

HTShape: Shape analysis of high-throughput experiments data

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Abstract

Given a dataset we first try to characterize its central value (location) with the sample mean (or sample median) and its spread around the location (variance) with the sample standard deviation (or sample range). When the sample size, n , is sufficiently large we can begin to assess the shape of the data in some meaningful way. Distributional shape is often characterized by two features (1) Skewness: a measure of how far the shape of the distribution deviates from symmetry around its location and (2) Kurtosis: a measure of how much weight is at the tails of the distribution relative to the weight around the location. This vignette describes the statistical analysis of the shape (skewness and kurtosis) of high-throughput experiments data (e.g. RNA-seq, microarray) using the **shape** package. It also demonstrates the detection of transcripts (e.g. genes) within a dataset whose sample shape is markedly different from the majority of transcripts in the same dataset. The ability to describe the shape of high-throughput genomics data is useful for two reasons: 1. It enriches the exploratory data analysis process, and 2. It provides a means of checking the distributional assumptions of statistical methods.

If you use **HTShape** (version 1.0.0) in your work, please cite:

- K. Okrah and H. Bravo. *HTShape analysis of high-throughput experiments data*. Under review.
- J. Hosking. *L-moments: analysis and estimation of distributions using linear combinations of order statistics*. Journal of the Royal Statistical Society. Series B (Methodological), **52**, 105–124, 1990.
- K. Okrah. **shape**: Shape analysis of high-throughput experiments data. R package version 1.0.0, 2014 (<https://github.com/kokrah/HTShape>).

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1 Introduction

Before we begin we first give a brief description of the theory on which our methods are based.

1.1 A numerical summary of shape

Similar to traditional moments, the theory of L-moments forms the basis of many statistical methods such as parameter estimation, hypothesis testing, and model selection. However, L-moments enjoy many theoretical and practical advantages over traditional moments (see [1–4]). In this vignette we focus on its ability to provide robust statistics that summarize a given dataset. The first four L-moments $\lambda_1, \lambda_2, \lambda_3$ and λ_4 measure location, variance, skewness, and kurtosis of data respectively. Unit free measures of relative variance, skewness, and kurtosis are defined as: L-CV $\tau = \lambda_2/\lambda_1$, L-skew $\tau_3 = \lambda_3/\lambda_2$, and L-kurt $\tau_4 = \lambda_4/\lambda_2$. The L-skew (τ_3) coefficient can take any value between (-1, 1); where $\tau_3 = 0$ implies symmetry and $\tau_3 > 0$ ($\tau_3 < 0$) implies skewness to the right (left). The symmetry-outlier plot (SO-plot) conveniently summarizes the shape of each gene as a point on a 2-dimensional plot (see L-moments ratio diagram in [2]). The interpretation of the L-kurt (τ_4) depends on L-skew (in general one would expect a highly skewed data to have a high kurtosis). At $\tau_3 = 0$ and $\tau_4 = 0$ the data has the shape of a uniformly distributed random variable. As τ_4 (positive) increases the data becomes bell shaped, for example at $\tau_4 = 0.1226$ the data is normally distributed. As τ_4 (negative) decreases the data becomes U-shaped (indicating two possible groups). See Figure 1 below for examples.

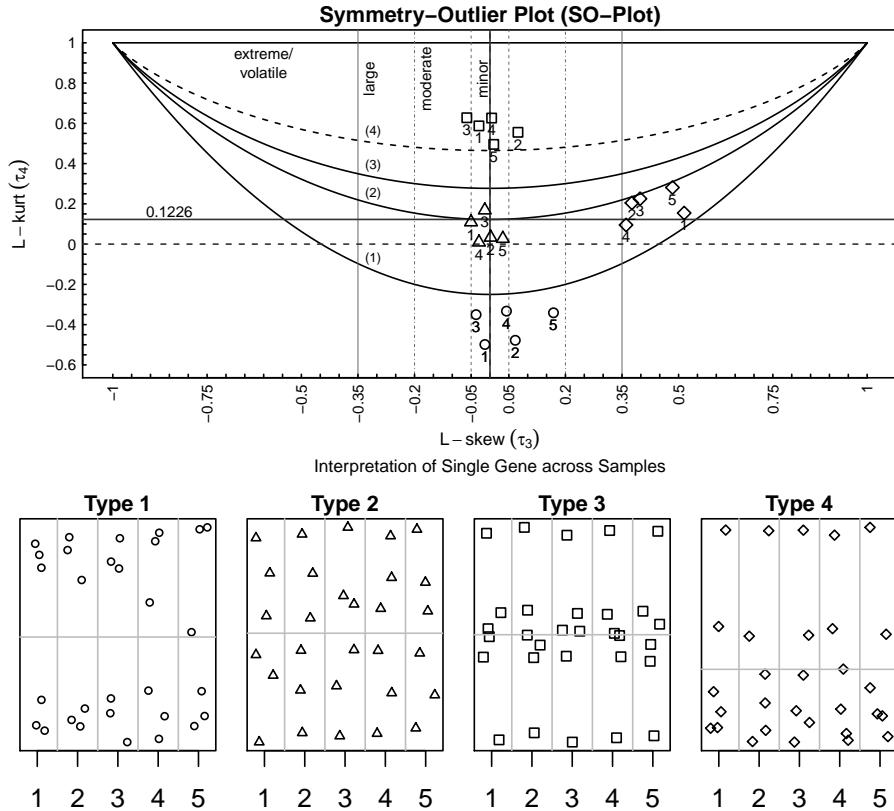


Figure 1: **Interpretation of the SO-plot.** We have shown examples, based on a sample of size 6, of the four main types of sample shape (bottom panels) and where they occur on the SO-plot (top panel). Every single point on the SO-plot corresponds to a summary of the shape of a single gene. The measure of skewness and kurtosis are not independent, as one would expect a highly skewed sample would tend to have a high kurtosis. This relationship is indicated by the parabolas on the SO-plot. Starting from below: (1) we have the theoretical lower bound for L-kurt, in terms of L-skew, $\tau_4 = 0.25(5\tau_3^2 - 1)$. Between the second (2) and third (3) parabolas is a region where the L-kurt measure is considered moderate. Between the third (3) and fourth (4) parabolas indicate a region where the L-kurt measure is considered to be high. Above the fourth curve is considered extreme. One should keep in mind that these descriptive measures are independent of the expression level and variance of the gene. Interpretation of the SO-plot ultimately rests on the context of the data.

