed file containing the same information would look like this:

```
1       242203      242862      XLOC_000002.1
1       242203      242646      XLOC_000002.2
1       254559      256717      XLOC_000002.3
1       254240      256717      XLOC_000002.4
1       341982      343630      XLOC_000003.1
1       342113      342607      XLOC_000003.2
1       342961      343494      XLOC_000003.3
1       3312599     3313720     XLOC_000024.1
1       3446776     3447142     XLOC_000024.2
1       9347375     9347527     XLOC_000047.1
```

For the identification of cross-species orthologs, the exon structure is not the primary interest. Rather, the whole genomic region that hosts a novel candidate gene is used here. Typically, not all novel gene candidates are considered and the lists are filtered according to some criteria, e.g. that a certain amount of genes is located within these regions, or a certain amount of samples have to have reads in that region.

The final set of regions of interest is then available in bed format, e.g. like this

```
1       242203      256717      XLOC_000002
1       341982      343630      XLOC_000003
1       3312599     3447142     XLOC_000024
1       9347375     9347527     XLOC_000047
```

For these loci, the nucleotide sequences have to be extracted. There are two way to do that. The first option is to do it outside of R using bedtools. For that, the bed file has to be saved to the HDD first. Assuming the *data.frame* that contains the bed information is called *novelBed* and we defined a system path to the project, called *projFolder*. The command to export the *data.frame* then is

```
  projFolder <- "/home/daniel/MyProjects/hoardeR-Example"
  write.table(novelBed, file.path(projFolder,"novel.bed"), row.names=FALSE,
              col.names=FALSE, sep="\t", quote=FALSE)
```

In the console this bed file can then be used to extract the fasta files, using the bedtools tool like this

```
bedtools getfasta -fi <input FASTA> -bed novel.bed -fo novel.fa
```

Here, the is a fasta file that contains the genome information of the species under investigation. If the resulting *novel.fa* file is empty or some other errors occur, a common source of error is a mislabeling of the chromosomes between the input fasta file and the corresponding bed file (e.g. leading CHR, Chr, etc.). This approach is especially then adviceable, when the species of interest is rare or the fasta file is not available from ensembl in the latest version.

However, if the species and also the genome assembly version is available at ensembl, the fasta information can be obtained straight with the hoardeR function *getFastaFromBed*. The hoardeR package is able to download the most common species genomes and annotations, a list of available combinations can be found in the *species* dataset, that comes with hoardeR

```
bedtools getfasta -fi <input FASTA> -bed novel.bed -fo novel.fa
```

```
> head(species)
```

```
        Common.name          Scientific.name Taxon.ID        Ensembl.Assembly        Accession Variation.database Regulation.database Pre.assembly
1   Aardvark (Pre)   Orycteropus afer afer  1230840                       -                -                  -                   -      OryAfe1
2           Alpaca           Vicugna pacos    30538                 vicPac1                -                  -                   -            -
3     Amazon molly        Poecilia formosa    48698 Poecilia_formosa-5.1.2 GCA_000485575.1                  -                   -            -
4      Anole lizard     Anolis carolinensis    28377               AnoCar2.0 GCA_000090745.1                  -                   -            -
5        Armadillo    Dasypus novemcinctus     9361               Dasnov3.0 GCA_000208655.2                  -                   -            -
6 Budgerigar (Pre) Melopsittacus undulatus    13146                       -                -                  -                   -    MelUnd6.3
```

Of particular interest are here the columns *Scientific.name* and *Ensembl.assembly*. If your species of interest matches your used assembly, you can use the automatic hoardeR function *getFastaFromBed* to obtain your fasta object like this. Here, we assume that our species of interest is cow/bos taurus and the fasta files should be downloaded to the folder */home/daniel/fasta/*

```
getFastaFromBed(novelBed, species="Bos taurus", fastaFolder="/home/daniel/fasta/")
```

It is also possible to obtain the genomic information with this command from assemblies that are not provided from Ensembl, or that have a newer/older version number. For that, the full syntax is

```
> getFastaFromBed(novelBed, species="Bos taurus", release = "84", fastaFolder=NULL, version=NULL)
```

