Species level resolution of female bladder microbiota from 16S rRNA amplicon sequencing

Carter Hoffman, MS[1]; Nazema Y Siddiqui, MD, MHSc[2]; Ian Fields, MD[3]; W. Thomas Gregory, MD[3]; Holly Simon, PhD[4]; Michael A. Mooney, PhD[1]; Alan J. Wolfe, PhD[5]; Lisa Karstens, PhD[1,3]*

Abstract

The human bladder contains bacteria in the absence of infection. Interest in studying these bacteria and their association with bladder conditions is increasing. However, the chosen experimental method can limit the resolution of the taxonomy that can be assigned to the bacteria found in the bladder. 16S rRNA amplicon sequencing is commonly used to identify bacteria in urinary specimens, but is typically restricted to genus-level identification. Our primary aim was to determine if accurate species-level identification of bladder bacteria is possible using 16S rRNA amplicon sequencing. We evaluated the ability of different classification schemes, each consisting of combinations of a reference database, a 16S rRNA gene variable region and a taxonomic classification algorithm to correctly classify bladder bacteria. We show that species-level identification is possible, and that the reference database chosen is the most important component, followed by the 16S variable region sequenced.

Importance

Species-level information may deepen our understanding of associations between bladder microbiota and bladder conditions, such as lower urinary tract symptoms and urinary tract infections. The capability to identify bacterial species depends on large databases of sequences, algorithms that leverage statistics and available computer hardware, and knowledge of bacterial genetics and classification. Taken together, this is a daunting body of knowledge to become familiar with before the simple question of bacterial identity can be answered. Our results show the choice of taxonomic database and variable region of the 16S rRNA gene sequence makes species level identification possible. We also show this improvement can be achieved through the more careful application of existing methods and use of existing resources.

Author Affiliations:

1. Department of Medical Informatics and Clinical Epidemiology, Oregon Health & Science University, Portland, OR USA
2. Department of Obstetrics and Gynecology, Duke University, Durham, NC USA
3. Department of Obstetrics and Gynecology Oregon Health & Science University, Portland, OR USA
4. AnimalBiome, Oakland, CA USA
5. Department of Microbiology & Immunology, Loyola University Chicago, Maywood, IL, USA

*Corresponding author: karstens@ohsu.edu

Introduction

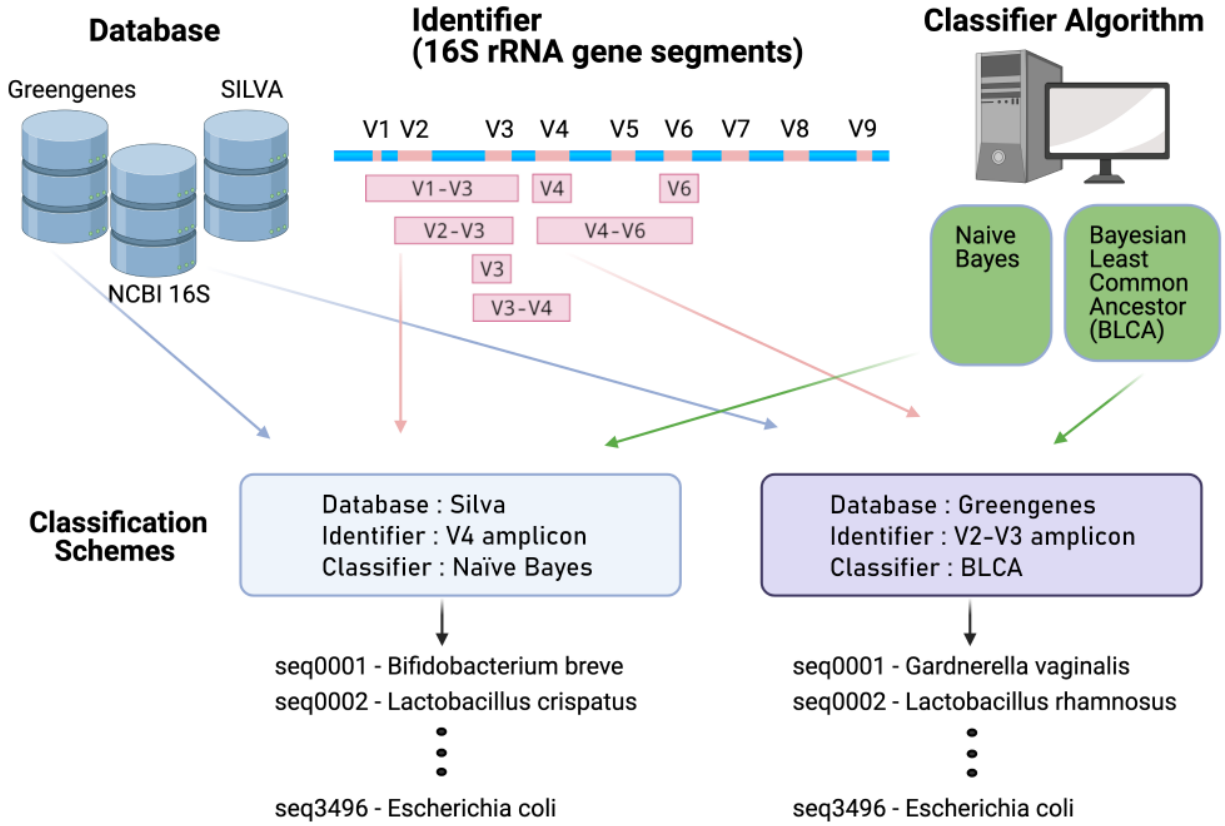
The human body provides a wide range of habitats, supporting a variety of microorganisms that include bacteria, archaea, viruses and fungi, collectively known as the human microbiome(1). Recent evidence from sequence-based and enhanced culturing techniques have revealed a population of microbes that exist in the bladder, even in the absence of infection(2–7). The discovery of the bladder microbiota (also known as the bladder urobiome) has led researchers to question how these microbes influence the health of the host. Studies have shown that altered urobiome diversity is associated with urinary tract infections (8–10), neurogenic bladder(7, 11, 12), bladder cancer(13, 14), and urgency urinary incontinence (UUI)(4, 15). These studies collectively provide evidence that the bladder urobiome, while previously overlooked, is clinically relevant and warrants further investigation.

To study the relationships between the bacteria found in the human bladder and health of the host, it is necessary to accurately identify bacteria in a rapid and large-scale manner. Reliable methods of determining the bacterial identity of an unknown bacterium include Matrix Assisted Laser Desorption/Ionization-time of flight (MALDI-TOF) analysis or whole genome sequencing (WGS) of purified colonies; both techniques permit species-level identification of bacteria(16). However, culturing specific bacterial species is time consuming and laborious. This limitation has been circumvented by adopting culture-independent methods of sequencing DNA directly from an environmental sample, such as shotgun metagenomic sequencing and targeted amplicon sequencing, the latter most commonly involving the 16S rRNA gene(17, 18). These culture-independent sequencing methods are an attractive strategy because they can more accurately reveal microbiota diversity by identifying bacteria that are difficult to grow in culture(19).

Targeted amplicon sequencing of the 16S rRNA gene is a common method for identifying bladder bacteria in a large-scale manner(20, 21). This is particularly true in urine where the low microbial biomass(9, 22) often results in lower bacterial DNA quantities than what is required for shotgun metagenomic sequencing(23). When performing targeted amplicon sequencing, DNA is extracted from all cells in a sample. Next, the polymerase chain reaction (PCR) is used to amplify a small segment of the bacterial genome, typically spanning one or more variable regions of the 16S rRNA gene. This amplified DNA segment is subject to high-throughput sequencing, such as Illumina MiSeq or HiSeq short read technology. Bioinformatics are used to process the resulting sequences and identify the taxonomy of the bacteria(24).

When identifying bacteria using targeted amplicon sequencing there are three important components (**Figure 1**). These components are: 1) a <u>database</u> of DNA sequences annotated with taxonomic information; 2) the <u>identifier</u>, or DNA sequence of the unknown bacterium; and 3) a <u>classifier</u>, which is the algorithm that compares the unknown sequence to those in a database until the closest match is found. These components work together as a *classification scheme*. One common classification scheme that can be implemented through the use of the mothur(25) or QIIME2(26, 27) bioinformatic pipelines uses the Silva database(28), the V4 region from the 16S rRNA gene sequence as the identifier(29), and the Naïve Bayes algorithm(27) as the classifier. A limitation of this and many other commonly used classification schemes is that the taxonomic resolution is usually constrained to the genus level. Recently, several new approaches to sequence processing and taxonomy assignment have become available (e.g. amplicon sequence

83 variant algorithms such as DADA2(30), and taxonomic classifiers such as Bayesian Lowest
84 Common Ancestor (BLCA)(31)), which may improve resolution to the species level.



85

86 **Figure 1: Model of the components that make up a classification scheme to assign**
87 **taxonomy to unknown sequences.** A classification scheme consists of a database, an identifier,
88 and a classifier. The databases used in this study are the Greengenes, Silva, and NCBI 16S. The
89 identifiers used in this study are subsequences of the 16S rRNA gene, computationally generated
90 using published primers as coordinates on the gene sequence. These targeted amplicons are the
91 V3, V4, and V6 variable regions of the gene, or span the V1-V3, V2-V3, V3-V4, and V4-V6
92 variable regions. The classifiers used in this study are the Naive Bayes and Bayesian Lowest
93 Common Ancestor (BLCA) algorithms. One example of a classification scheme is the Silva
94 database, V4 region identifier, and Naive Bayes classifier. Another example classification
95 scheme is the Greengenes database, V1-V2 region identifier, and BLCA classifier. These two
96 examples are distinct from each other and can have different outcomes when assigning
97 taxonomy.

98 Our primary aim was to determine if species-level identification of bladder bacteria is possible
99 using 16S rRNA amplicon sequencing studies. To achieve this aim, we used known DNA
100 sequences from a representative sample of bacteria found in the human female bladder,
101 published by Thomas-White and colleagues(32). This dataset includes bacterial species that have
102 been isolated from the bladder, cultured, and thoroughly characterized using WGS. We
103 computationally tested multiple classification schemes to see which schemes identified these
104 known DNA sequences at the species level. We evaluated several reference databases, variable

105 regions (i.e. potential identifiers), and taxonomic classification algorithms for their ability to
106 accurately identify bladder bacteria at the species level.