1.2 Package overview

The purpose of this package is to compute the shape statistics of each gene in a high-throughput dataset. Using these statistics we can find genes within a dataset whose sample shape is markedly different from the majority of genes in the same dataset. When put together these shape statistics give an overall description of the entire high-throughput dataset. The ability to describe the shape of high-throughput genomics data is useful for two reasons: 1. It enriches the exploratory data analysis step, and 2. It provides a means of checking the distributional assumptions of statistical methods.

There are three main functions in the **shape** package:

1. `fitShape()`
2. `computeDvals()`
3. `plotSO()`

Given a dataset such as a high-throughput expression matrix (or just a vector of measurements) the function `fitShape()` will compute and return the L-CV, L-skew, and L-kurt estimates for each gene.

Given the shape (i.e. L-skew and L-kurt) estimate of each gene, the function `computeDvals()` computes a dissimilarity distance (d-values) between each gene's shape estimate and the typical gene's shape estimate. The d-values range from 0 to 1; where 1 is very close and 0 is very far.

The function `plotSO()` shows each gene's shape estimate on a single plot (SO-plot).

1.3 Vignette overview

We will demonstrate the utility of the **shape** package as part of the data exploratory steps in the analysis of high-throughput experiments data. A total of three publicly available datasets will be used in this vignette :

1. The **Pickrell dataset** [5] is part of the International HapMap Project. RNA samples were extracted from the lymphoblastoid cell lines of 69 unrelated Nigerian individuals, 29 males and 40 females. See [6] for details on alignment and counting.
2. The **Hammoud dataset** [7] contains 10 samples of mRNA samples of 8-week old wild type mice (strain: C57BL/6). Of the 10 RNA samples 5 were obtained from spermatids (cells) and the other 5 from spermatocytes (cells). Summarized counts in the form of FPKM can be downloaded at GEO:GSE49622. For the details on alignment and counting please see [7].
3. The **Bottomly dataset** [8] was obtained from the ReCount webpage. See [6] for details on alignment and counting. It contains counts summarizing an RNA-seq experiment that includes 21 samples from inbred mouse strains. Eleven of the samples came from the strain DBA/2J and 10 from the strain C57BL/6J.

2 The Pickrell dataset

The datasets in this package are bundled together in the form of a list. Each component of the list contains the expression measures and its experimental design. We will use the Pickrell dataset to illustrate the main functions in this package.

We begin by loading the **shape** package into an R session and looking at the datasets available in the package.

```
> library(HTShape)
> data(examplesData) # Load datasets.
> names(examplesData)

[1] "bottomly" "hammoud"  "pickrell"
```

2.1 Filter counts and normalize

First we will filter out genes with low expression by only keeping genes whose counts per million (cpm) is more than 1 in at least 29 samples (where 29 is the minimum of the 29 male samples and 40 female samples).

Let us define a function to filter out the low count genes

```
> filterCounts <- function (pcounts, thresh, minSamples) {
  cpm <- t(t(pcounts) / colSums(pcounts)) * 1e+06
  keep <- rowSums(cpm > thresh) >= minSamples
  filteredPcounts <- pcounts[keep, ]
  filteredPcounts
}
```

and apply it to the Pickrell dataset.

```
> counts <- examplesData$pickrell$exprs
> gender <- examplesData$pickrell$cond
> (tab <- table(gender))

gender
female   male
  40     29

> pcounts <- counts + 1 # pseudo-counts
> minSamples <- min(tab)
> dim(pcounts) # Before filtration.

[1] 38415     69

> pcounts <- filterCounts(pcounts, 1, minSamples)
> dim(pcounts) # After filtration.

[1] 17604     69
```

After filtration we normalize for library size and transform the data to \log_2 scale. In this vignette we use the DESeq method [9], however any of the library size normalization methods can be used.

```
> ref <- exp(rowMeans(log(pcounts)))
> deseqScal <- apply(pcounts / ref, 2, median)
> pcounts <- t(t(pcounts) / deseqScal)
> y <- log2(pcounts)
```

2.2 `fitShape()`: computation of sample shape

We are now ready to compute the L-skew (τ_3) and L-kurt (τ_4) estimates (i.e. shape) of each gene. This is done by calling the function `fitShape()`.

```
> # Compute the L-skew (t3) and L-kurt (t4) of each gene.  
> res <- fitShape(t(y))  
> class(res)  
  
[1] "list"  
  
> names(res)  
  
[1] "lcv"    "lrats"  "lmoms"
```