Here, the Ensembl release version and also the assmebly version can be specified. However, if no release number is given (i.e. *release=NULL*), the function assumes to find a non-ensembl fasta file in the folder *fastaFolder*. These fasta files need then to be of the format, e.g. for the cow assembly version UMD3.1

```
Bos_taurus.UMD3.1.dna.chromosome.1.fa.gz
Bos_taurus.UMD3.1.dna.chromosome.2.fa.gz
Bos_taurus.UMD3.1.dna.chromosome.3.fa.gz
...
```

That means, first the scientific name, with underscores, then a dot, then the assembly identifier followed by another dot, then *dna.chromosome.* and the chromosome identifier, and then the file ending *fa.gz*. That means, the fasta files have to be gzipped.

As a working example, on Ensembl is only the sheep genome assembly 'Oar_v3.1', if one needs to work with the 'Oar_v4.0', one would download the chromosome wise files and then store them in some folder on the HDD as

```
Ovis_aries.Oar_v4.0.dna.chromosome.1.fa.gz
Ovis_aries.Oar_v4.0.dna.chromosome.2.fa.gz
Ovis_aries.Oar_v4.0.dna.chromosome.3.fa.gz
...
```

To obtain then the corresponding fasta objects, the command is

```
> getFastaFromBed(novelBed, species="Ovis aries", release = NULL,
                  fastaFolder="/home/daniel/fasta/", version="Oar_v4.0")
```

assuming again that the fasta files are located in */home/daniel/fasta/*

```
> bed <- data.frame(Chr=c(11,18,3),
                    Start=c(72554673, 62550696, 18148822),
                    End=c(72555273, 62551296, 18149422),
                    Gene=c("LOC1", "LOC2", "LOC3"))
> novelFA <- getFastaFromBed(bed, species="Bos taurus", release = "84",
                             fastaFolder="/home/ejo138/temp")
```

The option to extract the fasta sequences via bedtools is, however, much faster and is the recommended way. The internal function is especially useful for small bed files or fast testing purposes.

A previous stored fasta file can be imported to hoardeR with the *importFA()* function:

```
> novelFA <- importFA(file="/home/daniel/myFasta.fa")
```

The names of the fasta sequences should follow the form like

```
>Chr:Start-End
```

That meansm, for a sequence from chromosome 12 that starts at 123 and end at 456 the fasta sequence should be named like the following.

```
>12:123-456
```

Once the fasta object is available in R, it can be send to the NCBI blast service, using the central hoardeR function *blastSeq()*

```
>  blastSeq(novelFA,
            email="daniel.fischer@luke.fi",
            xmlFolder=file.path(projFolder,"hoardeROut/"),
            logFolder=file.path(projFolder,"hoardeRLog/"),
            keepInMemory=FALSE
            )
```

The main parameters are the fasta object, then for etiquette reasons a valid email address of the person who sends the data to NCBI and the required folder locations on the HDD. If they are not available, hoardeR will create them.

The *xmlFolder* stores the results delivered back from NCBI in xml format and the *logFolder* stores the log files. These files are especially then important, when the run crashes and should be continued or if the computer is switched off in between.

If a crashed run should be continued, it is enough to run the same command as the initially one using the same fasta object and hoardeR will continue from the point, where it crashed or it was interrupted. If the option *verbose=TRUE* is set (default), hoardeR keeps reporting status updates of the blast runs. There are still some finetuning parameters available, see the manual for details. However, there are some etiquette parameters of NCBI that cannot be changed. These are e.g the frequency and amounts of requests.

The verbose output of a *blastSeq* run looks as follows

```
Missing: 3
Running: 1
Finished: 0
Avg. Blast Time: 00:00:00
Total running time: 00:00:04
---------------------------------------------------------------
```

indicating e.g. here that there were 3 fasta sequences to be blasted, one of them is already running an NCBI, 0 are finished so far and the running time of the whole run is 4 seconds. After a while, *blastSeq* starts to check, if the active blast runs are finalized to spawn the next ones. In that case, the verbose output looks like this:

```
Run RW99J31C01R : 00:02:23
Missing: 1
Running: 1
Finished: 2
Avg. Blast Time: 00:01:10
Total running time: 00:02:40
---------------------------------------------------------------
```

Here, we see that there is still one sequence missing and one is also running. The ID number of that run is *RW99J31C01R* and it is active for 2 minutes and 23 seconds. So far, two sequence runs are ready and their average running time was 1 minute and 10 seconds.

