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Review paper

Amplification primers of SSU rDNA for soil protists

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ABSTRACT

We reviewed the literature and sequence data bases to evaluate primers used to identify SSU rDNA genes from protists commonly found in environmental soil samples. From our summary of the most common primers described in the literature, we performed *in silico* tests to determine their efficacy in identifying protists. We particularly noted the comprehensiveness of these primers for specific target taxa, and also noted the most common non-target SSUs amplified by the primers. Our review is intended to help non-specialists navigate through the literature, as the names used to describe protists have changed greatly over the past three decades. It provides a review of the various primers used to amplify soil protists, many of which have been published under multiple names, and their differences. It is also intended to serve as a comparative study for those analysing environmental samples.

1. Introduction

Soil protists are key components of the soil ecology, primarily as bacterivores (Crotty et al., 2011; De Ruiter et al., 1995) but also in other functional groups (Adl, 2003; Adl and Gupta, 2006). Over the past decade, it has become evident that reliance on morphological identification at the microscopic level hugely under-described their true diversity (Bass et al., 2007; Adl et al., 2007b). Their biogeographical distribution has been discussed recently, pointing to both cosmopolitan species, and a great deal of endemism with rare local species (Bates et al., 2013a,b; Foissner, 2006; Hillebrand et al., 2001). Although protist functional ecology and diversity studies are abundant from the marine and fresh water environments, these organisms have been largely neglected in soil, even though they perform the same key roles in the microbial loop (Caron et al., 2009; Cuvelier et al., 2010; Sherr and Sherr, 2002). One reason for this neglect was the lack of appropriate techniques to work with soil protists (Berthold and Palzenberger, 1995; Foissner, 1987) until more recently (Adl et al., 2007a). The other was the relatively late adoption of molecular techniques in soil ecology. Molecular studies (Bass et al., 2007; Ekelund et al., 2004) showed that soil protist communities exhibit high levels of cryptic diversity (reviewed in Adl and Gupta, 2006), much like those in aquatic environments, but these relatively recent studies have not yet had their full impact on the field.

Concomitant with these changes was a significant transformation of the classification of microbial eukaryotes, driven by new discoveries in cell ultrastructure and molecular analysis (Adl et al., 2005). These changes were not common knowledge among soil biologists since very few researchers were studying protists in this context. Below we summarize the main groups of soil protists that are encountered, and review useful PCR primers that can be used to identify protists in environmental samples using DNA sequence-based methods. This is followed by a discussion of their efficiency and specificity at identifying particular groups of protists.

2. Material and methods

2.1. Literature review

Primer names and sequences were harvested from publications identifying and describing microbial eukaryotes relevant to soil ecology (supplementary material). The search was performed at the genus level. Priority was given to primers which had been used to amplify SSU rDNA from morphologically identified specimens, either cultures or individual cells isolated from the environment. Multiple primer sets were identified for each taxon wherever possible. The review demonstrated that a variety of names and codes were used to designate primers, many of which were identical or strongly overlapping. These redundant primers were retained for analysis and comparison. If no SSU rDNA sequence was available for any member of a genus, that fact was noted (Supplementary material). Fungi were not reviewed as they are

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more appropriately surveyed with the "internal transcribed spacer" (ITS) region (Bates et al., 2013a).

2.2. Construction of evaluation alignments

The canonical taxonomy for this study is that presented in Adl et al. (2012) on behalf of the International Society of Protistologists. SSU rDNA sequences for each examined taxon were obtained by identifying the most concordant group in the Protist Ribosomal Reference Database (PR^2) (Guillou et al., 2012; http://ssu-rna.org), followed by individual searches for genera assigned to the taxon in Adl et al. (2012). Similar searches were performed on the Silva SSU reference ribosomal RNA database (http://www.arb-silva.de/) using its taxonomy, and referring to GenBank as necessary. It should be noted that in some cases, the genus names or other taxa listed in Silva have been superseded by recent publications. The degree of individual sequence overlap between the two datasets ranged from approximately 70–95% depending on the taxon. In order to test the comprehensiveness of the search methodology, we also confirmed that the sequences obtained using the primer sets in the original publications had been recovered.

