Evolution of Patchily Distributed Proteins Shared between Eukaryotes and Prokaryotes: *Dictyostelium* as a Case Study

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Key Words

Lateral gene transfer • Protist evolutionary genomics • Gene acquisition • Gene loss

Abstract

Protein families are often patchily distributed in the tree of life; they are present in distantly related organisms, but absent in more closely related lineages. This could either be the result of lateral gene transfer between ancestors of organisms that encode them, or losses in the lineages that lack them. Here a novel approach is developed to study the evolution of patchily distributed proteins shared between prokaryotes and eukaryotes. Proteins encoded in the genome of cellular slime mold *Dictyostelium discoideum* and a restricted number of other lineages, including at least one prokaryote, were identified. Analyses of the phylogenetic distribution of 49 such patchily distributed protein families showed conflicts with organismal phylogenies; 25 are shared with the distantly related amoeboflagellate *Naegleria* (Excavata), whereas only two are present in the more closely related *Entamoeba*. Most protein families show unexpected topologies in phylogenetic analyses; eukaryotes are polyphyletic in 85% of the trees. These observations suggest that gene transfers have been an important mechanism for the distribution of patchily distributed proteins across all domains of life. Fur-

ther studies of this exchangeable gene fraction are needed for a better understanding of the origin and evolution of eukaryotic genes and the diversification process of eukaryotes.

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Introduction

Only a small minority of all protein families is present in all living organisms [Charlebois and Doolittle, 2004; Ciccarelli et al., 2006], and the number of families present in the environment is larger than the number of genes in any extant genome. Lapierre and Gogarten [2009] explored this phenomenon and analyzed the presence and absence of gene families in 573 bacterial genomes. Three sets of genes were identified based on their frequencies of appearance: core genes, character genes and accessory genes. Proteins encoded by core genes are widely distributed and perform the basic common functions in cells. Character genes are rarer and for example enable the colonization of certain niches. Accessory genes are patchily distributed and found only in a few genomes and encode for functions that distinguish species and strains [Lapierre and Gogarten, 2009]. It is widely assumed that lateral gene transfer (LGT) is the main mechanism to distribute character and accessory genes among prokaryotes

[Baptiste et al., 2009; Dagan and Martin, 2007], whereas vertical inheritance is the main mechanism for maintaining the core genes in the genomes [Ciccarelli et al., 2006]. However, the impact of gene transfer on gene family dynamics in the evolution of microbial eukaryotes is still controversial [Baptiste et al., 2009]. Here, the author presents a case study of the evolution of patchily distributed proteins in a eukaryotic genome that suggests that gene transfer is a major evolutionary mechanism of distribution of accessory and character also in microbial eukaryotes.

Studies of LGT affecting eukaryotes have typically used phylogenomic approaches. Unexpected topologies with eukaryotes branching with prokaryotes have been identified among a large number of trees. These approaches have been effective in identifying LGT as a putatively important mechanism in eukaryotic genome evolution [Andersson, 2009a; Andersson et al., 2003, 2007; Archibald et al., 2003; Bowler et al., 2008; Carlton et al., 2007; Hall et al., 2005; Huang and Gogarten, 2008; Keeling and Palmer, 2008; Loftus et al., 2005; Nosenko and Bhattacharya, 2007; Ricard et al., 2006; Richards et al., 2006]. Many of these transfers are within patchily distributed protein families, typically present only in a few eukaryotic and prokaryotic lineages. However, phylogenomic approaches do not necessarily give balanced information about the frequencies of gene transfer for these genomes, because a failure to pass all the filters applied does not necessarily indicate that an individual gene family has evolved strictly via vertical inheritance. Another limitation for the studies of LGT, especially for accessory and character genes, is the limitation of data; there are only sequence information from a tiny fraction of the microbial diversity [Andersson, 2009b].

A circumstantial indication that we currently underestimate the impact of LGT on patchily distributed protein families in eukaryotic genome comes from a metagenomic study. Almost 300,000 clusters of protein sequences based on primary sequence similarity were found in a metagenomic project of ocean water [Yooseph et al., 2007]. Unexpectedly, many protein families previously only detected in eukaryotic organisms were found in prokaryotic organisms in the ocean, hinting at an important role of inter-domain gene transfer in the ocean. Unfortunately, the uncertainty of the taxonomic origin of individual sequences within metagenomic data precludes more detailed studies of LGT within this dataset.

