

cn.mops - Mixture of Poissons for CNV detection in NGS data

Günter Klambauer

Institute of Bioinformatics, Johannes Kepler University Linz
Altenberger Str. 69, 4040 Linz, Austria
cn.mops@bioinf.jku.at

Version 1.42.0, April 26, 2022

Contents

1	Introduction	3
2	Getting started and quick start	3
3	Input of <code>cn.mops</code>: BAM files, GRanges objects, or numeric matrices	4
3.1	Read count matrices as input	4
3.2	BAM files as input	5
4	Copy number estimation with <code>cn.mops</code>	6
4.1	Running <code>cn.mops</code>	6
4.2	The result object	7
5	Visualization of the result	9
5.1	Chromosome plots	9
5.2	CNV region plots	10
6	Exome sequencing data	11
7	Cases vs. Control or Tumor vs. Normal	14
8	Heterosomes and CNVs of tumor samples	14
9	<code>cn.mops</code> for haploid genomes	16
10	Adjusting sensitivity, specificity and resolution for specific applications	16
11	Overview of study designs and <code>cn.mops</code> functions	16
12	Exporting <code>cn.MOPS</code> results in tabular format	17
13	How to cite this package	18

1 Introduction

The `cn.mops` package is part of the Bioconductor (<http://www.bioconductor.org>) project. The package allows to detect copy number variations (CNVs) from next generation sequencing (NGS) data sets based on a generative model. Please visit <http://www.bioinf.jku.at/software/cnmops/cnmops.html> for additional information.

To avoid the false discoveries induced by read count variations along the chromosome or across samples, we propose a “Mixture Of PoissonS model for CNV detection” (`cn.MOPS`). The `cn.MOPS` model is not affected by read count variations along the chromosome, because at each DNA position a local model is constructed. Read count variations across samples are decomposed by the `cn.MOPS` model into integer copy numbers and noise by its mixture components and Poisson distributions, respectively. In contrast to existing methods, `cn.MOPS` model’s posterior provides integer copy numbers together with their uncertainty. Model selection in a Bayesian framework is based on maximizing the posterior given the samples by an expectation maximization (EM) algorithm. The model incorporates the linear dependency between average read counts in a DNA segment and its copy number. Most importantly, a Dirichlet prior on the mixture components prefers constant copy number 2 for all samples. The more the data drives the posterior away from the Dirichlet prior corresponding to copy number two, the more likely the data is caused by a CNV, and, the higher is the informative/non-informative (I/NI) call. `cn.MOPS` detects a CNV in the DNA of an individual as a segment with high I/NI calls. I/NI call based CNV detection guarantees a low false discovery rate (FDR) because wrong detections are less likely for high I/NI calls. We assume that the genome is partitioned into segments in which reads are counted but which need not be of constant length throughout the genome. For each of such a segment we build a model. We consider the read counts x at a certain segment of the genome, for which we construct a model across samples. The model incorporates both read count variations due to technical or biological noise and variations stemming from copy number variations. For further information regarding the algorithm and its assessment see the `cn.MOPS` homepage at <http://www.bioinf.jku.at/software/cnmops/cnmops.html>.

2 Getting started and quick start

To load the package, enter the following in your R session:

```
> library(cn.mops)
```

The whole pipeline will only take a few steps, if BAM files are available (for read count matrices directly go to step 2):