107 Results

108 **Representation of bladder bacteria in 16S rRNA gene sequence databases.** The Thomas-
109 White genome sequencing dataset consists of 149 bladder bacterial isolates, representing 78
110 bacterial species from 36 genera(32). There are several databases available for bacterial
111 identification using the 16S rRNA gene(33, 34). Of these, Greengenes (v.13_5)(35), Silva (v.
112 132)(36), and NCBI 16S Microbial (v. August 2019) were evaluated due to their widespread use
113 in amplicon sequencing studies and availability of species-level annotation. At the genus level,
114 all but one genus from the Thomas-White dataset (*Globicatella*) were present in the Greengenes
115 database and all genera were present in the Silva and NCBI 16S Microbial databases. At the
116 species level, all 78 bladder bacterial species were present in the Silva and NCBI 16S Microbial
117 databases, whereas only 21 species were present in the Greengenes database.

118 **Information contained in variable regions differs for bladder bacterial species.** Sequencing
119 studies frequently focus on a small segment of the 16S rRNA gene that can be rapidly sequenced
120 in a high throughput manner using short read sequencing technology, such as the Illumina HiSeq
121 or MiSeq. To evaluate the performance of different variable regions as identifiers, amplicons
122 were computationally generated from the Thomas-White genome sequencing dataset for the V1-
123 V3, V2-V3, V3-V4, V4-V6, V3, V4, and V6 variable regions using published primers (see
124 **Methods**). These computational amplicons were used to determine how well the currently
125 available classification schemes can distinguish bladder bacterial species (**Figure 2A**). To assess
126 different classification schemes, we tested multiple permutations of the variable regions listed
127 above with different databases (i.e. Greengenes, Silva, or NCBI 16S Microbial) and different
128 classifiers (i.e. Naive Bayes or BLCA, see **Figure 1**).

129 To quantify the amount of information contained across variable regions of the 16S rRNA gene
130 among commonly identified bladder bacteria, we performed a sliding window analysis on a
131 multiple sequence alignment (MSA) of all 16S rRNA gene sequences from the Thomas-White
132 dataset. We calculated entropy as a measure of information content along the MSA (**Figure 2B**).
133 As expected, the defined variable regions contained regions of high entropy, suggesting
134 variability across species, whereas variable regions were flanked by conserved regions with low
135 entropy containing sequences that are similar among species. The V1 and V2 regions contained
136 the highest entropy, while V7 and V8 contained the lowest.

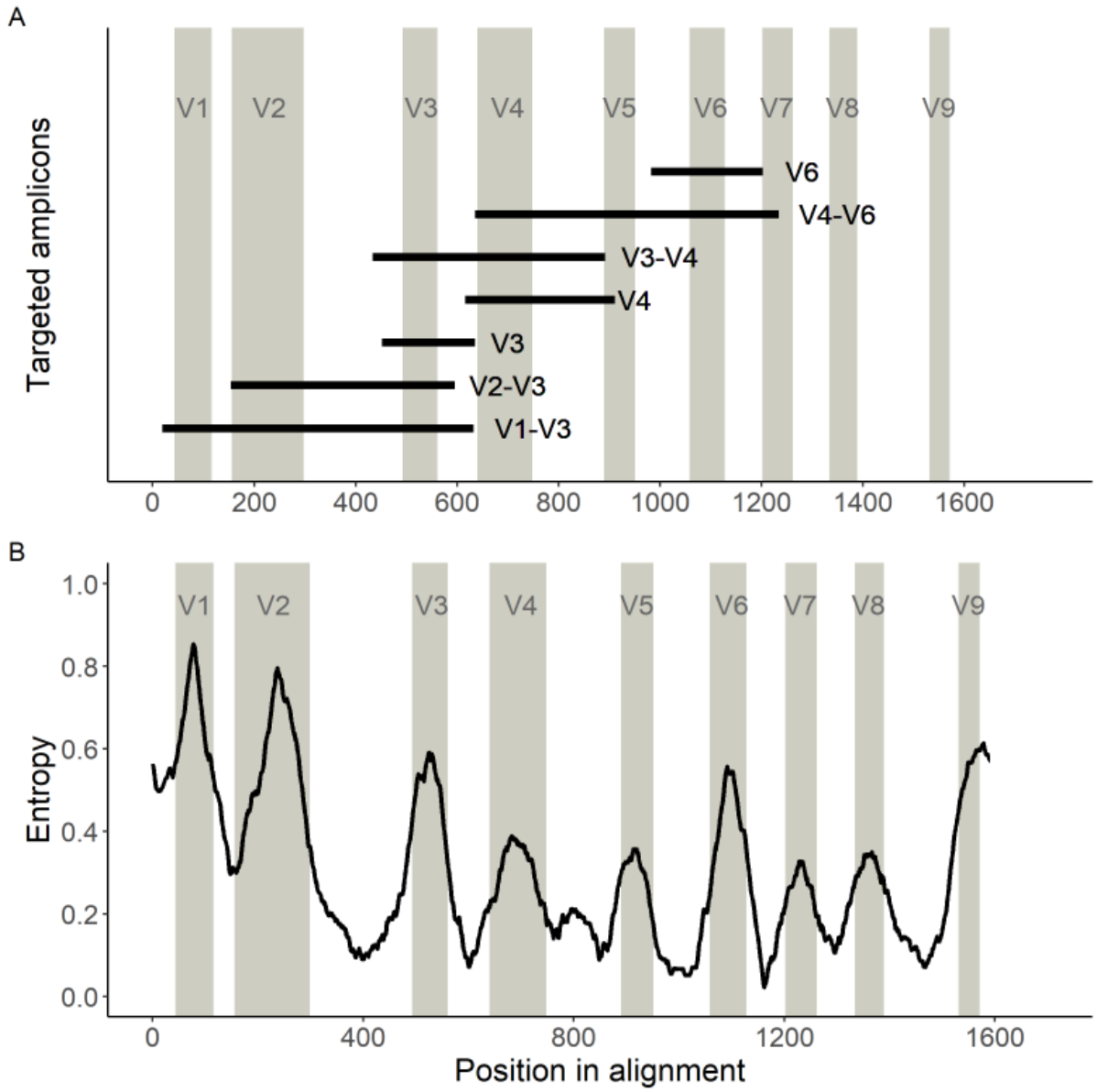
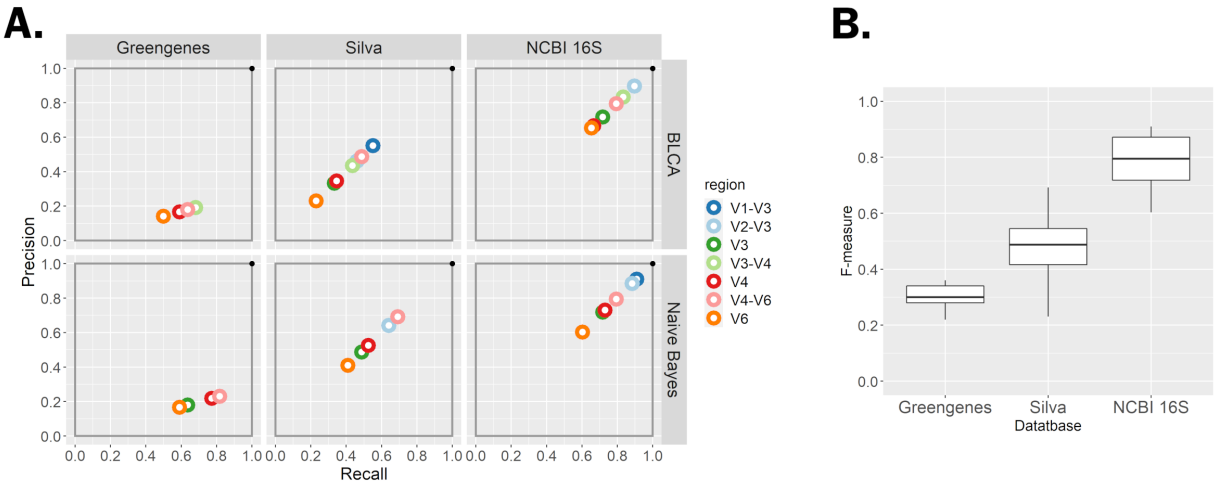


Figure 1. Variable regions of the 16S rRNA gene used in this study. A) Locations of the primers used in this study on the 16S rRNA gene. Locations of the predicted amplicons are shown as black bars in relation to the multi-sequence alignment (MSA) of the bacterial species described in Thomas-White et al. (2018). Gray columns are the locations of the known variable regions based on the sequence from *E. coli*. B) The information of variable regions, measured by entropy from a sliding window analysis of the MSA. Higher entropy indicates that the region has more variability across species, and therefore more information to identify a bacterial species. Lower entropy indicates that the region has little variability (i.e. is conserved) across species and therefore less information to identify a bacterial species.

Evaluation of classification scheme performance. To evaluate the ability of currently available resources to identify bladder species, we calculated the recall, precision and F-measure for each classification scheme implemented (see **Methods**). For these calculations, we compared results from computational classification schemes to known bacterial species

151 information from prior WGS. Each taxonomic classification was evaluated as a true match, true
152 non-match, false match or false non-match based on whether the taxonomic classification was
153 correctly assigned or not. Recall refers to the proportion of matches that the classification
154 scheme correctly identified out of all possible matches. Precision refers to the proportion of
155 matches that the classification scheme called correctly out of all classified matches. The F-
156 measure is the equally weighted harmonic mean of recall and precision.

157 In general, classification schemes that use the NCBI 16S Microbial database perform the best as
158 they have high recall and precision, thus the highest F-measures (median 0.79, range 0.60 –
159 0.91), regardless of which classifier is used (**Figure 3**). Classification schemes using the Silva
160 database show reduced recall and precision, thus lower F-measures (median 0.48 range 0.23 –
161 0.69). Because the Greengenes database is missing many of the bacterial species found in the
162 bladder, it is less precise. As such, classification schemes using the Greengenes database can
163 have good recall but low precision resulting in a low F-measure (median 0.30, range 0.22 –
164 0.36).



165
166 **Figure 2: Overall classification scheme evaluation. A.** The performance of each classification
167 scheme is summarized by the precision (y axis) and recall (x axis) for each variable region
168 (color). The best classification scheme would lie in the upper right-hand corner. **B.** Box plots
169 depicting the median, quartiles, and range of the F-measure of all classification schemes by
170 database. Overall, classification schemes using the NCBI 16S Microbial database had the
171 highest F-measures and those using the Greengenes databases had the lowest.

172 While the choice of database appears to have the largest effect on the outcome of a classification
173 scheme, the combination of identifiers and classifiers with specific databases show differences as
174 well. For example, when using the Silva database, the classifier has an impact on the
175 classification scheme outcome. With the Naive Bayes classifier, the identifiers yielding the
176 highest F-measures are the V3-V4 (0.69) and V4-V6 (0.69) targeted amplicons. Using the BLCA
177 classifier, the V1-V3 targeted amplicon has the highest F-measure (0.55).

178 **Confidence scores affect classification**.

