Selection of Marker Genes Using Whole-Genome DNA Polymorphism Analysis

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Abstract: Molecular markers serve to assign individual samples to specific groups. Such markers should be easily identified and have a high discrimination power, being highly conserved within groups while showing sufficient variability between the groups that are to be distinguished. The availability of a large number of complete genomic sequences now enables the informed selection of genes as molecular markers based on the observed patterns of variability. We derived a new scoring system based on observed DNA polymorphic differences, and which uses the Bayes theorem as adapted by Wilcox. For validation, we applied this system to the problem of identifying individual species within a prokaryotic (*Vibrio*) and a eukaryotic (*Diphyllobothrium*) genus for validation. Top-scoring candidates genes Chromosome segregation ATPase and ATPase-subunit 6 showed better discrimination power in *Vibrio* and *Diphyllobothrium*, respectively, as compared to standard molecular markers (*recA, dnaJ* and *atpA* for *Vibrio*, and 18s rRNA, ITS and COX1 for *Diphyllobothrium*).

Keywords: molecular marker, genome analysis, Bayes’s theorem, DNA polymorphism

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Background

Molecular methods to assign biological samples to specific groups (e.g., taxonomic groups) have largely replaced morphological comparisons, allowing hundreds or even thousands of characters to be compared across samples. Historically, numerous DNA-based approaches encompassing random whole-genomic analysis have been used to discriminate groups of organisms. These include methods like, among many others, restriction fragment length polymorphism (RFLP), or random amplification of polymorphic DNA (RAPD). Alternatively, sequences from genes, usually selected by their conserved, housekeeping roles, can be used. However, it is often the case that existing markers provide insufficient resolution or are confounded by homoplasy, homologous recombination and lateral gene transfer. In recent years, thanks to great advances in sequencing technologies, the number and diversity of completely sequenced genomes is growing exponentially. This provides the basis for optimizing the selection of marker genes based on the analysis of the whole genetic complement of a given set of organisms. Earlier attempts to use whole-genome information to select marker genes that could best serve as predictors of phylogenetic relatedness include the use of scores based on the level of sequence identities from whole-genome alignments, or the selection of unique sequence signatures present in a few species. These methods, however, do not exploit the information from sequence variability within a species. Here we propose and evaluate an alternative algorithm for the selection of optimal genetic markers, which is based on the comparison of complete genomes. In brief, the basis of our strategy is to rank different genes according to the level of DNA polymorphism within and between defined taxonomic groups. More specifically, DNA polymorphism is measured as the average number of nucleotide differences per site, and a conditional probabilistic statistic based on Bayes’s Theorem as adapted by Willcox is used to prioritize genes, so that genes presenting higher levels of polymorphism between groups but lower variation within a group receive higher scores. In order to validate the methodology, we apply it to the problem of selecting marker genes for the identification of individual species within a prokaryotic (Vibrio) and a eukaryotic (Diphyllobothrium) genus. Publicly available genomic sequences were analyzed to select high-scoring marker genes, which were subsequently amplified and sequenced in a set of additional, non-sequenced strains of these groups. The discrimination power (DP) of these newly obtained sequences was compared to that of traditional marker genes.

Methods

Sequence data

Complete genome sequences were downloaded from the National Center of Bioinformatics Information (NCBI) in Genbank (.GBK) format. These were: (i) chromosome I from the following Vibrio species and strains: V. cholerae (NC_002505), V. vulnificus (NC_004459), V. parahaemolyticus (NC_004603), V. harveyi (NC_009783), V. fischeri (NC_006840), Alivibrio salmonicida (NC_011312), V. splendidus (NC_011753), V. cholerae (NC_009457), V. cholerae (NC_012578), and V. cholerae (NC_012668); (ii) Whole mitochondrial genomes from different Diphyllobothrium species and strains: D. latum (NC_008945), D. nihonkaiense (NC_009463), D. latum (AB269325) and D. latum (DQ985706).