1. Getting the input data from BAM files (also see Section 3.2 and Section 3).

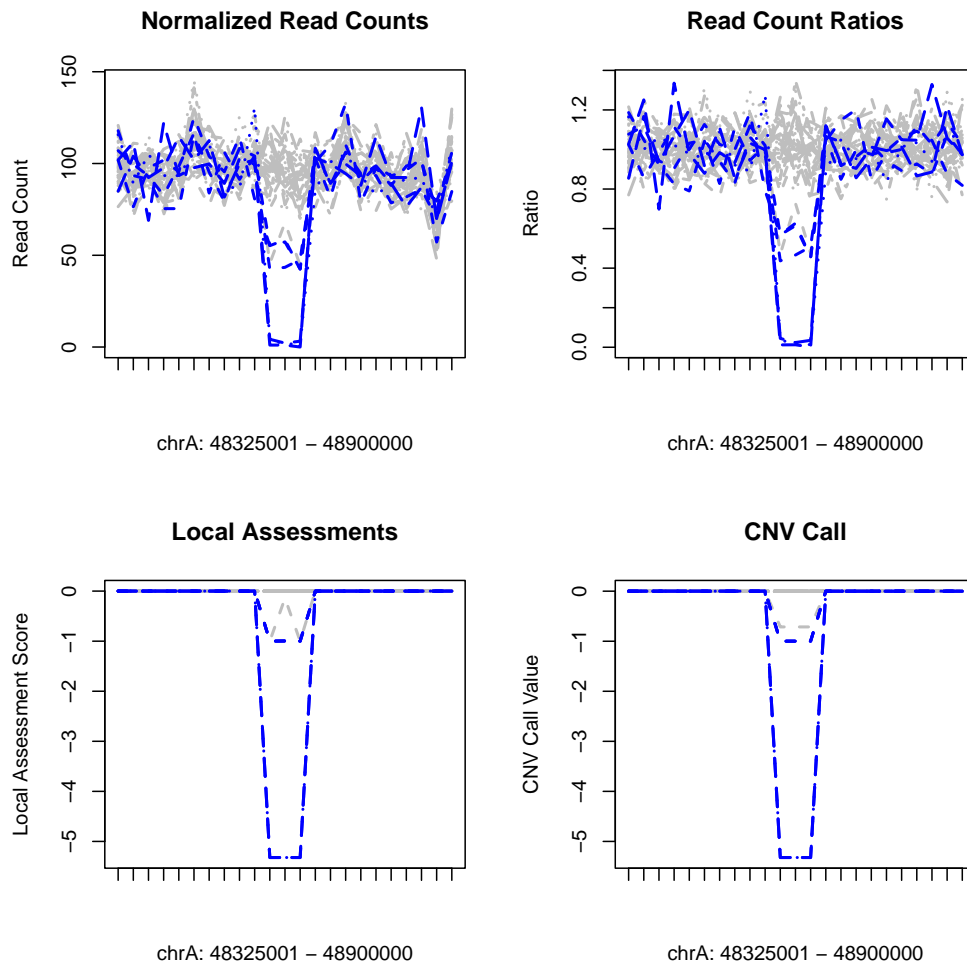
```
> BAMFiles <- list.files(pattern=".bam$")
> bamDataRanges <- getReadCountsFromBAM(BAMFiles)
```

2. Running the algorithm (also see Section 4.2).

```
> res <- cn.mops(bamDataRanges)
```

3. Visualization of the detected CNV regions. For more information about the result objects and visualization see Section 4.2.

```
> plot(res, which=1)
```



3 Input of `cn.mops`: BAM files, GRanges objects, or numeric matrices

3.1 Read count matrices as input

`cn.mops` does not require the data samples to be of any specific kind or structure. `cn.mops` only requires a *read count matrix*, i.e., given N data samples and m genomic segments, this is an $m \times N$ real- or integer-valued matrix \mathbf{X} , in which an entry x_{ij} corresponds to the read count of sample j in the i -th segment. E.g. in the following read count matrix sample three has 17 reads in the second segment: $x_{23} = 17$.

$$\mathbf{X} = \begin{matrix} & \text{Sample 1} & \text{Sample 2} & \text{Sample 3} & \text{Sample 4} \\ \text{Segment 1} & \left(\begin{array}{cccc} 88 & 82 & 79 & 101 \\ 83 & 78 & 71 & 99 \\ 43 & 50 & 55 & 37 \\ 47 & 58 & 48 & 42 \\ 73 & 86 & 95 & 91 \\ 92 & 90 & 80 & 71 \end{array} \right) \\ \text{Segment 2} \\ \text{Segment 3} \\ \text{Segment 4} \\ \text{Segment 5} \\ \text{Segment 6} \end{matrix}$$

`cn.mops` can handle numeric and integer matrices or GRanges objects, in which the read counts are stored as values of the object.

