

# segmentSeq: methods for identifying small RNA loci from high-throughput sequencing data

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October 17, 2016

## 1 Introduction

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High-throughput sequencing technologies allow the production of large volumes of short sequences, which can be aligned to the genome to create a set of *matches* to the genome. By looking for regions of the genome which to which there are high densities of matches, we can infer a segmentation of the genome into regions of biological significance. The methods we propose allows the simultaneous segmentation of data from multiple samples, taking into account replicate data, in order to create a consensus segmentation. This has obvious applications in a number of classes of sequencing experiments, particularly in the discovery of small RNA loci and novel mRNA transcriptome discovery.

We approach the problem by considering a large set of potential *segments* upon the genome and counting the number of tags that match to that segment in multiple sequencing experiments (that may or may not contain replication). We then adapt the empirical Bayesian methods implemented in the `baySeq` package [1] to establish, for a given segment, the likelihood that the count data in that segment is similar to background levels, or that it is similar to the regions to the left or right of that segment. We then rank all the potential segments in order of increasing likelihood of similarity and reject those segments for which there is a high likelihood of similarity with the background or the regions to the left or right of the segment. This gives us a large list of overlapping segments. We reduce this list to identify non-overlapping loci by choosing, for a set of overlapping segments, the segment which has the lowest likelihood of similarity with either background or the regions to the left or right of that segment and rejecting all other segments that overlap with this segment. For fuller details of the method, see Hardcastle *et al.* [2].

## 2 Preparation

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We begin by loading the `segmentSeq` package.

```
> library(segmentSeq)
```

Note that because the experiments that `segmentSeq` is designed to analyse are usually massive, we should use (if possible) parallel processing as implemented by the `parallel` package. If using this approach, we need to begin by define a *cluster*. The following command will use eight processors on a single machine; see the help page for 'makeCluster' for more information. If we don't want to parallelise, we can proceed anyway with a `NULL` cluster.

```
> if(require("parallel"))
+ {
+   numCores <- min(8, detectCores())
+   cl <- makeCluster(numCores)
+ } else {
+   cl <- NULL
+ }
```

The `readGeneric` function is able to read in tab-delimited files which have appropriate column names, and create an `alignmentData` object. Alternatively, if the appropriate column names are not present, we can specify which columns to use for the data. In either case, to use this function we pass a character vector of files, together with information on which data are to be treated as replicates to the function. We also need to define the lengths of

the chromosome and specify the chromosome names as a character. The data here, drawn from text files in the 'data' directory of the segmentSeq package are taken from the first million bases of an alignment to chromosome 1 and the first five hundred thousand bases of an alignment to chromosome 2 of *Arabidopsis thaliana* in a sequencing experiment where libraries 'SL9' and 'SL10' are replicates, as are 'SL26' and 'SL32'. Libraries 'SL9' and 'SL10' are sequenced from an Argonaute 6 IP, while 'SL26' and 'SL32' are an Argonaute 4 IP.

A similar function, readBAM performs the same operation on files in the BAM format. Please consult the help page for further details.

```
> chrlens <- c(1e6, 2e5)
> datadir <- system.file("extdata", package = "segmentSeq")
> libfiles <- c("SL9.txt", "SL10.txt", "SL26.txt", "SL32.txt")
> libnames <- c("SL9", "SL10", "SL26", "SL32")
> replicates <- c("AG06", "AG06", "AG04", "AG04")
> aD <- readGeneric(files = libfiles, dir = datadir,
+                 replicates = replicates, libnames = libnames,
+                 chrs = c(">Chr1", ">Chr2"), chrlens = chrlens,
+                 polyLength = 10, header = TRUE, gap = 200)
> aD
```

An object of class "alignmentData"  
13765 rows and 4 columns

Slot "libnames":  
[1] "SL9" "SL10" "SL26" "SL32"