Alignments, polymorphism analysis, and molecular marker score calculation

Genome sequences mentioned above were divided into four different groups: (1) VibrioDS, containing only one representative genome for each Vibrio species, using the Vibrio cholerae strain (NC_002505); (2) VibrioSS, comprising the four different Vibrio cholerae strains; (3) DiphyllobothriumDS containing one genome per Diphyllobothrium species; and strains: D. latum containing one genome per Diphyllobothrium species using NC_008945 as D. latum representative; (4) DiphyllobothriumSS containing all D. latum strains. Each group was aligned using MAUVE v2.3.1 using the progressiveAligner option. Output files were re-formatted to Variscan—extended multi-FASTA (XMFA) format with a custom PERL Script (XMFA.pl) and analyzed using Variscan v2.0. The resulting files were used as an input for the molecular marker score calculation implemented in a custom PERL script (SCORE.pl), and using two different window sizes of 300 pb and 500 pb, for Vibrio and Diphyllobothrium, respectively. The final output
consists of a plain text file listing the potential marker genes, sorted in a descending order of their scores.

Algorithm
The Bohle-Gabaldón (BG) score calculation is based on the level of DNA polymorphism in the Distinct Species (DS) group and Same Species (SS) groups, as inferred from the average of nucleotide differences per site ($\hat{\pi}$). Not more than one SS group may be considered. The Bayes’s theorem as adapted by Willcox is used as follows. If the number of genome sequences in DS group is lower than 4 and there is no length constraint for the marker, formula (1) is used.

If molecular marker with specific size is required ($S_{ref}$) formula (2) is used, $S_i$ is the nucleotides length of gene $i$. Also, if the amount of whole-genomes for DS group is 4 or more, is possible include Tajima’s $D$ ($\pi$) without specific size requirement (3) or with (4), which better account for the possibility of rare haplotypes. Based on Willcox conditions, higher $\hat{\pi}$ in Different Species ($\pi_{i(DS)}$) and lower in Same Species ($\pi_{i(SS)}$) is better. For ($D_{i(DS)}$) in DS group more negative values are preferred and, finally, the size of molecular marker ($S_{ref}$) is arbitrary. In order to reduce sequencing costs we selected rather small sizes (300 pb–500 pb).

BG score using DNA polymorphism (less than 4 genomes):

$$\text{Score}_{i} = \hat{\pi}_{i(DS)} (1 - \hat{\pi}_{i(SS)})$$

(1)

Scoring using DNA polymorphism and Size (less than 4 genomes)

$$\text{Score}_{i}^{\text{Size}} = \hat{\pi}_{i(DS)} (1 - \hat{\pi}_{i(SS)}) \left( \frac{S_i}{S_i + |S_{ref} - S_i|} \right)$$

(2)

Scoring using DNA polymorphism and Tajima’s $D$ (4 genomes and more):

$$\text{Score}_{i}^{\text{Tajima}} = \hat{\pi}_{i(DS)} (1 - \hat{\pi}_{i(SS)}) \left( - \frac{\hat{D}_{i(DS)}}{2} \right)$$

(3)

Experimental validation analysis
Additional Vibrio sequences for the candidate genes were obtained from biological samples stored in the Collection of Aquatic Important Microorganism (CAIM) at the Center of Research for Nutrition and Development (Mexico). Collected strains were: V. ordalii CAIM608, V. aestuarianus CAIM592, V. orientalis CAIM332, V. tubiashii CAIM313, V. splendidus CAIM319, V. cyclitrophicus CAIM 596, V. fortis CAIM629, V. parahaemolyticus CAIM320, V. harveyi CAIM513, V. rotiferianus CAIM577, V. mytili CAIM528, V. navarrensis CAIM609, V. fluvialis CAIM593, V. agarivorans CAIM615, V. mimicus CAIM602, V. metschnikovii CAIM317, V. vulnificus CAIM610, V. orientalis CAIM906 and V. neptunius CAIM532. Similarly, additional sequences for candidate Diphyllobothrium marker genes were obtained from samples fixed in ethanol at the Parasitology Institute of Biology Center of the Czech Republic. These included the strains D. latum TS-07/17, D. pacificum TS-06/30a.b., D. dendriticum TS-04/39, D. nihonkaiense TS-06/236, D. polyergusus TS-05/58 and D. ditremum TS-02/32.