This case study uses an approach in which gene families are included based on their phylogenetic distribution, rather than the topology of the phylogenetic trees. The

intention is not primarily to determine the impact of gene transfer in any particular eukaryotic genome. The genome of the social amoeba *Dictyostelium discoideum* is selected as a case study because it is completely sequenced [Eichinger et al., 2005] with a well-maintained genome database (<http://dictybase.org/>), and because the slime mold has not been identified as a protist for which LGT is unusually common using other approaches [Eichinger and Noegel, 2005; Watkins and Gray, 2006]. The distribution of the members of each of these families across the tree of life were analyzed, and complemented with phylogenetic analyses to distinguish LGT scenarios from gene duplication and losses. If gene transfer is rare in eukaryotic genome evolution, the patchily distributed datasets are expected to be the results of differential gene loss (or unequal rates of evolution). This would result in monophyletic eukaryotic sequences in the phylogenetic analyses. If, on the other hand, LGT is relatively common, eukaryotic sequences are expected to be polyphyletic. The analyses presented below indeed strongly suggest gene transfer to be an important evolutionary mechanism to spread patchily distributed genes between distantly related organisms, including eukaryotes.

Methods

The total set of 13,522 putative protein sequences encoded by the genome of the slime mold *D. discoideum* (<http://dictybase.org/>) was used to search for protein families (clusters of proteins with primary sequence similarity) that showed patchy distribution. Figure 1 shows an overview of completed genome sequences included in the study. Putative protein sequences from TBestDB, a taxonomically broad database of expressed sequence tag (EST) data from 54 diverse lineages of microbial eukaryotes [O'Brien et al., 2007], were also included to increase the sampling of protist proteins in the study. This dataset has been carefully checked for potential contamination [O'Brien et al., 2007]. The inclusion of putative protein sequences from ESTs precluded the use of any method based on reciprocal best BLAST hits [Tatusov et al., 2000]. Therefore, the program package PhyloGenie [Frickey and Lupas, 2004] was used on the 9,952 of the 13,522 *D. discoideum* protein sequences longer than 200 amino acids. PhyloGenie automatically performs similarity searches against the collected databases, creates a Hidden Markov Model based on the matches that fulfill user-defined criteria, and uses this Hidden Markov Model to search a larger set of matches and finally produces an aligned dataset. See the original PhyloGenie publication for further details [Frickey and Lupas, 2004].

In this study, a protein family is defined as a group of sequences with detectable primary sequence similarity that is distinct from other such groups. A patchy distribution was defined as homologs being present in *D. discoideum* and at least one prokaryote, but not in more than 20 archaeal, 20 eukaryotic, or 60 bacterial species, for the purpose of this analysis. These numbers were

Fig. 1. Distribution of 49 genes present in *Dictyostelium* and prokaryotes among sequenced eukaryotic genomes. A schematic reference tree of completed genome sequences included in the study. Numbers indicate the number of genomes available from the taxonomic group. The topology is based on a current hypothesis for phylogenetic relationships of eukaryotes [Adl et al., 2005; Baldauf, 2003; Hampl et al., 2009], but see Parfrey et al. [2010]. Eukaryotes are labeled according to their classification into proposed super-

groups [Adl et al., 2005]: Amoebozoa (purple), Archaeplastida (green), Chromalveolata (red), Excavata (brown), and Opisthokonta (orange; colors refer to the online version). The presences of genes in individual genomes and taxonomic groups are shown with filled circles. A dot for a group indicates the presence in at least one of the genome sequences available. See table 1 for further characteristics of the datasets.