The function stops, when there are no missing sequences anymore. The results of the run are stored in the *xmlFolder*. These results can already be analyzed while the *blastSeq* run is still active, so intermediate results can be obtained on the fly.

To analyze the blast results, first the xml files have to be imported to hoardeR. For that there is the *importXML()* function. It expects the folder address where the xml files are stored, in our example it is the *hoardeROut* folder in the *projFolder* folder.

```
> xmls <- importXML(folder=file.path(projFolder,"hoardeROut/"))
```

One of the first steps is to check what hit organisms were found. As naturally all sequences are also found in Bos Taurus/Cow, the host organism can be excluded from the table

```
> tableSpecies(xmls, exclude="Bos taurus")
```

This table can be displayed as barplot e.g. in the following way

```
> par(oma=c(5,0,0,0))
> barplot(sort(tableSpecies(xmls, exclude="Bos taurus"), decreasing=TRUE), las=2)
```

Next the hits for the Sus scrofa/Pig will be visualized. For that, the xml results will be first filtered accordingly. For that also the tableSpecies command is used. This time, however, a species is not excluded, but explicitely defined. Further, instead of just counting the occurances, we request in addition the location information:

```
> tableSpecies(xmls, species="Sus scrofa", locations = TRUE)
```

```
                                      Organism hitID hitLen hitChr  hitStart    hitEnd origChr origStart  origEnd
28 Sus scrofa breed mixed chromosome 4, Sscrofa10.2   494    644      4 105815870 105816509       3  18148822 18149422
```

without the *locations=TRUE* option, the output is be very minimalistic, as it just gives the frequencies:

```
> tableSpecies(xmls)

Bos taurus Equus caballus     Sus scrofa     Ovis aries 
         6              1              1              3 

> tableSpecies(xmls, species="Sus scrofa")
Sus scrofa 
         1 
```

The column *Organism* indicates the hit organism and the corresponding assembly. In this case it is Sus scrofa and SScrofa10.2. Cross-checking with the *species* data table reveals, that this assembly is the default assembly at Ensembl:

```
> species[grepl("Sus scrofa", species$Scientific.name),]
```

```
        Common.name Scientific.name Taxon.ID Ensembl.Assembly       Accession Variation.database Regulation.database Pre.assembly
57              Pig      Sus scrofa     9823      Sscrofa10.2 GCA_000003025.4                  Y                   Y            -
58 Pig FPC_map (Pre)  Sus scrofa map       NA                -               -                  -                   -          MAP
```

that means that the assembly can be obtained automatically from hoardeR for further analysis. The command *getAnnotation* download automatically the corresponding annotation into the folder */home/daniel/annotation*:

```
> ssannot <- getAnnotation(species = "Sus scrofa", annotationFolder="/home/daniel/annotation")
```

Having the annotation inforamtion available from Ensembl, we can intersect the findings with the annotation. First we check if the found loci intersect with an intergenic region in the Sus scrofa genome. Here, we assume that more than one hit was found and results would be stored in a list

```
> pigHits <- tableSpecies(xmls, species="Sus scrofa", locations = TRUE)
> pigInter <- list()
> for(i in 1:nrow(pigHits)){
>   pigInter[[i]] <- intersectXMLAnnot(pigHits[i,], ssannot)
> }
```

However, unfortunately there is no intersection:

```
> pigInter
[[1]]
Empty data.table (0 rows) of 15 cols: V1,V2,V3,V4,V5,V6...
```

Hence, we allow for a larger seach area and add flanking sites of 100kB to each side of the search area:

```
> pigInter.flank <- list()
> for(i in 1:nrow(pigHits)){
>   pigInter.flank[[i]] <- intersectXMLAnnot(pigHits[i,], ssannot, flanking=100)
> }
# This part transforms the list into a data frame and remove those 'hits' that
# do not report any intersection
> pigInter.flank <- pigInter.flank[sapply(pigInter.flank,nrow)>0]
> pigInter.flank <- do.call(rbind, pigInter.flank)
```

