TaxAss: Leveraging Custom Databases Achieves Fine-Scale Taxonomic Resolution

RUNNING TITLE
TaxAss achieves fine-scale taxonomic resolution

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WORD COUNTS:
Abstract (250)
Importance (147)
Text (5325)
ABSTRACT

Taxonomy assignment for microbial community composition studies can be limited by a lack of relevant reference organisms in large taxonomy databases. TaxAss is a taxonomy assignment workflow to classify 16S rRNA gene amplicon data using two taxonomy reference databases: a large comprehensive database such as Greengenes or SILVA, and a small ecosystem-specific database curated by scientists within a field. To test TaxAss performance, we classified five different freshwater datasets using the comprehensive Greengenes database and the freshwater-specific FreshTrain database. TaxAss increased the percent of the dataset classified compared to using only Greengenes. The increase in classifications was highest at fine-resolution taxa levels, where across the freshwater test-datasets the classifications at species-level increased by 24-40 percent reads. A similar increase in classifications was not observed in a control mouse gut dataset, which was not expected to contain freshwater bacteria. TaxAss maintained taxonomic richness compared to using only the FreshTrain. Richness was maintained across all taxa-levels from phylum to species. Without TaxAss, the majority of organisms not represented in the FreshTrain were unclassified, but at finer taxa levels incorrect classifications were also significant. TaxAss splits a dataset’s sequences into two groups based on their percent identity to reference sequences in the ecosystem-specific database. Highly similar sequences are classified using the ecosystem-specific database and the others are classified using the comprehensive database. TaxAss metrics help users choose a percent identity cutoff
appropriate for their data. TaxAss is free and open source, and available at

www.github.com/McMahonLab/TaxAss.

IMPORTANCE

Microbial communities drive ecosystem processes, but microbial community composition analyses using 16S rRNA gene amplicon datasets is limited by the lack of fine-resolution taxonomy classifications. Course taxonomic groupings at phylum, class, and order level lump ecologically distinct organisms together. To avoid this, many researchers cluster similar sequences into operational taxonomic units (OTUs). These fine-resolution groupings are more ecologically relevant, but OTU definitions are dataset-dependent and therefore cannot be compared between datasets. Microbial ecologists studying a variety of environments have curated small, ecosystem-specific taxonomy databases to provide consistent and up-to-date terminology. We created TaxAss, a workflow that leverages these ecosystem-specific databases to assign taxonomy. We found that TaxAss improves fine-resolution taxonomic classifications (family, genus and species). Fine taxonomic groupings are also ecologically relevant, so they provide an alternative to OTU-based analyses that is consistent and comparable between datasets. TaxAss enables researchers to compare data using ecologically relevant terminology.
INTRODUCTION

Microbial communities form the foundations of all ecosystems, yet interpretation of community data is limited by the difficulty of comparing across datasets. With the rapid development of massively parallel sequencing technology, scientists are increasingly able to fingerprint microbial communities using amplicon sequencing of marker genes such as the 16S rRNA gene. The resulting sequences are typically clustered into sequence identity-based Operational Taxonomic Units (OTUs). Comparison between amplicon datasets is difficult because sequence identity-based OTUs are specific to each analysis. For clarity, this paper refers to 16S rRNA gene amplicon sequencing datasets as “datasets” and defines OTUs as a dataset’s sequence unit of measure, irrespective of whether those units represent clustered sequences or unique sequences.

77 Taxonomy Allows Cross-Study Analyses

Sequence identity-based OTUs are widely accepted as good representations of coherent ecological entities (1), however these OTUs represent study-specific phylotypes that cannot be compared between datasets. This is because sequence identity-based OTU clustering is specific to each analysis, resulting in arbitrary OTU names. Additionally, the clustering algorithm and the similarity cutoff chosen for curation of each dataset impacts the ecological relationships observed (2). For these reasons, direct comparisons of sequence identity-based OTUs between multiple datasets are impossible without the computationally expensive task of combining and re-curating.
Taxonomic naming systems allow comparisons between datasets by creating consistent terminology and consistent phylogeny-determined boundaries between organisms. However, taxonomic naming is most useful when sequences can be classified to a fine level (e.g. family, genus, or species). Many abundant taxa are poorly represented in reference taxonomy databases (hereafter “databases”), resulting in only coarse classifications for large proportions of amplicon datasets (e.g. phylum, class, or order). Coarse taxonomic groupings often include diverse organisms with differing ecological roles, so analyses at coarse taxa levels miss underlying ecological dynamics (3). Fine-resolution taxonomic names are required to bridge the gap between ecologically relevant OTU-based analyses and consistent, comparable taxonomy-based analyses.

