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Comparison of Primer Sets for Use in Automated Ribosomal Intergenic Spacer Analysis of Aquatic Bacterial Communities: an Ecological Perspective[▽]

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Two primer sets for automated ribosomal intergenic spacer analysis (ARISA) were used to assess the bacterial community composition (BCC) in Lake Mendota, Wisconsin, over 3 years. Correspondence analysis revealed differences in community profiles generated by different primer sets, but overall ecological patterns were conserved in each case. ARISA is a powerful tool for evaluating BCC change through space and time, regardless of the specific primer set used.

Studies conducted within an ecological framework often involve broad-scale research questions whose answers depend less on obtaining a complete census of microbial populations than on the ability to sample a system of interest with high spatial or temporal resolution. Current molecular microbial ecology methods have allowed environmental microbiologists to ask important ecological questions that previously could not be addressed through culture-based approaches. Culture-independent methods also suffer from well-established limitations (7), however, and as such are useful but always imperfect. This is particularly true for PCR-based methods, since PCR amplification of microbial DNA can be subject to bias for a variety of reasons (10). In spite of these limitations, great advances in the study of microbial ecology have been made through the use of molecular methods.

A key component of any PCR-based method is the PCR primer set. Ideally, a universal primer set would amplify the target DNA from all taxonomic groups with equal efficiencies. No known primer sets meet this criterion. As a result, inconsistencies in DNA amplification are likely to be observed between particular primer sets.

The purpose of this study was to compare two recently evaluated automated ribosomal intergenic spacer analysis (ARISA) primer sets (3) for use in aquatic microbial ecology. ARISA distinguishes microbial populations based on the length heterogeneity in the ribosomal intergenic spacer region and provides a sensitive analysis of microbial communities at a relatively high level of taxonomic resolution with significant reproducibility (3, 6). In addition, the automated nature of the method enables rapid analysis of a large number of samples collected over space or time. The efficacy of this method for ecological research has been demonstrated by our research group and others (8, 9, 11). Here we sought to answer two important questions: (i) do different ARISA primer sets pro-vide dissimilar snapshots of bacterial community composition (BCC), and (ii) do these primer sets result in different conclusions about the ecology of an aquatic system?

Sampling and environmental data. Integrated water samples were taken from Lake Mendota (Dane County, WI) to a depth of 12 m approximately every 2 weeks during the ice-off season from April 2002 to October 2004. Microbial communities from 250 to 500 ml of lake water were captured on 0.2-μm filters (Pall Life Sciences; catalog no. 63025). Filters were stored at $-80°C$ until DNA was extracted using a FastPrep DNA purification kit (Bio 101). Environmental data were collected by the North Temperate Lakes Long Term Ecological Research program (http://lter.limnology.wisc.edu). Complete methodology and data are available at http://lterquery.limnology .wisc.edu.

ARISA. ARISA was conducted on all samples using two primer sets. Primer set 1406f/23Sr consisted of 5′-TGYACAC ACCGCCCGT-3′ (forward primer sequence) and 5′-GGGTT BCCCCATTCRG-3′ (reverse primer sequence). Primer set ITSF/ITSFReub consisted of 5′-GTCGTAACAAGGTAGCC GTA-3′ (forward primer sequence) and 5′-GCCAAGGCATC CACC-3′ (reverse primer sequence). The conditions for ARISA PCR using the 1406f/23Sr primer set were described previously (9). ARISA PCR conditions for the ITSF/ITSReub primer set were previously described by Cardinale et al. (3). Denaturing capillary electrophoresis was carried out for each PCR using an ABI 3730 genetic analyzer (PE Biosystems) as described previously (9).

ARISA profiles were analyzed using a 100- to 2,000-bp custom internal size standard (Bioventures), Genescan 3.1.2 (Applied Biosystems), and Genotyper 2.5 (Applied Biosystems), as described by Kent et al. (9). To include the maximum number of peaks while excluding background fluorescence, a threshold of 50 fluorescence units greater than the baseline was used. Profiles obtained with each primer set were independently analyzed and compared for each sample. In addition, profiles generated using ITSF/ITSReub were converted to the fragment length that would theoretically be detected using 1406f/ 23Sr by adding 197 base pairs and aligned using Matlab 5.0 (The Mathworks). The 197-bp addition corresponds to the