arbitrarily chosen to obtain a suitable number of datasets with a rather limited distribution among sequenced genomes. No criterion for the maximum number of paralogs within a species was applied. A setting where the maximum identity allowed for two sequences in the final dataset as 95% was used to avoid that many closely related sequences from species and genera overrepresented among genome projects are included in the dataset. Inspection of the result of the similarity searches revealed that these settings did not exclude any sequences with high similarity to *Dictyostelium* proteins which could represent a putatively very recent gene transfer event. Only datasets where the *D. discoideum* protein longer than 200 amino acids yielded a hit with an E-value better than $1e^{-20}$ to at least one non-*Dictyostelium* sequence in the initial similarity searches were considered for further analyses. This approach identified 94 proteins as patchily distributed, corresponding to 49 unique families (table 1). All alignments were inspected and a few misaligned and short sequences were removed (a total of 24 sequences in ten different datasets, corresponding to 1.7% of all sequences). The datasets are listed in table 1 and the alignments are available on request from the author.

The PhyloGenie package has been found to generate datasets with levels of sequence similarities suitable for phylogenetic reconstructions [Andersson et al., 2007; Frickey and Lupas, 2004; Hackett et al., 2007; Huang and Gogarten, 2007, 2008; Li et al.,

2006; Nosenko and Bhattacharya, 2007; Reyes-Prieto et al., 2006]. The 49 *D. discoideum* protein sequences were searched against the Pfam protein family database, version 23.0 [Finn et al., 2008] to test the consistency with another clustering method. Eight of the proteins had matches against Pfam families with E values better than $1e^{-55}$ (table 1). All these showed identical, or nearly identical (one species difference), phylogenetic distribution among eukaryotes as identified using PhyloGenie (fig. 1). An additional four *D. discoideum* proteins had Pfam matches with E values between $1e^{-55}$ and $1e^{-10}$ (table 1). These Pfams showed a wider phylogenetic distribution among eukaryotes than identified in this study. The boundary of the PhyloGenie clusters correlated with drastic drops in scores in the similarity searches, suggesting that the sequences represent sub-families distinct from the other sequences within the respective matching Pfam.

Automatically, maximum likelihood bootstrap analyses were performed on the 48 datasets with four or more sequences using phym.pl. This is a PhyloGenie [Frickey and Lupas, 2004] script modified by Peter Cordes (Dalhousie University) which uses PHYML, version 2.4.4 [Guindon and Gascuel, 2003]. The WAG substitution matrix was used together with models for among site rate and invariable sites (WAG + I + Γ) and a total of 100 bootstrap replicates were analyzed for each dataset. The codon usage and G+C content of their genes were compared to the total set of 9,952