**Ecosystem-Specific Taxonomy Databases**

Microbial ecologists from diverse ecosystems have created fine-resolution reference taxonomies by curating databases specific to their ecosystems. These ecosystem-specific databases are small compared to the large comprehensive databases compiled by Greengenes (4), Silva (5), and the Ribosomal Database Project (6), but they are generally well-curated with more finely resolved phylogenies for ecosystem-specific lineages. Examples of ecosystems with curated databases include the human oral cavity (7), the cow rumen (8), the honey bee gut (9), the cockroach and termite gut (10), activated sludge (11), and freshwater lakes (12). Ecosystem-specific databases are created to establish consistent vocabulary for common uncultured bacteria, create
monophyletic reference structures, incorporate new reference information, and understand what the “typical” organisms are in a given ecosystem. Additionally, ecosystem-specific databases can be used to assign taxonomy to a finer resolution than can be achieved with a large comprehensive database.

**Taxonomy Assignment Algorithm**

Classification algorithms assign taxonomic names to OTUs based on their similarity to reference sequences in a database. The most commonly used classification algorithm was developed by Wang et. al (13) for the Ribosomal Database Project and is implemented in both mothur (14) and QIIME (15). This naïve Bayesian classifier (hereafter “Wang classifier”) assigns taxonomy to OTUs based on 8-mer signatures and reports a bootstrap confidence estimate for each assignment (13). This bootstrap confidence value is based on the repeatability of the OTU’s assignment with subsampled 8-mers, not on an absolute similarity measure. In a large database an OTU dissimilar to any reference sequences will not be classified repeatably as any one taxon, resulting in a low bootstrap confidence. However, in a small database an OTU dissimilar to any reference sequences nevertheless can be classified repeatably because there are fewer references from which to choose. This pitfall is referred to as “forcing” in this paper, because OTUs are forced into incorrect classifications by the small database.
Introducing TaxAss

We aimed to obtain fine-level taxonomy classifications by leveraging an ecosystem-specific database, while at the same time maintaining the full biological diversity of an amplicon dataset. To this end, we developed an open source taxonomy assignment workflow (TaxAss) that uses the popular Wang classifier as implemented in mothur and employs both an ecosystem-specific database and a comprehensive database. The workflow maintains taxonomic richness and accuracy by only classifying OTUs that share high percent identity with ecosystem-specific reference sequences using the ecosystem-specific database. The remaining OTUs are classified using the comprehensive database.

Ideally, an ecosystem-specific database would be merged with a comprehensive database and TaxAss would not be necessary. However in reality, updates to comprehensive databases usually occur only every few years, during which time both databases diverge further from each other as they continue to be curated. TaxAss allows researchers to effectively leverage the most current version of both databases to achieve the maximum, most accurate, and finest-resolution classifications possible.

The FreshTrain

This paper demonstrates TaxAss’s efficacy using a variety of freshwater amplicon datasets, the comprehensive Greengenes database (4), and the ecosystem-specific Freshwater Training Set (FreshTrain) (12). The FreshTrain database was created in
2012 and was originally curated alongside Greengenes. It matches Greengenes at the phylum, class, and order levels, but at finer taxonomic levels it was curated based on additional information such as the geographical distribution of sequences. These finer levels are referred to as lineage, clade, and tribe and approximate the Linnaean family, genus, and species (12). This workflow and a newly updated version of the FreshTrain are available online at www.github.com/McMahonLab/TaxAss.

RESULTS

Methods Summary

TaxAss uses both an ecosystem-specific database and a large comprehensive database to improve taxonomic assignment resolution while maintaining richness. To classify the maximum possible number of OTUs and avoid forcing inaccurate ecosystem-specific classifications onto OTUs, the amplicon dataset is split into two groups prior to classification: OTUs with high percent identity to ecosystem-specific reference sequences and OTUs with low percent identity to ecosystem-specific reference sequences. The two groups are then classified separately using the Wang classifier and the appropriate database (Figure 1).

Fine-Resolution Classifications Increased

To test whether TaxAss improved taxonomic classification over solely using a comprehensive database, we assigned taxonomy to a Lake Mendota amplicon dataset first by using Greengenes alone and then by using TaxAss to leverage both
Greengenes and the FreshTrain (Figure 2a and Table 1). We compared the percent of reads classified by both methods and observed a marked improvement in the percent of the dataset classified to the fine taxa levels of family/lineage, genus/clade, and species/tribe. At species/tribe level, the percent of reads classified increased from 1.4% to 41%, at genus/clade level they increased from 23% to 63%, and at family/lineage level they increased from 64% to 78%. In addition to these increases in classifications, TaxAss also improved the quality of classifications because the FreshTrain is curated with terminology consistent with the freshwater microbial ecology literature. At family/lineage level, Greengenes alone could classify a majority of the dataset, but 75% of those Greengenes-classified reads received more meaningful ecosystem-specific nomenclature when using TaxAss.