179 The BLCA and Naïve Bayes classifiers used in this study will classify an unknown sequence
180 even when the posterior probability for that taxon is very low. To account for this situation, a

confidence score is calculated. This confidence score measures how much the classification changes through random permutation (bootstrapping) and the value reflects the "goodness of fit" of that classification. When lacking any knowledge of how to choose the best confidence score that minimizes the number of errors of a given classification scheme, using a predefined confidence score threshold is an option. We evaluated the performance of classification schemes against our test set when confidence score thresholds were used. For this analysis, matches that returned with confidence scores less than the designated confidence threshold were considered non-matches. **Figure 4** shows the effect of increasing the confidence score on the number of true matches returned by each classification scheme.

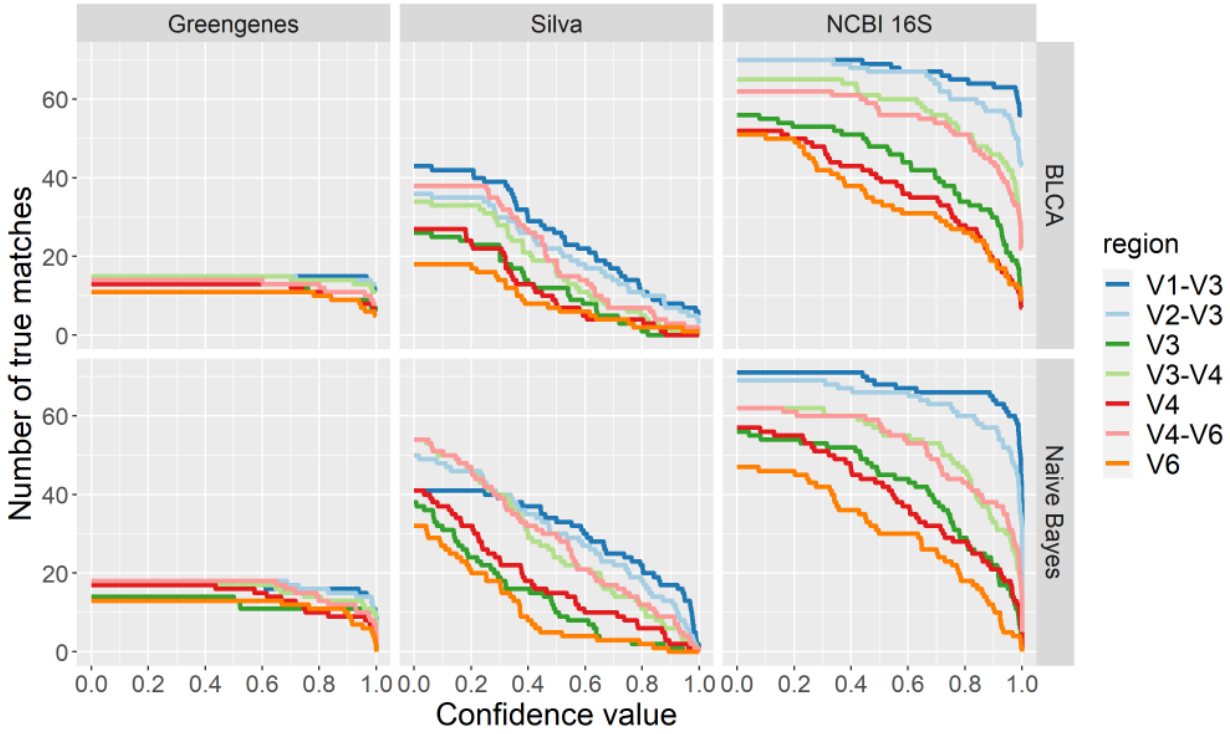


Figure 3: The number of true matches returned for each classification scheme across all confidence score values. As the confidence score value is increased where there is higher confidence of an accurate taxonomic assignment, the number of true matches dramatically decreases. For Greengenes and NCBI 16S databases, true matches especially decrease with confidence scores higher than 80%. With schemes using the Silva database, true matches steadily decrease with confidence scores as low as 30% .

Almost all classification schemes had a decrease in recall when using a default confidence score of 80% (**Supplemental Figure 1**). This effect is especially marked for the classification schemes that use the Silva database, which shows an average 79.3% reduction in recall with higher confidence scores. Classification schemes that use the NCBI 16S database are unequally affected by increasing confidence scores; for example the V1-V3 identifier shows a slight reduction in recall (7.1% on average), while the V6 identifier shows the largest (43.3% on average). Classification schemes that use the Greengenes database are only slightly affected with different confidence scores resulting in smaller magnitude differences in recall.

205 Changes in the precision of classification schemes are mostly affected by the database used
206 (**Supplemental Figure 1**), though confidence scores can further affect precision. For the
207 classification schemes that use the NCBI 16S database, precision is generally improved with
208 increasing confidence score, but at unequal amounts. For example, using the 80% threshold, the
209 V1-V3 identifier shows a slight average increase of 3.5%, while the V6 identifier shows a large
210 average increase of 39.7%. Classification schemes that use the Greengenes database show only
211 minimal changes in precision. In contrast, classification schemes using the Silva database are
212 differentially affected, with both reduction and gains in precision depending on the identifier and
213 classifier combinations that are chosen. In general, for the Silva database, precision is reduced
214 when using any 16S rRNA identifier with the BLCA classifier. However, a dramatic increase in
215 precision is shown by the classification scheme composed of the Silva database, V4 identifier,
216 and the Naive Bayes classifier. When ignoring a confidence score, this classification scheme has
217 a precision of 52.6%, but increases to 85.7% when using a confidence score threshold of 80%.

218 The overall changes in how these classification schemes perform when using a 50% or 80%
219 confidence score can be summarized by comparing the F-measure values shown in
220 **Supplemental Figure 2**. In almost every classification scheme, the F-measure value decreases
221 when a threshold is used. The classification schemes that use the Silva database clearly
222 demonstrate this effect, which show a 66.9% reduction in F-measure values on average when
223 higher confidence scores are used. The classification schemes that use the NCBI 16S database
224 show slight decreases in the F-measure values, with the exceptions of those that use the V3, V4
225 and V6 regions as identifiers. Those classification schemes show a large 27.2% reduction in F-
226 measure values on average. Finally, the classification schemes that use the Greengenes database
227 have slight changes in their F-measure values, regardless of whether a threshold is used or not.

228 **Amplicons spanning more than one variable region identify a higher number of bladder**
229 **bacterial species.** Amplicons spanning more than one variable region identified more unique
230 bladder bacteria at the species level than amplicons spanning a single variable region. For
231 example, with the commonly used V4 variable region and Naïve Bayes classifier, 21.8% of
232 bladder bacteria are correctly identified with the Greengenes database, whereas 52.6% are
233 identified with the Silva database and 73.1% with the NCBI 16S database (**Error! Reference**
234 **source not found.**A). In contrast, using amplicons spanning more than one variable region such
235 as the V1-V3 region, 91.0% of bacteria are correctly identified at the species level when using
236 the NCBI 16S database.

237 **Species identified depends on choice of database and variable region.** While the results thus
238 far have focused on summarizing overall performance of classification schemes for identifying
239 bladder bacteria at the species level, we also sought to determine which classification schemes
240 could be used to identify specific bacteria (**Figure 5B, Supplemental Figure 3**). Although the
241 NCBI 16S database contains the largest representation of bladder species, some species were not
242 identified with certain variable regions, if at all. For example, *Lactobacillus* species, which are
243 commonly found in the female bladder and vagina (32, 37, 38), were overall best represented
244 within the NCBI 16S database, with 8 out of 9 species being identified with the V1-V3 and V2-
245 V3 variable regions (**Figure 5C**). However, the other variable regions only identified between 4
246 and 6 *Lactobacillus* species when using the NCBI 16S database. Interestingly, *L. crispatus* and *L.*
247 *rhamnosus* were only detectable with specific combinations of variable regions and databases,
248 and *L. iners* was not correctly identified from our dataset with any classification scheme.

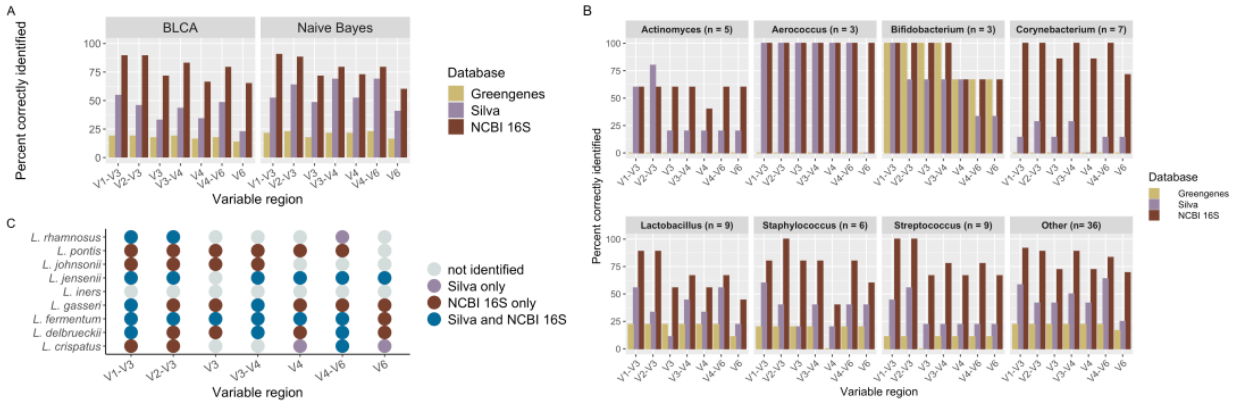


Figure 4: Percent of bladder bacteria correctly identified for each classification scheme. A. Overall results by classifier. With the commonly used V4 variable region and BLCA classifier, 17% of bladder bacteria are correctly identified using the Greengenes database, compared with 35% correctly identified using the Silva database and 67% using the NCBI 16S database. A similar trend is seen with the Naïve Bayes classifier. Using other variable regions can lead to improved species-level resolution to a maximum number of 91% correctly identified. **B.** The percent of bladder bacterial species identified with each database by variable region (BLCA). Genera with less than 3 species are grouped as Other. **C**. Lactobacillus species depends on the variable region and database used, with the NCBI 16S database having the most coverage, regardless of variable region chosen. The Greengenes database is not shown since it only classified two species (*L. pontis* and *L. delbrueckii*).

Additionally, we found that there were important discrepancies for bacteria that are thought to play a role in bladder health and disease(39) (**Supplemental Figure 3**). Several bladder species, such as *Gardnerella vaginalis*, were only detected with the NCBI 16S and Silva databases. *Staphylococcus* species were poorly identified with the V4 region but were distinguishable with all other regions. *Streptococcus* and *Corynebacterium* species were best identified with NCBI 16S. *Escherichia coli*, a common uropathogen(40), is not well represented in any of the databases, and was only detected with the V4 region and the NCBI 16S database.

