Lecture 6: Generalized multivariate analysis of variance

Measuring association of the ‘entire’ microbiome with other variables

- Distance matrices capture some aspects of the data (e.g. microbiome composition, relative abundance, phylogenetic relationships).
- Euclidean distance (square-root of sums of square differences between components of the centered data) captures the covariances of the variables.
- Can these characteristics be used to draw association of the entire microbiome with other variables of interest (e.g. treatment group, locus of sampling, etc.)?
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A general strategy for multivariate analysis

- Apply a normalization to the data (e.g. relative abundance);
- Calculate a distance metric between the observations (e.g. Unifrac, Jensen-Shannon, Chi-Square);
- Perform ordination and/or clustering analysis to visualize relationships between observations;
- **Test for differences between predefined groups (e.g. treatment levels, phenotypes)**
ANOVA

- Idea: $SS_{\text{total}} = SS_{\text{error}} + SS_{\text{treatments}}$
- F test: $F = [SS_{\text{treatments}}/(I - 1)]/[SS_{\text{error}}/(n_T - I)]$
- $F = \text{(variance between)/(variance within treatments)}$
- $I$ – number of treatments
- $n_T$ – total number of cases

ANOVA example

<table>
<thead>
<tr>
<th>a1</th>
<th>a2</th>
<th>a3</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>8</td>
<td>13</td>
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<td>8</td>
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<td>7</td>
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<tr>
<td>4</td>
<td>8</td>
<td>12</td>
</tr>
</tbody>
</table>

1. Within group means
   - $Y_1 = (6+8+4+5+3+4)/6 = 5$
   - $Y_2 = \ldots = 9$
   - $Y_3 = \ldots = 10$

2. Overall mean $Y = 8$

3. Between group sum of squares
   - $SS_{\text{treatments}} = n_1(Y_1-Y)^2 + n_2(Y_2-Y)^2 + n_3(Y_3-Y)^2 = 84$
   - $(k - 1) = 3 - 1 = 2$

4. Within group sum of squares
   - $SS_{\text{error}} = 68$
   - $(nT - k) = 18 - 3 = 15$

5. $F = (84/2)/(68/15) = 42/4.5 = 9.3$

6. $F_{\text{critical}}(2, 15) = 3.68$

7. Conclusion: The group effects are statistically significantly different.

8. Next: perform post-hoc pairwise tests to detect the pairs that are different
Euclidean MANOVA

- A direct extension of the univariate ANOVA to multiple variables.
- $SS = \Sigma (Y_i - \overline{Y})^T(Y_i - \overline{Y})$
- $SS = \Sigma d^2$, where $d$ is the Euclidean distance from the center.

Geometric representation of MANOVA
(Anderson, 2001)

$$F = \frac{SS_A/(a - 1)}{SS_W/(N - a)}$$

$SS_A$ – between group sums of squares  
$SS_W$ – within group sums of squares  
$SS_T$ – total sum of squares

$SS_T = SS_W + SS_A$

Key: Mean within group squared distance is equal to sum of squared distances to the centroid.
Calculating F-statistic from arbitrary distance matrices

\[ F = \frac{SS_A/(a - 1)}{SS_W/(N - a)} \]

Fig. 3. Schematic diagram for the calculation of (a) a distance matrix from a raw data matrix and (b) a non-parametric MANOVA statistic for a one-way design (two groups) directly from the distance matrix. \( SS_A \) is sum of squared distances in the half matrix shaded (divided by \( N \) (total number of observations)), \( SS_W \) is sum of squared distances within groups (divided by \( a \) (number of observations per group)). 

Obtaining p-values

- The F-statistic does not follow Fisher’s F-ratio under null, therefore we need to evaluate it’s distribution under null.

- Null hypothesis: there is no difference between groups; therefore, we can compute null distribution empirically by shuffling the group labels.

- For each reshuffling of labels compute F statistic, the p-value is then

\[ P = \frac{\text{No. of } F^* \geq F}{\text{Total no. of } F^*} \]
Post-hoc tests for multi-level factors

- When a factor has more than 2 levels, it is not immediately clear which pair of groups are different from each other.
- To figure this out a post-hoc **pairwise** tests need to be carried out.
- Pairwise p-values are calculated with additional permutations.
- Multiple comparison correction may be necessary.