A final reference dataset was obtained by pruning sequences that were either designated as "chimeric", or which were curated into genera that have since been re-assigned to other groups. However, environmental sequences which had been curated into an appropriate taxon were retained, and duplicate sequences were not pruned. No sequence quality cut-off beyond that employed by PR^2 or Silva was used, as primer mismatches due to imperfect sequencing could be assessed *ad hoc*. The final reference dataset was aligned using MUSCLE (Edgar, 2004) as implemented in MEGA 5.1 (Tamura et al., 2011).

2.3. Primer evaluation

The primer sets (reverse and forward) from the literature were tested against genera represented in the sequence data bases from each target group using BLAST (Altschul et al., 1990) (determined at % similarity). Each identified primer was also tested against the reference alignment. The assessment was based on the relative representation of the target region in the reference dataset, and the likely effect of any mismatches on PCR efficiency. For example, indels and mismatches near the 3′ end of the primer were penalized more heavily than isolated mismatches in the centre or 5′ end of the primer. Mismatches at the very 5′ or 3′ end of a reference sequence were also given less weight, especially if the context suggested that the mismatch to the deposited sequence could have been generated by a primer rather than by the underlying genomic sequence. Taxonomic comprehensiveness was given preference over taxonomic specificity in the final choice of primer sets. Sets that would amplify all members of a target taxon were favoured over those which would not, even if the more comprehensive primers would also amplify sequence from other taxa.

3. Results

3.1. History of protist names and classification changes

The supra-generic rank names used to refer to the various groups of protist changed tremendously and repeatedly from the early 1980's to 2005 (Levine et al., 1980; Lee et al., 1985, 2000; Adl et al., 2012). The taxonomy and the classification were unstable throughout this period due to new techniques that improved our understanding of the phylogenetic relatedness among genera. These innovations included a series of molecular procedures for obtaining and sequencing nucleic acids, as well as new statistical tools and software to analyse the information, and conceptual progress in how we understand sequence comparisons for phylogenetic analysis. The name changes could be stabilized once the outline of the main groups and ranks became clearer in the early 2000's. This new classification and taxonomy of protists (Adl et al., 2005) was recently revised (Adl et al., 2012) with some changes to incorporate new information, but it remains largely the same, so that we may be in a period of relative taxonomic stability (Table 1).

One early name casualty of the results was the term protozoa, as it became clear that photosynthetic species were intermingled with non-photosynthetic species, at every rank and throughout the protists (Palmer, 2003; Archibald, 2009; Chan and Bhattacharya, 2010). The term protist replaced the term protozoa in journal titles and society names over that period. The term phycology has retained some usefulness in ecology as it refers to primary producers. However the term protozoa no longer groups species that are ancestrally related, or functionally similar, as they are too diverse. One of the complicating factors was the displacement of several groups historically thought to be fungi into the protists; these included the oomycetes (now Peronosporomycetes), the hyphochytrids, and the slime moulds (now in Amoebozoa and several other places in the classification). Another was the transfer of the fungi out of the botanical realm into the zoological realm, as they are at the base of animals (Metazoa) with which they share a common ancestor. Both fungi and animals have an external digestion of substrates with mixed enzymes (internalised as a digestive tube in the animals), glycogen as storage polysaccharide, chitin as a structural polymer, and many metabolic pathways and cell-biological synapomorphies. The nomenclature implications of this reality are not trivial, especially for parasites, as they affect terminology and the validity of species type specimens (Redhead et al., 2006). The problems caused by having species traditionally described under separate, and incompatible, codes of nomenclature then being moved into another group were previously reviewed and discussed (Adl et al., 2007b).

The correct placement of species in the phylogeny and their historical relatedness is not just about taxonomy and classification. Correctly placing a species in the phylogeny matters, because it tells

Table 1
The highest rank classification of protists according to the International Society of Protistologists (Adl et al., 2012). *indicates lineages with genera commonly found in soils.

	Super-groups	Examples
Amorphea	Amoebozoa	Tubulinea*
		Mycetozoa*
	Opisthokonta	Fungi*
		Choanomonada
		Metazoa*
		Apusomonadida*
		Breviatea*
	Excavata	Metamonada
		Malawimonas
		Discoba*
Diaphoretickes		Cryptophyceae
		Centrohelida
		Telonemia
		Haptophyta
	Sar	Cercozoa*
		Foraminifera
		"Radiolaria"
		Alveolata*
		Stramenopiles*
	Archaeplastida	Glaucophyta
		Rhodophyceae
		Chloroplastida
Incertae sedis Eukaryota		Ancyromonadida*