Validation of computational findings on V4 amplicon data. To evaluate the performance of our computational findings on actual data, we acquired targeted amplicon sequencing data from 24 urine samples. These urine samples were a subset of those that were used to derive cultures in the Thomas-White dataset and thus should contain the same bacteria. Sequencing data were generated as part of two other studies using Illumina sequencing of the V4 region of the 16S rRNA gene(4, 41). We reprocessed the raw sequencing data (see **Methods**) and performed taxonomic classification to assess the performance of our computational findings. Since 16S rRNA amplicon sequencing will detect many more bacteria than those identified even with enhanced culture, we restricted the evaluation to only the bacteria that grew in culture from a given sample (see **Supplemental Figure 4**). We used Cohen's Kappa to assess the agreement between species assignment in the V4 computational dataset to the experimental V4 dataset (see **Methods)**. All classification schemes had moderate agreement (**Table 1**).

282 **Table 1.** Cohen's Kappa demonstrates moderate agreement of the computational and
283 experimental amplicon datasets.

Scheme	Cohen's Kappa
V4 – Silva – BLCA	0.70
V4 – Greengenes – BLCA	0.59
V4 – NCBI 16S – BLCA	0.58
V4 – Silva – Naïve Bayes	0.47
V4 – Greengenes – Naïve Bayes	0.65
V4 – NCBI 16S – Naïve Bayes	0.50

284

285 ## Discussion

286 Our study demonstrates that it is possible to attain results at the species level with existing
287 resources when performing targeted amplicon sequencing of urinary specimens. Though species-
288 level taxonomic resolution is possible, it requires a carefully chosen classification scheme.
289 Within the classification scheme, we found that the classifier chosen did not drastically affect the
290 identification of species, but the database and identifier does. Importantly, we found that the
291 reference database strongly influences the identification of bacteria at the species level. Overall,
292 the NCBI 16S database performed the best, whereas the Greengenes database performed the
293 worst using our approach.
294
295 While database selection is recognized as an important decision in taxonomy assignment for
296 amplicon sequencing studies, few studies have compared the effect of different databases on
297 taxonomy assignment. Furthermore, to our knowledge none have explored their use in
298 identifying bacteria originating from the human bladder. Park and Won recently compared
299 database performance on mock microbial communities and similarly found that the Silva
300 database outperformed the Greengenes database(42). Several groups have also demonstrated that
301 niche-specific databases perform the best for species level identification(43), an approach that
302 has been taken in several microbiome research communities, such as the vaginal(44), oral(45)
303 and gut(46).

304 For species level taxonomy assignments, the reference database must contain species-level
305 information of the bacteria to be identified. We found that the Greengenes database does not
306 currently contain many bacterial species that are found in the human bladder. In contrast, the
307 NCBI 16S Microbial and Silva databases had representation of all species that were identified
308 from prior studies of bladder bacteria. Thus, the latter two databases are better choices for
309 evaluating bacterial species from the bladder. Even though both of these databases contain
310 species of interest, the NCBI 16S database consistently outperformed the Silva database.

311 The classifiers used in this study are examples of two different strategies designed to overcome
312 the common challenges of searching an extremely large dataset in order to find matching pairs of
313 query sequences and reference records. While these two approaches are different in concept, we
314 did not find significant differences in their performance for species-level classification of bladder
315 bacteria. BLCA is an example of sequence comparison by pairwise alignment. The strength of

316 this method is due to the fact that the similarities between two DNA samples are directly
317 compared. This is a highly effective approach; however, until recent advances in computer
318 technology, it remained impractical because of the computational burden. The Naive Bayes
319 classifier is an example of a *k*-mer-based classification approach, and was designed to
320 circumvent the computational challenges that are faced with use of a pairwise alignment
321 classifier. However, there are limitations when using Naive Bayes for species-level
322 identification. The first limitation arises from the database training process. If one taxon has
323 more training examples than another, Naive Bayes generates unfavorable weights for the
324 decision boundary(47). The second limitation is that all features (i.e. the *k*-mers generated from
325 the DNA sequences) are assumed to be independent, and weights for taxa with strong
326 dependencies among the associated *k*-mers are larger than those taxa with weakly dependent *k*-
327 mers(47). A third limitation was that the available Naive Bayes Classifiers (such as those
328 available through the RDP classifier(48) and QIIME2(27)) only had genus level information for
329 the Silva and NCBI 16S databases. To use these for species-level assignment, it was necessary to
330 retrain the classifier to use the specified databases at the species level. This training converts the
331 DNA sequences to the calculated frequency that each *k*-mer occurs in a taxon.

332 Affordable sequencing of large-scale data is presently done with short read sequencing
333 technology, such as Illumina MiSeq. This is currently limited to sequencing reads up to 300
334 nucleotides in length. Until full-length 16S rRNA gene sequencing can be achieved affordably
335 on a large scale (e.g. technologies such as Oxford Nanopore and PacBio technologies), choosing
336 the optimal region of the 16S rRNA gene for identification purposes remains a significant part of
337 the experimental design. Thus, the variable regions that are used as identifiers require some
338 consideration.

339 Our findings show that use of the V2-V3, and V1-V3 regions of the 16S rRNA gene allowed for
340 the correct identification of the most bladder bacterial species when combined with the NCBI
341 16S database and either classifier. In general, amplicons that span more than one variable region
342 perform better than those that contain single variable regions. This is likely due to the increased
343 information available with longer reads. It is important to note that longer reads can have
344 experiment limitations(49). While shorter variable regions, such as the V4 region, did not
345 perform as well as longer amplicons, they were able to identify many bladder bacteria at the
346 species level if the NCBI 16S database was used (52 out of 78). These shorter amplicons are
347 widely used with Illumina sequencing and may be valid, depending on the study design and level
348 of precision desired. However, other variable regions may be employed when more detailed
349 information is desired.

350 This study is adds to a growing body of literature informing and improving experimental design
351 for urobiome research. Several studies have now informed how the method of sample
352 collection(2, 50), sample handling and storage(51), sample chemical composition(52), and DNA
353 extraction protocol(52, 53) can influence downstream results and interpretations. By taking a
354 computational approach to evaluating classification schemes, we were able to thoroughly assess
355 the ability of various combinations of databases, identifiers, and classifiers to identify known
356 bladder bacterial species. However, there are several practical limitations of amplicon
357 sequencing that were not captured in this approach. We used published primers to create
358 computational amplicons, but this may not reflect the efficiency of primers binding in actual
359 experiments. Additionally, targeted amplicon sequencing generates a large number of

overlapping reads and provides the data for methods to correct for errors introduced in the lab by the polymerase enzyme and through sequencing. Our method did not account for the experimental noise that is found in experimental data that may decrease the ability of the classification schemes to differentiate between bacterial species with similar sequences.

Conclusion

Species level taxonomy assignment will greatly benefit studies focused on the urobiome and its relationship to bladder health and disease. Our results show that it is possible to reliably classify bladder bacterial species using targeted amplicon sequencing of the 16S rRNA gene variable regions with existing classification algorithms and databases. We determined that the most important component of the classification scheme is the database used, and that among those that were tested, the NCBI 16S database allows for the best identification of bladder species. Our validation with V4 amplicon data demonstrates that the predicted computational outcomes are a reasonable approximation for how a classification scheme will perform on real data. It can be expected that the alternate variable regions covered in this study, such as the V2-V3 region of the 16S rRNA gene, would have similar outcomes.

Importantly, we found that no single variable region gives 100% coverage of all bladder bacteria species. Thus, the choice of variable region may significantly affect the results of a given study. One approach to resolve this could be to use multiple amplicon sequencing or long read sequencing technology. These technologies are emerging and may prove to be beneficial for the urobiome community. Furthermore, no database has 100% coverage across a variable region. This could be resolved by using more than one database for classification, though this approach is complicated by differences in databases in terms of formatting, as well as conflicting classifications(54). Both of these components are important for planning experimental and computational aspects of urobiome studies, and should be considered when comparing results across studies.

Material and Methods

Code resources. All scripts that were written for this project can be found in the GitHub repository (https://github.com/KastensLab/BladderBacteriaSpecies). All scripts sourced from this repository are referred to as "custom."

The Thomas-White dataset. The 78 species of bladder bacteria used in this study were identified by culturing 149 bacterial isolates from 77 urine samples and performing whole-genome sequencing, as described in Thomas-White et al.(32). This set of identified species served as the basis for our computational analysis and is referred to as the Thomas-White dataset. For each species identified, the 16S rRNA gene sequence of the corresponding type strain was downloaded from the Silva v132 release (https://www.arb-silva.de/) on 4/27/2019. A *type strain* is the sequence of the cultured isolate that was subject to the metabolic, genotypic and phenotypic evaluations taken to define the bacterial species(55), and is the agreed bacterial organism to which the taxonomic name refers. Sequences were searched using the "[T]" filter setting, and sequences longer than 1450 nt with alignment and pintail quality scores greater than 95% were selected. For the species that had no hits, the taxonomic synonym (see below) was

400 used as the search query, if available. One unidentified *Corynebacterium* species had no type
401 strain available, and was excluded from the analysis.

402 **The V4 validation dataset.** Targeted amplicon sequences from 24 urine samples, using the
403 V4 region of the 16S rRNA gene sequence, is referred to as the V4 validation dataset. These 24
404 urine samples originated from a subsample of the women whose samples comprised the Thomas-
405 White dataset. Sequencing data were generated as part of two other published studies using
406 Illumina sequencing of the V4 region of the 16S rRNA gene(4, 41). The raw sequence reads
407 were processed with DADA2 version 1.14.1(30) to generate amplicon sequence variants (ASVs).
408 The ASVs were subjected to taxonomic classification with the BLCA algorithm.

409 **Synonyms of species.** Species names have changed in response to advances in bacterial
410 systematics. All currently known species synonyms were downloaded from the Prokaryotic
411 Nomenclature Up-to-Date(56) (PNU) website on 1/5/2020. PNU includes information down to
412 the strain level, but these entries were consolidated to the species level. For example, entries like
413 *Enterobacter cloacae* and *Enterobacter cloacae dissolvens* are treated as synonyms of
414 *Enterobacter cloacae*. Classification results were then checked for synonyms using the custom
415 "validate_match_batch.py" script.