3.2 BAM files as input

The most widely used file format for aligned short reads is the Sequence Alignment Map (SAM) format or in the compressed form the Binary Alignment Map (BAM). We provide a simple function that makes use of the `Rsamtools` package to obtain the alignment positions of reads. The result object of the function can directly be used as input for `cn.mops`. The author can provide functions for input formats other than BAM upon request: *cn.mops@bioinf.jku.at*.

```
> BAMFiles <- list.files(system.file("extdata", package="cn.mops"), pattern=".bam$",
+                         full.names=TRUE)
> bamDataRanges <- getReadCountsFromBAM(BAMFiles,
+                                       sampleNames=paste("Sample", 1:3))
```

In `bamDataRanges` you have now stored the genomic segments (left of the `|`'s) and the read counts (right of the `|`'s):

```
> (bamDataRanges)
```

GRanges object with 2522 ranges and 3 metadata columns:

	seqnames	ranges	strand	Sample 1	Sample 2	Sample 3
	<Rle>	<IRanges>	<Rle>	<integer>	<integer>	<integer>
[1]	20	1-25000	*	0	0	0
[2]	20	25001-50000	*	0	0	0
[3]	20	50001-75000	*	762	848	784
[4]	20	75001-100000	*	0	0	0
[5]	20	100001-125000	*	0	0	0
...
[2518]	20	62925001-62950000	*	0	0	0
[2519]	20	62950001-62975000	*	0	0	0
[2520]	20	62975001-63000000	*	0	0	0
[2521]	20	63000001-63025000	*	0	0	0
[2522]	20	63025001-63025520	*	0	0	0

seqinfo: 1 sequence from an unspecified genome

4 Copy number estimation with `cn.mops`

To get a first impression, we use a data set, in which CNVs have been artificially implanted. The simulated data set was generated using distributions of read counts as they appear in real sequencing experiments. CNVs were implanted under the assumption that the expected read count is linear dependent on the copy number. For example in a certain genomic we expect λ reads for copy number 2, then we expect 2λ reads for copy number 4. The linear relationship was confirmed in different studies, like Alkan *et al.* (2009), Chiang *et al.* (2008) and Sathirapongsasuti *et al.* (2011).

4.1 Running `cn.mops`

The read counts are stored in the objects `X` and `XRanges`, which are the two basic input types that `cn.mops` allows:

```
> data(cn.mops)
> ls()

[1] "BAMFiles"          "CNVRanges"         "X"                  "XRanges"
[5] "bamDataRanges"     "cn.mopsVersion"    "exomeCounts"        "resCNMOPS"
```

The same data is stored in a `GRanges` object, in which we see the genomic coordinates, as well as the read counts (values):

```
> head(XRanges[,1:3])

GRanges object with 6 ranges and 3 metadata columns:
      seqnames      ranges strand |      S_1      S_2      S_3
      <Rle>       <IRanges> <Rle> | <integer> <integer> <integer>
[1]      chrA      1-25000      * |      102       93      109
[2]      chrA    25001-50000      * |      118       99      100
[3]      chrA    50001-75000      * |       85       81       82
[4]      chrA   75001-100000      * |       87      116      106
[5]      chrA  100001-125000      * |       87       68       89
[6]      chrA  125001-150000      * |       87       91       91
-----
seqinfo: 1 sequence from an unspecified genome; no seqlengths
```

We are now ready to run `cn.mops` on the `GRanges` object:

```
> resCNMOPS <- cn.mops(XRanges)
```

To calculate integer copy number use the command `calcIntegerCopyNumbers`:

```
> resCNMOPS <- calcIntegerCopyNumbers(resCNMOPS)
```

Alternatively, it is possible to use an integer matrix, in which the genomic coordinates can be stored as rownames and the entries are the read counts. For example the data from above represented by an integer matrix `X`:

```
> head(X[,1:3])
```

```

              S_1 S_2 S_3
Chr_A_1_25000    102  93 109
Chr_A_25001_50000 118  99 100
Chr_A_50001_75000   85  81  82
Chr_A_75001_100000  87 116 106
Chr_A_100001_125000 87  68  89
Chr_A_125001_150000 87  91  91
```