2.3 `computeDvals()`: finding outlier genes

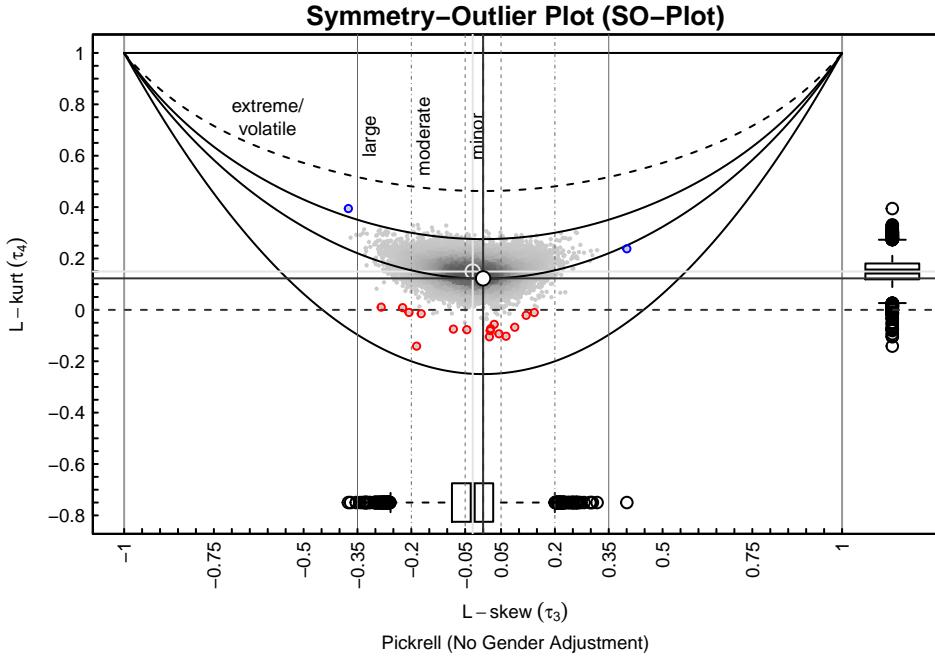
Given the shape of each gene in the dataset the function `computeDvals()` computes the dissimilarity score (d-values) between each gene's shape and the typical gene's shape. The d-values range from 0 to 1; where 1 is very close and 0 is very far. See section 2.5 for details.

```
> # Compute d-values  
> t3 <- res$lrats["LR3",] # Grab L-skew estimates.  
> t4 <- res$lrats["LR4",] # Grab L-kurt estimates.  
> dvals <- computeDvals(t3, t4)
```

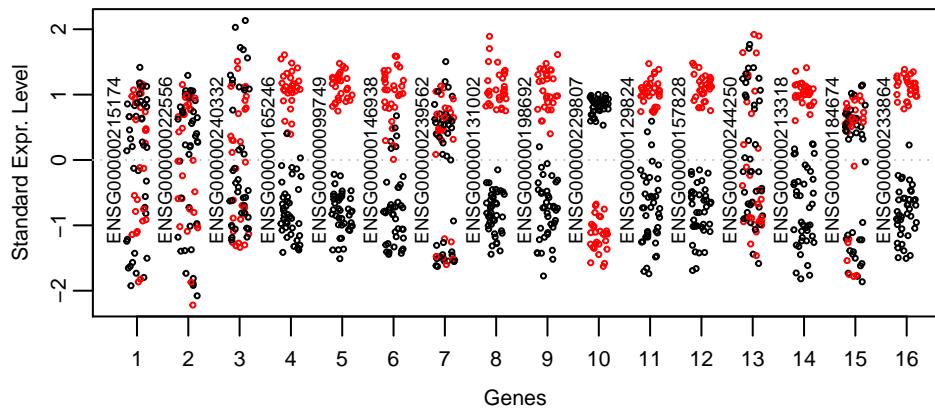
2.4 `plotSO()`: the symmetry outlier plot (SO-plot)

We now construct the SO-plot. On the SO-plot we highlight genes that have very low ($< 10^{-4}$) d-values (aka. outlier genes). This criterion is arbitrary and is at the users discretion. For illustrative reasons we separate the outlier genes into two groups; those with the extreme skew (blueGroup) from the rest (redGroup).

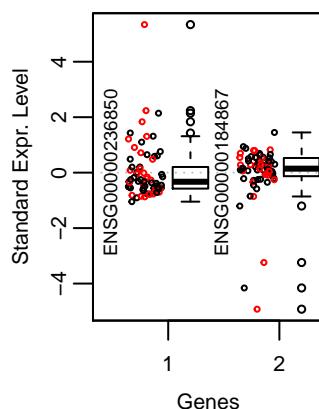
```
> # Symmetry-Outlier plot.  
> plotSO(t3, t4, dataName="Pickrell (No Gender Adjustment)", verbose = TRUE)  
  
[1] "Pickrell (No Gender Adjustment) L-skew: (25%, 50%, 75%) = (-0.09, -0.03, 0.03)"  
  
> # Pick volatile / outlier genes.  
> sel <- which(dvals < 0.0001) # select 0.01% cutoff  
>  
> # Separate outlier genes into 2 groups for illustration purposes  
> blueGroup <- sel[abs(t3[sel]) > 0.3]  
> redGroup <- sel[abs(t3[sel]) <= 0.3]  
> points(t3[blueGroup], t4[blueGroup], cex=0.5, col="blue")  
> points(t3[redGroup], t4[redGroup], cex=0.5, col="red")
```



Let us take a closer look at the genes called outliers. Keep in mind that outlier here means that the shape of the gene is different from the majority of gene shapes in the data; independent of the gene's variance and expression level. First we begin with the redGroup (contains 16 genes).



As we can see some of these genes exhibit two groups. The genes are colored by sex. Black is female and red is male. Genes 4, 5, 6, 8, 9, 10, 11, 12, 14, and 16 probably form two groups due to gender differences. Genes 7, 13, and 15 show two groups but probably not due to gender. Perhaps they are due to some other unknown factors (biological or technical) or they are just due to chance. For the blueGroup



gene 1 appears to be skewed systematically whereas gene 2 appears to be influenced by three extreme levels.