416 **Databases.** The Greengenes database version 13_5 was downloaded on 9/23/19 from
417 (http://greengenes.secondgenome.com/?prefix=downloads/greengenes_database/gg_13_5/). The
418 Silva database version 132 was downloaded on 9/14/19 from (https://www.arb-
419 silva.de/no_cache/download/archive/release_132/Exports/) as a FASTA formatted file. The
420 NCBI 16SMicrobial database is bundled with the BLCA package, and is also available from
421 (ftp://ftp.ncbi.nlm.nih.gov/blast/db/). For use with BLCA, each database was processed with
422 provided scripts (`1.subset_db_gg.py` from BLCA for greengenes, `makeblastdb` utility
423 from BLAST + suite combined with custom `write_taxonomy.py` for Silva, and
424 `1.subset_db_acc.py` from BLCA for NCBI 16S Microbial). For use with Naïve Bayes,
425 the FASTA file for each database was reformatted to work with QIIME2 using the custom
426 "write_qiime_train_db.py" script. The Naive Bayes classifier was then trained with the "fit-
427 classifier-naive-bayes" script QIIME2 script.

428 **Presence of Thomas-White species in databases.** To verify that all species from the
429 Thomas-White dataset were present in the databases used in this study, each database was first
430 converted to a FASTA file (if needed) using the "blastdbcmd" utility included in the Blast+ suite.
431 The FASTA file was then searched using regular expressions and the Linux command-line
432 program *grep* for a match of each species in the dataset. The commands were implemented using
433 the custom "species_in_db.bash" script. The presence or absence of each species was recorded.

434 **Multisequence alignment**. The 16S gene sequences from the Thomas-White dataset were
435 formed into a multi-sequence alignment using the T-coffee program(57). T-coffee version
436 12.00.7fb08c2 was downloaded from (http://tcoffee.org/Packages/Stable/Latest/) on 4/5/2019.
437 Alignments were performed using the default settings.

438 **Sliding window analysis**. Comparing the 16S rRNA gene sequences of the species in the
439 Thomas-White dataset reveals regions of conserved sequence and regions of variability. The
440 degree that variable regions of species differ from each other can aid the identification of each

species; therefore, quantifying the amount of variability of a region across a set of species is important.

Sliding window analysis (SWA) is the method by which a list of subsequences are generated by taking successive groups of equal size, in the manner of a window of fixed length sliding across the full sequence. Quantifying the amount of variability along a MSA is achieved by combining SWA with calculating the Shannon Entropy contained in each column framed by the window.

The minimum Shannon entropy occurs when all nucleotides in a position (column) of the MSA are the same. The maximum occurs when all possible nucleotides in the MSA are present at that position. However, the Shannon Entropy treats gaps in a sequence as relevant, where in practice gaps reflect an absence of useful information. Multisequence alignments can generate many columns of gap characters due to insertions or deletions (indels) in the respective sequences that make up the MSA. A consequence of treating gaps as relevant is the Shannon Entropy will interpret these indel regions as conserved sequence. This limitation was overcome by weighting the entropy scores against gaps(58). The locations of known variable regions of the 16S gene sequence were validated, and the relative amount of variability was quantified, using the custom "weighted_ent.py" script.

Primers. Amplicons were computationally generated from the Thomas-White genome sequencing dataset for the V1-V3, V2-V3, V3-V4, V4-V6, and V4 variable regions using published primers and the V3 and V6 regions using designed primers. The primer sequences used, listed in order of amplicon spanning variable region(s), forward primer name and sequence, reverse primer name and sequence are: **V1-V3**: A17F 5'-GTT TGA TCC TGG CTC AG-3', 515R 5'-TTA CCG CGG CMG CSG GCA-3'(59, 60). **V2-V3**: 16S_BV2f 5'-AGT GGC GGA CGG GTG AGT AA-3', HDA-2 5'-GTA TTA CCG CGG CTG CTG GCA C-3'(61, 62). **V3:** v3_579F 5'-THT TSS RCA ATG GRS GVA-3', v3_779R 5'-GKN SCR AGC STT RHY CGG-3'. **V3-V4:** V3F 5'-CCT ACG GGA GGC AGC AG-3', V4R 5'-GGA CTA CHV GGG TWT CTA AT-3'(63). **V4**: F515 5'-GTG CCA GCM GCC GCG GTA A-3', R806 5'-CCT GAT GHV CCC AWA GAT TA-3'(18). **V4-V6:** 519F 5'-GTG CCA GCT GCC GCG GTA ATA-3', 1114R 5'-GGG GTT GCG CTC GTT GC-3'(60). **V6:** v6_1183F 5'-CCG CCT GGG GAS TAC GVH-3', v6_1410R 5'-AGT CCC RYA ACG AGC GCA-3'. Degenerate primer design was employed to generate primer sets for the V3 and V6 regions of the 16S rRNA gene that would anneal to as many species in the Thomas-White dataset as possible with DegePrime(64) (https://github.com/EnvGen/DEGEPRIME.git). DegePrime has the option to ignore columns of a MSA if the number of "-" characters exceed a user-defined threshold. The MSAs were preprocessed with this threshold set to .01. The main script of DegePrime was run using a degeneracy setting of 4096 and a window length of 18.

Extracting computational amplicons. For each primer set, the DNA sequence bracketed by the forward and reverse primers was extracted from the multisequence alignment. Coordinates of the MSA were identified by searching the *E. coli* sequence (accession number EU014689.1.1541) included in the MSA for a match to the forward and reverse primer sequences, and then mapping those position to the MSA of the Thomas-White dataset. This procedure was done using the custom "extract_16s_vr.py" script and output as a multi-record FASTA formatted file.

Taxonomic classifiers. Taxonomic classification was performed with Bayesian lowest common ancestor (BLCA) and Naïve Bayes classifiers. BLCA(31) was cloned from the GitHub repository https://github.com/qunfengdong/BLCA.git. For the 16S variable regions, the BLCA was run using default settings but pointing to the selected reference database, either Greengenes, Silva, or NCBI 16S. The Naïve Bayes classifier as implemented by QIIME2(27) was used with the Greengenes, Silva, and NCBI 16S databases and a confidence setting of 0, 50, and 80, but otherwise default settings.

Evaluating computational results. To evaluate the taxonomic classification results of each classification scheme on the computational amplicon dataset, the custom "new_taxonomy_results_2020-3-14.Rmd" file was used. These scripts compare the results of each record pair (each comparison between the query sequence and sequence held in the reference database) from the classification scheme to the known identify of the query sequence from the Thomas-White dataset. All record pairs that were assigned a match by the classification schemes were evaluated according to the following definitions (**Figure 6**):

> ***True match*** - All record pairs assigned as a match that have identical genus and species labels.
> ***False match*** - All record pairs assigned as a match that did not have identical genus and species labels.
> ***False non-match*** - If a record representing a species in the Thomas-White dataset was present in the database, but was not assigned as a match, the record was evaluated as a false non-match.
> ***True non-match*** - All records in the reference database that were not in the Thomas-White dataset. While records assigned to this category were not used in evaluating the classification schemes in this manuscript, the definition is still included for completeness.

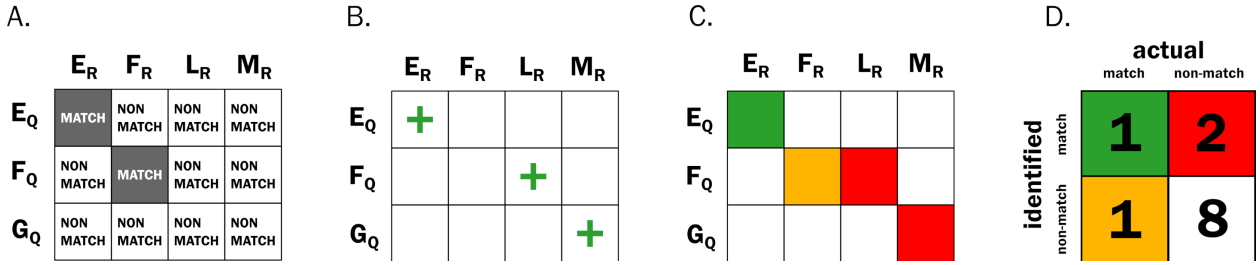


Figure 6. Example of classification evaluation used in this study. Suppose there is a classification scheme comprising a set of query sequences (the rows E,F,G) and the set of reference sequences (the columns E,F,L,M) held in a reference database. In this example, the number of reference records is greater than the query records, and the reference is missing a corresponding G record from the query set. **A**) If the query and reference record letters are the

same, then they are designated as a **match**. If they are different they are designated as a **non-match**. **B**) Next, the classifier is allowed to assign record pairs as matches or non-matches for all query sequences, represented as green plus signs for matches and blank cells as non-matches. Some results are correct, and some are not. Note that despite the lack of a matching record in the reference database, the classifier still designated the (G:M) pair as a match. **C**) Using the definitions for assigning the classifications to the confusion matrix, there is one **true match** (green square), two **false matches** (red squares), one **false non-match** (yellow square), and 8 **true non-matches** (white squares). D) The cell values of the confusion matrix are then filled out, and performance measurements can be calculated. For this classification scheme, the precision is 1/(1+2)=.33, recall is 1/(1+1)=.5, and the F-measure is (2*.33*.5)/(.33+.5)=.40.

Performance measures. Recall, precision and the F-measure were used to evaluate the performance of each classification scheme implemented. Recall refers to the proportion of matches that the classification scheme correctly identified (true matches) out of all possible matches (true matches plus false non-matches). Precision refers to the proportion of matches that the classification scheme called correctly (true matches) out of all classified matches (true matches plus false matches). The F-measure is the equally weighted harmonic mean of recall and precision. For this study, we chose to maximize recall over precision, because the number of true matches impacts the subsequent work on diversity measures, such as species richness and evenness(65).

Evaluating V4 validation results. To determine the expected bacterial species in each sample, the results of the whole genome sequencing on the isolates cultured from the corresponding subject was used. The species of bacteria in the V4 sequencing data were identified using classification schemes composed of 1) the Greengenes, Silva, and NCBI 16S microbial databases 2) the V4 sequencing results as the identifier, and 3) the BLCA and Naive Bayes classifiers. For each classification scheme, the species identification from the computational and validation datasets was scored as a '1' if correct, and '0' otherwise. This resulted in a table for each classification scheme consisting of bacterial species as the rows, and the scores from the computational or validation outcomes as columns. Cohen's kappa was then calculated for each table using the "kappa2" function from the "irr" R package (https://CRAN.R-project.org/package=irr).

Data Availability.
This project used previously acquired publicly available data(20). All code that was written for this project can be found in the GitHub repository:
https://github.com/KarstensLab/BladderBacteriaSpecies.

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Author contributions.

557 CH and LK conceived and designed experiments, and wrote the manuscript. CH performed
558 analyses, LK reviewed analyses. AJW supplied the data. LK, CH, MM, HS, TG, IF, NS, and
559 AJW interpreted results and revised the manuscript.

560 **References**

561 1. Lederberg J, McCray AT. 2001. 'Ome Sweet 'Omics-- A Genealogical Treasury of Words.
562 The Scientist 15:2.