We are now ready to run `cn.mops` on the integer matrix:

```
> resCNMOPsx <- cn.mops(X)
```

To calculate integer copy number use the command `calcIntegerCopyNumbers`:

```
> resCNMOPsx <- calcIntegerCopyNumbers(resCNMOPsx)
```

Note that the two results `resCNMOPS` and `resCNMOPSRanges` identify the same CNVs:

```
> all(individualCall(resCNMOPsx)==individualCall(resCNMOPS))
```

4.2 The result object

To get a summary of the CNV detection result, just enter the name of the object (which implicitly calls `show`):

```
> (resCNMOPS)
```

The CNVs per individual are stored in the slot `cnvs`:

```
> cnvs(resCNMOPS)[1:5]
```

GRanges object with 5 ranges and 4 metadata columns:

	seqnames	ranges	strand	sampleName	median
	<Rle>	<IRanges>	<Rle>	<character>	<numeric>
[1]	chrA	1775001-1850000	*	S_1	-1.00000
[2]	chrA	53300001-53400000	*	S_1	-5.32193
[3]	chrA	113175001-113325000	*	S_1	-0.99991
[4]	chrA	48575001-48650000	*	S_2	-5.32193
[5]	chrA	70625001-70750000	*	S_2	-5.32193
	mean	CN			

```

      <numeric> <character>
[1] -0.9999999      CN1
[2] -5.321928      CN0
[3] -0.999682      CN1
[4] -5.321928      CN0
[5] -5.321928      CN0
-----
seqinfo: 1 sequence from an unspecified genome; no seqlengths

```

Segments, in which individual CNVs accumulate, are called CNV regions and can be accessed by `cnvr`:

```

> cnvr(resCNMOPS)[1,1:5]

GRanges object with 1 range and 5 metadata columns:
      seqnames      ranges strand |      S_1      S_2
      <Rle>      <IRanges> <Rle> | <character> <character>
[1]      chrA 1775001-1850000      * |      CN1      CN2
      S_3      S_4      S_5
      <character> <character> <character>
[1]      CN2      CN2      CN2
-----
seqinfo: 1 sequence from an unspecified genome; no seqlengths

```

We now want to check, whether `cn.mops` found the implanted CNVs. We have stored the implanted CNVs (see beginning of Section) in the object `CNVRanges`.

```

> (CNVRanges[15,1:5])

GRanges object with 1 range and 5 metadata columns:
      seqnames      ranges strand |      S_1      S_2      S_3
      <Rle>      <IRanges> <Rle> | <integer> <integer> <integer>
[1]      chrA 1751017-1850656      * |          1          2          2
      S_4      S_5
      <integer> <integer>
[1]          2          2
-----
seqinfo: 1 sequence from an unspecified genome; no seqlengths

```

Next we identify overlaps between CNVs that were detected by `cn.mops` and CNVs that were implanted. Towards this end we use the functions of the `GenomicRanges` package.

```

> ranges(cnvr(resCNMOPS))[1:2]

```


IRanges object with 2 ranges and 0 metadata columns:

	start	end	width
	<integer>	<integer>	<integer>
[1]	1775001	1850000	75000
[2]	5525001	5600000	75000

```
> ranges(cnvr(resCNMOPS)) %over% ranges(CNVRanges)
```

```
[1] TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE TRUE
[15] TRUE TRUE TRUE TRUE TRUE
```

The detected CNV regions all overlap with the known CNV regions contained in CNVRanges.

The function `cn.mops` creates an instance of the S4 class `CNVDetectionResult` that is defined by the present package. To get detailed information on which data are stored in such objects, enter

```
> help(CNVDetectionResult)
```

5 Visualization of the result

5.1 Chromosome plots

`cn.mops` allows for plotting the detected segments of an individual at one chromosome by a plot similar to the ones produced by DNACopy:

```
> segplot(resCNMOPS, sampleIdx=13)
```



Figure 1: The x-axis represents the genomic position and on the y-axis we see the log ratio of the read counts (green) and the copy number call of each segment (red).