The FreshTrain reference sequences come exclusively from temperate lake epilimnia, and many of them come from Lake Mendota itself. Lake Mendota is a eutrophic, temperate lake in Wisconsin, USA; and the Lake Mendota amplicon dataset consists of 95 epilimnetic samples collected by the North Temperate Lakes Microbial Observatory over 11 years. To test TaxAss’s efficacy when the ecosystem-specific database is less representative of the ecosystem under investigation, we classified amplicon datasets from a range of freshwater ecosystems first by using Greengenes alone and then by using TaxAss to leverage Greengenes and the FreshTrain (Figure 2b). The additional ecosystems we chose included the epilimnion of oligotrophic Lake Michigan (16), the eutrophic Danube River (17), and the epilimnion and hypolimnion of dystrophic Trout...
Bog (WI, USA) (18). We also used a mouse gut dataset (19) as a negative control to ensure that TaxAss would not assign FreshTrain classifications erroneously. All freshwater datasets showed improvements at all fine taxa levels (Figure 2b and Supplemental Figure 2), with the amount of improvement reflecting the similarity of each ecosystem to the FreshTrain reference sequences. For example, the temperate lake Mendota and Lake Michigan epilimnia improved most (54 and 52% at genus/clade level), while the dystrophic bog hypolimnion improved least (28% at genus/clade level). Only 0.1, 0.3, and 0.2% of the mouse gut control dataset received FreshTrain classifications at the species, genus, and family levels.

Richness Maintained

To test whether TaxAss improved taxonomic classification over solely using an ecosystem-specific database, we assigned taxonomy to the Lake Mendota dataset first by using the FreshTrain alone and then by using TaxAss to leverage both the FreshTrain and Greengenes. TaxAss maintained taxonomic richness at all taxa levels by classifying OTUs into a larger variety of taxonomic names (Figure 3 and Table 1). At the same time TaxAss prevented inaccuracies in fine-resolution classifications that were introduced by FreshTrain-only classifications (Figure 3b).

The FreshTrain is a more specific database with less taxonomic richness than Greengenes, so a decrease in taxonomic richness in a FreshTrain-only classification was expected. For example, the FreshTrain focuses on heterotrophic bacteria and does
not include any Cyanobacteria, which comprised 8.5% of the Lake Mendota dataset. All
of Lake Mendota’s cyanobacterial OTUs were classified as something else (99% as
unclassified), which resulted in a loss of phylum-level richness in the FreshTrain-only
classification (Figure 3a). In contrast, TaxAss maintained the taxonomic richness of a
Greengenes-only classification and reduced unclassified reads at all taxa levels (Table
1).

In the FreshTrain-only classification scheme, we also observed occasions when an
OTU was incorrectly given a taxonomic assignment when it should have been labeled
unclassified (Figure 3b). This “forcing” of OTUs into incorrect classifications by the small
FreshTrain database was less common than OTUs being called “unclassified,” but had
significant effects on taxa relative abundances at finer-resolution taxa levels. Lake
Mendota’s 5th most abundant lineage, the Bacteroidetes bacl, gained 27% more reads
in a FreshTrain-only classification compared to using TaxAss. The forcing errors
TaxAss prevented were significant enough to change basic attributes such as rank
abundances of top taxa, and had an even larger impact on the freshwater test-datasets
that differed more from the FreshTrain references (Supplemental Figure 3).

Percent Identity Cutoff
An OTU is classified taxonomically in the ecosystem-specific database only if it matches
a sequence in that database at a sequence identity above the threshold set by the user.
Therefore, the percent identity cutoff choice for taxonomic classification is central to the
proper functioning of TaxAss because it determines which OTUs are classified in each
database (ecosystem-specific vs comprehensive). If the percent identity cutoff is set too
high, ecosystem-specific OTUs are passed to the comprehensive database for
classification; while if it is set too low, non-ecosystem-specific OTUs are passed to the
ecosystem-specific database for classification.

We found that a percent identity cutoff of 98-99% was appropriate for the analyzed
freshwater datasets, and we applied a cutoff of 98% when processing all data used in
this paper. We chose the percent identity cutoff to maximize the percent of each dataset
receiving taxonomic classification at every taxa level (Figure 4 and Supplemental Figure
4). TaxAss allows users to choose a cutoff specific to their data by generating the plots
shown in Figure 4, but users who wish to save computational time can simply choose a
percent identity cutoff and only run the classification once.

**BLAST Conversion Validated**

The calculation of percent identity for use in database selection is based on the percent
identity returned by The National Center for Biotechnology Information’s Basic Local
Alignment Search Tool (BLAST) (20). The default megablast settings are appropriate
for our application because they have been highly optimized to find short, highly similar
alignments. However, BLAST finds areas of local similarity and there is no way to
require BLAST to align the entire length of a query OTU’s sequence. 16S rRNA gene
amplicon sequences are highly similar, and differences in taxonomic classification can
be based on even a single mismatch in the amplified region. Therefore, we recalculated the percent identities BLAST returned into “full-length” percent identities for the entire query OTU’s sequence (Supplemental Document 1 and Equation 1).

We found that recalculating percent identity was necessary to prevent dissimilar OTUs from inclusion in the ecosystem-specific classification. For example, the FreshTrain does not include any reference sequences from the major freshwater phylum *Cyanobacteria*, so no cyanobacterial OTUs have high true percent identities to any references in the FreshTrain. We found that the percent identity recalculation was necessary to prevent some cyanobacterial OTUs from meeting the percent identity cutoff due to the original BLAST percent identities being based on only a short aligned section of the OTU sequence (Supplemental Figure 1).

