Collapsed Haplotype Pattern Method for Linkage Analysis of Next-Generation Sequence Data

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[sleal@bcm.edu](mailto:sleal@bcm.edu)**Abstract**

Recent advances in next generation sequencing (NGS) make it possible to directly sequence genomes and exomes of individuals with Mendelian diseases and screen the sequence data for causal variants. With the reduction in cost of NGS, DNA samples from entire families can be sequenced and linkage analysis can be performed directly using NGS data. Inspired by “burden” tests which are used for complex trait rare variant association studies, we developed a collapsed haplotype pattern (CHP) method for linkage analysis. Using data from several deafness genes we demonstrate that the CHP method is substantially more powerful than analyzing individual variants. Unlike applying NGS data filtering approaches, the CHP method provides statistical evidence of a gene’s involvement in disease etiology and is also less likely to exclude causal variants in presence of phenocopies and/or reduced penetrance. The CHP method was implemented in the SEQLinkage software package which can perform linkage analysis on NGS data or can generate data compatible with many linkage analysis tools, reviving them for use in NGS era.

**Keywords**

Parametric linkage analysis, LOD scores, Mendelian diseases and traits, Next-generation sequence data, Statistical method.**Introduction**

The advent and advance of NGS in recent years has led to the identification of a large number of Mendelian disease genes. The typical approach to identifying Mendelian disease causal variants using either whole genome sequence (WGS) or exome sequence (WES) data is to filter variants in an affected individual or shared by affected family members, excluding those which are found at higher frequencies, e.g. >0.1% in variant databases. Sometimes unaffected family member(s) are also used in the filtering process. While filtering is straightforward and has been successful1, such efforts rely on limited family information, e.g. mode of inheritance, sharing between a subset of family members and information from external resources on variant functional characterization and frequencies. On the other hand, linkage analysis, which incorporates information on mode of inheritance, penetrance, allele frequencies and genetic map information, remains a powerful tool to localize Mendelian disease loci. As a result, combined SNP array based linkage analysis and sequence based filtering method is becoming popular2. Although it has been shown that analyzing single nucleotide variants (SNVs) from WES data provides acceptable linkage results, due to low heterozygosity of SNVs and allelic heterogeneity this approach can be less powerful than analysis of SNPs from genotyping arrays3.

Here we describe a collapsed haplotype pattern (CHP) method to create markers that are more heterozygous and informative for linkage analysis than individual SNVs. Unlike when SNPs are analyzed the CHP method does not require linkage disequilibrium (LD) pruning to avoid spurious associations4. The CHP method is particularly powerful in the presents of intra- (e.g. compound heterozygotes) and inter-family allelic heterogeneity, a phenomenon commonly observed for Mendelian diseases. When causal variants are missing from samples, CHP method can still maintain some power due to transmission information retained by other variants. We have developed the SEQLinkage software package implementing the CHP method. Since SEQLinkage can test for linkage heterogeneity and calculate Heterogeneity LOD (HLOD) scores the CHP method remains powerful when there is locus heterogeneity, i.e. the underlying genetic etiology is not due to the same gene/region in all families.

**Materials and Methods**

For the CHP method instead of analyzing each variant separately, multiple variants which form haplotypes within a genetic region, e.g. gene, are analyzed. This is done by constructing a marker which reflects the transmission pattern of the entire region and is numerically compatible with currently available linkage analysis methods and software. These markers incorporate allelic heterogeneity between and within families in a region and often have higher heterozygosity than SNVs, making them more informative and powerful to detect linkage.

To generate regional markers, haplotypes for the region must be obtained for all samples with sequence data. NGS data from family members are first checked for Mendelian errors; variants with Mendelian inconsistencies are treated as missing data if they cannot be rectified based on apparent allele transmission patterns. An improved version of the Lander-Green algorithm for genetic phasing is then applied to reconstruct haplotypes in the pedigrees5. For each pedigree, we first cluster variants on regional haplotypes by “bins”, e.g. LD blocks, and collapse variants in a bin into an indicator variable with values 0 or 1 for having no minor allele or at least one minor allele within the bin, which is similar to the collapsing method for association analysis with rare variants6. We then assign each collapsed haplotype a single numeric value so that different patterns of collapsed haplotypes in all samples are uniquely represented (Figure 1). The choice of coding for patterns are arbitrary, although we use continuous positive integers and assign a smaller value for collapsed haplotypes having more 0’s than 1’s. The sample haplotypes thus represented can be directly used for parametric linkage analysis with many existing linkage software packages.