This results in an intersection:

```
> pigInter.flank
   V1      V2  V3        V4        V5 V6 V7 V8                                                                                                    V9 origChr origStart  origEnd hitChr  hitStart    hitEnd
1:  4 ensembl gene 105858080 105858409  .  -  . gene_id "ENSSSCG00000006603"; gene_version "2"; gene_source "ensembl"; gene_biotype "protein_coding";       3  18148822 18149422      4 105815870 105816509
```

In order to visualize the results the *plotHit* function can be used. In its basic appearance, it only plots the similarity between the original and the hit organism like this:

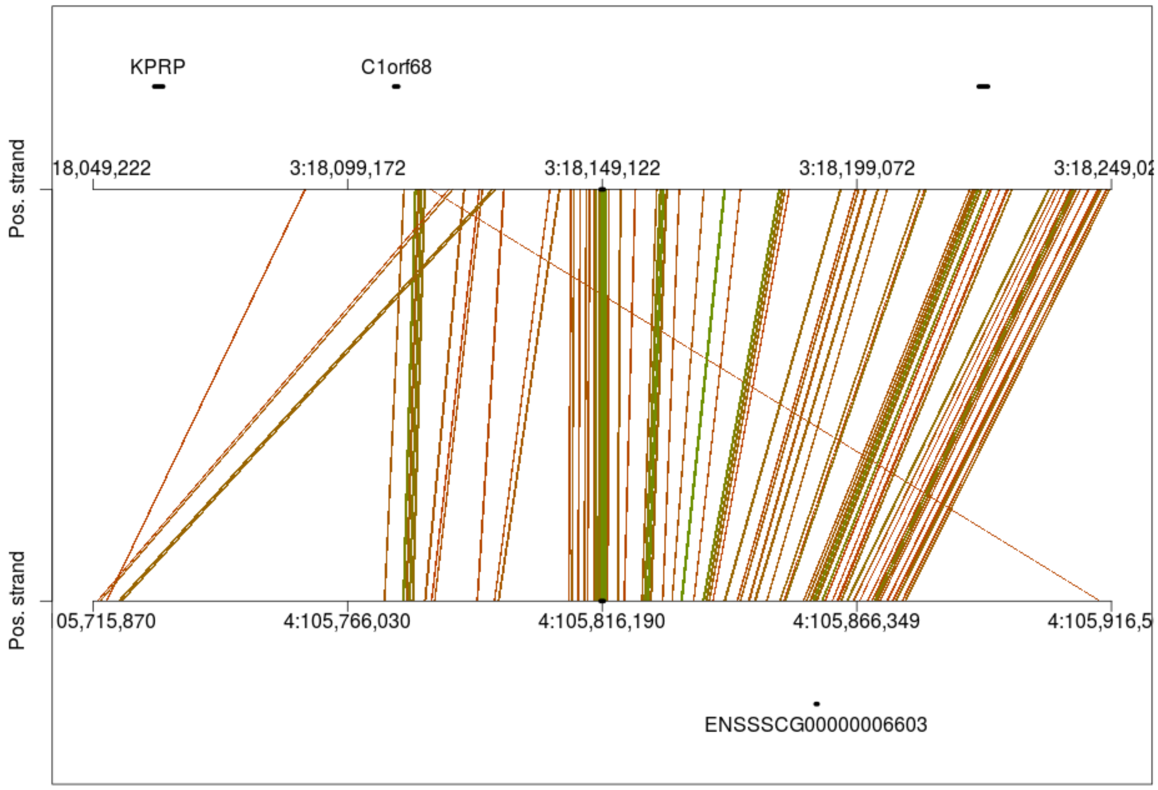
```
>        plotHit(
+            hits=pigInter.flank,
+            flanking=100,
+            diagonal=0.25 ,
+            hitSpecies = "Sus scrofa",
+            origSpecies = "Bos taurus",
+            fastaFolder = "/home/ejo138/fasta/",
         # The following options are optional
+            window=NULL ,
+            which=NULL,
+            figureFolder = "/home/daniel/figures/",
+            figurePrefix = "pigIS"
+            )
```