5.2 CNV region plots

`cn.mops` allows for plotting the detected CNV regions:

```
> plot(resCNMOPS, which=1)
```



Figure 2: The x-axis represents the genomic position and on the y-axis we see the read counts (left), the call of the local model (middle) and the CNV call produced by the segmentation algorithm. Blue lines mark samples having a copy number loss.

6 Exome sequencing data

To apply `cn.mops` to exome sequencing data requires a different preprocessing, since constant windows spanning the whole genome are not appropriate. The initial segments in which the reads are counted should be chosen as the regions of the baits, targets or exons. The read count matrix can now be generated by using the function `getSegmentReadCountsFromBAM` that requires the genomic coordinates of the predefined segments as `GRanges` object. The resulting read count

matrix can directly be used as input for `cn.mops`. A possible processing script could look like the following:

```
> library(cn.mops)
> BAMFiles <- list.files(pattern=".bam$")
> segments <- read.table("targetRegions.bed", sep="\t", as.is=TRUE)
> gr <- GRanges(segments[,1], IRanges(segments[,2], segments[,3]))
> X <- getSegmentReadCountsFromBAM(BAMFiles, GR=gr)
> resCNMOPS <- exomecn.mops(X)
> resCNMOPS <- calcIntegerCopyNumbers(resCNMOPS)
```

We included an exome sequencing data set in this package. It is stored in `exomeCounts`.

```
> resultExomeData <- exomecn.mops(exomeCounts)
> resultExomeData <- calcIntegerCopyNumbers(resultExomeData )
```

```
> plot(resultExomeData, which=5)
```