Slot "replicates":  
[1] AG06 AG06 AG04 AG04  
Levels: AG04 AG06

Slot "alignments":  
GRanges object with 13765 ranges and 2 metadata columns:

	seqnames	ranges	strand	tag	multireads
	<Rle>	<IRanges>	<Rle>	<character>	<numeric>
[1]	>Chr1	[600, 623]	+	AAGGATTGGTGGTTGAAGACACA	1
[2]	>Chr1	[665, 688]	+	ATCCTTGTAGCACACATTTGGCA	1
[3]	>Chr1	[762, 785]	+	AGAGTGATTTGGATGATTCAAGAC	1
[4]	>Chr1	[762, 786]	+	AGAGTGATTTGGATGATTCAAGACT	1
[5]	>Chr1	[774, 794]	+	ATGATTCAAGACTTCTCGGTA	1
...	...	...	...	...	...
[13761]	>Chr2	[179857, 179872]	-	TACGGAAAGAAAGGAA	1
[13762]	>Chr2	[179964, 179987]	-	AGAGAGAGCCATTCATGAACCATT	1
[13763]	>Chr2	[179978, 180000]	-	GAGATTCTCCGCTAGAGAGAGCC	1
[13764]	>Chr2	[179999, 180022]	-	ATTAATATTAATTCATCGGGAAGA	1
[13765]	>Chr2	[180002, 180022]	-	ATTAATATTAATTCATCGGGA	1

-----  
seqinfo: 2 sequences from an unspecified genome

Slot "data":  
Matrix with 13765 rows.

	SL9	SL10	SL26	SL32
1	0	1	0	0
2	7	1	0	0
3	0	0	17	0
4	1	0	0	0
5	0	1	0	0
...	...	...	...	...
13761	0	1	0	0
13762	0	0	0	7
13763	0	1	0	0

```
13764  0  1  0  0
13765  0  1  0  0
```

Slot "libsizes":

```
[1] 4447 6531 9666 6675
```

Next, we process this alignmentData object to produce a segData object. This segData object contains a set of potential segments on the genome defined by the start and end points of regions of overlapping alignments in the alignmentData object. It then evaluates the number of tags that hit in each of these segments.

```
> sD <- processAD(aD, gap = 100, c1 = c1)
> sD
```

An object of class "segData"

2109 rows and 4 columns

Slot "replicates":

```
[1] AG06 AG06 AG04 AG04
```

Levels: AG04 AG06

Slot "coordinates":

GRanges object with 2109 ranges and 0 metadata columns:

	seqnames	ranges	strand
	<Rle>	<IRanges>	<Rle>
[1]	>Chr1	[600, 623]	*
[2]	>Chr1	[600, 688]	*
[3]	>Chr1	[600, 830]	*
[4]	>Chr1	[600, 916]	*
[5]	>Chr1	[665, 688]	*
...	...	...	...
[2105]	>Chr2	[179708, 179872]	*
[2106]	>Chr2	[179708, 180037]	*
[2107]	>Chr2	[179738, 179872]	*
[2108]	>Chr2	[179738, 180037]	*
[2109]	>Chr2	[179923, 180037]	*

-----  
seqinfo: 2 sequences from an unspecified genome

Slot "locLikelihoods" (stored on log scale):

Matrix with 0 rows.

<0 x 0 matrix>

Slot "data":

Matrix with 0 rows. Matrix with 0 rows.

```
SL9 SL10 SL26 SL32
```

Slot "libsizes":

```
[1] 4447 6531 9666 6675
```

We can now construct a segment map from these potential segments.

## Segmentation by heuristic methods

A fast method of segmentation can be achieved by exploiting the bimodality of the densities of small RNAs in the potential segments. In this approach, we assign each potential segment to one of two clusters for each replicate group, either as a segment or a null based on the density of sequence tags within that segment. We then combine these clusterings for each replicate group to gain a consensus segmentation map.

```
> hS <- heuristicSeg(sD = sD, aD = aD, RKPM = 1000, largeness = 1e8, getLikes = TRUE, c1 = c1)
```

.....