Table 1. Summary of the 49 datasets with patchily distributed proteins

dictyBaseID	Figure	Taxa, No. ^a	Pos., No. ^b	Eukaryotes		<i>D. discoideum</i>		Gene product	GO biological process ^f	Pfam ^g	E value ^g
				No. ^c	Clan ^d	No. ^e	Clan ^d				
DDB0167972	S1AA	7	496	6	ND	5	++			PF01841	1e ⁻²¹
DDB0168057	S1AB	85	211	3	---	2	+++	similar to fusolin precursor and chitinases	–	PF03067	6e ⁻⁵⁹
DDB0168523	S1AC	40	196	12	--	1	ND			PF09348	5e ⁻⁸⁶
DDB0168585	S1AD	5	560	1	ND	1	ND	similar to amylovoran biosynthesis protein precursor	–		
DDB0168966	S1AE	11	208	6	++	1	ND				
DDB0168996	S1AF	18	375	12	++	1	ND				
DDB0169183	S1AG	65	157	5	--	3	+++	prespore-specific protein	–	PF06555	2e ⁻⁸²
DDB0183821	S1AH	27	214	14	---	1	ND				
DDB0183949	S1AI	12	373	6	--	1	ND			PF09513	2e ⁻²⁰⁴
DDB0184040	S1AJ	8	308	7	ND	2	+++			PF10354	6e ⁻⁴⁵
DDB0184156	S1AK	34	302	3	–	3	–	similar to acetyltransferases, GNAT family	metabolic process		
DDB0184339	S1AL	78	375	75	–	8	---				
DDB0185044	S1AM	64	248	46	--	2	--	GlcNAc transferase ^h	protein amino acid glycosylation		
DDB0186510	S1AN	24	208	23	ND	2	+++				
DDB0186797	S1AO	33	348	7	---	2	+++	similar to glycoside hydrolases	carbohydrate metabolic process		
DDB0186941	S1AP, 2D	11	220	4	--	1	ND				
DDB0187838	S1AQ	25	561	6	--	5	+++			PF10462	3e ⁻¹⁷³
DDB0188311	S1AR	37	365	16	--	2	+++			PF09511	2e ⁻¹³⁴
DDB0188511	S1AS	78	364	41	--	1	ND	ADP-ribosylhydrolase	protein amino acid ADP-ribosylation	PF03747	1e ⁻⁵²
DDB0189107	S1AT	46	278	39	--	10	+				
DDB0189492	S1AU	61	207	8	–	1	ND			PF08857	3e ⁻⁹⁶
DDB0190317	S1AV, 2A	38	350	1	ND	1	ND	aminoglycoside phosphotransferase	response to antibiotic		
DDB0191097	S1AW, 2C	8	672	5	+++	1	ND	conditioned medium factor ^h			
DDB0202584	S1AX	47	264	22	---	2	+++	similar to esterases	–		
DDB0203248	S1AY	11	262	1	ND	1	ND				
DDB0203511	–	2	458	1	ND	1	ND				
DDB0203574	S1AZ	47	206	1	ND	1	ND				
DDB0204284	S1BA	33	378	26	--	1	ND				
DDB0204814	S1BB	63	305	1	ND	1	ND	galactose-1-phosphate uridylyltransferase	galactose metabolic process		
DDB0205754	S1BC	46	294	7	--	1	ND				
DDB0205899	S1BD	40	644	13	---	5	---				
DDB0206017	S1BE	51	494	7	---	2	+++	similar to permeases	transport		
DDB0206279	S1BF, 2F	58	370	8	---	2	---				
DDB0206432	S1BG	44	431	19	---	1	ND	similar to amidohydrolases	metabolic process	PF04909	1e ⁻¹⁵
DDB0206556	S1BH	76	315	20	--	2	–	similar to amidinotransferases	metabolic process		
DDB0217056	S1BI, 2E	29	357	16	++	2	--				
DDB0217068	S1BJ	76	173	71	--	5	--				

Table 1 (continued)

dictyBaseID	Figure	Taxa, No. ^a	Pos., No. ^b	Eukaryotes		<i>D. discoideum</i>		Gene product	GO biological process ^f	Pfam ^g	E value ^g
				No. ^c	Clan ^d	No. ^e	Clan ^d				
DDB0217198	S1BK	59	193	29	–	2	---	similar to 2-phospho- glycerate kinase	–		
DDB0218286	S1BL	99	324	8	--	2	++	similar to glycosyl hydrolase	carbohydrate metabolic process		
DDB0218308	S1BM	49	336	24	+++	1	ND	similar to glucokinase	glycolysis		
DDB0231036	S1BN	56	422	27	--	5	++	AprA ^h	regulation of aggregate size; negative regulation of cell proliferation; sporulation	PF10142	9e ⁻²⁵⁸
DDB0231655	S1BO	142	153	122	---	108	---	large family containing prespore cell-inducing factor, extracellular matrix proteins, etc.	–		
DDB0231670	S1BP	5	377	3	+++	3	+++				
DDB0232076	S1BQ	38	181	29	–	4	---	similar to N-acylsphing- osine amidohydrolase	–		
DDB0234056	S1BR	6	544	5	ND	3	+++	vacuolin ^h			
DDB0235278	S1BS, 2B	72	270	15	--	2	+++	similar to patatins	lipid metabolic process		
DDB0235282	S1BT	46	711	4	–	1	ND	similar to patatins	lipid metabolic process		
DDB0237649	S1BU	20	300	18	--	3	---				
DDB0238183	S1BV	50	252	6	---	2	–	similar to chitinases	carbohydrate metabolic process		

