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Gene expression

DEGseq: an R package for identifying differentially expressed genes from RNA-seq data

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ABSTRACT

Summary: High-throughput RNA sequencing (RNA-seq) is rapidly emerging as a major quantitative transcriptome profiling platform. Here, we present DEGseq, an R package to identify differentially expressed genes or isoforms for RNA-seq data from different samples. In this package, we integrated three existing methods, and introduced two novel methods based on MA-plot to detect and visualize gene expression difference.

Availability: The R package and a quick-start vignette is available at http://bioinfo.au.tsinghua.edu.cn/software/degseq

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Supplementary information: Supplementary data are available at *Bioinformatics* online.

1 INTRODUCTION

High-throughput sequencing technologies developed rapidly in recent years. These technologies can generate millions of reads in a relatively short time and at low cost. Using such platforms to sequence cDNA samples (RNA-seq) has been shown as a powerful method to analyze the transcriptome of eukaryotic genomes (Wang *et al.*, 2009). RNA-seq can provide digital gene expression measurement and is regarded as an attractive approach competing to replace microarrays for analyzing transcriptome in an unbiased and comprehensive manner.

Up to now, there are few handy programs for comparing RNA-seq data and identifying differentially expressed genes from the data, although some recent publications have described their methods for this task (Bloom *et al.*, 2009; Marioni *et al.*, 2008; Tang *et al.*, 2009). Here, we present DEGseq, a free R package for this purpose. Two novel methods along with three existing methods have been integrated into DEGseq to identify differentially expressed genes. The input of DEGseq is uniquely mapped reads from RNA-seq data with a gene annotation of the corresponding genome, or gene (or transcript isoform) expression values provided by other programs like RPKM (Mortazavi *et al.*, 2008). The output of DEGseq includes a text file and an XHTML summary page. The text file contains the expression values for the samples, a *P*-value and two kinds of *Q*-values for each gene to denote its expression difference between libraries. The XHTML summary page contains statistic summary report graphs as shown in Figure 1A.

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2 METHODS

RNA sequencing could be modeled as a random sampling process, in which each read is sampled independently and uniformly from every possible nucleotide in the sample (Jiang and Wong, 2009). Under this assumption the number of reads coming from a gene (or transcript isoform) follows a binomial distribution (and could be approximated by a Poisson distribution). Based on this statistical model, Fisher's exact test and likelihood ratio test were proposed to identify differentially expressed genes (Bloom *et al.*, 2009; Marioni *et al.*, 2008). The two methods have been integrated into DEGseq.

2.1 MA-plot-based method with random sampling model

Using the statistical model described above, we proposed a novel method based on the MA-plot, which is a statistical analysis tool having been widely used to detect and visualize intensity-dependent ratio of microarray data (Yang, *et al.*, 2002). Let C_1 and C_2 denote the counts of reads mapped to a specific gene obtained from two samples, with $C_i \sim$ binomial (n_i, p_i), $i = 1,2$, where n_i denotes the total number of mapped reads and p_i the probability of a read coming from that gene. We define $M = \log_2 C_1 - \log_2 C_2$, and $A = (\log_2 C_1 + \log_2 C_2)/2$. It can be proved that under the random sampling assumption the conditional distribution of M given that $A = a$ (a is an observation of A), follows an approximate normal distribution (see Supplementary Methods Section 1). For each gene on the MA-plot, we do the hypothesis test of $H_0: p_1 = p_2$ versus $H_1: p_1 \neq p_2$. Then a *P*-value could be assigned based on the conditional normal distribution (see Supplementary Materials for detail).

2.2 MA-plot-based method with technical replicates

Though it has been reported that sequencing platform has low background noise (Marioni *et al.*, 2008; Wang *et al.*, 2009), technical replicates would still be informative for quality control and to estimate the variation due to different machines or platforms. We proposed another MA-plot-based method which estimates the noise level by comparing technical replicates in the data (if available). In this method, a sliding-window is first applied on the MA-plot of the two technical replicates along the *A*-axis to estimate the random variation corresponding to different expression levels. A smoothed estimate of the intensity-dependent noise level is done by loess regression, and converted to local standard deviations (SDs) of M conditioned on A, under the assumption of normal distribution. The local SDs are then used

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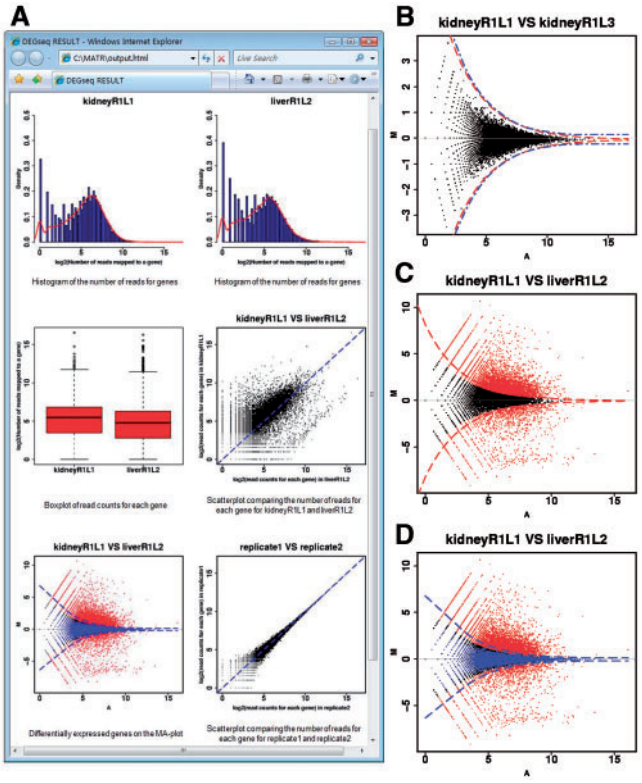