We also found that it was necessary to recalculate the percent identity from several BLAST alignments (“hits”) for each OTU because the best BLAST hit did not always have the highest recalculated percent identity. TaxAss examines the top five BLAST hits, recalculates the percent identity of each, and then uses the highest recalculated percent identity to determine if an OTU meets the cutoff. To ensure enough BLAST hits were examined to consistently arrive at the highest possible recalculated percent identity, we calculated the proportion of times each BLAST hit number had the highest recalculated percent identity. In the Lake Mendota amplicon dataset the first BLAST hit almost always also had the best recalculated score, and the contribution of additional
BLAST hits was very low, especially when only "good" hits above a stringent percent identity cutoff were considered (Table 2). In the Lake Mendota dataset at the chosen 98 percent identity cutoff, 99.69% of the best hits found by BLAST were also the best re-calculated hits and only 0.01% of BLAST's 5th hits were used. TaxAss generates a version of Table 2 for users' individual datasets, and if they observe more high-number BLAST hits contributing to the best re-calculated hit they can increase the number of BLAST results used for the calculation.

DISCUSSION

Ecosystem-Specific Databases

The need for curated ecosystem-specific databases has been recognized by microbial ecologists studying many ecosystems. TaxAss was developed specifically to leverage the Freshwater Training Set (FreshTrain) (12), but it could be applied to custom databases curated for other ecosystems: the dictyopteran gut microbiota reference database (DictDb) (10), the rumen and intestinal methanogen database (RIM-DB) (8), the honey bee database (HBDB) (9), the microbial database for activated sludge (MiDAS) (11), and the human oral microbiome database (HOMD) (7). These databases were created by starting with a comprehensive database such as SILVA or Greengenes and then re-curating the reference sequences from the study ecosystem, sometimes also incorporating new reference sequences. Often during curation, phylogenies were collapsed to be monophyletic and incorporate new organisms, and abundant but unnamed organisms were given placeholder names to allow for consistent terminology among researchers.
DictDB, HBDB, and MiDAS are fully integrated with modified versions of the entire SILVA database, so a workflow like TaxAss that leverages two databases is not needed because the single merged database can be used in one step for taxonomy assignment. However fully integrated databases can be difficult to maintain over time because new versions of each database will diverge from each other, and TaxAss provides a means to circumvent this divergence. The FreshTrain is an example of this divergence in action. The FreshTrain was originally integrated into the Hugenholtz database that eventually became Greengenes, and Greengenes was last updated in May 2013. In addition, SILVA now contains more total references and has been updated as recently as September 2016, so some researchers prefer to use the more recently updated SILVA as their comprehensive database. Similarly, the FreshTrain has been updated almost annually since its creation as new full-length 16S rRNA gene sequences from freshwater ecosystems became available. TaxAss allows microbial ecologists to use the most up-to-date versions of their preferred databases without performing or waiting for reconciliation of each release.

Once an ecosystem-specific database has diverged from the comprehensive database, as occurred with the FreshTrain, leveraging the ecosystem-specific database for taxonomy assignment is no longer straightforward. Reintegrating the ecosystem-specific database into the comprehensive database is more involved than simply concatenating databases and removing duplicated references because conflicting phylogenetic
structures must be resolved. Analysis of community amplicon data is a fairly routine part of many studies for which extensive phylogenetic curation would fall outside the scope.

The FreshTrain has been used in a variety of ways since it diverged from the current version of Greengenes, and it is often difficult to discern the specifics from cursory sentences in a paper’s methods section. TaxAss provides a well-documented and rigorously tested workflow to leverage two conflicting databases without extensive curation.

Current FreshTrain Usage

The simplest way the FreshTrain has been used to assign taxonomy to amplicon datasets is as part of a separate, complementary analysis. For example, in a study of the River Thames Basin (21), FreshTrain and Greengenes classifications were displayed side by side and separate metrics such as diversity indices were calculated for each. However, the bulk of the taxonomic analyses were carried out at the coarse phylum level despite most abundant OTUs having FreshTrain nomenclature. When the FreshTrain is used independently, the loss of richness in taxonomic classifications (Figure 3) makes it difficult to use ecosystem-specific classifications for entire-dataset analyses. TaxAss provides ecosystem-specific classifications without loss of taxonomic richness, thus allowing for a single comprehensive analysis.

Another straightforward approach has been to classify amplicon datasets sequentially, first using the FreshTrain and then re-classifying the unclassified sequences using a
comprehensive database. For example, in a study of Lake Erken, Sweden (22), OTUs were first classified with the FreshTrain, and then unclassified OTUs were reclassified using SILVA. While this approach allows for a single analysis, the initial classification of all sequences with the small FreshTrain database can cause forcing of OTUs into incorrect classifications (Figure 3b). TaxAss prevents forcing by splitting the OTUs into two groups prior to classification.