^a Total number of taxa included in the dataset. ^b Number of positions in the *Dictyostelium* protein used in the phylogenetic analyses. ^c Total number of eukaryotic sequences. ^d +++, ++, and + indicate whether the sequences formed a clan [Wilkinson et al., 2007] with ≥95, ≥50, <50% bootstrap support, respectively (monophyly). ---, --, and – indicate that the sequences are split into two or more clans with sequences from other domains with ≥95, ≥50, <50% bootstrap support, respectively (polyphyly). ND indi-

cates that monophyly cannot be tested since only a single sequence from the group, or a single sequence from outside the group is present. ^e Number of *D. discoideum* sequences. ^f Biological processes - Gene Ontology terms [Ashburner et al., 2000]. ^g Results of Pfam searches [Finn et al., 2008]. ^h These gene products have been functionally characterized (see main text for details).

genes coding for proteins longer than 200 amino acids analyzed using the program CodonW (<http://codonw.sourceforge.net/>).

Potential functions for the proteins were retrieved by searching dictyBase by all *D. discoideum* proteins included in the 49 datasets. References to functional characterizations were found for proteins from four of the datasets. Putative gene functions for the remaining protein families were retrieved either from dictyBase in combination with similarity searches against the non-redundant database. Biological processes Gene Ontology terms [Ashburner et al., 2000] of the protein families were retrieved from dictyBase or the Gene Ontology web page (<http://www.geneontology.org/>).

Results and Discussion

The screening procedure identified 49 patchily distributed proteins shared between the slime mold *D. discoideum* and at least one prokaryote present in the databases. Two complementary evolutionary analyses were

performed on these protein families. Firstly, the phylogenetic distributions were compared to the expected organismal relationships between the genomes included in the study. Secondly, the phylogenetic relationships within the protein families were studied.

Unexpected Phylogenetic Distribution of the 49 Protein Families

The compositions within the 49 datasets vary greatly; the total number of taxa is between 2 and 142, and the number of *D. discoideum* paralogs varies between 1 and 108 (table 1). The occurrences of other taxa in the dataset were analyzed using the PHATG program within PhyloGenie [Frickey and Lupas, 2004] in order to study patterns of phylogenetic distribution among patchily distributed *D. discoideum* genes (fig. 1 and online suppl. table 1; for all online supplementary material, see www.karger.com/doi/10.1159/000324505). *Entamoeba histolyti*-

ica is the only other completely sequenced member of Amoebozoa (fig. 1). Interestingly, this amoeba is present in only two of the 49 datasets (online suppl. table 1), indicative of loss of genes in the lineages leading to the human parasite *E. histolytica*, or gain in the lineage leading to *Dictyostelium*. Furthermore, the genus *Naegleria*, represented by the draft genome of *Naegleria gruberi*, an aerobic amoeboflagellate classified within Excavata [Adl et al., 2005], is present in half of the datasets (fig. 1), while the parasite *Leishmania major* is represented only in five (fig. 1). No additional homologs were found when the 49 patchily distributed *D. discoideum* proteins were used in similarity searches against their translated nucleotide *Entamoeba* and *Leishmania* genome sequences, indicating that the differences are not due to erroneous annotations of nucleotide sequences. However, they may reflect gene losses in the lineages leading to the parasites. The pattern could also be produced by lineage variations in evolutionary rates that lead to problems in identifying members of the protein families in some branches of the tree. In both cases the identified eukaryotes are expected to form a distinct (monophyletic) group to the exclusion of all other sequences in a phylogenetic analysis.

Phylogenetic Analyses Indicate Inter- and Intra-Domain LGT in Patchily Distributed Proteins

Maximum likelihood analyses were performed for the datasets in order to gain more insights into the evolution of the individual genes. The phylogenetic trees show complex topologies often at odds with organismal relationships. The complex topologies are likely the results of gene duplications, losses, LGT and perhaps endosymbiotic gene transfers in the evolution of the patchily distributed proteins. All trees are found in online supplementary figure 1, and six trees representing the diversity of topologies obtained are shown in figure 2. Inferences based on alignments of single genes may be prone to artifacts due to the use of suboptimal models of the sequence evolution in the analyses and a limited number of sites in the alignments. However, it is unlikely that phylogenetic artifacts could have produced the anomalous topologies on a large scale. Many trees show sequences from distantly related organisms clustering tightly together (fig. 2 and online suppl. fig. 1), which is very difficult to explain solely by methodological artifacts.