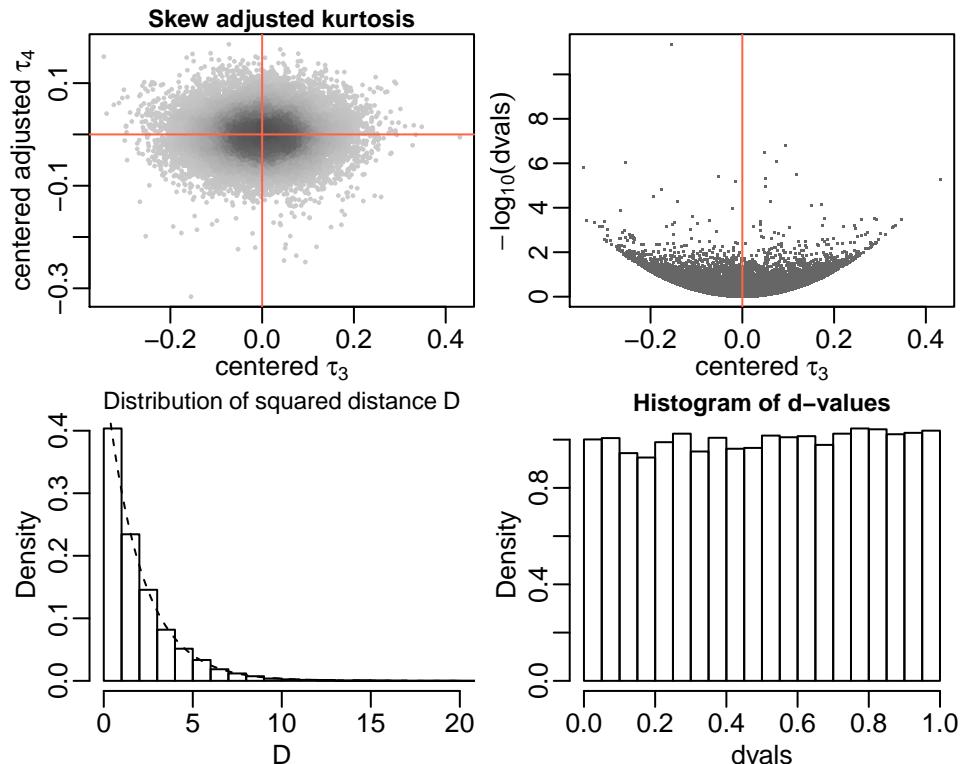
2.5 Steps in outlier computation

We know describe how we assign d-values to the genes. There are three main steps:

1. Estimate the dependence of L-kurt (τ_4) on L-skew (τ_3) with a lowess fuction. And adjust the L-kurt estimates by subtracting the predicted lowess values.
2. Model the adjusted (τ_4) estimates and (τ_3) estimates with a bivariate Gaussian. And compute the statistical distance of each point from the mean.
3. From the statistical distance obtain the exceedance probalitiy using a chi-square distribution with 2 degrees of freedom.

The background steps can be shown when calling `computeDvals()` by setting the argument `plot=TRUE`.

```
> head(computeDvals(t3, t4, plot=TRUE))
```



ENSG00000127720	ENSG00000242018	ENSG00000051596	ENSG00000236211
0.47352534	0.99400885	0.21526143	0.08799414
ENSG00000213697	ENSG00000135541		
0.79532942	0.74748301		

In the top left panel we have shown the adjusted L-kurt and L-skew estimates (both are centered). These points are assumed to be generated from a bivariate Gaussian distribution. See [2] for the basis of this assumption. Statistical distances are computed for each point. The square of these distances follow a chi-square distribution with 2 degrees of freedom. In the bottom left panel we have shown the histogram of the squared distances obtained from the Pickrell dataset. On the top of this histogram we have shown the density of the chi-square 2-df distribution (broken curve). The d-value for a gene is defined as the $\Pr(\chi^2 \text{ 2df} > \text{gene's squared distance})$. In the top right panel we show the $-\log_{10}(d\text{-values})$ versus the centered L-skew estimates. In the bottom right we show a histogram of the d-values. Also shown are the d-values for the first 6 genes in the Pickrell dataset. We have called the statsitics d-values instead of p-values in order to avoid the confusion that it is a formal statistical test. The d-value is used here as a descriptive measure.

3 The Hammoud dataset

Let us now analyze the Hammoud dataset. It contains 10 samples of mRNA profiles of 8-week old wild type mice (strain: C57BL/6). Of the 10 RNA samples 5 were obtained from spermatids (cells) and the other 5 from spermatocytes (cells). The data has been normalized and are in RPKM units.

```
> hammoud <- examplesData$hammoud
> rpkm <- hammoud$exprs
> cond <- hammoud$cond
> (tab <- table(cond))
```

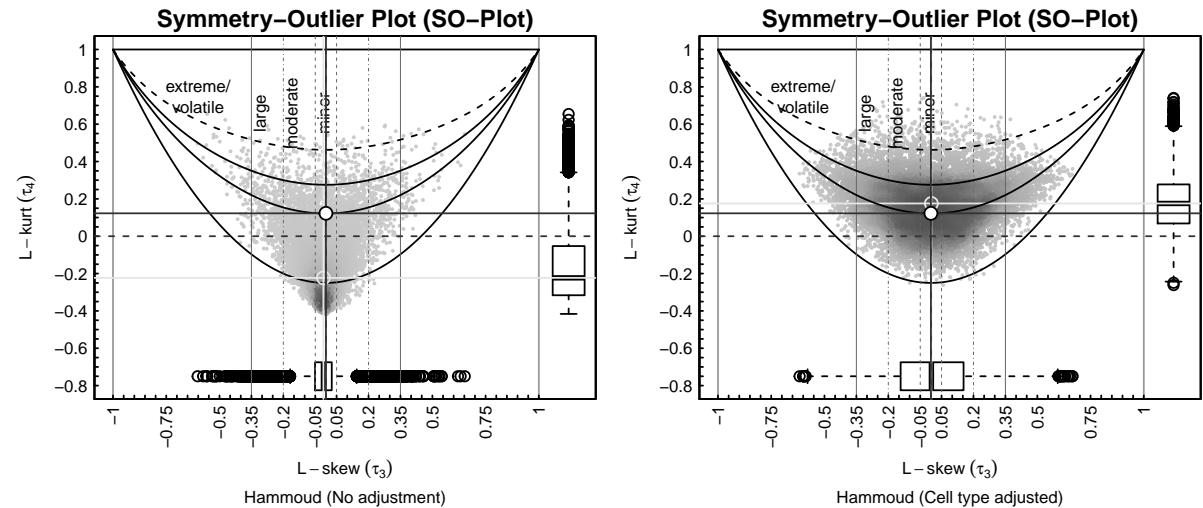
```
cond
Spermatids Spermatocytes
5 5
```