For WES data genes can be used as regional markers. Within each region, commonly used bin size options for variants collapsing are 1) LD based collapsing, which uses estimated LD blocks as bins, 2) complete collapsing, whose bin size equals gene/region length and 3) no collapsing, whose bin size equals one. For regions where recombination events occur within a family, the sub-unit that shows the strongest evidence of linkage among all sub-units created by recombination breakpoints is used as the LOD score for the family on the region so that results from multiple families can be combined.

In order to reconstruct genotypes for family members missing sequence data, linkage analysis requires marker allele frequencies. Frequencies of regional markers generated by CHP method can be derived from minor allele frequencies (MAF) of variants and pair-wise LD between variants. For rare variants with MAF derived from large samples (see Discussion), the minor allele counts can be approximated by a multivariate Poisson distribution with joint probability mass function  where  is expected allele counts for the *M* variants and  is the variance-covariance matrix7. The covariance between variants  and  can be computed by  where  is the LD coefficient,  is population MAF and  is the sample size based on which population MAF are estimated. Therefore for given haplotype pattern the corresponding frequency  can be computed from the probability mass function. When collapsing is applied, MAF for the collapsed unit is given as  by definition. Calculations of haplotype pattern frequencies are based on population MAF of rare variants; but in a given family where only limited haplotypes are observed, haplotype pattern frequency should be adjusted to reflect their relative probability of occurrence in the family. Therefore for haplotypes in a family we normalize the frequencies such that they sum to 1. The normalized collapsed haplotype pattern frequencies are then used as the allele frequencies for the corresponding regional genotype markers.

To facilitate linkage analysis using sequence data in VCF format, we developed the SEQLinkage software that uses the Elston-Stewart algorithm as incorporated in FASTLINK8. It provides results in text format and high quality graphical reports for both LOD and HLOD scores. Additionally SEQLinkage supports output of regional genotype data into formats compatible with linkage software such as LINKAGE9 and Merlin10, with which two-point and multipoint parametric linkage analysis can be performed. Additionally MEGA211 format is supported, which can be used to transform data to the required input for a number of linkage programs.

To evaluate performance of our method we performed empirical type I error and power calculations of two-point linkage analysis using data on four non-syndromic hearing impairment (NSHI) genes: two autosomal recessive genes *GJB2* and *SLC26A4*, and two autosomal dominant genes *MYO7A* and *MYH9*. Two-generation pedigrees were simulated, allowing for 3 up to 8 offspring in the last generation with the proportions determined by the distribution of number of children per family in the United States in 2012, rescaled such that these proportions add up to 100% (3 children: 69.34%, 4 children: 20.52%, 5 children: 6.84%, 6 children: 2.28%, 7 children 0.76%, 8 children 0.26%). Genotypes are simulated for the four genes based on the variant sites and the corresponding minor allele frequencies in European Americans recorded in Exome Variant Server (Table S2).

For power evaluations we annotate these variants using Deafness Variation Database (DVD) and NCBI ClinVar, labelling variants as “causal” if they are deemed “HI-causal” by both databases. Disease status for individuals are determined by genotypes on those causal sites under dominant mode of inheritance for *MYO7A* and *MYH9*, and recessive (compound heterozygotes and homozygotes) for *GJB2* and *SLC26A4*, assuming complete penetrance. Additionally for each mode of inheritance we allow for allelic heterogeneity among families, i.e., the causal variant site in a gene may not be the same for different families. We ascertain simulated families having two or more affected offspring for linkage analysis. To introduce locus heterogeneity we mix families having causal variants in one gene but not others, so that each simulated gene contributes to etiology of only a proportion of families in the entire dataset. We simulate 500 replicates under each different setting of sample size, mode of inheritance, presence of allelic heterogeneity and locus heterogeneity. For each replicate we compute LOD and HLOD scores using the CHP method and also for SNV markers for comparison purposes. Power is estimated by  where the denominator is the total number of replicates and the numerator is the number of tests that successfully detected the linkage signal, i.e. LOD score greater than 3.3 or HLOD score greater than 3.6 which provides a genome wide significance level of p<0.0512.

