Gowinda: unbiased analysis of gene set enrichment for genome-wide association studies

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ABSTRACT
Summary: An analysis of gene set [e.g. Gene Ontology (GO)] enrichment assumes that all genes are sampled independently from each other with the same probability. These assumptions are violated in genome-wide association (GWA) studies since (i) longer genes typically have more single-nucleotide polymorphisms resulting in a higher probability of being sampled and (ii) overlapping genes are sampled in clusters. Herein, we introduce Gowinda, a software specifically designed to test for enrichment of gene sets in GWA studies. We show that GO tests on GWA data could result in a substantial number of false-positive GO terms. Permutation tests implemented in Gowinda eliminate these biases, but maintain sufficient power to detect enrichment of GO terms. Since sufficient resolution for large datasets requires millions of permutations, we use multi-threading to keep computation times reasonable.

Availability and implementation: Gowinda is implemented in Java (v1.6) and freely available on http://code.google.com/p/gowinda/

Supplementary information: Manual: http://code.google.com/p/gowinda/wiki/Manual. Test data and tutorial: http://code.google.com/p/gowinda/wiki/Tutorial. Validation: http://code.google.com/p/gowinda/wiki/Validation.

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1 INTRODUCTION
The advent of high-throughput analysis such as single-nucleotide polymorphism (SNP) arrays and next-generation sequencing enabled large-scale genome-wide association (GWA) studies (Nordborg and Weigel, 2008) or GWA-like studies, such as selective genotyping (Darvasi and Soller, 1994) and experimental evolution (Turner et al., 2011), for almost any phenotype of interest. These studies typically yield hundreds of candidate SNPs associated with the studied trait. A wide-spread approach to shed light on the biological implications of these SNPs is a test for gene set enrichment [e.g. Gene Ontology (GO)] (Ashburner et al., 2000; Wang et al., 2010).

Such an analysis of gene set enrichment is based on the assumptions that all genes are sampled independently from each other with the same probability. These assumptions are violated with data from GWA studies as (i) longer genes usually have more SNPs resulting in a higher probability of being sampled and (ii) overlapping genes are sampled in clusters (Holmans et al., 2009). For these reasons, we developed Gowinda, a user-friendly and multi-threaded software specifically designed for detecting unbiased enrichment in gene sets from large datasets such as generated by GWA studies. By relying on standard file formats, any species with a sequenced and annotated genome may be analyzed, hence it favorably compares to a similar tool that is restricted to the analysis of GWA datasets in humans (Holmans et al., 2009). For a review of different available methods, see Wang et al. (2010). We validated Gowinda and show that the biases inherent to GWA dataset could result in a substantial number of false-positive GO terms and that Gowinda eliminates these biases while still yielding highly reliable results.

2 IMPLEMENTATION
Gowinda calculates the significance of overrepresentation for each gene set with permutation tests. Gowinda randomly samples SNPs from the total set of SNPs and records the associated genes. After repeating this permutation multiple times, an empirical null distribution of gene abundance for every gene set is obtained. The significance of overrepresentation of the candidate SNPs is estimated from the empirical null distribution. To account for multiple testing, an empirical false discovery rate (FDR) is calculated, by dividing the number of expected gene sets for a given P-value (averaged from the simulations) by the number of observed gene sets.

Gowinda requires four input files, all of which widely used standard formats: a file with the annotation of the species of interest (.gtf or .gff), a file with the total set of SNPs used for the GWA study [.vcf, .mpileup or similar (Danecek et al., 2011; Li et al., 2009)], a file containing the candidate SNPs (must be a subset of all SNPs) and a file with the mapping of genes to gene sets. Such files with the mapping between genes and GO terms can, for example, be obtained either from FuncAssociate2 (Berziz et al., 2009) or from High-Throughput (HT) GoMiner (Zeeberg et al., 2005). In addition to this, Gowinda can be used to identify enrichment of SNPs in any user-defined gene set (Manual: http://code.google.com/p/gowinda/wiki/Manual; Test data and walkthrough: http://code.google.com/p/gowinda/wiki/Tutorial).

Gowinda does not reproduce the exact pattern of linkage disequilibrium (LD) between SNPs but offers two complementary test strategies making two extreme assumptions about LD:

• SNPs are in linkage equilibrium: Gowinda randomly samples the same number of SNPs as candidate SNPs. Subsequently, the corresponding genes are identified and overrepresentation is estimated as described above. Note that the number of randomly sampled SNPs is kept constant in the simulations.

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Another important feature of Gowinda is a flexible definition of a gene. We randomly picked five small GO categories (5–10 genes) and introduced a candidate SNP into every gene associated with these GO categories. Subsequently, we randomly sampled 1000 SNPs and computed the significance for the overrepresentation of every GO category, either on the basis of SNPs using Gowinda or based on the corresponding genes using HT GoMiner. We found that Gowinda yields almost identical results as HT GoMiner (Fig. 1A; Spearman’s rank correlation, $\rho = 0.99$; $P$-value $< 2.2 \times 10^{-16}$). We also assessed the bias introduced in GWAS data and to what extent Gowinda corrects for this bias. We created a biased dataset by introducing a SNP every 100 bp into all genes and again randomly sampled 1000 SNPs. We calculated the significance of overrepresentation using Gowinda and HT GoMiner. Consistent with the expected bias due to different gene lengths and overlapping genes, HT GoMiner reports a significant enrichment for 341 GO categories (FDR < 0.05), whereas Gowinda correctly reports zero (FDR < 0.05). We also found that the correlation of the $P$-values of overrepresentation for Gowinda and HT GoMiner dramatically decreased with the biased dataset (Fig. 1B; Spearman’s rank correlation; $\rho = 0.56$; $P$-value $< 2.2 \times 10^{-16}$).

Finally, we tested whether Gowinda correctly identifies overrepresentation for five randomly preselected GO categories. We randomly picked five small GO categories (5–10 genes) and introduced a candidate SNP into every gene associated with these GO categories. Subsequently, we randomly sampled SNPs from the biased dataset until a total of 1000 candidate SNPs were obtained.}

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