563 2. Wolfe AJ, Toh E, Shibata N, Rong R, Kenton K, FitzGerald M, Mueller ER,
564 Schreckenberger P, Dong Q, Nelson DE, Brubaker L. 2012. Evidence of Uncultivated
565 Bacteria in the Adult Female Bladder. J Clin Microbiol 50:1376–1383.

566 3. Khasriya R, Sathiananthamoorthy S, Ismail S, Kelsey M, Wilson M, Rohn JL, Malone-Lee
567 J. 2013. Spectrum of Bacterial Colonization Associated with Urothelial Cells from Patients
568 with Chronic Lower Urinary Tract Symptoms. J Clin Microbiol 51:2054–2062.

569 4. Pearce MM, Hilt EE, Rosenfeld AB, Zilliox MJ, Thomas-White K, Fok C, Kliethermes S,
570 Schreckenberger PC, Brubaker L, Gai X, Wolfe AJ. 2014. The Female Urinary
571 Microbiome: a Comparison of Women with and without Urgency Urinary Incontinence.
572 mBio 5:e01283-14.

573 5. Price TK, Dune T, Hilt EE, Thomas-White KJ, Kliethermes S, Brincat C, Brubaker L,
574 Wolfe AJ, Mueller ER, Schreckenberger PC. 2016. The Clinical Urine Culture: Enhanced
575 Techniques Improve Detection of Clinically Relevant Microorganisms. J Clin Microbiol
576 54:1216–1222.

577 6. Ackerman AL, Underhill DM. 2017. The mycobiome of the human urinary tract: potential
578 roles for fungi in urology. Ann Transl Med 5:31–31.

579 7. Fouts DE, Pieper R, Szpakowski S, Pohl H, Knoblach S, Suh M-J, Huang S-T, Ljungberg I,
580 Sprague BM, Lucas SK, Torralba M, Nelson KE, Groah SL. 2012. Integrated next-
581 generation sequencing of 16S rDNA and metaproteomics differentiate the healthy urine
582 microbiome from asymptomatic bacteriuria in neuropathic bladder associated with spinal
583 cord injury. J Transl Med 10:174.

584 8. Thomas-White KJ, Gao X, Lin H, Fok CS, Ghanayem K, Mueller ER, Dong Q, Brubaker
585 L, Wolfe AJ. 2018. Urinary microbes and postoperative urinary tract infection risk in
586 urogynecologic surgical patients. Int Urogynecology J 29:1797–1805.

587 9. Moustafa A, Li W, Singh H, Moncera KJ, Torralba MG, Yu Y, Manuel O, Biggs W, Venter
588 JC, Nelson KE, Pieper R, Telenti A. 2018. Microbial metagenome of urinary tract infection.
589 Sci Rep 8:4333.

590 10. Neugent ML, Hulyalkar NV, Nguyen VH, Zimmern PE, De Nisco NJ. 2020. Advances in
591 Understanding the Human Urinary Microbiome and Its Potential Role in Urinary Tract
592 Infection. mBio 11:1–15.

593 11. Groah SL, Pérez-Losada M, Caldovic L, Ljungberg IH, Sprague BM, Castro-Nallar E,
594 Chandel NJ, Hsieh MH, Pohl HG. 2016. Redefining Healthy Urine: A Cross-Sectional
595 Exploratory Metagenomic Study of People With and Without Bladder Dysfunction. J Urol
596 196:579–587.

597 12. Bajic P, Wolfe AJ, Gupta GN. 2019. The Urinary Microbiome: Implications in Bladder
598 Cancer Pathogenesis and Therapeutics. Urology 126:10–15.

599 13. Zeng J, Zhang G, Chen C, Li K, Wen Y, Zhao J, Wu P. 2020. Alterations in Urobiome in
600 Patients With Bladder Cancer and Implications for Clinical Outcome: A Single-Institution
601 Study. Front Cell Infect Microbiol 10:555508.

602 14. Bučević Popović V, Šitum M, Chow C-ET, Chan LS, Roje B, Terzić J. 2018. The urinary
603 microbiome associated with bladder cancer. Sci Rep 8:12157.

604 15. Karstens L, Asquith M, Davin S, Stauffer P, Fair D, Gregory WT, Rosenbaum JT,
605 McWeeney SK, Nardos R. 2016. Does the Urinary Microbiome Play a Role in Urgency
606 Urinary Incontinence and Its Severity? Front Cell Infect Microbiol 6.

607 16. Kliem M, Sauer S. 2012. The essence on mass spectrometry based microbial diagnostics.
608 Curr Opin Microbiol 15:397–402.

609 17. Pace NR. 1997. A Molecular View of Microbial Diversity and the Biosphere. Science
610 276:734–740.

611 18. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, Fierer
612 N, Knight R. 2011. Global patterns of 16S rRNA diversity at a depth of millions of
613 sequences per sample. Proc Natl Acad Sci 108:4516–4522.

614 19. The Human Microbiome Project Consortium, Huttenhower C, Gevers D, Knight R,
615 Abubucker S, Badger JH, Chinwalla AT, Creasy HH, Earl AM, FitzGerald MG, Fulton RS,
616 Giglio MG, Hallsworth-Pepin K, Lobos EA, Madupu R, Magrini V, Martin JC, Mitreva M,
617 Muzny DM, Sodergren EJ, Versalovic J, Wollam AM, Worley KC, Wortman JR, Young
618 SK, Zeng Q, Aagaard KM, Abolude OO, Allen-Vercoe E, Alm EJ, Alvarado L, Andersen
619 GL, Anderson S, Appelbaum E, Arachchi HM, Armitage G, Arze CA, Ayvaz T, Baker CC,
620 Begg L, Belachew T, Bhonagiri V, Bihan M, Blaser MJ, Bloom T, Bonazzi V, Paul Brooks
621 J, Buck GA, Buhay CJ, Busam DA, Campbell JL, Canon SR, Cantarel BL, Chain PSG,
622 Chen I-MA, Chen L, Chhibba S, Chu K, Ciulla DM, Clemente JC, Clifton SW, Conlan S,
623 Crabtree J, Cutting MA, Davidovics NJ, Davis CC, DeSantis TZ, Deal C, Delehaunty KD,
624 Dewhirst FE, Deych E, Ding Y, Dooling DJ, Dugan SP, Michael Dunne W, Scott Durkin
625 A, Edgar RC, Erlich RL, Farmer CN, Farrell RM, Faust K, Feldgarden M, Felix VM, Fisher
626 S, Fodor AA, Forney LJ, Foster L, Di Francesco V, Friedman J, Friedrich DC, Fronick CC,
627 Fulton LL, Gao H, Garcia N, Giannoukos G, Giblin C, Giovanni MY, Goldberg JM, Goll J,
628 Gonzalez A, Griggs A, Gujja S, Kinder Haake S, Haas BJ, Hamilton HA, Harris EL,
629 Hepburn TA, Herter B, Hoffmann DE, Holder ME, Howarth C, Huang KH, Huse SM, Izard
630 J, Jansson JK, Jiang H, Jordan C, Joshi V, Katancik JA, Keitel WA, Kelley ST, Kells C,
631 King NB, Knights D, Kong HH, Koren O, Koren S, Kota KC, Kovar CL, Kyrpides NC, La
632 Rosa PS, Lee SL, Lemon KP, Lennon N, Lewis CM, Lewis L, Ley RE, Li K, Liolios K, Liu

633 B, Liu Y, Lo C-C, Lozupone CA, Dwayne Lunsford R, Madden T, Mahurkar AA, Mannon
634 PJ, Mardis ER, Markowitz VM, Mavromatis K, McCorrison JM, McDonald D, McEwen J,
635 McGuire AL, McInnes P, Mehta T, Mihindukulasuriya KA, Miller JR, Minx PJ, Newsham
636 I, Nusbaum C, O'Laughlin M, Orvis J, Pagani I, Palaniappan K, Patel SM, Pearson M,
637 Peterson J, Podar M, Pohl C, Pollard KS, Pop M, Priest ME, Proctor LM, Qin X, Raes J,
638 Ravel J, Reid JG, Rho M, Rhodes R, Riehle KP, Rivera MC, Rodriguez-Mueller B, Rogers
639 Y-H, Ross MC, Russ C, Sanka RK, Sankar P, Fah Sathirapongsasuti J, Schloss JA, Schloss
640 PD, Schmidt TM, Scholz M, Schriml L, Schubert AM, Segata N, Segre JA, Shannon WD,
641 Sharp RR, Sharpton TJ, Shenoy N, Sheth NU, Simone GA, Singh I, Smillie CS, Sobel JD,
642 Sommer DD, Spicer P, Sutton GG, Sykes SM, Tabbaa DG, Thiagarajan M, Tomlinson CM,
643 Torralba M, Treangen TJ, Truty RM, Vishnivetskaya TA, Walker J, Wang L, Wang Z,
644 Ward DV, Warren W, Watson MA, Wellington C, Wetterstrand KA, White JR, Wilczek-
645 Boney K, Wu Y, Wylie KM, Wylie T, Yandava C, Ye L, Ye Y, Yooseph S, Youmans BP,
646 Zhang L, Zhou Y, Zhu Y, Zoloth L, Zucker JD, Birren BW, Gibbs RA, Highlander SK,
647 Methé BA, Nelson KE, Petrosino JF, Weinstock GM, Wilson RK, White O. 2012.
648 Structure, function and diversity of the healthy human microbiome. Nature 486:207–214.

649 20. Karstens L, Asquith M, Caruso V, Rosenbaum JT, Fair DA, Braun J, Gregory WT, Nardos
650 R, McWeeney SK. 2018. Community profiling of the urinary microbiota: considerations for
651 low-biomass samples. Nat Rev Urol 15:735–749.

652 21. Aragón IM, Herrera-Imbroda B, Queipo-Ortuño MI, Castillo E, Del Moral JS-G, Gómez-
653 Millán J, Yucel G, Lara MF. 2018. The Urinary Tract Microbiome in Health and Disease.
654 Eur Urol Focus 4:128–138.

655 22. Adebayo AS, Ackermann G, Bowyer RCE, Wells PM, Humphreys G, Knight R, Spector
656 TD, Steves CJ. 2020. The Urinary Tract Microbiome in Older Women Exhibits Host
657 Genetic and Environmental Influences. Cell Host Microbe 28:298-305.e3.

658 23. Oechslin CP, Lenz N, Liechti N, Ryter S, Agyeman P, Bruggmann R, Leib SL, Beuret CM.
659 2018. Limited Correlation of Shotgun Metagenomics Following Host Depletion and
660 Routine Diagnostics for Viruses and Bacteria in Low Concentrated Surrogate and Clinical
661 Samples. Front Cell Infect Microbiol 8:375.

