SHORT COMMUNICATION

Evaluating amplified rDNA restriction analysis assay for identification of bacterial communities

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Abstract Amplified ribosomal DNA restriction analysis (ARDRA) and restriction fragment length polymorphism were originally used for strain typing and for screening clone libraries to identify phylogenetic clusters within a microbial community. Here we used ARDRA as a model to examine the capacity of restriction-based techniques for clone identification, and the possibility of deriving phylogenetic information from ARDRA-based dendrograms. ARDRA was performed in silico on 48,759 sequences from the Ribosomal Database Project, and it was found that the fragmentation profiles were not necessarily unique for each sequence in the database, resulting in different species sharing fragmentation profiles. Although ARDRA-based clusters separated clones into different genera, these phylogenetic clusters did not overlap with trees constructed according to sequence alignment, calling into question the intra-genus ARDRA-based phylogeny. It is thus suggested that the prediction power of ARDRA clusters in identifying clone phylogeny be regarded with caution.

Keywords ARDRA · In-silico fragmentation ·
16S subunit ribosomal DNA ·
Ribosomal database project

Abbreviations
ARDRA   Amplified ribosomal DNA restriction analysis
RE   Restriction endonuclease

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Amplified ribosomal DNA restriction analysis (ARDRA) is a commonly used tool to study microbial diversity that relies on DNA polymorphism (Deng et al. 2008). Clones containing 16S rDNA gene fragments, obtained by applying either universal or genus-specific primer sets, are amplified and digested

by restriction endonucleases (REs), followed by separation of the resulting fragments on high-density agarose or acrylamide gels. The emerging profiles are then used either to cluster the community into genotypic groups or for strain typing (Tiedje et al. 1999).

Attempts to use ARDRA to identify species within particular genera have only been partially successful. *Mycoplasma* species isolated from cats (Criado-Fornelio et al. 2003) and humans (Stakenborg et al. 2005) were identified using the ARDRA technique, though their identity was not confirmed by sequencing. ARDRA fingerprinting could not distinguish between genomic species of *Acinetobacter*, even at a low cut-off level (Koeleman et al. 1998), while identification of *Bdellovibrio* and *Bdellovibrio*-like microorganisms using ARDRA grouping mostly reflected diversity and phylogenetic affiliation when compared to sequencing of the 16S rDNA gene (Davidov and Jurkevitch 2004). Rhizobia species isolated from nodules of *Vicia* were identified using 16S ARDRA, restriction fragment length polymorphism (RFLP) of 16S–23S internally transcribed spacer (ITS), and sequencing of the 16S rDNA. The derived phylogenetic relationships mostly supported the relationships estimated by the ARDRA and ITS-RFLP, albeit some discrepancies were detected (Lei et al. 2008). *Lactobacillus* strains isolated from grape must and wine (Rodas et al. 2003), dairy products (Giraffa et al. 1998), and faecal samples (Ventura et al. 2000) were identified by ARDRA fingerprinting. However, the tested isolates were verified by partial sequencing and by comparing the ARDRA patterns to predicted profiles of known strains of lactobacilli. Attempts to apply ARDRA profiles using a panel of six enzymes in order to discriminate *Ralstonia* and *Pandoraea* strains isolated from the respiratory tract of cystic fibrosis patients showed that all the *Ralstonia*, but not the *Pandoraea* strains tested could be differentiated (Segonds et al. 2003). *Brevibacillus* species isolated from clinical, dairy and industrial environments were distinguished using ARDRA (the amplicons were digested with five REs), but comparison of the emerging profiles to those obtained with several phenotypic methods and sequence analysis revealed inconsistencies (Logan et al. 2002). However, a careful choice of REs enabled the use of the ARDRA technique to discriminate among *Lactobacillus*, *Streptococcus*

and *Bifidobacterium* at the genus, but not species, level (Collado and Hernandez 2007).

Heyndrickx et al. (1996) studied the application of ARDRA in the clarification of the phylogeny and taxonomy of the genus *Bacillus*. They found several inter-specific phylogenetic relationships, as well as inter-group phylogenetic relationships, to be in accordance with 16S rDNA sequence analysis; thus, the ARDRA technique, based on the combination of five selected REs, was deemed reliable and valuable for phylogenetic and taxonomic studies of large sets of strains. However, some apparent phylogenetic relationships indicated by ARDRA were not supported by the sequence analysis results. It was postulated that this stemmed from the small phylogenetic distance between these rDNA groups (Vaneechoutte and Heyndrickx 2001).