## Segmentation by empirical Bayesian methods

A more refined approach to the problem uses an existing segment map (or, if not provided, a segment map defined by the `hS` function) to acquire empirical distributions on the density of sequence tags within a segment. We can then estimate posterior likelihoods for each potential segment as being either a true segment or a null. We then identify all potential segments in the with a posterior likelihood of being a segment greater than some value `'lociCutoff'` and containing no subregion with a posterior likelihood of being a null greater than `'nullCutoff'`. We then greedily select the longest segments satisfying these criteria that do not overlap with any other such segments in defining our segmentation map.

```
> classSegs <- classifySeg(sD = sD, aD = aD, cD = hS, cl = cl)
```

.....

```
> classSegs
```

GRanges object with 336 ranges and 0 metadata columns:

	seqnames	ranges	strand
	<Rle>	<IRanges>	<Rle>
[1]	>Chr1	[ 1, 599]	*
[2]	>Chr1	[600, 916]	*
[3]	>Chr1	[917, 940]	*
[4]	>Chr1	[941, 967]	*
[5]	>Chr1	[968, 17054]	*
...	...	...	...
[332]	>Chr2	[178506, 178513]	*
[333]	>Chr2	[178514, 178636]	*
[334]	>Chr2	[178637, 179707]	*
[335]	>Chr2	[179708, 180037]	*
[336]	>Chr2	[180038, 200000]	*

-----

seqinfo: 2 sequences from an unspecified genome

An object of class "lociData"

336 rows and 4 columns

Slot "replicates"

AG06 AG06 AG04 AG04

Slot "groups":

list()

Slot "data":

	AG06.1	AG06.2	AG04.1	AG04.2
[1,]	1	1	0	2
[2,]	41	39	65	83
[3,]	0	0	0	0
[4,]	13	7	0	0
[5,]	2	3	0	0

331 more rows...

Slot "annotation":

data frame with 0 columns and 336 rows

Slot "locLikelihoods" (stored on log scale):

Matrix with 336 rows.

	AG04	AG06
1	0.15246	0.097146
2	0.7997	0.94194

```

3 0.098218 0.14411
4 0.096572 0.98552
5 0.073621 0.031097
... ..
332 0.1216 0.21412
333 0.90295 0.99585
334 0.071039 0.061141
335 0.85766 0.93969
336 0.074168 0.13408

```

Expected number of loci in each replicate group

```

AG04 AG06
132.1335 188.5964

```

By one of these methods, we finally acquire an annotated lociData object, with the annotations describing the co-ordinates of each segment.

We can use this lociData object, in combination with the alignmentData object, to plot the segmented genome.

```

> par(mfrow = c(2,1), mar = c(2,6,2,2))
> plotGenome(aD, hS, chr = ">Chr1", limits = c(1, 1e5),
+           showNumber = FALSE, cap = 50)
> plotGenome(aD, classSegs, chr = ">Chr1", limits = c(1, 1e5),
+           showNumber = FALSE, cap = 50)