For type I error evaluations we use the same gene sequences and demographic data, yet simulate disease pedigrees under the null, i.e., affection status not caused by any of the rare variants in the gene of interest. We consider different genetic architectures under the null including situations when 1) variants in the gene region are independent, 2) there is complete LD between variants and 3) there are recombination events within a gene at each meiosis yet the recombination breakpoints are different across sample families. Additionally we simulate scenarios when parental genotypes are missing at random to evaluate type I error when CHP marker frequencies have to be calculated using population MAF and LD estimated from data. Type I errors are computed for cumulative HLOD scores on gene *SLC26A4* across 20 families using 500 replicates.

**Results**

Empirical type I error estimates are constantly zero for all scenarios previously described, suggesting that there is no inflation of test statistic in the presence of within-gene recombination, strong inter-marker LD or missing genotype data. Empirical power calculations for several known non-syndromic hearing loss genes using the CHP method as well as for individual SNV are summarized by contour plots as displayed in Figures 2. Power analysis on LOD and HLOD suggests that CHP is substantially more powerful for all models in the presence of intra- (Figure 2C) and inter-family allelic heterogeneity (Figures 2A–2C). Specifically for an autosomal recessive model with allelic heterogeneity, i.e. compound heterozygotes, and also with locus heterogeneity of 50%, it requires 12 families for CHP to achieve a power of 90% for gene *SLC26A4*, while analyzing individual SNVs requires >50 families to achieve the same power at a significance level of α=0.05.

We provide sample size estimates for the simulated NSHI linkage study based on HLOD scores, assuming 50% locus heterogeneity. Specifically we estimated sample size requirement for CHP when causal variants are missing from sequence data in a large proportion of the participating families. Table 1 demonstrates that comparing to SNV method, CHP method can tolerate some extend of missing data, and in certain scenarios the required sample size for CHP is still smaller than SNV method even when causal variants from 75% participating families are missing.

**Discussion**

For linkage analysis, correct specification of marker allele frequency is crucial to controlling for type I error and reducing type II error13. The number of founders with available genotypes in data for linkage analysis might often be too small to obtain a sufficiently accurate allele frequency estimate, thus we recommend the input VCF file be annotated with external source of MAF information such as from 1000 genomes or Exome Variant Server. For some populations MAF information may not be available and frequencies estimated from founders have to be used.

In the context of Mendelian disease mapping it is reasonable to assume that common variants are not directly causal. Therefore including common variants will neither contribute to nor reduce power when causal rare variants are sequenced and haplotypes are directly used as markers. However, common variants should be excluded from analysis when variants in a region are binned (see Figure 1) because they may mask the contribution of rare variants and attenuate linkage signals. If common variants are excluded from CHP analysis, we suggest that these variants should be analyzed separately because for regions where rare causal variants are missing from sequence data, common variants might capture linkage signals if they are linked to the nearby missing causal variants.

We have also compared power of CHP with multipoint linkage analysis performed by Genehunter14. Although multipoint linkage is more powerful than analyzing individual marker, our method still remains the most powerful (Table S1). Regardless of power, cautions have to be used when performing multipoint linkage analysis on sequence data because when genotypes are missing for some samples (common for NGS based family data) and haplotype frequencies estimations are performed, tightly linked variants may lead to inflated type I error for multipoint linkage analysis programs that ignore inter-marker LD in their models15,16. Even for programs that model inter-marker LD, e.g., LINKAGE/FASTLINK and Merlin, the haplotype frequency estimates involving rare variants can be inaccurate for studies with limited number of founders, leading to inflated type I error.

The SEQLinkage package, freely available at URL <http://bioinformatics.org/seqlink>, can efficiently extract genotypes from VCF files and uses the CHP method described here to perform linkage analysis as well as data format conversion on sequence data so that other programs can also be used to perform linkage analysis if desired. It provides a novel and effective approach that brings back well established linkage analysis techniques for use with the growing wealth of genomic data of human pedigrees. Unlike the commonly practiced filtering approaches used for sequence data, SEQLinkage provides statistical evidence of the involvement of variants in the etiology of Mendelian diseases. Additionally because it incorporates mode of inheritance information and penetrance models it is less likely than filtering to exclude causal variants in the presence of phenocopies and/or reduced penetrance. For Mendelian traits for which the penetrance model is not well established but the mode of inheritance is known, an affected-only analysis can be performed where all unaffected individuals are made unknown to avoid decreased power due the use of an incorrect penetrance model. We recommend the use of SEQLinkage in parallel to filtering methods on the same sequence data to take full advantage of the power of NGS in families.**Acknowledgements**