A study assessing the applicability of ARDRA to the identification of operational taxonomic units based on their ARDRA profiles was carried out more than a decade ago (Moyer et al. 1996), in which a detailed analysis of the types of REs that provide the best differentiating power was performed. In addition, Moyer et al. (1996) compared phylogenetic trees based on 16S rDNA sequences and on ARDRA profiles, and reached the conclusion that using ten REs will yield 76–100% success in obtaining accurate phylogenetic affiliations. However, that study used the very narrow range of sequences available at the time, while today the databases have increased many-fold and the questions have once again arisen: Are ARDRA profiles sufficient for clone identification? Are phylogenetic relationships described by ARDRA sufficiently representative of the "true" relationships determined by the 16S rDNA sequences?

In the current study, we re-evaluated the predictive power of ARDRA, by assessing two ways in which ARDRA can be used to foresee the identity or phylogeny of clones: (a) environmental clone identification via profile matching to theoretically computed fragmentation profiles and (b) clustering of ARDRA fragmentation profiles in comparison to parallel sequence-based clustering.

A total of 48,759 sequences from the Ribosomal Database Project (RDP) (Maidak et al. 2001) were taken for in-silico ARDRA (see "Supplementary material" for detailed methods). Profiles were found to be unique to genera for two or more REs (Fig. 1a),
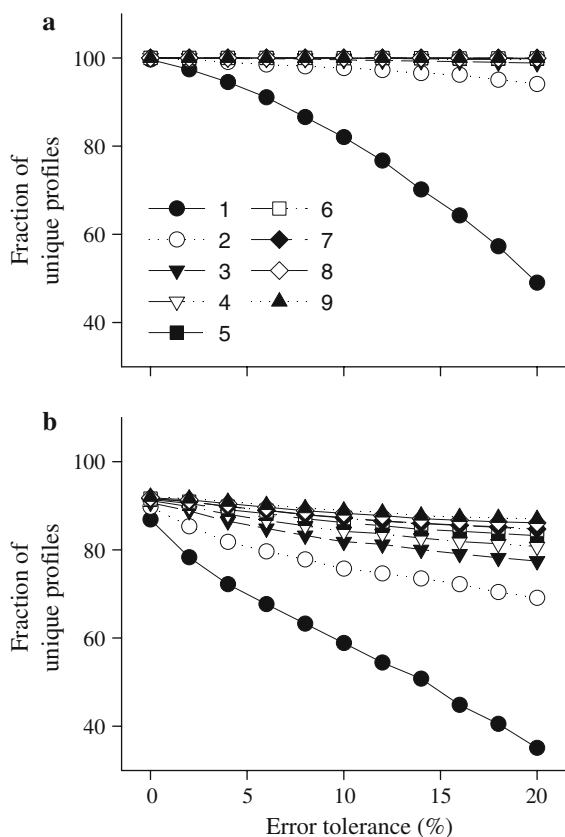
Fig. 1 Proportion of profiles unique to a single genus (**a**) and to a single species (**b**), defined as the number of profiles specific to a single genus or species, respectively, divided by the total number of profiles. Both are dependent on the error tolerance (the *x*-axis) and on the number of restriction enzymes used. The *number* indicates the number of restriction enzymes used

et al. (1996); thus, we found their conclusions to be applicable, even when a large number of sequences is considered (48,759 vs. 106 sequences), for several combinations of the tested REs (data not shown). It is important to point out that the conclusions reached above come from a global perspective; it is possible that for specific genera, different sets of three REs would be better for inter-genera differentiation. When a specific genus is of interest, a specific set of REs may produce a higher resolution even with less than three REs. The scripts written by the authors can be used to this end and can be obtained on request.

Dendrograms calculated from the theoretical fragmentation profiles (see "Supplementary material") of random collections of species, both intra and inter-genus, were found to have little relationship with the phylogenetic clustering based on the corresponding 16S rDNA sequence. Trees based on 16S rDNA sequences and on ARDRA fragmentation were compared, and the average distance between the ARDRA-based and sequence-based trees was 77 ± 2.6, out of a maximum distance of 94 (see "Supplementary material"). In a parallel study, 20 groups of 85 sequences each were used for comparison of ARDRA to 16S rDNA sequence-based phylogenies (see details in "Supplementary material"). The average similarity between the sequence- and ARDRA-based clusters was 2.9%, while the maximum similarity was 7.3%.