```

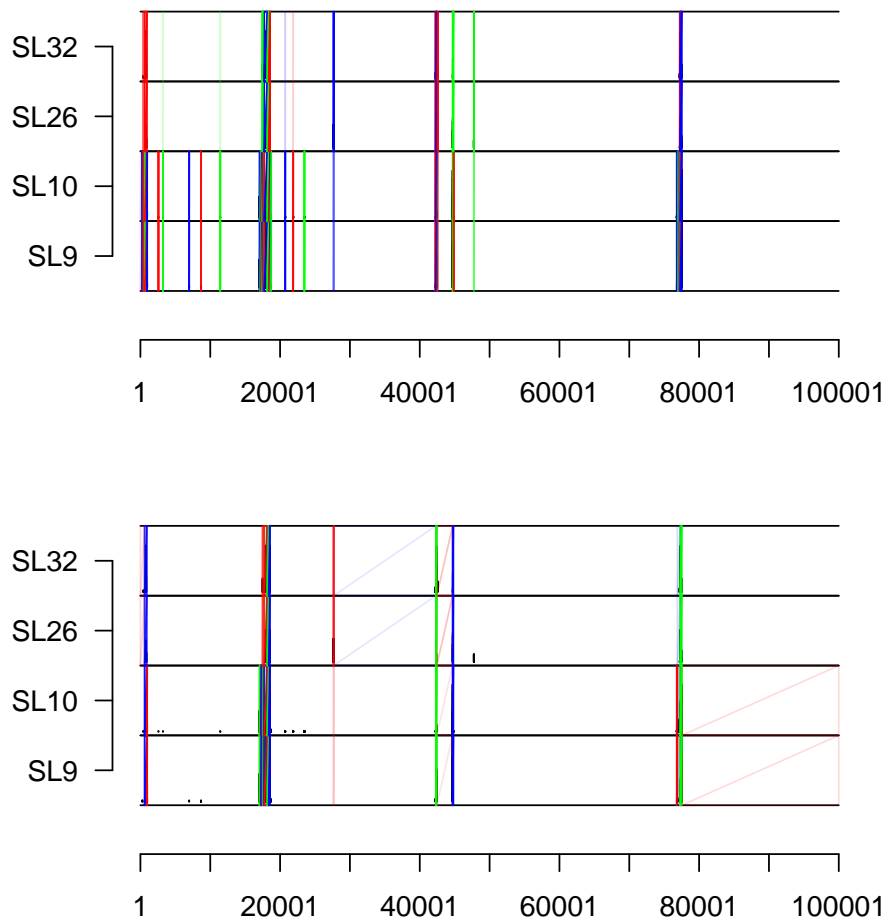


Figure 1: The segmented genome (first  $10^5$  bases of chromosome 1.

Given the calculated likelihoods, we can filter the segmented genome by controlling on likelihood, false discovery rate, or familywise error rate

```
> loci <- selectLoci(classSegs, FDR = 0.05)
> loci
```

GRanges object with 137 ranges and 0 metadata columns:

	seqnames	ranges	strand
	<Rle>	<IRanges>	<Rle>
[1]	>Chr1	[ 600, 916]	*
[2]	>Chr1	[ 941, 967]	*
[3]	>Chr1	[17055, 17111]	*
[4]	>Chr1	[17275, 17331]	*
[5]	>Chr1	[17484, 17527]	*
...	...	...	...
[133]	>Chr2	[152795, 152829]	*
[134]	>Chr2	[169196, 169230]	*
[135]	>Chr2	[178389, 178505]	*
[136]	>Chr2	[178514, 178636]	*
[137]	>Chr2	[179708, 180037]	*

-----

```
seqinfo: 2 sequences from an unspecified genome
An object of class "lociData"
137 rows and 4 columns
```

```
Slot "replicates"
AG06 AG06 AG04 AG04
Slot "groups":
list()
```

```
Slot "data":
  AG06.1 AG06.2 AG04.1 AG04.2
[1,]    41    39    65    83
[2,]    13     7     0     0
[3,]    30    36     0     0
[4,]    30    36     0     0
[5,]    14    13     0    15
132 more rows...
```

```
Slot "annotation":
data frame with 0 columns and 137 rows
```

```
Slot "locLikelihoods" (stored on log scale):
Matrix with 137 rows.
```

	AG04	AG06
1	0.7997	0.94194
2	0.096572	0.98552
3	0.088014	0.99836
4	0.088014	0.99836
5	0.52937	0.98722
...	...	...
133	0.52745	0.95106
134	0.093257	0.98092
135	0.92566	0.41093
136	0.90295	0.99585
137	0.85766	0.93969

```
Expected number of loci in each replicate group
  AG04    AG06
```

84.84949 131.89284

The lociData objects can now be examined for differential expression with the baySeq package.

First we define the possible models of differential expression on the data. In this case, the models are of non-differential expression and pairwise differential expression.

```
> groups(classSegs) <- list(NDE = c(1,1,1,1), DE = c("AG06", "AG06", "AG04", "AG04"))
```

Then we get empirical distributions on the parameter space of the data.

```
> classSegs <- getPriors(classSegs, cl = cl)
```

Then we get the posterior likelihoods of the data conforming to each model. Since the 'classSegs' object contains null regions as well as true loci, we will use the 'nullData = TRUE' option to distinguish between non-differentially expressed loci and non-expressed regions. By default, the loci likelihoods calculated earlier will be used to weight the initial parameter fit in order to detect null data.

```
> classSegs <- getLikelihoods(classSegs, nullData = TRUE, cl = cl)
```

.