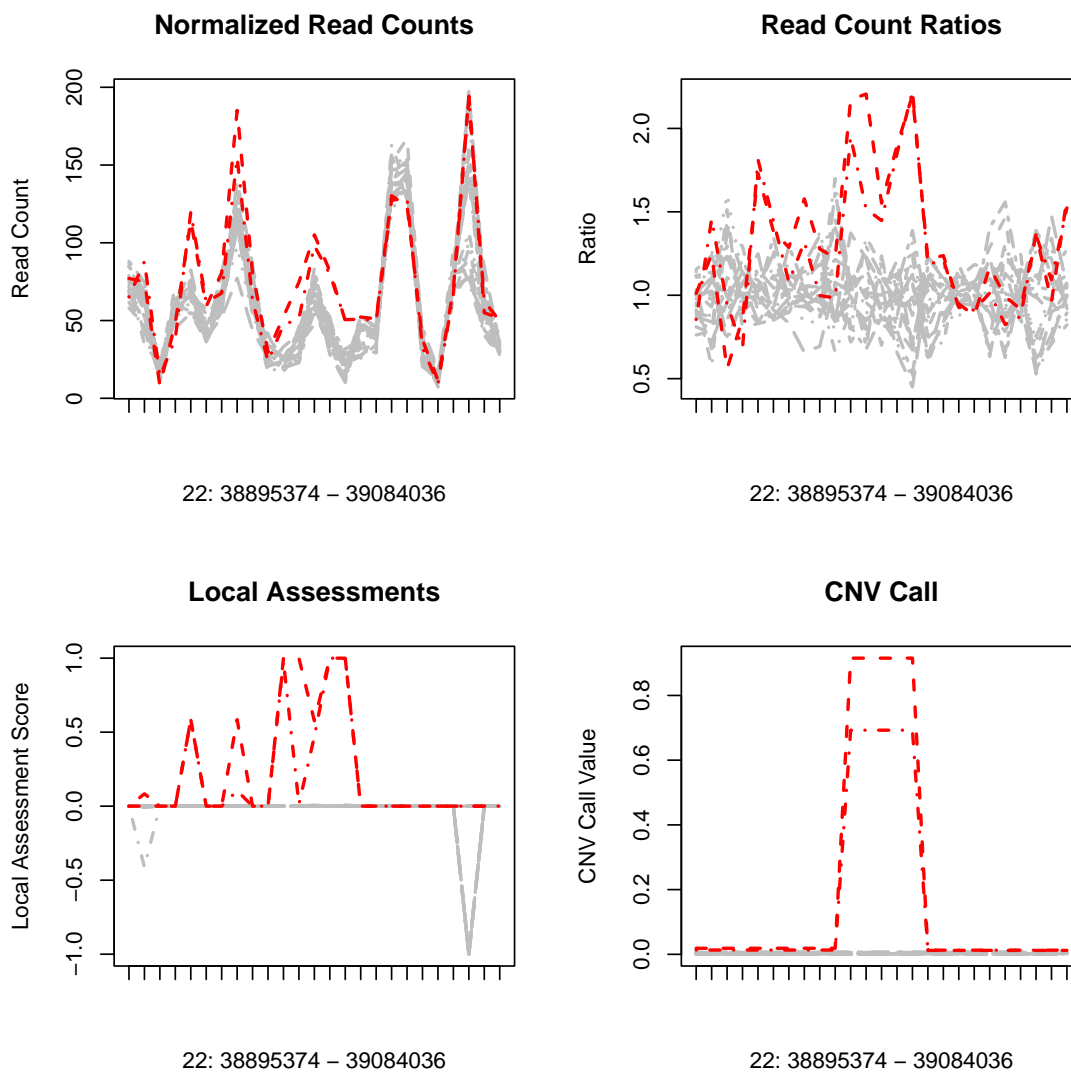


Figure 3: ExomeSeq data results.

Possible issues and notes A problem can occur, if the names of the reference sequences, e.s. chromosomes, are inconsistent between the bed file and the bam file. For example "chr1", "chr2", ..., "chrX", "chrY" and "1", "2", ..., "X", "Y". This can easily be solved by replacing the seqlevels of the GRanges object:

```
> #the following removes the "chr" from reference sequence names
> library(GenomeInfoDb)
> seqlevels(gr) <- gsub("chr","",seqlevels(gr))
```

Results can also be improved if you extend your target regions by a small amount of bases to the left and to the right (in the following case it is 30bp):

```
> gr <- GRanges(segments[,1],IRanges(segments[,2]-30,segments[,3]+30))
> gr <- reduce(gr)
```

7 Cases vs. Control or Tumor vs. Normal

For detection of CNVs in a setting in which the normal state is known, the function `referencecn.mops` can be applied. It implements the `cn.MOPS` algorithm adjusted to this setting. For tumor samples very high copy numbers can be present – the maximum copy number with the default setting is 8 – and `cn.mops` has to be adjusted to allow higher copy numbers.

```
> resRef <- referencecn.mops(cases=X[,1],controls=rowMeans(X),
+                           classes=c("CN0", "CN1", "CN2", "CN3", "CN4", "CN5", "CN6",
+                                     "CN7", "CN8", "CN16", "CN32", "CN64", "CN128"),
+                           I = c(0.025, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 8, 16, 32, 64),
+                           segAlgorithm="DNACopy")
```

Analyzing: Sample.1

```
> resRef <- calcIntegerCopyNumbers(resRef)
> (cnvs(resRef))
```

GRanges object with 4 ranges and 4 metadata columns:

	seqnames	ranges	strand	sampleName	median	mean
	<Rle>	<IRanges>	<Rle>	<character>	<numeric>	<numeric>
[1]	undef	2029-2032	*	Case_1	0.584963	0.5850
[2]	undef	2133-2136	*	Case_1	-5.321928	-5.3219
[3]	undef	4051-4054	*	Case_1	-2.660964	-2.6610
[4]	undef	4528-4533	*	Case_1	-1.000000	-1.0000