More sophisticated designs

- Two-way MANOVA
  - Straightforward extension with all interactions considered.
- Stratification/block design
  - When an effect is to be determined within the levels of another factor
  - E.g. Location of sampling vs. treatment
More sophisticated regression scenarios

• Based on Zapala & Schork, PNAS 2006.
• Suppose we have M predictor variables
• We treat the multivariate \((N \times P)\) data (microbiome abundance, gene expression, etc.) as the response variable \(Y\)
• The basic multivariate regression model is \(Y = X\beta + \varepsilon\),
• where \(\beta\) is the coefficient matrix, and \(\varepsilon\) is an error term.
• Define the hat matrix as usual \(H = (X'X)^{-1}X'\).

Regression scenario (continued)

\[ G = -\frac{1}{2} \left( I - \frac{1}{n} 11' \right) D^{(2)} \left( I - \frac{1}{n} 11' \right); \]
• Then \( F = \frac{\text{tr}(HGH)/(M-1)}{\text{tr}[(I-H)G(I-H)]/(N-M)}. \)
• This is how PERMANOVA is implemented in R/vegan package, function adonis().
Assumptions of PERMANOVA

- PERMANOVA is defined for balanced sample sizes, but can be rewritten for \( n_x \neq n_y \).
- Homoscedasticity is an underlying assumption.
- Do violations of these assumptions lead to undesired behaviors?

Simulation to test these assumptions:

- Let \( X \) be 1,000 dimensional uncorrelated standard normal
- Let \( Y \) be 1,000 dimensional uncorrelated multivariate normal with each component
  - mean = \( 1/\sqrt{1000} \)*e
  - S.D. = 0.8
- Simulate data with \( n_x, n_y \) \( \in \{5,10,15,20\} \)
- Compute Euclidean distances, PERMANOVA p-values

Empirical robustness of PERMANOVA to heteroscedasticity and unbalanced sample sizes
Robustness of PERMANOVA

- When both homoscedasticity and balanced sample sizes are violated adverse statistical behavior can be observed.
- If $X$ is the more dispersed sample then
  - $n_x < n_y$ leads to type I error inflation,
  - $n_x > n_y$ leads to loss of power,
  - where $n_x$ is the number of observations in the more dispersed sample.

Idea: Univariate approach to heteroscedasticity issues

- Consider the square of Welch $t$-statistic $T_W^2 = \frac{(\bar{x} - \bar{y})^2}{s_x^2/n_x + s_y^2/n_y}$.
- If we can write $T_W^2$ in terms of pairwise distances, we can generalize it to multivariate data.
- We can use permutation testing to assess the significance.
Key equations for $T_W^2$ derivation

• $s^2 = \frac{1}{n_x(n_x - 1)} \sum_{i<j} (x_i - x_j)^2 = \frac{1}{n_x(n_x - 1)} \sum_{i<j} d_{ij}^2$.

• Where $\sum_{i<j}^n$ denotes double summation $\sum_{i=1}^n \sum_{j=i+1}^n$.

• Let $Z = (z_1, \ldots, z_{n_x+n_y}) = (x_1, \ldots, x_{n_x}, y_1, \ldots, y_{n_y})$,

  \[
  (\bar{x} - \bar{y})^2 = \frac{n_x + n_y}{n_x n_y} \left[ \frac{1}{n_x} \sum_{i<j} (z_i - z_j)^2 - \frac{1}{n_y} \sum_{i<j} (x_i - x_j)^2 - \frac{1}{n_x} \sum_{i<j} (y_i - y_j)^2 \right].
  \]

Pseudo-F vs $T_W^2$

\[
F = \frac{1}{n_x + n_y} \sum_{i,j=1}^{n_x+n_y} d_{ij}^2 - \frac{1}{n_x} \sum_{i<j}^{n_x} d_{ij}^2 - \frac{1}{n_y} \sum_{i<j}^{n_y} d_{ij}^2 \frac{1}{n_x + n_y} \sum_{i,j=1}^{n_x+n_y} (d_{ij}^2) / (n_x - n_y - 2)
\]

\[
T_W^2 = \frac{n_x + n_y}{n_x n_y} \times \frac{1}{n_x + n_y} \sum_{i,j=1}^{n_x+n_y} d_{ij}^2 - \frac{1}{n_x} \sum_{i<j}^{n_x} d_{ij}^2 - \frac{1}{n_y} \sum_{i<j}^{n_y} d_{ij}^2 \frac{1}{n_x + n_y} \sum_{i,j=1}^{n_x+n_y} (d_{ij}^2) / \left( \frac{1}{n_x(n_x-1)} \sum_{i,j=1}^{n_x+n_y} d_{ij}^2 + \frac{1}{n_y(n_y-1)} \sum_{i,j=1}^{n_x+n_y} d_{ij}^2 \right)
\]