We can examine the highest likelihood non-expressed ('null') regions

```
> topCounts(classSegs, NULL, number = 3)
```

	seqnames	start	end	width	strand	AG06.1	AG06.2	AG04.1	AG04.2	Likelihood	FDR.
1	>Chr1	447910	512945	65036	*	3	6	0	4	0.9483169	0.05168307
2	>Chr1	670986	696964	25979	*	0	4	0	2	0.9472338	0.05222462
3	>Chr1	754198	758593	4396	*	1	0	1	0	0.9469059	0.05251445

FWER.

1 0.05168307  
2 0.10172212  
3 0.14941538

The highest likelihood expressed but non-differentially expressed regions

```
> topCounts(classSegs, "NDE", number = 3)
```

	seqnames	start	end	width	strand	AG06.1	AG06.2	AG04.1	AG04.2	Likelihood	FDR.NDE
1	>Chr2	4903	6834	1932	*	2464	4524	5775	4190	0.9795807	0.02041929
2	>Chr2	6879	7404	526	*	421	1559	1255	751	0.9627162	0.02885157
3	>Chr1	446881	447380	500	*	739	490	1173	1110	0.9583247	0.03312616

FWER.NDE

1 0.02041929  
2 0.05694182  
3 0.09624409

And the highest likelihood differentially expressed regions

```
> topCounts(classSegs, "DE", number = 3)
```

	seqnames	start	end	width	strand	AG06.1	AG06.2	AG04.1	AG04.2	Likelihood	ordering
1	>Chr1	17055	17111	57	*	30	36	0	0	0.9976820	AG06>AG04
2	>Chr1	17275	17331	57	*	30	36	0	0	0.9976820	AG06>AG04
3	>Chr1	634297	634350	54	*	65	90	12	17	0.9945872	AG06>AG04

FDR.DE      FWER.DE

1 0.002317980 0.002317980  
2 0.002317980 0.004630588  
3 0.003349574 0.010018286

Finally, to be a good citizen, we stop the cluster we started earlier:

```
> if(!is.null(cl))
+   stopCluster(cl)
```

## Session Info

---

```
> sessionInfo()

R version 3.3.1 (2016-06-21)
Platform: x86_64-pc-linux-gnu (64-bit)
Running under: Ubuntu 16.04.1 LTS

locale:
 [1] LC_CTYPE=en_US.UTF-8      LC_NUMERIC=C              LC_TIME=en_US.UTF-8
 [4] LC_COLLATE=C             LC_MONETARY=en_US.UTF-8  LC_MESSAGES=en_US.UTF-8
 [7] LC_PAPER=en_US.UTF-8     LC_NAME=C                 LC_ADDRESS=C
[10] LC_TELEPHONE=C          LC_MEASUREMENT=en_US.UTF-8 LC_IDENTIFICATION=C

attached base packages:
[1] parallel stats4 stats graphics grDevices utils datasets methods
[9] base

other attached packages:
 [1] segmentSeq_2.8.0          ShortRead_1.32.0         GenomicAlignments_1.10.0
 [4] SummarizedExperiment_1.4.0 Biobase_2.34.0           Rsamtools_1.26.0
 [7] Biostrings_2.42.0        XVector_0.14.0           BiocParallel_1.8.0
[10] baySeq_2.8.0             abind_1.4-5             GenomicRanges_1.26.0
[13] GenomeInfoDb_1.10.0      IRanges_2.8.0           S4Vectors_0.12.0
[16] BiocGenerics_0.20.0

loaded via a namespace (and not attached):
 [1] edgeR_3.16.0             zlibbioc_1.20.0         lattice_0.20-34         hwriter_1.3.2
 [5] tools_3.3.1             grid_3.3.1             latticeExtra_0.6-28     Matrix_1.2-7.1
 [9] RColorBrewer_1.1-2     bitops_1.0-6           limma_3.30.0           locfit_1.5-9.1
[13] BiocStyle_2.2.0
```

## References

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- [1] Thomas J. Hardcastle and Krystyna A. Kelly. *baySeq: Empirical Bayesian Methods For Identifying Differential Expression In Sequence Count Data*. BMC Bioinformatics (2010).
- [2] Thomas J. Hardcastle and Krystyna A. Kelly and David C. Baulcombe. *Identifying small RNA loci from high-throughput sequencing data*. Bioinformatics (2012).