DNA purification and amplification
Genomic DNA from Vibrio species was purified using E.Z.N.A. Bacterial DNA Kit (Omega Biotek, USA). Diphyllobothrium samples were diluted (1) in nuclease-free water, macerated with mortar, to subsequently purify DNA using E.Z.N.A. Tissue DNA Kit (Omega Biotek, USA), following manufacturer’s instructions. The final volume for PCR were 50 µL with 5 µL Buffer 10x (20 mM Tris-HCl pH 8.0, 40 mM NaCl, 2 mM Sodium phosphate, 0.1 mM EDTA, 1 mM DTT, stabilizers, 50% (v/v) glycerol), 1 µL
dNTPs (10 mM), 6 µL MgCl₂ (50 mM), 1 µL primers (10 µM), 0.5 µL Platinum Taq DNA polymerase (2.5 U), 5 µL template DNA and 31.5 µL free nuclease water. Primers for target gene amplification were designed based on the level of observed sequence conservation. The primers used for *Vibrio* were forward 5′-ATG GTT TCA ATT AAN GGN TTR CCK-3′ and reverse 5′-TTA GAT GTA RAK ATC GAC MCC NA-3′ and for *Diphyllobothrium* target gene were forward 5′-ATG ATC TTT AGT GGT TAT TCA-3′ and reverse 5′-CTA ATG GTC CAC TGA AAA TGA TAA TAT-3′. The thermal profile used was the following: initial activation (2 min, 95 °C), followed by 35 cycles of denaturation (1 min, 95 °C), annealing (1 min, 55 °C) and extension (1 min, 72 °C), and a final extension (4 min, 72 °C). Electrophoresis agarose gel (1.5%) stained with Ethidium bromide was used to identify the PCR products from *Vibrio* (∼300 pb) and *Diphyllobothrium* (∼500 pb). PCR products were purified using Minielute gel extraction kit (QIAGEN, USA) and cloned using CloneJET PCR cloning kit (Fermentas, USA). This kit includes the positive selection cloning vector pJET1.2/blunt that contains a lethal gene which is disrupted by ligation of a DNA insert into the cloning site. As a result, only cells with recombinant plasmids are able to propagate. Finally, DNA from the *E. coli* top 10 colonies was purified using E.Z.N.A. bacterial DNA Kit (Omega Biotek, USA). Total DNA obtained from clones was amplified using primers pJET1.2 forward and reverse (CloneJET, Fermentas, USA) with BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystem, USA) using manufacturer’s instructions. The PCRs products were purified for Dyes using Dye Terminator Removal kit (Omega Biotek, USA) and sequenced using ABI PRISM 310 machine (Applied Biosystem, USA). The sequences obtained were edited, assembled, aligned and compared using CLC Genomics Workbench v3.5.5 (CLC Bio, Denmark).

### Molecular marker discrimination power analysis

To prioritize the markers, we developed a simple Discrimination Power (DP) score (5) based in Bayes’s Theorem adapted by Willcoxii which evaluates the maximum identity ($\Delta I_{\text{max}}^x$) for each species in each molecular marker gene ($x$) analyzed.

$$DP_x = \prod_{i=1}^{n} (1 - \Delta I_{\text{max}}^x)$$

The maximum value for DP is 1 (ie, perfect molecular marker), if maximum difference of identity for the closest species in each species for each molecular marker tends to 0. The minimum value for DP is 0.