CN

	<character>
[1]	CN3
[2]	CN0
[3]	CN1
[4]	CN1

seqinfo: 1 sequence from an unspecified genome; no seqlengths

8 Heterosomes and CNVs of tumor samples

With the default settings the normalization procedure assumes that the ploidy of each sample is the same. However, it is possible to account for different karyotypes. When analyzing CNVs on the X or Y chromosome one possibility is to treat males and females separately. The second option is to provide the normalization function with the information about the gender, that is different

ploidy states of the X and Y chromosome. This can be handled by the `ploidy` parameter of the normalization function. In the following we show the normalization for the X chromosome, if the first 10 individuals are males (ploidy set to 1) and the next 30 individuals are females (ploidy set to 2):

```
> XchrX <- normalizeChromosomes(X[1:500, ],ploidy=c(rep(1,10),rep(2,30)))
> cnvr(calcIntegerCopyNumbers(cn.mops(XchrX,norm=FALSE)))
```

GRanges object with 1 range and 40 metadata columns:

	seqnames	ranges	strand	S_1	S_2	S_3
	<Rle>	<IRanges>	<Rle>	<character>	<character>	<character>
[1]	undef	1-500	*	CN1	CN1	CN1
	S_4	S_5		S_6	S_7	S_8
	<character>	<character>	<character>	<character>	<character>	<character>
[1]	CN1	CN1		CN1	CN1	CN1
	S_9	S_10		S_11	S_12	S_13
	<character>	<character>	<character>	<character>	<character>	<character>
[1]	CN1	CN1		CN2	CN1	CN2
	S_14	S_15		S_16	S_17	S_18
	<character>	<character>	<character>	<character>	<character>	<character>
[1]	CN2	CN1		CN2	CN1	CN2
	S_19	S_20		S_21	S_22	S_23
	<character>	<character>	<character>	<character>	<character>	<character>
[1]	CN2	CN2		CN2	CN2	CN2
	S_24	S_25		S_26	S_27	S_28
	<character>	<character>	<character>	<character>	<character>	<character>
[1]	CN2	CN2		CN2	CN1	CN1
	S_29	S_30		S_31	S_32	S_33
	<character>	<character>	<character>	<character>	<character>	<character>
[1]	CN2	CN2		CN2	CN2	CN2
	S_34	S_35		S_36	S_37	S_38
	<character>	<character>	<character>	<character>	<character>	<character>
[1]	CN2	CN2		CN2	CN2	CN0
	S_39	S_40				
	<character>	<character>				
[1]	CN2	CN1				

seqinfo: 1 sequence from an unspecified genome; no seqlengths

Karyotype information can also improve results of CNV detection in tumor samples. The best results can be reached, if for each chromosome the number of appearances in the cell is known. In this case normalization should be applied to each chromosome separately.

9 `cn.mops` for haploid genomes

For haploid genomes the prior assumption is that all samples have copy number 1. The function `haplocn.mops` implements the `cn.MOPS` algorithm adjusted to haploid genomes.

```
> resHaplo <- haplocn.mops(X)
> resHaplo <- calcIntegerCopyNumbers(resHaplo)
```

10 Adjusting sensitivity, specificity and resolution for specific applications

The default parameters of both the local models of `cn.mops` and the segmentation algorithm were optimized on a wide ranged of different data sets. However, you might want to adjust sensitivity and specificity or resolution to your specific needs.

upperThreshold The calling threshold for copy number gains, a positive value. Lowering the threshold will increase the detections, raising will decrease the detections.

lowerThreshold The calling threshold for copy number losses, a negative value. Raising the threshold will increase the detections, lowering will decrease the detections.

priorImpact This parameter should be optimized for each data set, since it is influenced by number of samples as well as noise level. The higher the value, the more samples will have copy number 2, and consequently less CNVs will be detected.

minWidth The minimum length of CNVs measured in number of segments. The more adjacent segments with a high or low copy number call are joined, the higher the confidence in the detections. A lower value will lead to more shorter segments, and a higher value will yield to less, but longer segments.

The length of the initial segments is also crucial. They should be chosen such that on average 50 to 100 reads lie in one segment. The `WL` parameter of `getReadCountsFromBAM` determines this resolution.