Thirty-nine of the datasets contain multiple eukaryotic and multiple non-eukaryotic sequences (table 1). The topology of these were analyzed for the presence or absence of eukaryotic clans (the term 'clade' is problematic for these unrooted phylogenies [Wilkinson et al., 2007]).

A single clan is expected if only vertical inheritance has occurred, while inter-domain gene transfers could have created a larger number of clans. At any rate, in most trees (33 of 39) the eukaryotic sequences are found in two or more clans, ten of which with >95% support for the association of eukaryotic and non-eukaryotic sequences (fig. 2b, d, f; table 1). This indicates that LGT is likely to have affected the evolution of these patchily distributed proteins identified in *Dictyostelium*. An alternative explanation for the occurrence of multiple eukaryotic clans could be that paralogs of the gene were present in the last universal common ancestor. If these were differentially lost in eukaryotes they could create topologies falsely interpreted as being the result of gene transfer. However, such a scenario would often require maintenance of paralogs in many lineages for long evolutionary time scales followed by recent independent losses. It would also require the presence of multiple copies in ancestral organisms. In many cases, the eukaryotic clans containing one or a few eukaryotic species group with bacterial or archaeal sequences with rather strong statistical support (fig. 2d, f). This strongly favors inter-domain gene transfer scenarios, since it requires a large number of recent gene losses in the absence of gene transfer. Together with the analyses of phylogenetic distribution (fig. 1), the phylogenetic analyses strongly suggest that LGT has affected the majority of the identified patchily distributed protein families to some extent. However, the evolutionary events cannot be reconstructed in detail for most of the families due to methodological limitations and incomplete sampling [see Andersson, 2009b, for further discussion].

The evolutionary histories for the six families showing a single eukaryotic clan are more difficult to interpret (table 1). Interestingly, all five of those that contain multiple eukaryotic species (the sixth contains three *D. discoideum*

Fig. 2. Phylogenetic trees of six patchily distributed *D. discoideum* proteins. Maximum likelihood trees of conceptually translated aligned amino acid positions of aminoglycoside phosphotransferase (a), a protein with similarity to patatins (b), conditioned medium factor (c), and conserved hypothetical proteins (d-f) (datasets DDB0186941, DDB0217056 and DDB0206279, respectively). Bootstrap support values >50% are shown. Accession numbers are found in online supplementary figure 1. The unrooted trees are arbitrarily rooted for the presentation. Archaeal, bacterial and viral names are labeled red, black and light blue, respectively. Eukaryotes are in bold and labeled according to their classification into super-groups [Adl et al., 2005]: Amoebozoa (purple), Archaeplastida (green), Chromalveolata (red), Excavata (brown), Opisthokonta (orange), and Rhizaria (dark blue; colors refer to the online version).

paralogs) fail to reproduce the expected organismal relationships within the eukaryotic clan (fig. 2c, e, online suppl. fig. 1AE, AF and BM). This could indicate a spread of the gene in eukaryotes via intra-domain gene transfer rather than vertical inheritance since the last common eukaryotic ancestor. However, vertical inheritance followed by differential loss is also a plausible explanation; additional sampling of diverse eukaryotes is needed to further test how these proteins have been distributed.