3.1 SO-plot: with and without cell type adjustment

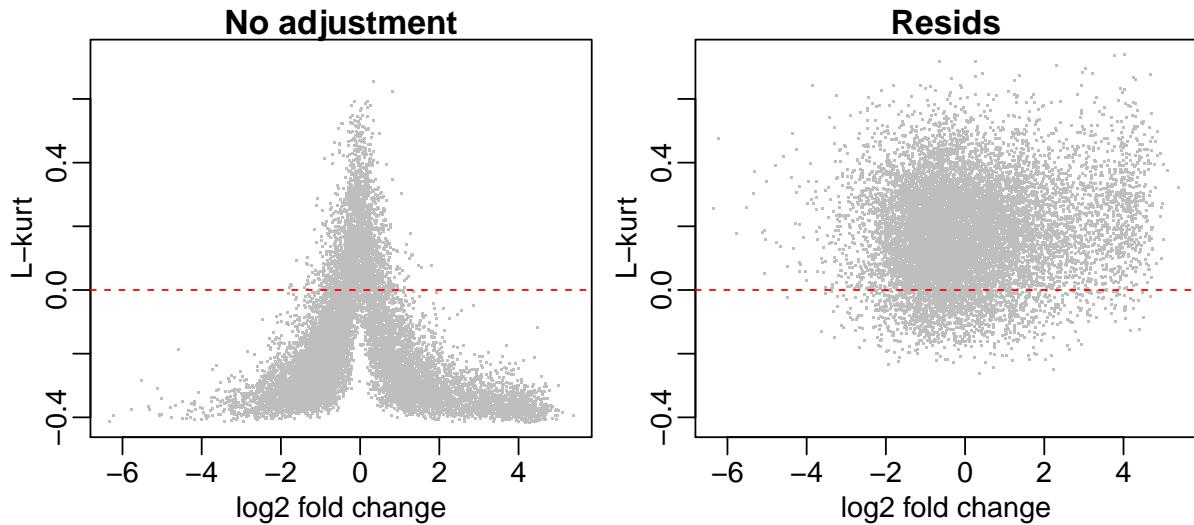
First we filter out genes with low expression by only keeping genes whose RPKM is more than 1 in at least 5 samples (the minimum number of samples per group). Next we transform the RPKM to $\log_2(RPKM + 1)$.

In this analysis we will look at the shape of the genes with and without adjustment for cell type. Let us denote the filtered log transformed RPKM with no cell type adjustment as $\log_2\text{RPKM}$. The adjusted data is then obtained by subtraction the group (spermatid group and spermatocyte group) means from the corresponding samples. We will denote this data as `resids`.

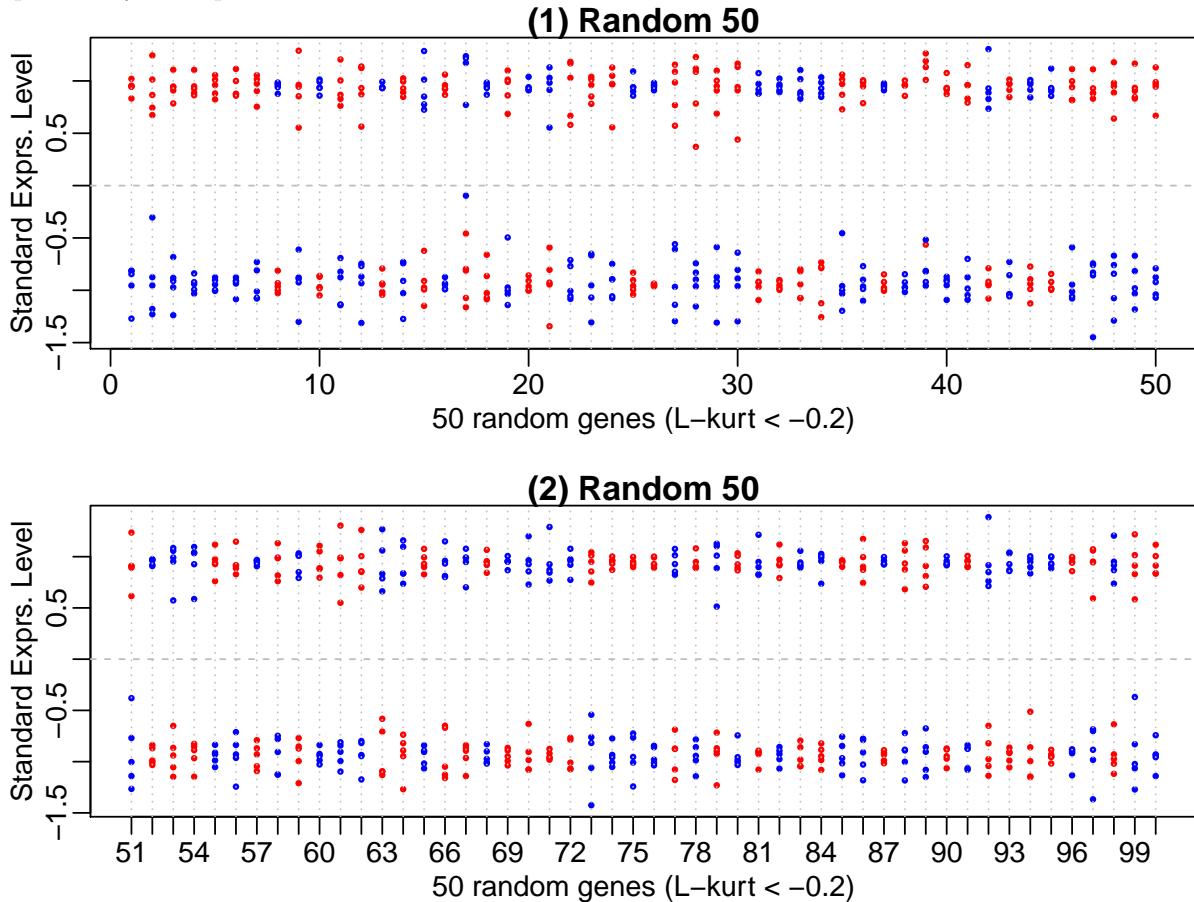
```
> par(mgp=c(1.5, 0.5, 0), mar=c(2.5, 2.5, 1, 0.5), mfrow=c(1, 2))
>
> res1 <- fitShape(t(log2RPKM)) # Without adjustment for cell type.
> res2 <- fitShape(t(resids)) # With adjustment for cell type.
>
> plotSO(res1$lrats["LR3"], res1$lrats["LR4"],
  dataName = "Hammoud (No adjustment)")
> plotSO(res2$lrats["LR3"], res2$lrats["LR4"],
  dataName="Hammoud (Cell type adjusted)")
```



These two SO-plots are very different. The unadjusted SO-plot has the bulk of its genes below $\tau_4 = 0$ and concentrated at $\tau_3 = 0$ (symmetry). This suggests that a lot of genes are differentially expressed across the cell type. Let us investigate further by exploring the relationship between L-kurt and log fold change.