This forcing of OTUs into incorrect classifications when using the FreshTrain was observed in a study of cyanobacterial blooms in Yanga Lake, Australia (23), where the authors observed that cyanobacterial OTUs were forced into heterotrophic classifications. To prevent this, Greengenes was used for an initial classification, then only OTUs assigned to phyla included in the FreshTrain were reclassified and renamed with confidently assigned FreshTrain nomenclature. This approach prevented the forcing of Yanga Lake’s abundant cyanobacterial OTUs, but it would not prevent forcing of OTUs that belong to phyla included in the FreshTrain (Verrucomicrobia, Bacteroidetes, Proteobacteria, and Actinobacteria). In freshwater datasets such as bogs, rivers, and lake hypolimnia many organisms belonging to FreshTrain phyla differ significantly from the lake epilimnion references included in the FreshTrain. TaxAss prevents forcing classifications onto OTUs of any phyla.

Another way to avoid the forcing observed with the Wang classifier is to use BLAST-based taxonomy assignment algorithms that determine assignments based on
sequence similarity. Since the BLAST algorithm calculates an absolute similarity instead of a relative one, a similarity cutoff prevents classifications to dissimilar sequences. The BLAST method to assign taxonomy has been used with the FreshTrain to classify sequences from boreal lakes in Quebec, Canada (24). However, unlike the Wang classifier, BLAST only takes into account individual reference sequences and ignores their encompassing phylogenetic structure. The BLAST-based algorithm from Classification Resources for Environmental Sequence Tags (CREST) (25) addresses this by taking a lowest common ancestor approach. Each query OTU is classified to the finest taxa level that its top BLAST hits share. The CREST algorithm also has been used to assign taxonomy using the FreshTrain to sequences obtained from the Danube River in southeastern Europe (17). This approach avoided forcing and incorporated phylogenetic information in the taxonomy assignments, however it does not maintain diversity by also leveraging a comprehensive database. Additionally, the Wang classifier is more robust at coarser taxa levels and for shorter sequences (25), and it is implemented in common tools like mothur and QIIME. TaxAss allows users to leverage both ecosystem-specific and comprehensive databases using the highly trusted and conveniently implemented Wang classifier.

Future TaxAss Usage

We recommend all microbial ecologists studying freshwater systems use the FreshTrain and TaxAss to classify their 16S rRNA gene amplicon datasets. This will result in a consistent, specific, and comparable vocabulary throughout the field, and will improve
classification for analysis of individual datasets. We also recommend that microbial ecologists with different ecosystem-specific databases consider TaxAss when their databases diverge from the most up-to-date comprehensive database and phylogenetic curation is outside the scope of their project.

We recommend microbial ecologists create ecosystem-specific databases if one does not already exist, since they provide improved analysis and enhanced collaboration for the entire field. Phylogenies must be created from full-length 16S rRNA gene sequences, which are not collected as routinely as short amplicon sequences. However, we believe the benefit of these databases as a reference for the field and to improve taxonomic classification of amplicon sequences justifies the effort to create them, especially since TaxAss allows their use without constant re-curation. Additional full-length sequences to flesh-out the existing phylogenetic structure of organisms can be created with clone libraries, as was done for the FreshTrain. New sequencing technologies, such as the long reads produced by Nanopore (26) and PacBio (27, 28) instruments, promise even easier reference sequence generation in the future.

**Practical Guidance for Using TaxAss**

TaxAss includes detailed descriptions of its constituent scripts including argument options and descriptions, so users are able to customize their analyses. The most important decision users make is the cutoff percent identity that determines which database is used to classify each OTU. If an OTU is above the cutoff (i.e. has high
percent identity to an ecosystem-specific reference sequence) then it will be classified
with the ecosystem-specific database. When the cutoff is higher, fewer OTUs are
classified with the ecosystem-specific database and users run the risk of leaving some
ecosystem-specific OTUs poorly classified by the comprehensive database. If the cutoff
is lower, more OTUs will be classified with the ecosystem-specific database and users
run the risk of forcing incorrect classifications onto OTUs and losing taxonomic richness.
Users can decide on a percent identity cutoff at the beginning and run only one
classification, or they can run TaxAss with several cutoffs and generate versions of
Figure 4 to help guide their choice.

We found that a percent identity cutoff of 98-99% optimized classifications in our test-
datasets. The finding that most OTUs match their ecosystem-specific reference
sequences with such high percent identity suggests that the commonly chosen 97%
sequence identity clustering is too coarse to observe fine taxa level dynamics. This is
supported by previous findings that sequence identity-based OTUs can impose artificial
delineations between organisms that affect results differently depending on the lineage
(29), and that sequence identity-based OTUs can contain temporally discordant
sequences (30). We recommend that users planning a taxonomy-centric analysis
classify unique sequences without sequence identity-based clustering and use fine-level
taxonomic assignment to group their data. This will make the classification take longer,
but likely users will save computational time overall by not clustering. For users who
also want to emphasize OTU-based analyses and whose datasets are too large for
unclustered analyses, we recommend choosing a finer sequence identity-based OTU definition such as 98 or 99% to best leverage the fine-level classification provided by TaxAss and a detailed ecosystem-specific database. When OTUs have been clustered based on sequence identity, we recommend that users choose the same or lower percent identity cutoff in TaxAss to prevent OTUs with constituent sequences falling on either side of the percent identity cutoff.