Unexpected Groupings of Eukaryotes Suggest Additional Transfers

The phylogenies of many of the patchily distributed proteins show clans containing distantly related eukaryotes (fig. 2b–e). Such unexpected grouping of protists could either be explained by an inter-domain LGT followed by an intra-domain transfer or by multiple independent inter-domain transfers from not-yet-sampled or extinct prokaryotic lineages [Andersson, 2009a; Keeling and Palmer, 2008]. For example, the close relationships between the *Capsaspora* and *Naegleria* sequences in the tree of patatin-like proteins (fig. 2b) and between *Karlodinium*, *Bigelowiella* and *Naegleria* sequences in figure 2e possibly represent intra-eukaryotic gene transfers. The grouping of the *Naegleria* and *Dictyostelium* sequences in figure 2d, on the other hand, could very well be the result of two independent prokaryote-to-eukaryote gene transfers. Further taxon sampling is obviously needed to resolve the patterns of putative transfers, and/or gene losses, for each individual protein family. Nevertheless, these topologies are part of an emerging trend; many similar topologies have recently been found in phylogenetic studies of protist genes of prokaryotic origin [Andersson, 2009a; Andersson et al., 2006, 2007; Hampl et al., 2008; Keeling and Palmer, 2008; Nosenko and Bhattacharya, 2007; Richards et al., 2006; Rogers et al., 2007; Stechmann et al., 2006]. They are usually interpreted as sequential transfers between eukaryotes, after an initial transfer from a prokaryote. This could possibly be rationalized by a higher probability for a foreign eukaryotic gene to be functional once incorporated into the eukaryotic nucleus because of the differences in transcription and translational machineries between the domains of life [Andersson, 2009a]. Although these are interesting observations, studies targeting this particular aspect of gene transfer are needed to get a better understanding.

Directions and Timing of Transfers for Patchily Distributed Genes

The direction of a gene transfer event can only be determined beyond any reasonable doubt if the recipient

lineage is nested within a clade of donor lineages that show the expected organismal phylogeny. Such identification requires that the transfer is an exception in the evolution of the protein family in question. This seldom seems to be the case for patchily distributed proteins at the current depth of sampling of organismal diversity. Accordingly, it cannot be excluded that some of the putative transfers have been from endosymbionts (e.g. mitochondria or chloroplasts) to the nucleus of the host lineage, although the sources and directions of most of the putative transfers in these datasets are elusive. A much denser taxon sampling is needed in order to determine donor and recipient lineages for individual transfers [Andersson, 2009b].

An alternative approach to determine the direction and timing of a gene transfer event takes advantage of the observation that genomes of distantly related organisms have different properties. For example, a gene G+C content that differs significantly from the majority of the genes in the genome could be hypothesized to have been recently introduced into the genome [Lawrence and Ochman, 1997; Ragan, 2001]. No indications of recent acquisitions of any of the 49 patchily distributed genes were found using this approach. Similarly, neither correspondence analysis of the relative synonymous codon usage, nor analysis of intron density identified any obvious differences between the patchily distributed genes and the total set of *D. discoideum* genes (online suppl. table 2 and online suppl. fig. 2). These observations probably indicate that the majority of the patchily distributed genes have been present in the *Dictyostelium* lineage long enough to have ameliorated to its genomic characteristics. In fact, 47 of the 49 proteins do have orthologs in the *Dictyostelium purpureum* genome data made available at the US Department of Energy Joint Genome Institute (<http://www.jgi.doe.gov/>) after the analyses were completed.

Proteins Involved in Cellular Functions Shared between Distantly Related Microbes

Potential functions for the proteins were retrieved from dictyBase and by similarity searches against the non-redundant (nr) database at NCBI (table 1). Four of the 49 protein families have members with characterized functions in *D. discoideum* and five proteins have strong similarities to characterized proteins from other organisms. Another 15 *D. discoideum* gene products could be assigned a putative function based on weak similarities. 25 of the 49 genes encode conserved hypothetical proteins with unknown function. Roles in metabolic processes dominate among the genes that do have assigned

putative functions (table 1). Over-representation of metabolic genes is usually observed in phylogenomic studies of eukaryotic LGT, which has been rationalized by metabolic adaptation in the recipient eukaryotic lineage [Andersson et al., 2007; Bowler et al., 2008; Carlton et al., 2007; Loftus et al., 2005; Ricard et al., 2006]. Interestingly, functions in protein glycosylation, cell communication, and regulation of cell development also are present among the patchily distributed genes (table 1).