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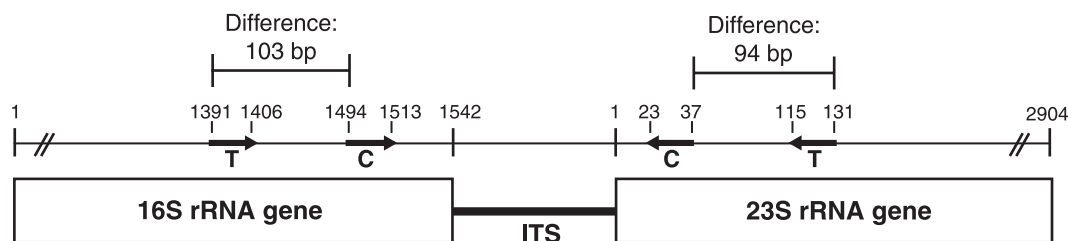
FIG. 1. The positions of the primers used in this study are shown on the prokaryotic rRNA operon. Bold lines indicate primer annealing sites using *E. coli* numbering. T, 1406f/23Sr primers utilized by Fisher and Triplett (6); C, ITSF/ITSReub primers introduced by Cardinale et al. (3); ITS, 16S-23S rRNA operon internal transcribed spacer. Length heterogeneity in the ITS sequence is used to distinguish microbial populations in ARISA. The 1406f/23Sr primer set amplifies an additional 197 bases compared to that amplified by the ITS/ITSReub primer set (based upon standard *E. coli* numbering). This value was added to ITSF/ITSReub data to generate the cITSF/ITSReub data set and allowed direct comparison of data produced from the two primer sets.

difference in length obtained by amplifying the 16S-23S intergenic spacer region using primer set ITSF/ITSReub versus that obtained with primer set 1406f/23Sr, based on *Escherichia coli* numbering (1) (Fig. 1). This "converted" data set will be referred to as cITSF/ITSReub.

Multivariate statistical analyses. Correspondence analysis (CA) was carried out on the 1406f/23Sr, ITSF/ITSReub, and cITSF/ITSReub data sets. This approach is useful for summarizing and graphically representing the multivariate data collected over time and enables comparisons of ecological patterns apparent in the data generated by each primer set. Samples with similar community structures plot close together in a CA ordination, and axes represent theoretical environmental gradients, which can be compared to measured environmental variables. Axes were selected to explain the greatest variability in the community data. CA biplots and correlation coefficients were generated using Canoco for Windows, version 4.51 (Biometris-Plant Research International; 1997 to 2003).

Analysis of similarity (ANOSIM) is a method to test for differences among defined groups in multivariate data sets that is analogous to analysis of variance in univariate statistics (4). ANOSIM was used to directly compare Lake Mendota BCC data produced by the 1406f/23Sr primer set with the cITSF/ITSReub profiles, testing the hypothesis that community profiles produced by the same primer set are more similar to each other than to profiles produced by different primer sets. Successive ANOSIM analyses were conducted with the data set after peaks falling below a threshold of 1, 2, 3, 4, or 5% of total fluorescence were removed. ANOSIM produces a statistic, R, which indicates the magnitude of difference among groups of samples. An R of 1 indicates that the communities completely differ among defined groups, and an R of 0 indicates no difference among groups (4, 5). The statistical significance of R was tested by Monte Carlo randomization.

Profile composition. BCC data generated by the two primer sets (1406f/23Sr and cITSF/ITSReub) were combined in a sin-
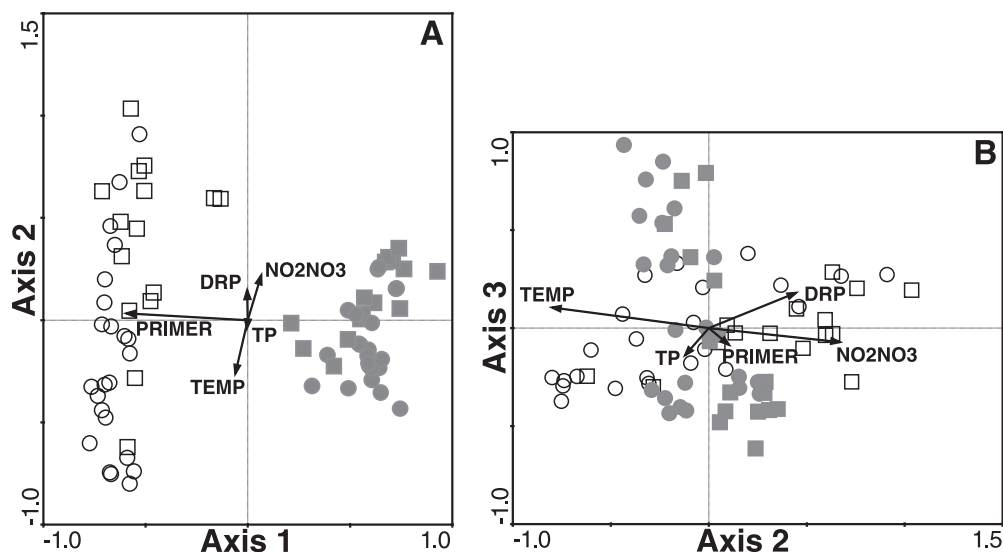