Since clustering is based on pairwise distance or similarity between sequences, two distance
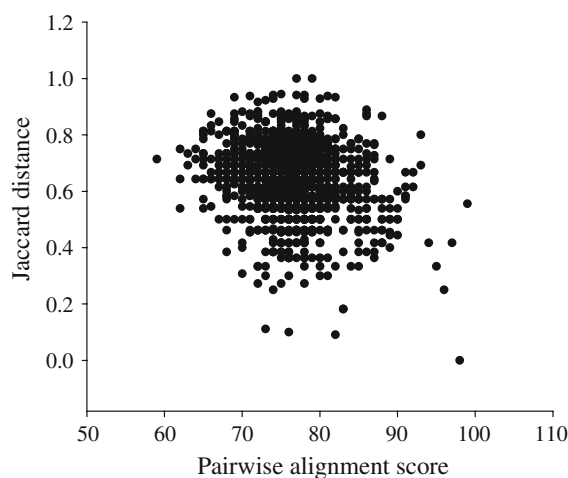
even at a high error level of 20% in the sizing of the restriction fragments (as might occur in agarose gels). However, at least three enzymes were necessary to differentiate species (Fig. 1b). Even then, while most patterns referred to unique sequences, exceptions were observed, e.g. species of *Salmonella* (one species), *Citrobacter* (3), *Klebsiella* (3) and *Enterobacter* (7) shared patterns, as did species of *Kocuria* (1), *Micrococcus* (3), *Arthrobacter* (4) and *Streptomyces* (3). When all ten REs were used, only four patterns were shared by more than one genus: *Citrobacter* (4) and *Klebsiella* (1); *Legionella* (3) and *Fluoribacter* (1); *Saccharothrix* (1) and *Actinosynnema* (1); *Raoultella* (1) and *Citrobacter* (1). These results agree with those obtained by Moyer

Fig. 2 Correlation between the pairwise sequence analysis scores and the calculated Jaccard distances for a random group of 50 species
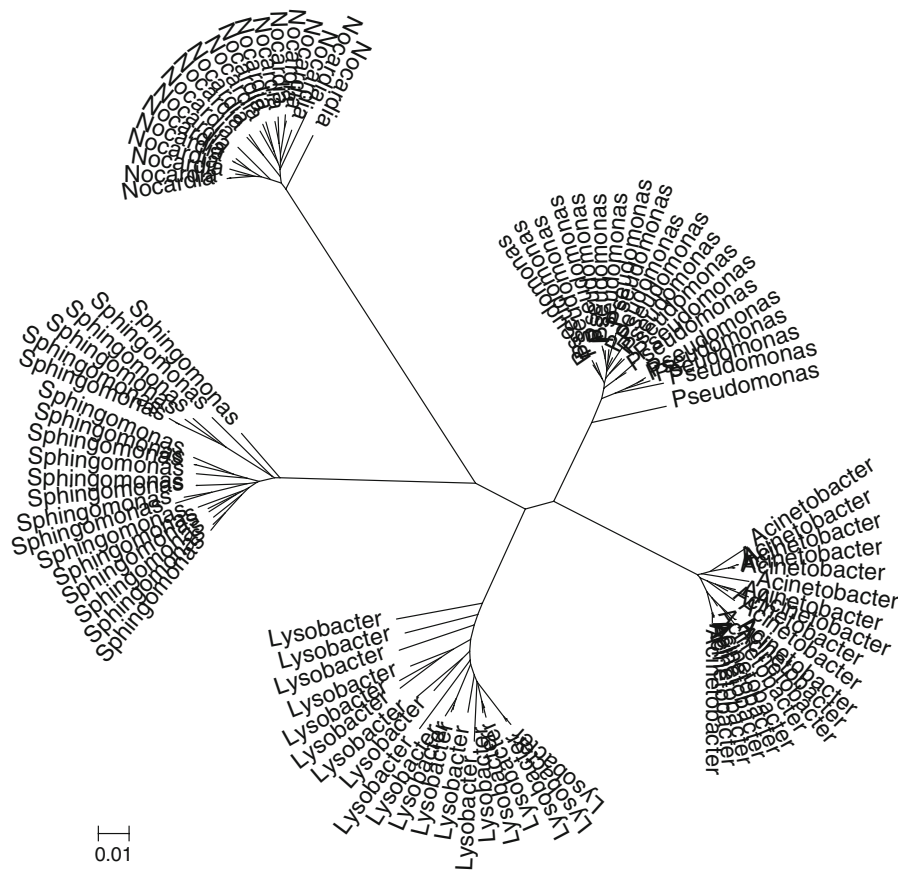
Fig. 3 Sequence-based phylogenetic tree of 20 representatives from each of the genera listed in Fig. S1

parameters will result in similar trees if the two parameters correlate to a certain degree. When random groups of 50 species were used, a slight negative correlation was found between the pairwise alignment scores and the calculated Jaccard distances (for details see "Supplementary material"), with an average Pearson correlation coefficient of -0.23 ± 0.078 (Fig. 2). The negative direction of the correlation was expected, since pairwise alignment scores measure similarity and the Jaccard distance measures dissimilarity; however, the low value of the Pearson correlation coefficient indicated a weak correlation. Consequently, the phylogenetic information that could be attributed to the ARDRA clustering, based on the Jaccard distance between profiles, was limited.