662 24. Prodan A, Tremaroli V, Brolin H, Zwinderman AH, Nieuwdorp M, Levin E. 2020.
663 Comparing bioinformatic pipelines for microbial 16S rRNA amplicon sequencing. PLOS
664 ONE 15:e0227434.

665 25. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA,
666 Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ,
667 Weber CF. 2009. Introducing mothur: Open-Source, Platform-Independent, Community-
668 Supported Software for Describing and Comparing Microbial Communities. Appl Environ
669 Microbiol 75:7537–7541.

670 26. Navas-Molina JA, Peralta-Sánchez JM, González A, McMurdie PJ, Vázquez-Baeza Y, Xu
671 Z, Ursell LK, Lauber C, Zhou H, Song SJ, Huntley J, Ackermann GL, Berg-Lyons D,

672 Holmes S, Caporaso JG, Knight R. 2013. Advancing our understanding of the human
673 microbiome using QIIME. Methods Enzymol 531:371–444.

674 27. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, Alexander H,
675 Alm EJ, Arumugam M, Asnicar F, Bai Y, Bisanz JE, Bittinger K, Brejnrod A, Brislawn CJ,
676 Brown CT, Callahan BJ, Caraballo-Rodríguez AM, Chase J, Cope EK, Da Silva R, Diener
677 C, Dorrestein PC, Douglas GM, Durall DM, Duvallet C, Edwardson CF, Ernst M, Estaki
678 M, Fouquier J, Gauglitz JM, Gibbons SM, Gibson DL, Gonzalez A, Gorlick K, Guo J,
679 Hillmann B, Holmes S, Holste H, Huttenhower C, Huttley GA, Janssen S, Jarmusch AK,
680 Jiang L, Kaehler BD, Kang KB, Keefe CR, Keim P, Kelley ST, Knights D, Koester I,
681 Kosciolek T, Kreps J, Langille MGI, Lee J, Ley R, Liu Y-X, Loftfield E, Lozupone C,
682 Maher M, Marotz C, Martin BD, McDonald D, McIver LJ, Melnik AV, Metcalf JL,
683 Morgan SC, Morton JT, Naimey AT, Navas-Molina JA, Nothias LF, Orchanian SB,
684 Pearson T, Peoples SL, Petras D, Preuss ML, Pruesse E, Rasmussen LB, Rivers A, Robeson
685 MS, Rosenthal P, Segata N, Shaffer M, Shiffer A, Sinha R, Song SJ, Spear JR, Swafford
686 AD, Thompson LR, Torres PJ, Trinh P, Tripathi A, Turnbaugh PJ, Ul-Hasan S, van der
687 Hooft JJJ, Vargas F, Vázquez-Baeza Y, Vogtmann E, von Hippel M, Walters W, Wan Y,
688 Wang M, Warren J, Weber KC, Williamson CHD, Willis AD, Xu ZZ, Zaneveld JR, Zhang
689 Y, Zhu Q, Knight R, Caporaso JG. 2019. Reproducible, interactive, scalable and extensible
690 microbiome data science using QIIME 2. Nat Biotechnol 37:852–857.

691 28. Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, Peplies J, Glockner FO. 2007.
692 SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA
693 sequence data compatible with ARB. Nucleic Acids Res 35:7188–7196.

694 29. Hugenholtz P, Skarshewski A, Parks DH. 2016. Genome-Based Microbial Taxonomy
695 Coming of Age. Cold Spring Harb Perspect Biol 8.

696 30. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. 2016.
697 DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods
698 13:581–583.

699 31. Gao X, Lin H, Revanna K, Dong Q. 2017. A Bayesian taxonomic classification method for
700 16S rRNA gene sequences with improved species-level accuracy. BMC Bioinformatics
701 18:247.

702 32. Thomas-White K, Forster SC, Kumar N, Kuiken MV, Putonti C, Stares MD, Hilt EE, Price
703 TK, Wolfe AJ, Lawley TD. 2018. Culturing of female bladder bacteria reveals an
704 interconnected urogenital microbiota. Nat Commun 9:1557.

705 33. Breitwieser FP, Lu J, Salzberg SL. 2019. A review of methods and databases for
706 metagenomic classification and assembly. Brief Bioinform 20:1125–1136.

707 34. Hugerth LW, Andersson AF. 2017. Analysing Microbial Community Composition through
708 Amplicon Sequencing: From Sampling to Hypothesis Testing. Front Microbiol 8:22.

709 35. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D,
710 Hu P, Andersen GL. 2006. Greengenes, a Chimera-Checked 16S rRNA Gene Database and
711 Workbench Compatible with ARB. Appl Env Microbiol 72:5069–5072.

712 36. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO.
713 2012. The SILVA ribosomal RNA gene database project: improved data processing and
714 web-based tools. Nucleic Acids Res 41:D590–D596.

715 37. Ravel J, Gajer P, Abdo Z, Schneider GM, Koenig SSK, McCulle SL, Karlebach S, Gorle R,
716 Russell J, Tacket CO, Brotman RM, Davis CC, Ault K, Peralta L, Forney LJ. 2011. Vaginal
717 microbiome of reproductive-age women. Proc Natl Acad Sci 108:4680–4687.

718 38. Martinez RM, Hulten KG, Bui U, Clarridge JE. 2014. Molecular Analysis and Clinical
719 Significance of Lactobacillus spp. Recovered from Clinical Specimens Presumptively
720 Associated with Disease. J Clin Microbiol 52:30–36.

721 39. Mueller ER, Wolfe AJ, Brubaker L. 2017. Female urinary microbiota. Curr Opin Urol
722 27:282–286.

723 40. Flores-Mireles AL, Walker JN, Caparon M, Hultgren SJ. 2015. Urinary tract infections:
724 epidemiology, mechanisms of infection and treatment options. Nat Rev Microbiol 13:269–
725 284.

726 41. Thomas-White KJ, Hilt EE, Fok C, Pearce MM, Mueller ER, Kliethermes S, Jacobs K,
727 Zilliox MJ, Brincat C, Price TK, Kuffel G, Schreckenberger P, Gai X, Brubaker L, Wolfe
728 AJ. 2016. Incontinence medication response relates to the female urinary microbiota. Int
729 Urogynecology J 27:723–733.

730 42. Park S-C, Won S. 2018. Evaluation of 16S rRNA Databases for Taxonomic Assignments
731 Using a Mock Community. Genomics Inform 16:e24.

732 43. Myer PR, McDaneld TG, Kuehn LA, Dedonder KD, Apley MD, Capik SF, Lubbers BV,
733 Harhay GP, Harhay DM, Keele JW, Henniger MT, Clemmons BA, Smith TPL. 2020.
734 Classification of 16S rRNA reads is improved using a niche-specific database constructed
735 by near-full length sequencing. PLOS ONE 15:e0235498.

736 44. Fettweis JM, Serrano MG, Sheth NU, Mayer CM, Glascock AL, Brooks JP, Jefferson KK.
737 2012. Species-level classification of the vaginal microbiome. BMC Genomics 13:9.

738 45. Chen T, Yu W-H, Izard J, Baranova OV, Lakshmanan A, Dewhirst FE. 2010. The Human
739 Oral Microbiome Database: a web accessible resource for investigating oral microbe
740 taxonomic and genomic information. Database 2010:baq013–baq013.

741 46. Ritari J, Salojärvi J, Lahti L, de Vos WM. 2015. Improved taxonomic assignment of human
742 intestinal 16S rRNA sequences by a dedicated reference database. BMC Genomics
743 16:1056.

744 47. Rennie JDM, Shih L, Teevan J, Karger DR. 2003. Tackling the Poor Assumptions of Naive
745 Bayes Text Classifiers, p. 8. *In* Proceedings of the Twentieth International Conference on
746 Machine Learning. The AAAI Press, Washington, DC.

747 48. Wang Q, Garrity GM, Tiedje JM, Cole JR. 2007. Naive Bayesian Classifier for Rapid
748 Assignment of rRNA Sequences into the New Bacterial Taxonomy. Appl Environ
749 Microbiol 73:5261–5267.

750 49. Abellan-Schneyder I, Matchado MS, Reitmeier S, Sommer A, Sewald Z, Baumbach J, List
751 M, Neuhaus K. 2021. Primer, Pipelines, Parameters: Issues in 16S rRNA Gene Sequencing
752 6:22.

753 50. Pohl HG, Groah SL, Pérez-Losada M, Ljungberg I, Sprague BM, Chandal N, Caldovic L,
754 Hsieh M. 2020. The Urine Microbiome of Healthy Men and Women Differs by Urine
755 Collection Method. Int Neurourol J 24:41–51.

756 51. Jung CE, Chopyk J, Shin JH, Lukacz ES, Brubaker L, Schwanemann LK, Knight R, Wolfe
757 AJ, Pride DT. 2019. Benchmarking urine storage and collection conditions for evaluating
758 the female urinary microbiome. Sci Rep 9:13409.

759 52. Munch MM, Chambers LC, Manhart LE, Domogala D, Lopez A, Fredricks DN, Srinivasan
760 S. 2019. Optimizing bacterial DNA extraction in urine. PLOS ONE 14:e0222962.

761 53. Ackerman AL, Anger JT, Khalique MU, Ackerman JE, Tang J, Kim J, Underhill DM,
762 Freeman MR, the NIH Multidisciplinary Approach to the Study of Chronic Pelvic Pain
763 (MAPP). 2019. Optimization of DNA extraction from human urinary samples for
764 mycobiome community profiling. PLOS ONE 14:e0210306.

765 54. Balvočiūtė M, Huson DH. 2017. SILVA, RDP, Greengenes, NCBI and OTT — how do
766 these taxonomies compare? BMC Genomics 18:114.

767 55. Rainey FA. 2011. How to Describe New Species of Prokaryotes, p. 7–14. *In* Methods in
768 Microbiology. Elsevier.

769 56. Parte AC. 2018. LPSN – List of Prokaryotic names with Standing in Nomenclature
770 (bacterio.net), 20 years on. Int J Syst Evol Microbiol 68:1825–1829.

771 57. Notredame C, Higgins DG, Heringa J. 2000. T-coffee: a novel method for fast and accurate
772 multiple sequence alignment. J Mol Biol 302:205–217.

773 58. Valdar WSJ. 2002. Scoring residue conservation. Proteins Struct Funct Bioinforma 48:227–
774 241.

775 59. Kumar PS, Griffen AL, Moeschberger ML, Leys EJ. 2005. Identification of Candidate
776 Periodontal Pathogens and Beneficial Species by Quantitative 16S Clonal Analysis. J Clin
777 Microbiol 43:3944–3955.