**Table 1.** 10 top-scoring marker genes for *Vibrio* species discrimination using $S_i = 300$ pb.

| Score | Locus tag | Size (pb) | $\pi_{(DS)}$ | $\pi_{(SS)}$ | Tarima’s $D_{(DS)}$ |
|-------|-----------|-----------|--------------|--------------|-------------------|
| 0.00308 | VC1988 | 0.98387 | 0.03469 | 0.00000 | -0.09022 |
| 0.00252 | VC1954 | 0.33667 | 0.05809 | 0.00000 | -0.12885 |
| 0.00238 | VC2163 | 0.78667 | 0.03703 | 0.00000 | -0.08185 |
| 0.00237 | VC2354 | 0.47667 | 0.04847 | 0.00000 | -0.10258 |
| 0.00233 | VC2665 | 0.96667 | 0.03374 | 0.00000 | -0.07132 |
| 0.00222 | VC2189 | 0.59667 | 0.04396 | 0.00000 | -0.08477 |
| 0.00212 | VC1986 | 0.60653 | 0.04145 | 0.00000 | -0.08437 |
| 0.00208 | VC2658 | 0.82189 | 0.03318 | 0.00000 | -0.07621 |
| 0.00207 | VC2652 | 0.56667 | 0.03689 | 0.00000 | -0.09897 |
| 0.00207 | VC1534 | 0.59817 | 0.04150 | 0.00000 | -0.08352 |

**Table 2.** 10 top-scoring marker genes for *Diphyllobothrium* species discrimination using $S_i = 500$ pb.

| Score | Gen | Size (pb) | $\pi_{(DS)}$ | $\pi_{(SS)}$ |
|-------|-----|-----------|--------------|--------------|
| 0.01175 | ATP6 | 509 | 0.01196 | 0.00013 |
| 0.01066 | ND6 | 458 | 0.01156 | 0.00015 |
| 0.00733 | ND3 | 356 | 0.00944 | 0.00019 |
| 0.00563 | ND4L | 260 | 0.00833 | 0.00028 |
| 0.00524 | COX2 | 569 | 0.00596 | 0.00023 |
| 0.00479 | ND2 | 879 | 0.00841 | 0.00015 |
| 0.00433 | ND4 | 1250 | 0.01083 | 0.00017 |
| 0.00404 | ND1 | 890 | 0.0079 | 0.00022 |
| 0.00355 | ND5 | 1568 | 0.01115 | 0.00047 |
| 0.00230 | COX1 | 1565 | 0.00720 | 0.00004 |
### Table 3. Prokaryotic molecular markers genes comparison using Discrimination power scoring.

| Species                  | Accession number | SC | recA     | DNAJ | ATPase | Chromosome segregation ATPase |
|--------------------------|------------------|----|----------|------|--------|-------------------------------|
|                          |                  |    | CSC      | Id   | (1-Id) | CSC      | Id   | (1-Id) | CSC    | Id   | (1-Id) |
| *V. aestuarianus*        | JN040521         | 1  | 2        | 0.999| 0.001  | 5        | 0.801| 0.199  | 8      | 0.899| 0.101  |
| *V. alginolyticus*       | NZ_AAPS01000071  | 2  | 1        | 0.999| 0.001  | 15       | 0.883| 0.117  | 14     | 0.967| 0.033  |
| *V. cholerae*            | NC_002505        | 3  | 11       | 0.921| 0.079  | 11       | 0.932| 0.068  | 11     | 0.958| 0.042  |
| *V. coralliilyticus*     | NZ_ACZN01000015  | 4  | 12       | 0.971| 0.029  | 12       | 0.904| 0.096  | 20     | 0.957| 0.043  |
| *V. cyclitrophicus*      | JN040526         | 5  | 18       | 0.924| 0.076  | 18       | 0.904| 0.096  | 18     | 0.973| 0.027  |
| *V. fischeri*            | NZ_AAPS01000071  | 6  | 16       | 0.876| 0.124  | 16       | 0.852| 0.148  | 16     | 0.918| 0.082  |
| *V. fluvialis*           | JN040529         | 7  | 3, 11    | 0.861| 0.139  | 2        | 0.848| 0.152  | 4      | 0.889| 0.111  |
| *V. fortis*              | JN040527         | 8  | 5        | 0.882| 0.118  | 19       | 0.853| 0.147  | 15     | 0.942| 0.058  |
| *V. harveyi*             | JN040517         | 9  | 15       | 0.979| 0.021  | 15       | 0.925| 0.075  | 15     | 0.979| 0.021  |
| *V. metschnikovi*        | JN040531         | 10 | 15       | 0.845| 0.155  | 7        | 0.825| 0.175  | 20     | 0.835| 0.165  |
| *V. mimicus*             | JN040530         | 11 | 3        | 0.921| 0.079  | 3        | 0.932| 0.068  | 3      | 0.958| 0.042  |
| *V. neptunius*           | JN040535         | 12 | 4        | 0.971| 0.029  | 4        | 0.904| 0.096  | 4      | 0.957| 0.043  |
| *V. orientalis*          | JN040523         | 13 | 17       | 0.893| 0.107  | 13       | 0.851| 0.149  | 19     | 0.974| 0.026  |
| *V. parahaemolyticus*    | JN040516         | 14 | 9        | 0.917| 0.083  | 2        | 0.867| 0.133  | 15     | 0.970| 0.030  |
| *V. rotiferianus*        | JN040518         | 15 | 9        | 0.979| 0.021  | 9        | 0.925| 0.075  | 9      | 0.979| 0.021  |
| *V. salmonicida*         | NC_011312        | 16 | 6        | 0.876| 0.124  | 6        | 0.852| 0.148  | 6      | 0.918| 0.082  |
| *V. shilonii*            | NC_ABCh01000040  | 17 | 13       | 0.893| 0.107  | 13       | 0.826| 0.174  | 15     | 0.912| 0.088  |
| *V. splendidus*          | JN040524         | 18 | 5        | 0.924| 0.076  | 5        | 0.904| 0.096  | 5      | 0.973| 0.027  |
| *V. tubiashii*           | JN040522         | 19 | 9        | 0.886| 0.114  | 14, 8   | 0.853| 0.147  | 13     | 0.935| 0.065  |
| *V. vulnificus*          | JN040533         | 20 | 9, 11    | 0.859| 0.141  | 9        | 0.842| 0.158  | 17     | 0.904| 0.096  |