The parameters are then as follows. *hits* expects the matrix from above with the hits. If this is a matrix (i.e. several hits were found) a figure folder should be provided, as for each hit a separate plot will be created. For example, we the above optional parameters a figure for each hit will be created in the folder */home/daniel/figures/*, using the prefix *pigIS*. However, if only a single figure should be created, without storing it directly to the HDD, one would drop the *figureFolder* and *figurePrefix* parameters (they are *NULL* by default) and instead specify in the *which* option, which hit should be plotted. An alternative would be to restrict the matrix given to the *hits* option, e.g. for seeing the first hit only, one would use the option *hits=pigInter.flank[1,]*.

The other option control the behavior of the plot. The flanking option defines the plotting area around the hit in Mb and the diagonal option defines the threshold after that a similarity line should be plotted. As higher that value (between 0 and 1) as more restrictive it is and as less similarity lines will be used in the plot. The two parameters *hitSpecies* and *origSpecies* define the two organisms that should be compared. In our case the original organism was Bos taurus/cow and the hit was Sus scrofa/pig. The location of the corresponding fasta files (or to where they should be stored), is defined in the *fastaFolder* option. Again, if the assembly is the same as in the *species* dataset, the fasta files will be automatically fetched from the Ensembl page. In case a tailored assembly is required, it can be specified with additional options, see the manual for further instructions on that.

The similarity is calculated using a shifting window approach. That means, the plotting area is divided into chunks of a certain length, defined with the *window* option. By default that window has the similar lengths as the hit-sequence has, but an own value can be defined in the *window option*. Our function then tests all pairwise combinations between the chunks of the origianl organism and the hit organism and calculates the similarity between them and stores the best result. That way, each chunk of original organism gets a chunk from the hit organism assigned. Only those combinations are then considered further, that are at least having a threshold value as defined in *diagonal*.

With that, the figure for the pig would look like this:



Chromosomal positions of original and target organism

On the top line the annotation of Bos taurus is plotted, on the bottom line the one of Sus scrofa, followed by the axes of the chromosomal regions. The vertical center line indicates the hit, further highlighted with the black spots on the chromosomal regions. It appears that this region is rather similar, although in the case of Bos taurus it seems that the genomic region is a bit stretched, as the lines are not parallel but open up towards the Bos taurus side. Also, it seems that the identified gene in the flanking region corresponds to an already annotated gene in Bos taurus.

```
>         plotHit(
+             hits=pigInter.flank,
+             flanking=100,
+             window=NULL ,
+             diagonal=0.25 ,
+             hitSpecies = "Sus scrofa",
+             origSpecies = "Bos taurus",
+             fastaFolder = "/home/ejo138/fasta/",
+             origAnnot=btannot,
+             hitAnnot=ssannot
+         #    coverage=TRUE,
+         #    bamFolder = "/mnt/data2/Data/Ruminomics Papilla/",
+             #indexOffset=94,
+          #    figureFolder = "/home/ejo138/figures/",
+          #    figurePrefix = "pigIS"
+                )
```

We continue with examining the same hit for Ovis aries/sheep. Here we have

```
> tableSpecies(xmls, species="Ovis aries", locations = TRUE)
```

```
                                                                Organism hitID hitLen hitChr   hitStart    hitEnd origChr origStart  origEnd
8   Ovis aries breed Texel chromosome 3, Oar_v4.0, whole genome shotgun sequence   577    616      3  33956005  33955391      11  72554673 72555273
16 Ovis aries breed Texel chromosome 14, Oar_v4.0, whole genome shotgun sequence   553    613     14  59259439  59260042      18  62550696 62551296
24  Ovis aries breed Texel chromosome 1, Oar_v4.0, whole genome shotgun sequence   539    604      1 101361981 101362569       3  18148822 18149422
```

Indicating that the hit comes from the Oar_v4.0 assembly. However, in Ensembl is only the Oar_v.3.1 assembly and hence, the automatic download would use the wrong assembly and consequently also would operate on the wrong coordiantes. Hence, the correct assembly has to be downloaded from the Ovis aries consortium or NCBI and stored as chromosome-wise fasta files in the fasta folder. Same holds also for the annotation files.