**TaxAss Improves Analyses**

Taxonomy-based analyses allow researchers to compare results across datasets. Leveraging an ecosystem-specific database for taxonomy assignment results in a high proportion of fine resolution classifications, and grouping sequences based on these classifications is a dataset-independent way to describe community composition. The resulting taxonomic terminology is consistent and comparable between analyses, and the finely resolved taxonomic groupings rival the resolution of sequence identity-based OTUs. TaxAss can replace or complement sequence identity-based OTU clustering, which results in dataset-dependent OTU clusters and names. Reclustering sequence identity-based OTUs to compare across datasets is computationally expensive, and is not possible for datasets created with differing amplification primers. When researchers use TaxAss to assign fine-level taxonomy to their datasets, colleagues can compare their results directly, without re-analysis and regardless of primer set. Additionally, taxonomic nomenclature can also bridge amplicon-based analyses and genomic analyses.
Leveraging ecosystem-specific databases for taxonomy assignment also improves researchers’ interpretations of individual datasets. Ecosystem-specific terminology is more meaningful because ecosystem-specific databases incorporate additional reference sequences, finer phylogenetic delineations, consistent nomenclature for uncultured organisms, and monophyletic structures. For example, the dominant lineage in freshwater is the FreshTrain’s actinobacterial lineage acl, which in Greengenes is usually classified as Ack-M1. Although a classification exists for this organism in both databases, the Greengenes lineage includes all sequences that match a single historically used primer, while the FreshTrain classification includes a slightly different subset of actinobacterial sequences and is based on a phylogeny manually curated from full-length 16S rRNA genes. Additionally, the FreshTrain includes finer-level phylogenetic information on acl’s constituent subgroups known as clades and tribes, which prior work suggests are ecologically and metabolically differentiated (12). The fine-resolution taxonomy assignments provided by TaxAss and an ecosystem-specific database allow researchers to link their amplicon datasets with known ecophysiological traits.

Ecosystem-specific phylogenies that are not fully incorporated into a comprehensive database are not straightforward to leverage for taxonomy assignment. The FreshTrain, for example, has diverged from Greengenes since it was created, and it has been used for taxonomy assignment with inconsistent and sometimes unreliable methods. TaxAss
is a well-documented, open source, and rigorously tested workflow that avoids the pitfalls of using a small database: forcing incorrect classifications onto sequences and losing taxonomic richness by leaving unrepresented organisms unclassified. At the same time, TaxAss achieves the benefits of an ecosystem-specific database: more meaningful nomenclature, larger proportions of the dataset classified, and finer-resolution classifications.

**METHODS**

**How to Use TaxAss**

TaxAss replaces only the taxonomy assignment step of users’ preferred amplicon dataset processing pipeline such as mothur or qiime. TaxAss consists of a series of scripts using R, Python, bash, mothur, and BLAST that are run from the terminal command line. The input to TaxAss is a quality controlled fasta file, and if users opt to run the optional percent identity cutoff metrics a relative abundance table is also required. The output of TaxAss is the fasta file’s sequence IDs followed by their 7-level taxonomy assignments. Scripts, step-by-step instructions, and detailed explanations of script argument options are available online at https://github.com/McMahonLab/TaxAss.

**Percent Identity Recalculation**

The naïve Bayesian algorithm used for taxonomy assignment (the Wang classifier) (13) can “force” incorrect classifications onto OTUs when a close match does not exist in a small reference database. TaxAss uses the well-accepted Wang classifier, but avoids
forcing when classifying with a small ecosystem-specific database by only classifying sequences for which a close reference exists. The National Center for Biotechnology Information’s Basic Local Alignment Search Tool (BLAST) (20) is utilized to split the amplicon dataset into two groups prior to classification: one is classified with the ecosystem-specific database, the other with the comprehensive database.

Blastn queries each OTU sequence against the ecosystem-specific database using the default megablast settings, which are optimized to find highly similar matches between sequences longer than 30 bp (31). However, blast returns the percent identity of the highest scoring pair (the “pident”), which does not necessarily include the full length of the query OTU sequence. OTU sequences are highly similar; a single mismatch can change a classification, so mismatches at the ends of OTU sequences (in the “overhang”) that BLAST leaves out of the alignment must be included in the percent identity cutoff used for classification. Therefore, the BLAST pident is recalculated to a full length percent identity with the following equation:

\[
\text{percent identity} = \frac{\text{pident} \times \text{length}}{\text{qlen} + \text{length} - (\text{qend} - \text{qstart} + 1)}
\]  
(Equation 1)

where “pident” is the percent identity returned by BLAST, “length” is the length of the alignment, “qlen” is the query length, “qend” is the query end, and qstart is the query start. All of these parameters are returned by BLAST output format 6, and detailed
descriptions of what they are, the equation derivation, and an example alignment and calculation are included in Supplemental Document 1.