Fig. 1. (**A**) An example of the summary report page generated by DEGseq. (**B**) The plot generated by DEGseq showing whether the variation between technical replicates can be largely explained by the random sampling model. The red lines correspond to the 'theoretical' 4-fold local SD of M conditioned on A according to the random sampling model calculated by the method described in Section 2.1, and the blue lines show the 4-fold local SD of M estimated by the comparison of technical replicates (as described in Section 2.2). See Supplementary Methods Section 3 for detail. (**C**) An example of differentially expressed genes (red points) identified between kidney and liver by the MA-plot-based method with random sampling model at an FDR of 0.1%. The red lines show the 'theoretical' 4-fold local SD of M according to the random sampling model. (**D**) An example of differentially expressed genes (red points) identified between kidney and liver by MA-plot-based method with technical replicates at an FDR of 0.1%. Blue points are from the replicates (kidneyR1L1 and kidneyR1L3), and the blue lines show the 4-fold local SD of M for the two technical replicates.

to identify the difference of the gene expression between the two samples (see Supplementary Materials for detail).

2.3 Multiple testing correction

For the above methods, the P-values calculated for each gene are adjusted to Q-values for multiple testing corrections by two alternative strategies (Benjamini and Hochberg, 1995; Storey and Tibshirani, 2003). Users can set either a P-value or a false discovery rate (FDR) threshold to identify differentially expressed genes.

2.4 Dealing with two groups of samples

To compare two sets of samples with multiple replicates or two groups of samples from different individuals (e.g. disease samples versus control samples), we employed the R package samr (Tibshirani *et al.*, 2009) in DEGseq. The package samr implemented the method described in Tusher *et al.* (2001), which assigns a score to each gene on the basis of change in gene expression relative to the SD of repeated measurements and uses permutations of the repeated measurements to estimate FDR.

3 APPLICATION EXAMPLES

We applied DEGseq on the RNA-seq data from Marioni *et al.* (2008). The RNA samples from human liver and kidney were analyzed using the Illumina Genome Analyzer sequencing platform. Each sample was sequenced in seven lanes, split across two runs of the machine, and two different cDNA concentrations (1.5 pM and 3 pM) were tested for each sample. We used the refFlat gene annotation file downloaded from UCSC Genome browser and chose the method proposed by Storey and Tibshirani (2003) to correct P-values for multiple testing.

We first checked whether the variation between technical replicates could be explained by the random sampling model. This was done with the 'checking' feature in DEGseq (Supplementary Material) on kidney sample sets kidneyR1L1 (sequenced in Run 1, Lane 1) and kidneyR1L3, which were generated at same cDNA concentration. Figure 1B shows that the variation can be almost fully explained by the random sampling model, which supports the notion that technical replicates of this dataset have little technical variation (Marioni *et al.*, 2008). And none of the gene was falsely identified as differentially expressed between the two replicates by each method at an FDR of 0.1%, respectively (Supplementary Table 1). However, samples sequenced at different concentrations showed larger variance (Supplementary Fig. S1A).

We next applied DEGseq to compare the samples from kidney (kidneyR1L1) and liver (liverR1L2). For the MA-plot-based method that needs technical replicates, we used kidneyR1L1 and kidneyR1L3. More than 6000 genes were identified as differentially expressed by each method at an FDR of 0.1%, respectively. And the lists of differentially expressed genes given by different methods are quite consistent with each other (Supplementary Table S2). Figure 1C and 1D shows the results given by the MA-plot-based method with random sampling model and with technical replicates, respectively. And Supplementary Figure S1 shows the results given by the likelihood ratio test and Fisher's exact test.

4 DISCUSSION

In some application, researchers may have several replicates sequenced under each condition. Current observations suggest that typically RNA-seq experiments have low technical background noise (which could be checked using DEGseq) and the Poisson model fits data well. In such cases, users could directly pool the technical replicates together to get higher sequencing depth and detect subtle gene expression changes. Otherwise the methods that estimate the noise by comparing the replicates are recommended. DEGseq also supports users to export gene expression values in a table format which could be directly processed by edgeR (Robinson, 2009), an R package implementing the method based on negative binominal distribution to model overdispersion relative to Poisson for digital gene expression data with small replicates (Robinson and Smyth, 2007).

DEGseq supports using expression values based on either the raw reads counts or normalized gene expression values like RPKM (Mortazavi *et al.*, 2008). But for the methods based on the random

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sampling model, we suggest using the raw counts, which better fits the random sampling model.

DEGseq can also be applied to identify differential expression of exons or pieces of transcripts. Users can define their own 'genes' and compare the expression difference of these 'genes' using DEGseq by simply providing their own annotation files in UCSC refFlat format.

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Conflict of Interest: none declared.

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