Phylogenetic trees were constructed for 20 representative OTUs from each of five genera with increasing inter-genera distances (Fig. S1), based on their 16S rDNA sequence (Fig. 3) and on the ARDRA

fragmentation profile (Fig. 4). The sequence-based tree (Fig. 3) produced a clear division between the genera, with inter-genera distances reflecting the expected differences based on the taxonomic identity of the genera. However, while the ARDRA tree (Fig. 4) did differentiate between the OTUs (with one exception), it did not maintain the taxonomic structure of the genera (Fig. S1).

To conclude, ARDRA can be a suitable tool for genus differentiation of environmental clones based on in-silico fragmentation. Moreover, in-silico profiles may be used for species identification provided caution is taken in the type and number of REs selected. Differentiation of strains requires more stringent measures, which are so time-consuming that the applicability of ARDRA to that end can be called into question. In addition, ARDRA-based dendrograms may not mirror 16S rDNA sequence-based phylogenetic trees.
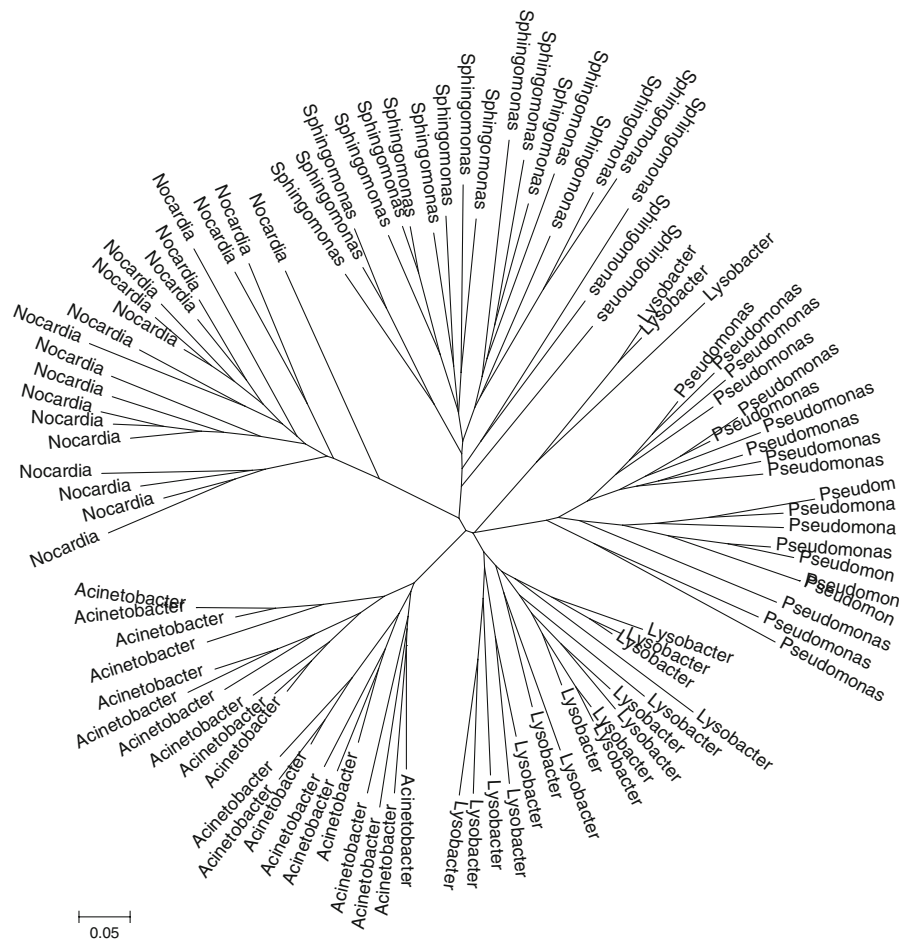
Fig. 4 ARDRA-based phylogenetic tree of 20 representatives from each of the genera listed in Fig. S1

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