The recalculaton to full length percent identity is conservative; all query nucleotides not included in the alignment (nucleotides in the “overhang”) are considered mismatches. This means that it would be possible to exclude an OTU from the ecosystem-specific classification when its true percent identity is above the cutoff due to matches in unaligned overhangs. An example of this situation is illustrated in Supplemental Document 1. When the highest scoring BLAST alignments contain matches on the overhangs, some of the lower-scoring alignments will be longer, and therefore have a higher recalculated percent identity. To correct for this TaxAss recalculates the percent identity of the top five blast hits and uses the best one for the cutoff decision. TaxAss also shows users the distribution of chosen hits, so that settings can be re-evaluated if BLAST is not primarily returning hits that have the best recalculated percent identities.

**Cutoff Choice**

OTUs with percent identities greater than or equal to the users’ specified cutoff are classified with the ecosystem-specific database using the Wang classifier as implemented by mothur. The remaining OTUs are classified with the comprehensive database, also using the Wang classifier. The choice of a percent identity cutoff is left to users so that they can balance their choices based on the structures of their datasets and their plans for analysis. If the percent identity cutoff is too low, dissimilar OTUs will
be classified in the ecosystem-specific database and may be left unclassified or forced into incorrect classifications, but if the percent identity cutoff is too high, OTUs similar to the ecosystem-specific database will be classified by the comprehensive database and may end up poorly classified.

Users have the option to choose a cutoff percent identity at the start, or they can classify their datasets with multiple cutoffs and TaxAss will provide metrics to guide their decisions. These metrics include versions of Figure 4, which shows the cutoff choices that maximize the proportion of dataset classified at different taxa levels.

As an additional optional check, users can also classify their datasets with only the comprehensive database and then compare the classifications. TaxAss provides metrics to check for forcing incorrect coarse-resolution classifications onto OTUs (Supplemental Table 1). Phylum- and class-level classifications are more reliable when assigned by a large comprehensive database that includes more diversity, so if ecosystem-specific classifications at these coarse taxa levels disagree with the comprehensive database’s assignments it suggests that the percent identity cutoff is too low. Only these coarse levels can be used to check for forcing, because at finer taxonomic levels too many OTUs end up unclassified by the comprehensive database to compare assignments.
The freshwater datasets used in this paper are all publicly available on the National Center for Biotechnology Information’s (NCBI) Sequence Read Archive (SRA). The accession numbers are: Lake Mendota (ERP016591), Trout Bog (ERP016854), Lake Michigan (SRP056973), and Danube River (SRP045083). The Lake Michigan and bog project accessions include additional sample types, so only the Lake Michigan and Trout Bog samples were used. The mouse gut dataset is the full version of the example data used by mothur’s miSeq SOP, and is available on the mothur website (https://www.mothur.org/wiki/MiSeq_SOP).

Quality control of fastq files was performed according to mothur's MiSeq SOP (19, accessed September 2017) through the chimera checking step. The resulting unique sequences were used as OTUs in all further analyses. The single-end sequencing datasets (Lake Mendota and Trout Bog) were also pre-processed with VSEARCH (32) to trim to uniform lengths and remove low quality sequences with > 0.5 expected errors. During TaxAss, the percent identity cutoff used for all datasets was 98%, and the Wang classifier’s bootstrap confidence was set at 80% for all classifications. Batch files that reproduce all download, quality control, and TaxAss processing for each dataset are available in Supplemental Document 2.

The taxonomy databases used in this paper are also publicly available. The Freshwater Training Set (FreshTrain) (12) is available from www.github.com/McMahonLab/TaxAss.
The Greengenes database (4) is available from greengenes.secondgenome.com/downloads. Further details on download, versions, and formatting can be found in Supplemental Document 2 and in the detailed directions provided at www.github.com/McMahonLab/TaxAss.

ACKNOWLEDGEMENTS

We thank the North Temperate Lakes Long Term Ecological Research Program (NTL-LTER) and the Microbial Observatory for their years of support collecting the Lake Mendota and Trout Bog time series. We also thank the Earth Microbiome Project for generously sequencing our Lake Mendota and Trout Bog datasets. We thank Benjamin Crary and Jason Woodhouse for sharing insights from their efforts to combine taxonomy databases for classification, and the many users of TaxAss who have reported bugs during development.

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Table 1. Classification of the Lake Mendota dataset

|                     | Percent Classified<sup>a</sup> | Taxonomic Richness<sup>b</sup> |
|---------------------|--------------------------------|---------------------------------|
|                     | Greengenes | TaxAss | FreshTrain | Greengenes | TaxAss | FreshTrain |
| Phylum              | 98         | 98     | 87         | 68         | 68     | 5          |
| Class               | 93         | 93     | 79         | 209        | 207    | 17         |
| Order               | 83         | 87     | 74         | 423        | 419    | 35         |
| Family/Lineage      | 64         | 78     | 68         | 699        | 733    | 65         |
| Genus/Clade         | 23         | 63     | 56         | 1161       | 1206   | 102        |
| Species/Tribae      | 1          | 41     | 42         | 1322       | 1405   | 154        |