We can see clearly that there is a strong relationship between L-kurt estimates and fold-change in the No adjustment plot whereas there is none in the Resids plot. Let us randomly select and plot a few (100) of the genes with L-kurt estimates less than -0.2. The spermatids samples are colored blue and the spermatocyte samples are colored red.



4 The Bottomly dataset

The Bottomly dataset contains counts summarizing an RNA-seq experiment that includes 21 samples from inbred mouse strains. Eleven of the samples came from the strain DBA/2J and 10 from the strain C57BL/6J.

```
> bottomly <- examplesData$bottomly
> counts <- bottomly$exprs
> cond <- bottomly$cond
> (tab <- table(cond))

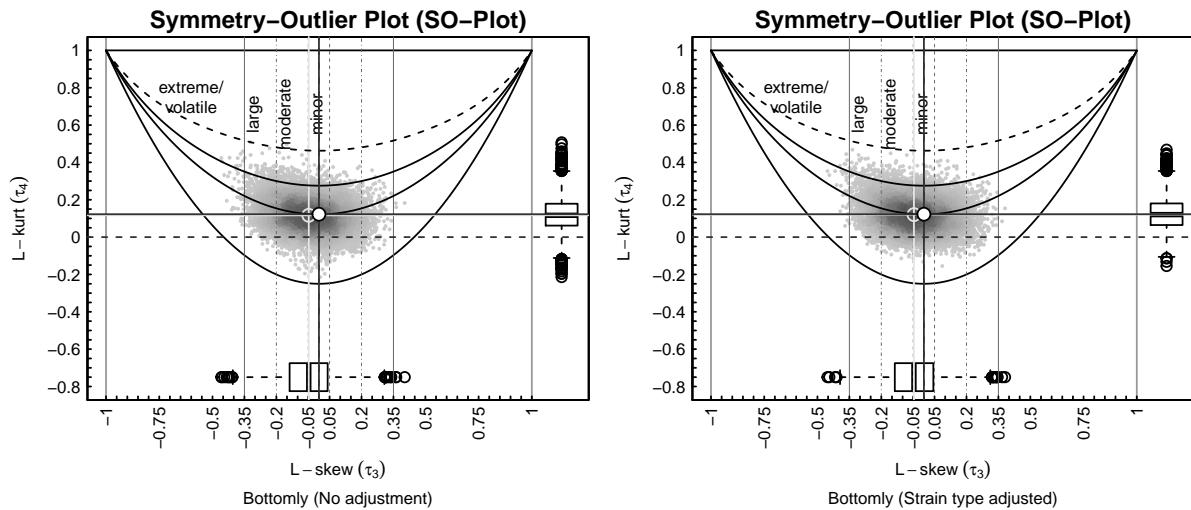
cond
C57BL/6J    DBA/2J
10          11
```

4.1 SO-plot: with and without strain type adjustment

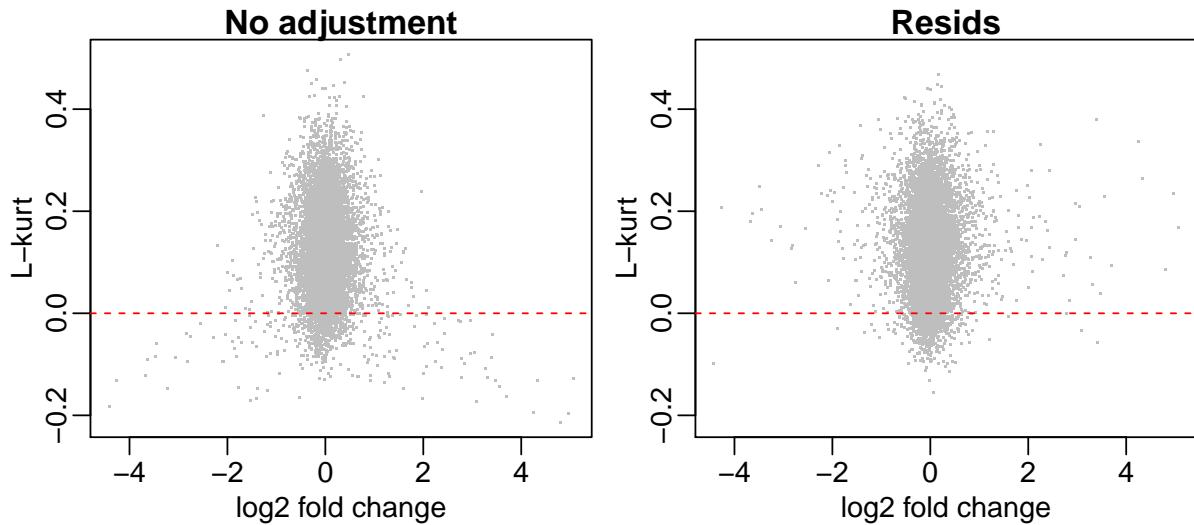
First we filter out genes with low expression by only keeping genes whose cpm is more than 1 in at least 10 samples (the minimum number of samples per condition). Next we normalize the counts + 1 (pseudo-counts) using DESeq's method and transform the normalized pseudo-counts to $\log_2(\text{normalized pseudo-counts})$.