FIG. 2. Correspondence analyses biplots directly comparing ARISA data sets generated by using two different primer sets. Axes 1 and 2 (A) and axes 2 and 3 (B) of the ordination comparing 1406f/23Sr and converted ITSF/ITSReub ARISA data sets. Because of the length differences in the amplicons generated by the two primer sets, 197 bp was added to each fragment generated by ITSF/ITSReub PCR to allow direct comparison of community compositions by correspondence analysis. Filled symbols represent bacterial community data generated from ITSF/ITSReub ARISA, and open symbols represent community data generated using 1406f/23Sr. Squares, samples collected in spring and fall; circles, samples collected in summer. Vectors represent environmental variables and the dummy variable "PRIMER" (1, 1406f/23Sr; 0, ITSF/ITSReub). TP, total phosphorus; TEMP, water temperature; DRP, dissolved reactive phosphorus; NO2NO3, nitrite and nitrate.

TABLE 1. ANOSIM *R* values for comparisons between ARISA
profiles generated from primer set 1406f/23Sr and converted
primer set ITSF/ITSReub

Data set[a]	*R*[b]	*P*	*n*[c]
Full	0.941	0.001	38
1%	0.925	0.001	38
2%	0.869	0.001	38
3%	0.770	0.001	38
4%	0.638	0.001	38
5%	0.379	0.001	36

[a] Comparisons of ARISA profiles were performed by analyses using various
thresholds (1 to 5%) for minimum relative fluorescence contributions by a single
"taxon." See text for details.

[b] An ANOSIM *R* value of 1 indicates complete dissimilarity between samples
analyzed with the two primer sets; 0 corresponds to complete similarity among
these groups of samples.

[c] *n*, number of samples included in the ANOSIM analysis.

gle correspondence analysis (Fig. 2). Samples amplified by the
two primer sets strongly separate along axis 1 (Fig. 2A). The
"primer" vector nearly parallel to axis 1 illustrates the strong
correlation between axis 1 and the primer set used for ARISA
(correlation coefficient, 0.975), indicating that the primer set
best describes the difference observed among the samples de-
spite correcting for the difference in amplicon length. These
differences could be important at the molecular and the meth-
odological level and may significantly influence conclusions
about the presence of taxa or their relative abundances.

To determine if the strong differences in BCC patterns due
to the primer set were driven by dominant or less-abundant
populations (based upon relative areas of ARISA peaks), we
gradually increased the minimum relative fluorescence thresh-
old for inclusion in the analysis. ANOSIM *R* values consistently

decreased as the threshold was increased from 0% (all peaks
included) to 5% (only dominant peaks included), indicating
that when minor community members are excluded the primer
effect is reduced (Table 1).

The observed differences between BCC determined using
1406f/23Sr and ITSF/ITSReub appear to be driven largely by
amplicons that do not contribute significantly to overall
ARISA profile fluorescence. If we assume that relative fluo-
rescence is proportional to relative abundance (supported by
(2), this observation could be the result of differential ampli-
fication of rare taxa. If each primer set preferentially amplifies
different groups of rare taxa, the simple conversion of the
ITSF/ITSReub profiles to 1406f/23Sr bins by adding 197 bp
would generate dissimilar fingerprints, as we observe in Fig.
2A. Even if relative fluorescence is not correlated with relative
abundance, the same subset of strongly amplified taxa is
represented in profiles generated by both primer sets. The
ANOSIM sensitivity analysis (Table 1) demonstrates that this
subset of taxa dictates our interpretation of the Lake Mendota
bacterial community dynamics with either primer set.