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**Web Resources**

America's Families and Living Arrangements, https://www.census.gov/prod/2013pubs/p20-570.pdf

Exome Variant Server (EVS), http://evs.gs.washington.edu/EVS

Deafness Variation Database (DVD), http://deafnessvariationdatabase.com

NCBI ClinVar, https://www.ncbi.nlm.nih.gov/clinvar

**Figure legends**

**Figure 1. The Collapsed Haplotype Pattern (CHP) method**. This figure illustrates the coding of regional markers for a group of 6 variants in pedigree with autosomal compound recessive disorder. Panel A displays the use of original haplotype patterns as regional markers, a special case of the CHP method using bins of size 1; panel B displays the *complete collapsing* theme with bin size equaling the length of the region; panel C displays a collapsing theme with bins of size 3 and panel D displays the *LD based collapsing* theme assuming the 2nd to 6th variant loci are in LD with each other. Collapsing themes B, C and D are useful when the region under consideration is too diverse to represent with a reasonable number of markers for the linkage analysis algorithm to work with large pedigrees.

**Figure 2. Power comparisons for LOD and HLOD statistics in two-point linkage analyses.** This figure shows the power for collapsed haplotype pattern markers (CHP) vs. single nucleotide variant (SNV) analysis under various modes of inheritance in the presence of intra- and inter-family allelic heterogeneity. X-axis is number of families, Y-axis is proportion of locus heterogeneity, i.e. the proportion of families with non-syndromic hearing impairment (NSHI) caused by detrimental variants in the gene under investigation, i.e. either *MYO7A* or *MYH9* for dominant model, or *GJB2* or *SLC26A4* for recessive model. Contour curves on the graphs are power estimates, dark orange lines for CHP and light blue lines for SNV. Panel A displays the power for the LOD and HLOD statistics under dominant model; panel B displays the power for the LOD and HLOD statistics under recessive model; panel C displays the power for the LOD and HLOD statistics under recessive model in the presence of intra-family allelic heterogeneity, i.e. affected individuals are compound heterozygous. CHP method is more powerful for both LOD and HLOD at a significance level of α=0.05, but the absolute power of HLOD is not significantly larger than LOD. This is because due to the very low MAFs for the genes under study for most families all variants in the non-causal gene are monomorphic and therefore are uninformative.

**Tables**

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| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Required Power | Gene | MOI | CHP1 | SNV2 | | CHP-M75%3 | | SNV-M75% |
| 0.8 | *SLC26A4* | recessive | 11 | 40 | 39 | | 160 | |
| 0.9 | *SLC26A4* | recessive | 13 | 45 | 46 | | 180 | |
| 0.8 | *SLC26A4* | compound recessive | 11 | 50 | 39 | | 200 | |
| 0.9 | *SLC26A4* | compound recessive | 13 | 55 | 46 | | 220 | |
| 0.8 | *GJB2* | recessive | 12 | 23 | 44 | | 92 | |
| 0.9 | *GJB2* | recessive | 14 | 28 | 52 | | 112 | |
| 0.8 | *GJB2* | compound recessive | 12 | 25 | 44 | | 100 | |
| 0.9 | *GJB2* | compound recessive | 14 | 34 | 52 | | 136 | |
| 0.8 | *MYO7A* | dominant | 12 | 16 | 31 | | 64 | |
| 0.9 | *MYO7A* | dominant | 14 | 20 | 36 | | 80 | |
| 0.8 | *MYH9* | dominant | 11 | 13 | 32 | | 52 | |
| 0.9 | *MYH9* | dominant | 14 | 18 | 41 | | 72 | |
| Note: 50% locus heterogeneity is assumed for all scenarios.  1Number of families required for CHP method.  2Number of families required for single variant method.  3“M-75%”: number of families required when causal variants in 75% participating families are missing. | | | | | | | | |

**Table 1: Sample size estimates for the simulated NSHI study.**