In this analysis we will look at the shape of the genes with and without adjustment for strain. Let us denote the filtered log transformed counts with no strain adjustment as `log2pcounts`. The adjusted data is then obtained by subtracting the group (DBA/2J group and C57BL/6J group) means from the corresponding samples. We will denote this data as `resids`.

```
> par(mgp=c(1.5, 0.5, 0), mar=c(2.5, 2.5, 1, 0.5), mflow=c(1, 2))
>
> res1 <- fitShape(t(log2pcounts)) # Without adjustment for strain.
> res2 <- fitShape(t(resids)) # With adjustment for strain.
>
> plotSO(res1$lrats["LR3"], res1$lrats["LR4"],
  dataName = "Bottomly (No adjustment)")
> plotSO(res2$lrats["LR3"], res2$lrats["LR4"],
  dataName="Bottomly (Strain type adjusted)")
```



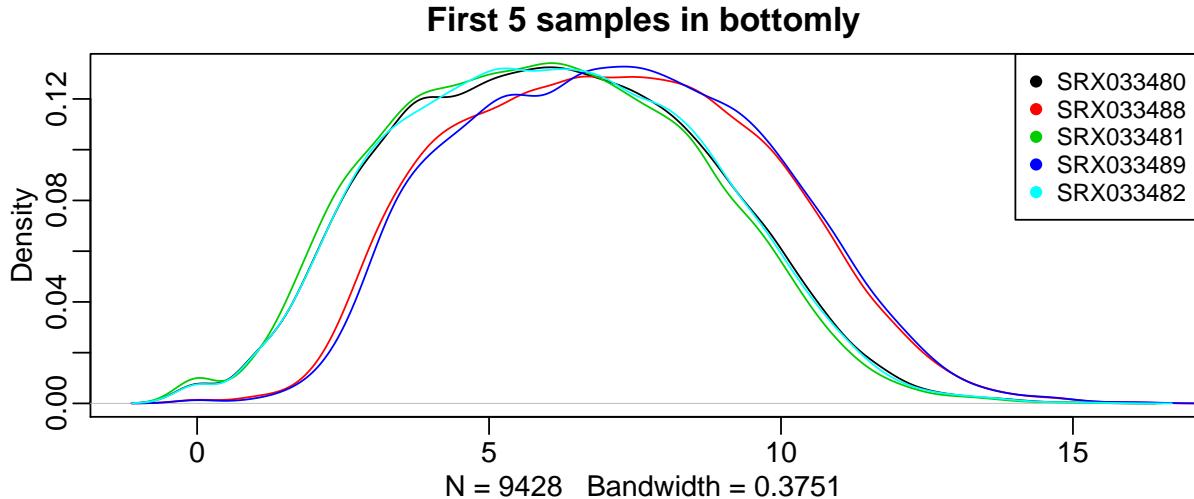
These two SO-plots are very similar. This suggests that most genes are not differentially expressed. Let us investigate further by exploring the relationship between L-kurt and log fold change.



Only a few genes change L-kurt estimates after we adjust for strain.

5 Shape manova

We can use L-moments to summarize the distribution of gene expressions for each sample. We will demonstrate this with the un-normalized bottomly dataset loaded from the previous section (currently defined as counts). Below are density plots of the first five samples.



We can summarize the shape of these samples with L-moments statistics as shown below.

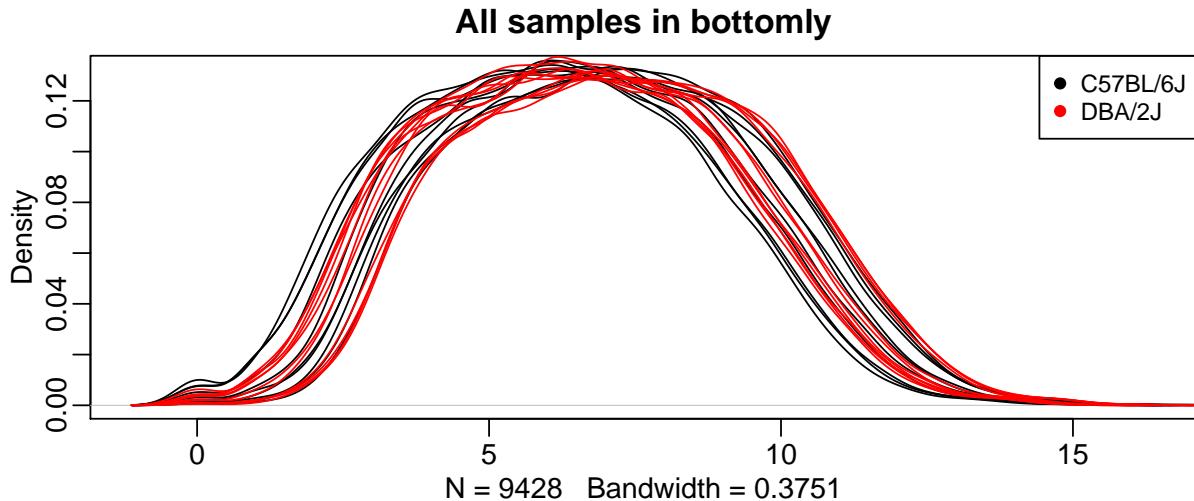
```
> fitShape(log2(counts+1) [,1:5]) # counts equals raw counts

$lcv
SRX033480  SRX033488  SRX033481  SRX033489  SRX033482
0.2439561  0.2088938  0.2474517  0.2046416  0.2430171

$lrats
SRX033480  SRX033488  SRX033481  SRX033489  SRX033482
LR3 0.02298013 0.02627616 0.02389291 0.02543849 0.02255072
LR4 0.07447406 0.06760287 0.07453042 0.06876221 0.07489465

$lmoms
SRX033480  SRX033488  SRX033481  SRX033489  SRX033482
L1 6.08659386 7.12305448 5.94364519 7.20841154 6.07479153
L2 1.48486173 1.48796205 1.47076498 1.47514057 1.47627806
L3 0.03412231 0.03909793 0.03514085 0.03752534 0.03329114
L4 0.11058368 0.10059051 0.10961673 0.10143392 0.11056534
```