Ecological conclusions and seasonal patterns. Within the
combined analysis, axes 2 and 3 of the CA ordination combine
to best explain the variance in BCC driven by environmental
parameters and reveal substantial overlap between the two
data sets (Fig. 2B). In addition, sample ordination along axes 2
and 3 demonstrates good agreement between the 1406f/23Sr
and cITSF/ITSReub data in seasonal separation and correla-
tion with environmental variables. Individual CA ordinations
of ARISA profiles generated by each primer set show similar
seasonal patterns and relationships to chemical and physical
variables (Fig. 3). The visual patterns in the ordinations are
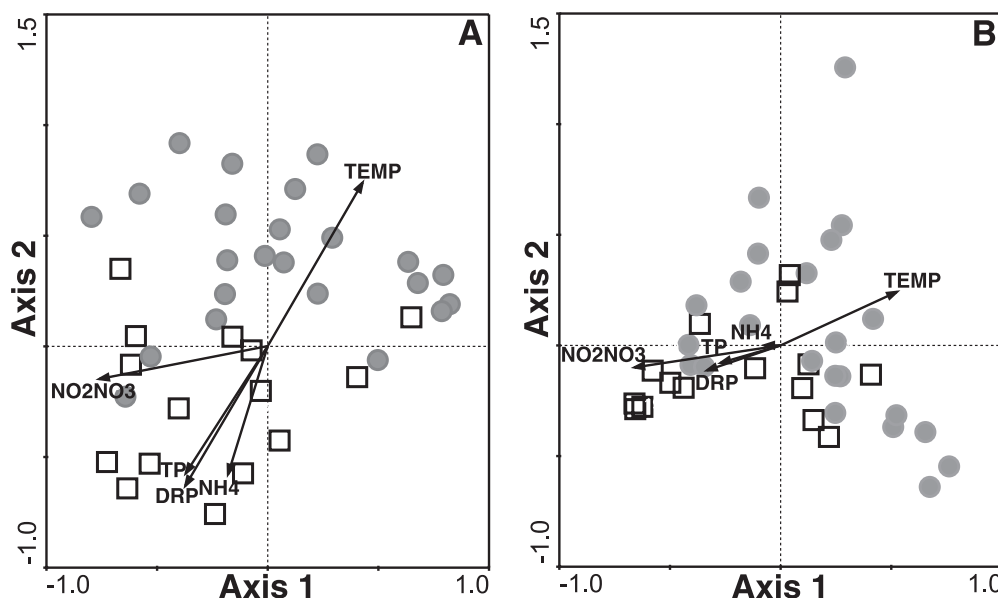supported by correlation coefficients between the axis 1 sample

FIG. 3. Correspondence analysis biplots of bacterial community compositions in Lake Mendota. Seasonal dynamics of bacterioplankton
communities were assessed from April 2002 to October 2004 using ARISA relative fluorescence values. ARISA data were generated with two
different primer sets: (A) 1406f/23Sr (6) and (B) ITSF/ITSReub (3). Squares, samples collected in spring and fall; circles, samples collected in
summer. Vectors represent environmental variables; the direction of increase for each variable and the length of each arrow indicate the degree
of correlation with the ordination axes. TP, total phosphorus; TEMP, water temperature; DRP, dissolved reactive phosphorus; NH4, ammonium;
NO2NO3, nitrite and nitrate.

TABLE 2. Correlation coefficients between sample scores on the first ordination axis and environmental vectors calculated for community data generated by each primer set

Factor	Correlation coefficient	
	ITSF/ITSReub	1406f/23Sr
Temperature	0.617	0.376
Nitrite/nitrate	−0.776	−0.667
Dissolved reactive phosphorus	−0.391	−0.326
Total phosphorus	−0.318	−0.325

scores from the independent correspondence analyses and environmental drivers that are shared among these data sets (Table 2). The signs and strengths of correlation were similar for bacterial community data generated by each primer set.

The predominant ecological patterns in BCC were evident regardless of the choice of primer set used for ARISA. Our two primer sets lead to similar conclusions about seasonal patterns in BCC dynamics in Lake Mendota over 3 years and the environmental factors that drive these patterns (Fig. 3).

A recent study evaluated three primer sets for use with ARISA, comparing the richnesses of ARISA profiles generated by each primer set from six environments (3). The results of this study indicated dramatic differences in measured bacterial richness among the three primer sets, but we suggest that this method of evaluation has limited ecological relevance, given the well-known limitations of molecular microbial ecology methods (7). The purpose of ARISA is not to evaluate richness and composition of single samples. ARISA provides rapid, reproducible community fingerprints that alone contain little information but, when applied in the context of an ecological question and compared to other fingerprints through time or across space (9, 11), offer insight about drivers of community composition change. Our study suggests that different primer sets provide dissimilar fingerprints from a given environmental sample, likely due to differential amplification of taxa with a small contribution to ARISA total fluorescence.

However, when fingerprints from different primer sets are compared along some ecological gradient (time, space, etc.) the pattern of change inferred by ARISA is remarkably robust with respect to primer set.

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