How do these compare when $n_x = n_y$ or $\frac{1}{n_x(n_x-1)} \sum_{i<j} d_{ij}^2 - \frac{1}{n_y(n_y-1)} \sum_{i<j} d_{ij}^2$?
Empirical performance of $T_W^2$ vs PERMANOVA

Typical experimental scenarios at $n \approx 10$ or $n \approx 50$
Performance in a real dataset

Table 2. Comparison of PERMANOVA and $T^2_{W}$ on mouse gut microbiome dataset.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>N obs.</th>
<th>$\omega$</th>
<th>d</th>
<th>PERMANOVA</th>
<th>$T^2_{W}$</th>
<th>N obs.</th>
<th>$\omega$</th>
<th>d</th>
<th>PERMANOVA</th>
<th>$T^2_{W}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. vs. All Abx.</td>
<td>10 vs. 40</td>
<td>1.4</td>
<td>0.22</td>
<td>1.21</td>
<td>0.040</td>
<td>0.0001</td>
<td>10 vs. 36</td>
<td>1.4</td>
<td>0.29</td>
<td>1.34</td>
</tr>
<tr>
<td>C. vs. Penicillin</td>
<td>10 vs. 10</td>
<td>0.85</td>
<td>0.12</td>
<td>1.90</td>
<td>0.00001</td>
<td>0.00002</td>
<td>10 vs. 9</td>
<td>1.1</td>
<td>0.07</td>
<td>1.94</td>
</tr>
<tr>
<td>C. vs. Vancomycin</td>
<td>10 vs. 10</td>
<td>1.8</td>
<td>0.08</td>
<td>2.26</td>
<td>0.00009</td>
<td>0.0001</td>
<td>10 vs. 9</td>
<td>1.6</td>
<td>0.21</td>
<td>2.70</td>
</tr>
<tr>
<td>C. vs. Tetracycline</td>
<td>10 vs. 10</td>
<td>1.2</td>
<td>0.12</td>
<td>2.05</td>
<td>0.00005</td>
<td>0.00005</td>
<td>10 vs. 10</td>
<td>1.0</td>
<td>0.07</td>
<td>1.89</td>
</tr>
<tr>
<td>C. vs. Van. + Tetr.</td>
<td>10 vs. 10</td>
<td>1.1</td>
<td>0.10</td>
<td>1.97</td>
<td>0.002</td>
<td>0.002</td>
<td>10 vs. 8</td>
<td>1.4</td>
<td>0.11</td>
<td>2.24</td>
</tr>
</tbody>
</table>

PERMANOVA-S: accommodates multiple distances

- Based on Tang et al. *Bioinformatics* 2016.
- Suppose we want to consider $K$ distances simultaneously, $D_1, \ldots, D_K$.
- We would like to know the significance of the entire ensemble
- Determine which individual distance performs best
PERMANOVA-S: Ensembling algorithm

1. For each $D_k$, compute the observed pseudo-F statistic $F_k$;
2. Obtain $B$ permutations and compute $F_k^{(1)}, ..., F_k^{(B)}$;
3. Compute p-value for each $k$, $p_k$, and $p_{min} = \min(p_1, ..., p_K)$;
4. For each $k$, compute the permutation p-value as $p_k^{(b)} = (B - \text{rank}(F_k^{(b)})/B$;
5. For each permutation $b$, obtain minimal permutation p-value $p_{min}^{(b)} = \min(p_1^{(b)}, ..., p_k^{(b)})$.
6. The final (unified) p-value is the proportion of $p_{min}^{(1)}, ..., p_{min}^{(b)}$ smaller than $p_{min}$.

Summary

• PERMANOVA is useful for omnibus hypothesis testing;
• PERMANOVA has undesirable behavior with unbalanced heteroscedastic data;
• $T_W^2$ corrects that behavior in two sample case;
• PERMANOVA testing can be done with ensembling multiple distances.