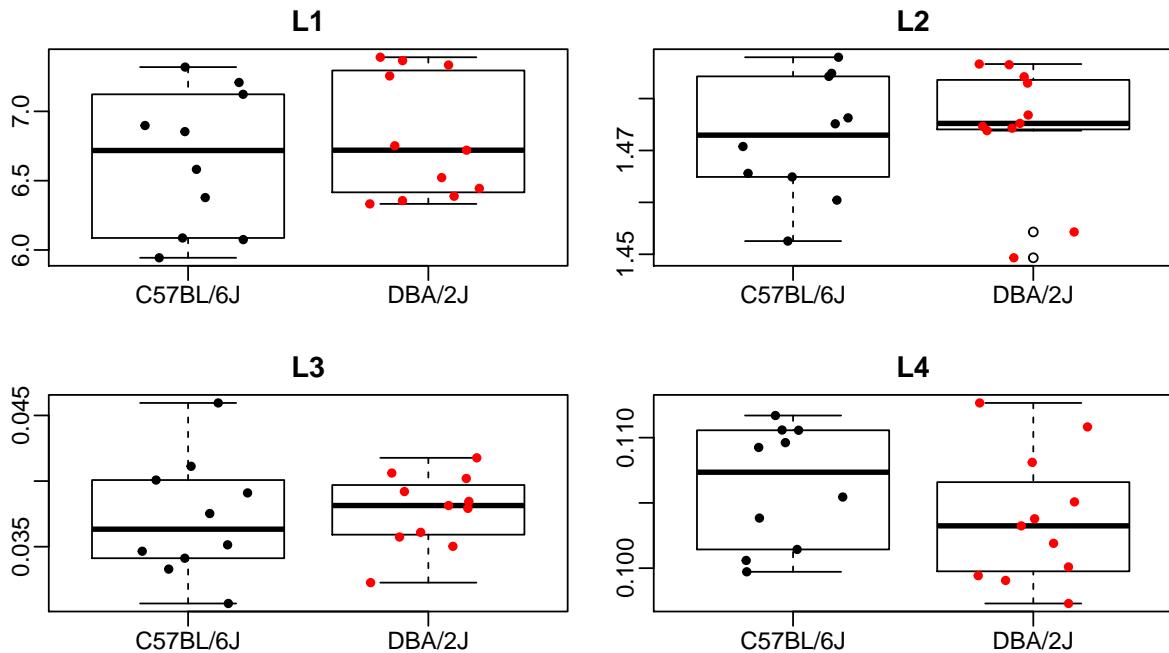
Based on these statistics we can test whether the sample distributions differ across biological groups. The shapeManova function tests this hypothesis by performing a multivariate anova (manova) on the first four L-moments. It also provides single anova p-values for each L-moment separately (adjusted for multiplicity using Bonferroni's method).



```
> table(cond)

cond
C57BL/6J    DBA/2J
10          11

> boxplotSLM(data=log2(counts+1), groups=cond, data.name="Bottomly",
   groupCol=rep(c("black", "red"), table(cond)))
```



```
> shapeManova(data=log2(counts+1), groups=cond)

$apvals
  L1     L2     L3     L4 
1.000 1.000 1.000 0.995 

$manova.pval
[1] 0.846
```

Individually, all the L-moments are not significantly different across the two mouse strains. Suggesting that the sample distributions are not significantly different across the mouse strains. Jointly, the manova p-value confirms this.

6 Summary and discussion

We have built on the sound statistical properties of the L-moments ratio estimators to provide a framework for exploring the distributional shapes of genes and the detection of genes (volatile/outlier genes) with shapes that are markedly different from the majority in a given high-throughput transcriptome dataset (SO-plot). The SO-plot (symmetry-outlier) is informative for samples sizes as little as $n \geq 6$. This makes the SO-plot a very powerful tool for exploratory purposes.

Although we analyzed RNA-seq data other types of high-throughput data can benefit from this kind of analysis. In the future examples of analyzing microarray data and methylation data will be included in this vignette.

7 Session Information

```
> sessionInfo()

R version 3.2.1 (2015-06-18)
Platform: x86_64-apple-darwin13.4.0 (64-bit)
Running under: OS X 10.10.5 (Yosemite)

locale:
[1] en_US.UTF-8/en_US.UTF-8/en_US.UTF-8/C/en_US.UTF-8/en_US.UTF-8

attached base packages:
[1] stats      graphics   grDevices utils      datasets   methods
[7] base

other attached packages:
[1] HTShape_1.0.0 knitr_1.12.3

loaded via a namespace (and not attached):
[1] magrittr_1.5       formatR_1.2.1       tools_3.2.1
[4] KernSmooth_2.23-15 stringi_1.0-1       highr_0.5.1
[7] stringr_1.0.0     evaluate_0.8
```

- [1] W. Kirby. Algebraic boundedness of sample statistics. *Water Resources Research*, 10:220–222, 1974.
- [2] J. Hosking. L-moments: analysis and estimation of distributions using linear combinations of order statistics. *Journal of the Royal Statistical Society. Series B (Methodological)*, 52:105–124, 1990.
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- [5] K. Pickrell, J. Marioni, A. Pai, J. Degner, B. Engelhardt, E. Nkadori, J. Veyrieras, M. Stephens, Y. Gilad, and J. Pritchard. Understanding mechanisms underlying human gene expression variation with RNA-sequencing. *Nature*, 464:768–772, 2010.
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- [7] S. Hammoud, D. Low, C. Yi, D. Carrell, E. Guccione, and B. Cairns. Chromatin and transcription transitions of mammalian adult germline stem cells and spermatogenesis. *Cell stem cell*, 2014.
- [8] D. Bottomly, N. Walter, J. Hunter, P. Darakjian, S. Kawane, K. Buck, R. Searles, M. Mooney, S. McWeeney, and R. Hitzemann. Evaluating gene expression in C57BL/6J and DBA/2J mouse striatum using RNA-seq and microarrays. *PloS one*, 6:e17820, 2011.
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