<sup>a</sup>Percent classified = total reads classified / total reads in dataset x 100%

<sup>b</sup>Taxonomic Richness = total unique classifications

Table 2. Agreement between BLAST and recalculated percent identities.

| BLAST result | Percent Identity Cutoff Applied<sup>a</sup> |
|--------------|---------------------------------------------|
|              | 95  | 96  | 97  | 98  | 99  | 100 |
| Hit 1        | 97.8<sup>b</sup> | 98.3 | 99.0 | 99.69 | 99.905 | 100 |
| Hit 2        | 1.3 | 1.0 | 0.6 | 0.22 | 0.066 | 0   |
| Hit 3        | 0.4 | 0.3 | 0.2 | 0.05 | 0.014 | 0   |
| Hit 4        | 0.2 | 0.2 | 0.1 | 0.03 | 0.009 | 0   |
| Hit 5        | 0.3 | 0.3 | 0.1 | 0.01 | 0.006 | 0   |

<sup>a</sup>Calculations performed only on sequences above listed recalculated percent identities

<sup>b</sup>e.g. 97.8% of BLAST’s first hits also had the highest recalculated percent identity
Figure 1. TaxAss Conceptual Diagram

TaxAss separates OTUs into two groups that are classified separately and then recombined. OTUs similar to any ecosystem-specific reference sequences are classified using the ecosystem-specific database, otherwise they are classified by the comprehensive database.
**Figure 2. TaxAss performance compared to Greengenes-only performance.**

**A and B:** Left bars represent the Greengenes-only classification and right bars represent the TaxAss classification that leveraged both Greengenes and the FreshTrain. Within the right bars, red reads were classified by the FreshTrain using TaxAss and were left unclassified using only Greengenes; yellow reads were classified by the FreshTrain using TaxAss but received Greengenes classifications using only Greengenes, and grey reads were classified by Greengenes when using TaxAss.

**A:** In the Lake Mendota dataset, TaxAss leveraged the FreshTrain and Greengenes to achieve improved fine-resolution classifications. **B:** TaxAss achieved improvements in a range of freshwater datasets despite the FreshTrain’s primary focus on temperate lake epilimnia. Few changes in classification were observed in the mouse gut control.

Versions of this figure across all datasets and taxa levels can be found in Supplemental Figure 2.
Figure 3. TaxAss performance compared to FreshTrain-only performance.

A and B: Lake Mendota reads represented by blue bars were incorrectly classified as red bars in the FreshTrain-only classification. Rank order of the bars follows the TaxAss-classification rank abundances. Only taxa with at least 0.5% relative abundance are included, and at lineage level the number of bars displayed is further truncated to 20. A: TaxAss maintained phylum richness by classifying the phyla (blue) that are not included in the FreshTrain using Greengenes. B: TaxAss similarly maintained lineage richness, and also prevented inaccuracies from forced taxonomic classifications (red bars). Versions of this figure across all test-ecosystems can be found in Supplemental Figure 3.
Figure 4. Percent identity cutoff choice.

The percent of reads classified in the Lake Mendota dataset at each taxa level. Faint vertical lines highlight the maxima. TaxAss users can use this visualization to identify a percent identity cutoff appropriate for their dataset. Versions of this figure across all test-ecosystems can be found in Supplemental Figure 4.
LIST OF SUPPLEMENTAL DOCUMENTS

Supplemental Table 1. Coarse-level classification disagreements in Lake Mendota dataset at 98 percent identity cutoff.

Supplemental Figure 1. Importance of percent identity recalculation.
The phylum Cyanobacteria exemplifies why the percent identity recalculation is necessary. The ecosystem-specific FreshTrain database contains no cyanobacterial references, so cyanobacterial reads serve as a control for something that should be classified in the comprehensive Greengenes group. However, BLAST returned hits with high percent identities for cyanobacterial OTUs due to including partial alignments (red bars). After the TaxAss percent identity recalculation, the cyanobacterial OTUs had lower percent identities and none were included in the FreshTrain classification group (grey bars).

Supplemental Figure 2. TaxAss compared to Greengenes-only performance.
Versions of Figure 2 for each test-dataset and taxa level.

Supplemental Figure 3. TaxAss compared to FreshTrain-only performance.
Versions of Figure 3 for each test-dataset.
Supplemental Figure 4. Percent Identity Cutoff Where Classifications Are Maximized.

Versions of Figure 4 for each test-dataset.

Supplemental Document 1. BLAST percent identity recalculation.

This 3-page document defines the blast terminology, derives the equation to recalculate percent identity, and provides an example alignment and calculation.

Supplemental Document 2. Data Processing Batch Files.

This zip file contains directions for reproducing all data processing in this paper. It includes batch scripts that download each dataset, batch scripts that quality control each dataset, and batch scripts that perform TaxAss on each dataset. It also contains the versions of TaxAss scripts used in this paper. A README file includes directions for how to download the versions of databases and programs used in processing, and how to source the included batch scripts. The zip file does not include the data itself, just the folder structure and scripts for download and processing; the uncompressed folder is 1 MB.