**Discrimination power score**

| Score | 7.980 × 10⁻²⁷ | 3.530 × 10⁻¹⁹ | 1.070 × 10⁻²⁶ | 6.310 × 10⁻¹⁴ |

**Notes:** Underline Score is highest. JN040516-JN040535: In this work.

**Abbreviations:** SC, Specie code; CSC, Closest specie code; Id, Identity (Match nucleotides/total nucleotides).
when the level of identity of that marker in the closest species tends to 1 for each species.

Results
Automated prioritization of marker genes
Publicly available genomes from *Vibrio* and *Diphyllobothrium* were downloaded and subjected to the selection of marker genes approach aforementioned. For each genus, a list of potential marker genes sorted in descending order of their BG scores was produced. For *Vibrio* species (Table 1), the best molecular marker is a protein-coding gene with locus tag VC1988 in chromosome 1 of the reference genome *V. cholerae* NC_002505. This gene encodes a chromosome segregation ATPase, a protein essential for cell division that forms part of a chromosomal segregation complex. In the case of *Diphyllobothrium*, the analysis of completely sequenced mitochondrial genomes revealed the gene encoding the subunit 6 of the ATPase complex as the best potential marker gene (Table 2). This enzyme is part of the mitochondrial oxidative phosphorylation and is essential for the generation of ATP.15

Experimental Validation
In order to validate the effectiveness of our approach we amplified these marker genes from additional strains of known taxonomic assignment but with no current genomic sequences available. The effectiveness of the markers, as measured by the Discrimination Power score (DP) described above, was compared to that of common markers used previously for these species. These were *atpA*, *dnaJ* and *recA* for *Vibrio* and 18S rRNA, COX1 and 18S rRNA + ITS + 5.8S rRNA for *Diphyllobothrium*. Twenty new sequences were obtained from the chromosome segregation ATPase gene in different *Vibrio* species. Remarkably, this gene showed the best Discrimination Power value (Table 3) with a DP score of $6.3 \times 10^{-14}$. Standard markers showed lower discrimination powers: *dnaJ* ($DP_{dnaJ} = 3.5 \times 10^{-19}$), *atpA* ($DP_{atpA} = 1.1 \times 10^{-26}$) finally *recA* ($DP_{recA} = 7.9 \times 10^{-27}$). In the case of *Diphyllobothrium*, seven new sequences were obtained from ATPase-subunit 6 (ATP6) gene in different species. Again, the marker gene selected by our approach presented the highest Discrimination