11 Overview of study designs and `cn.mops` functions

In Table1 we give an overview of the functions implemented in this package and present settings for which they are appropriate. All these functions work for multiple, at least two, samples. CNV detection for single samples usually yields many false detections, because of characteristics of genomic segments that lead to a higher or lower read count (and coverage). These biases can usually not be corrected for (except for the GC content and the mappability bias). Being aware of all these problems we have implemented the function `singlecn.mops` for cases in which only one sample is available.

Seq. Type	Ploidy	Study	Samples	Function
WGS	2n	cohort/non-tumor/GWAS	≥ 5	cn.mops
WGS	2n	tumor vs. normal	≥ 2	referencecn.mops
ES	2n	cohort/non-tumor/GWAS	≥ 5	exomecn.mops
ES	2n	tumor vs. normal	≥ 2	referencecn.mops
WGS	1n	cohort/non-tumor/GWAS	≥ 5	haplocn.mops
WGS	1n	tumor vs. normal	≥ 2	<i>not implemented</i>
ES	1n	cohort/non-tumor/GWAS	≥ 5	haplocn.mops
ES	1n	tumor vs. normal	≥ 2	<i>not implemented</i>

Table 1: **Seq. Type** reports the sequencing technology that was used: whole genome sequencing (WGS) or targeted/exome sequencing (ES). **Ploidy** gives the usual ploidy of the samples. In case of a tumor vs. control study the control sample is meant. **Study**: The type of study to be analyzed for CNVs: either a cohort study, such the HapMap or the 1000Genomes Project, or studies including a number of non-tumor samples or studies with both healthy and diseased individuals, i.e. genome wide association studies (GWAS). **Samples** reports the minimum number of samples needed for the analysis. **Function** gives the function of the cn.mops package that is appropriate for this setting.

12 Exporting cn.MOPS results in tabular format

Users can extract the segmentation, the CNVs and the CNV regions with the following:

```
> library(cn.mops); data(cn.mops)
> result <- calcIntegerCopyNumbers(cn.mops(XRanges))
> segm <- as.data.frame(segmentation(result))
> CNVs <- as.data.frame(cnvs(result))
> CNVRegions <- as.data.frame(cnvr(result))
```

The results can be exported with write.csv for Excel, LibreOffice Calc, etc. These files will include the genomic position, copy number and other information.

```
> write.csv(segm, file="segmentation.csv")
> write.csv(CNVs, file="cnvs.csv")
> write.csv(CNVRegions, file="cnvr.csv")
```

13 How to cite this package

If you use this package for research that is published later, you are kindly asked to cite it as follows: (Klambauer *et al.*, 2012).

To obtain BibTeX entries of the reference, you can enter the following into your R session:

```
> toBibtex(citation("cn.mops"))
```

References

- Alkan, C., Kidd, J. M., Marques-Bonet, T., Aksay, G., Antonacci, F., Hormozdiari, F., Kitzman, J. O., Baker, C., Malig, M., Mutlu, O., Sahinalp, S. C., Gibbs, R. A., and Eichler, E. E. (2009). Personalized copy number and segmental duplication maps using next-generation sequencing. *Nat. Genet.*, **41**(10), 1061–1067.
- Chiang, D. Y., Getz, G., Jaffe, D. B., Zhao, X., Carter, S. L., Russ, C., Nusbaum, C., Meyerson, M., and Lander, E. S. (2008). High-resolution mapping of copy-number alterations with massively parallel sequencing. *Nat. Methods*, **6**, 99–103.
- Klambauer, G., Schwarzbauer, K., Mitterecker, A., Mayr, A., Clevert, D.-A., Bodenhofer, U., and Hochreiter, S. (2012). cn.MOPS: Mixture of Poissons for Discovering Copy Number Variations in Next Generation Sequencing Data with a Low False Discovery Rate. *Nucleic Acids Research*, **40**(9), e69.
- Sathirapongsasuti, F. J., Lee, H., Horst, B. A., Brunner, G., Cochran, A. J., Binder, S., Quackenbush, J., and Nelson, S. F. (2011). Exome Sequencing-Based Copy-Number Variation and Loss of Heterozygosity Detection: ExomeCNV. *Bioinformatics*, **27**(19), 2648–2654.