60. Kumar P, Brooker M, Dowd S, Camerlengo T. 2011. Target Region Selection Is a Critical Determinant of Community Fingerprints Generated by 16S Pyrosequencing. PLOS ONE 6.

61. Bukin YuS, Galachyants YuP, Morozov IV, Bukin SV, Zakharenko AS, Zemskaya TI. 2019. The effect of 16S rRNA region choice on bacterial community metabarcoding results. Sci Data 6:190007.

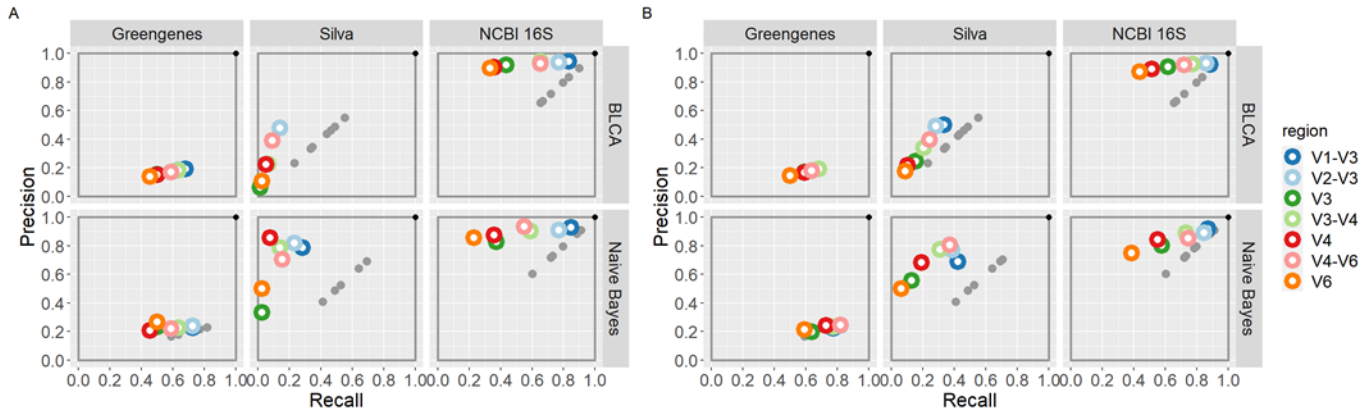
62. Walter J, Tannock GW, Tilsala-Timisjarvi A, Rodtong S, Loach DM, Munro K, Alatossava T. 2000. Detection and Identification of Gastrointestinal Lactobacillus Species by Using Denaturing Gradient Gel Electrophoresis and Species-Specific PCR Primers. Appl Environ Microbiol 66:297–303.

63. Graspeuntner S, Loeper N, Künzel S, Baines JF, Rupp J. 2018. Selection of validated hypervariable regions is crucial in 16S-based microbiota studies of the female genital tract. Sci Rep 8:9678.

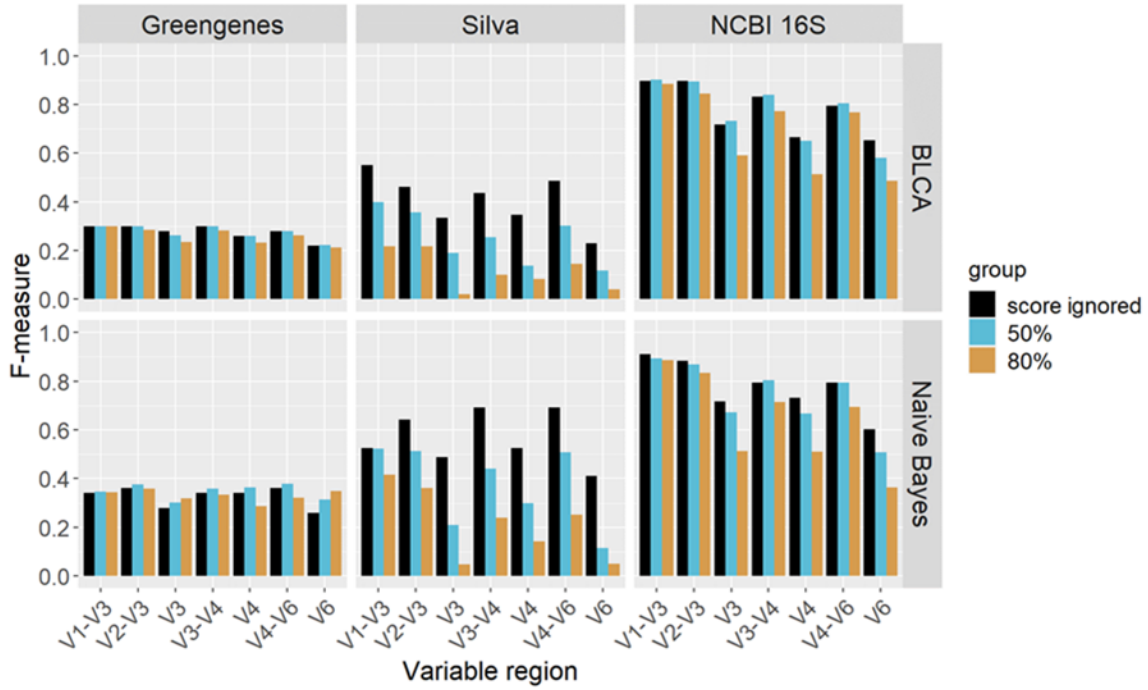
64. Hugerth LW, Wefer HA, Lundin S, Jakobsson HE, Lindberg M, Rodin S, Engstrand L, Andersson AF. 2014. DegePrime, a Program for Degenerate Primer Design for Broad-Taxonomic-Range PCR in Microbial Ecology Studies. Appl Environ Microbiol 80:5116–5123.

65. Morris EK, Caruso T, Buscot F, Fischer M, Hancock C, Maier TS, Meiners T, Müller C, Obermaier E, Prati D, Socher SA, Sonnemann I, Wäschke N, Wubet T, Wurst S, Rillig MC. 2014. Choosing and using diversity indices: insights for ecological applications from the German Biodiversity Exploratories. Ecol Evol 4:3514–3524.

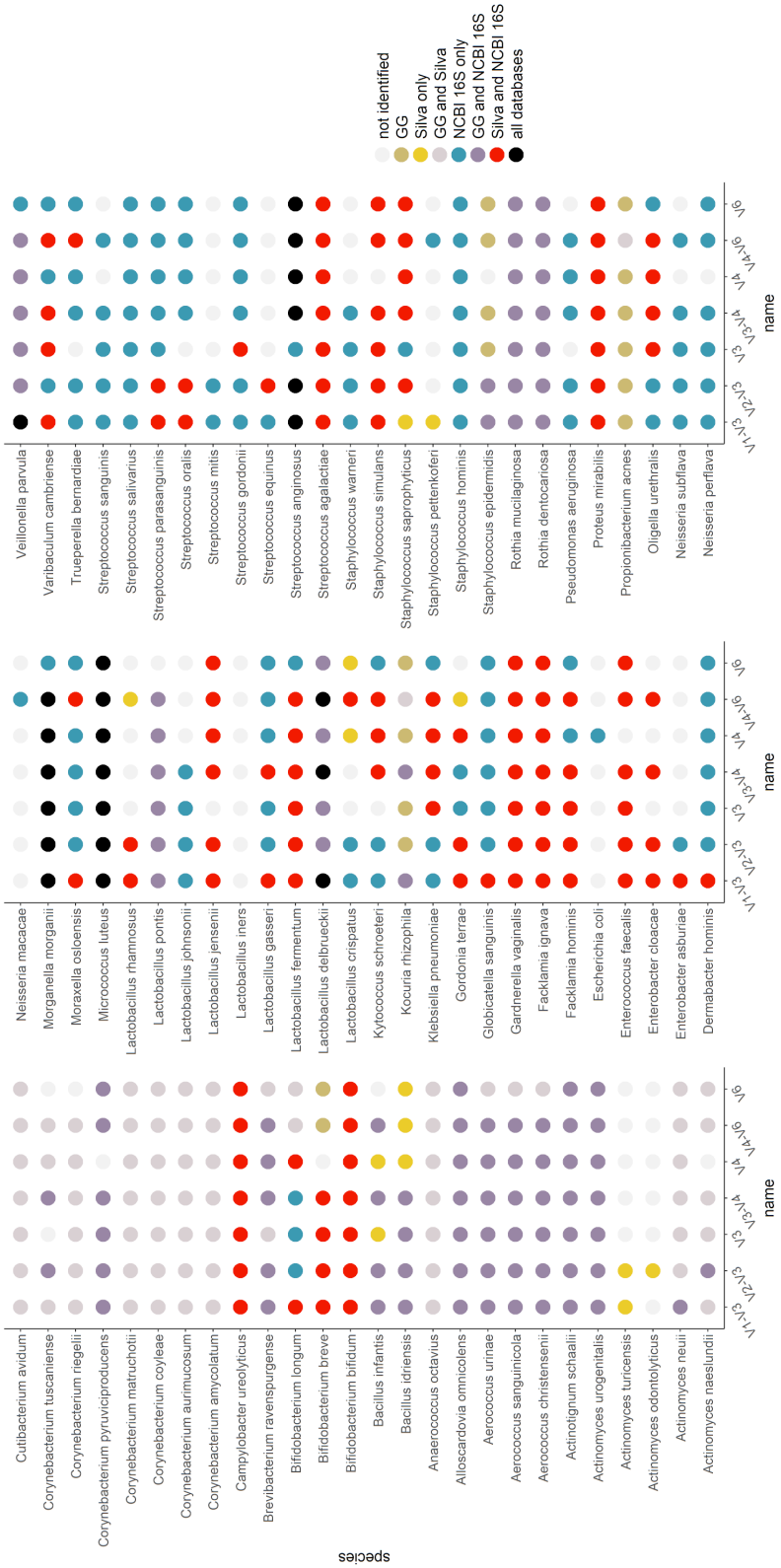
Supplemental Figures



Supplemental Figure 1. Classification scheme precision and recall when using a confidence scores of (A) 80% and (B) 50% as a threshold. The schemes that use the Silva database have very low recall compared to when the confidence score is ignored (gray dots), whereas schemes that use Greengenes and NCBI 16S are not as affected.



Supplemental Figure 2. F-measure values for all classification schemes. Values shown are for confidence scores of 50%, 80%, and when confidence scores are ignored.



Supplemental Figure 3. Bladder bacterial species of the Thomas-White dataset identified by database, variable region, and BLCA classifier.

Supplemental Figure 4. Summary of the NCBI 16S classification for *in silico* classification on the V4 validation dataset. Blue dots represent the species/sample pair that was identified by whole genome sequencing, but was not identified by the classification scheme. Yellow dots represent the species/sample pair that was successfully identified by the classification scheme and whole genome sequencing of the culture data.