| Species                  | Accession number | SC | COX1 | 18S rRNA | 18S rRNA + ITS + 5.8s rRNA + 5.8s rRNA | Discrimination power value |
|--------------------------|------------------|----|------|----------|---------------------------------------|---------------------------|
| *D. dendriticum*         | UN400538         | 1  |      |          |                                       |                           |
| *D. ditremum*            | UN400539         | 2  | 1    |          |                                       |                           |
| *D. latum*               | UN400540         | 3  | 1,2  |          |                                       |                           |
| *D. nihonkaiense*        | UN400541         | 4  | 1,2  |          |                                       |                           |
| *D. pacificum*           | UN400542         | 5  | 1,2,3|          |                                       |                           |

Notes: Underline Score is higher. JN040536-JN040541: In this work.
Abbreviations: SC, Species code; CSC, Closest species code; Id, Identity (Match nucleotides/total nucleotides).
power \( DP_{\text{ATP}} = 7.9 \times 10^{-6} \), followed by COX1 \( D_{\text{COX1}} = 5.8 \times 10^{-6} \), ITS rRNA \( DP_{\text{ITS}} = 4.4 \times 10^{-11} \) and 18s rRNA \( DP_{18s\text{rRNA}} = 1.4 \times 10^{-13} \) (Table 4).

**Discussion**

We have proposed and validated a novel approach for the informed selection of marker genes based on the observed levels of DNA polymorphism\(^{10}\) among whole genomic sequences. Our results indicate that our approach effectively selects marker genes for species differentiation. Besides having greater discrimination powers than traditional markers, our markers also reduced the number of species that showed identical sequences for the marker. Nevertheless, in both genera studies, there are still some species that are too closely related to be differentiated with a single marker. The use of a combination of markers, or the selection of specific markers for that group of species within the genus would be required. Our approach has some minimal requirements. For instance, if the goal is to obtain marker genes for species differentiation in a given genus, a minimum of three different strain genomes belonging to two different species within the genus is required. Moreover, the design of primers may present problems if the sequences are too divergent, although this problem is shared with other approaches.

Our approach and scoring system method provides a new, powerful tool for the exploitation of available genome sequences to assist in the selection of marker genes. In both the eukaryotic and prokaryotic genera tested, the theoretical analyses showed excellent correlation with empirical results and showed a better performance than molecular markers previously proposed by different authors for the same species. The adaptation of Bayes theorem permitted the use of a conditioned statistic that prioritizes genes showing low DNA polymorphism inside the same species (different strains), while displaying high DNA polymorphism between different species.

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**Author Contributions**

Conceived and designed the experiments: HB, TG. Analysed the data: HB, TG. Wrote the first draft of the manuscript: HB, TG. Contributed to the writing of the manuscript: HB, TG. Agree with manuscript results and conclusions: HB, TG. Jointly developed the structure and arguments for the paper: HB, TG. Made critical revisions and approved final version: HB, TG. All authors reviewed and approved of the final manuscript.

**Disclosures and Ethics**

As a requirement of publication author(s) have provided to the publisher signed confirmation of compliance with legal and ethical obligations including but not limited to the following: authorship and contributorship, conflicts of interest, privacy and confidentiality and (where applicable) protection of human and animal research subjects. The authors have read and confirmed their agreement with the ICMJE authorship and conflict of interest criteria. The authors have also confirmed that this article is unique and not under consideration or published in any other publication, and that they have permission from rights holders to reproduce any copyrighted material. Any disclosures are made in this section. The external blind peer reviewers report no conflicts of interest.

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Supplementary Data
The scoring system and the necessary re-formatting scripts have been implemented in PERL. The PERL scripts (SCORE.pl and XMFA.pl) and a user manual for Windows, Linux and Mac are available at http://www.bioinformatics.cl.