Two of the four proteins that have been functionally characterized in *D. discoideum* encode secreted proteins involved in regulation of cell development (table 1). The conditioned medium factor is an 80-kDa glycoprotein secreted by starved cells that is required for aggregation [Jain et al., 1992], and the autocrine proliferation repressor (AprA) inhibits cell proliferation [Brock and Gomer, 2005]. Although the conditioned medium factor was identified more than a decade ago, no homologs have been known; genes with sequence similarities were only recently described in bacterial phytopathogens of the genus *Xanthomonas* [Andrade et al., 2006]. Similarly, homologous genes coding for AprA have only been found in *Dictyostelium* and a few bacterial species [Brock and Gomer, 2005]. Fascinatingly, both these genes are now detected in the amoeboflagellate *N. gruberi* [Fritz-Laylin et al., 2010] and the choanoflagellate *Monosiga brevicollis* [King et al., 2008], as well as in the EST dataset from the tubulinean *Hartmanella vermiformis* (online suppl. fig. 1AW and BN). In addition, AprA homologs were detected in the excavates *Malawimonas californiana*, *Malawimonas jakobiformis*, *Jakoba libera* and *Histiona aroides*, the chlorarachniophyte *Bigelowiella natans*, and a number of bacterial lineages (online suppl. fig. 1BN). This indicates that these secreted cell communication proteins have a broader phylogenetic distribution than previously appreciated, suggesting similarities in cell development between distantly related microbes.

Skp1 is a protein found in several eukaryotic protein complexes [West, 2003]. It is glycosylated by UDP-N-acetylglucosamine(GlcNAc):hydroxyproline Skp1 GlcNAc transferase. In *D. discoideum*, this reaction is performed in the cytosol, whereas in animal cells the glycosylation is performed in the Golgi apparatus by distantly related enzymes [West, 2003]. The *Dictyostelium* sequence was initially found to be most similar to bacterial sequences [Van Der Wel et al., 2002]. Here, it is shown that a large part of the eukaryotic diversity actually encodes the *Dictyostelium* type of the enzyme (online suppl. fig. 1AM). The fourth gene with a functionally characterized gene product codes for vacuolin. This protein targets the vacu-

ole for exocytosis, and characterizes the post-lysosomal compartment [Brock and Gomer, 2005]. No homologs have previously been detected, but in this analysis two are found in amoeboflagellate *N. gruberi*, and another in the γ -proteobacteria *Coxiella burnetii*, strain Dugway 7E9-12 (online suppl. fig. 1BR). Intriguingly, *C. burnetii* – the etiological agent of Q fever – is highly adapted to a life in acidic eukaryotic phagolysosomes [Seshadri et al., 2003], and expression of a vacuolin might be a mechanism for host modulation.

Analyses of Gene Transfers Are Heavily Dependent on Experimental Approaches

The original analysis of the *D. discoideum* genome sequence identified 18 potential gene transfers [Eichinger et al., 2005]. The criterion used to classify a gene as a putative transfer was that the *D. discoideum* protein domain matched a bacteria-specific Pfam domain and had phylogenetic relationships indicative of LGT [Eichinger et al., 2005]. Consequently, only datasets where *D. discoideum* is the only eukaryote, such as in figure 2a, were considered. All proteins that have homologs in other eukaryotic genomes were excluded. This study suggests a conservative approach to find gene families that have participated in gene transfer events because the vast majority of the patchily distributed proteins do have eukaryotic homologs outside Amoebozoa, yet most have likely been transferred relatively recently (fig. 2b–f). Only one of the 18 previously identified putative gene transfers [Eichinger et al., 2005] is included among the 49 datasets of patchily distributed proteins identified in this analysis (a pre-spore specific protein). This is a reflection of the different approaches; the number of genes affected by LGT in the *D. discoideum* genome is likely much larger than the sum of the candidate genes in the two analyses. Likewise, the 191 gene transfer candidates reported from the genome of the amoeboflagellate *N. gruberi* are very likely only the tip of the iceberg of LGTs affecting this heterolobosean lineage; only genes with homologies in prokaryotes, but no other eukaryotic group, were considered [Fritz-Laylin et al., 2010].

Interestingly, this study includes 14 phylogenetic trees where *D. discoideum* sequences are found in multiple clans, seven of which with bootstrap support values above 95% for their separation (fig. 2e, f; table 1). For several of these, the most likely explanation is that the slime mold has acquired genes coding for homologous proteins repeatedly from different sources, sometimes followed by gene duplications within the lineage (i.e. fig. 2f, online suppl. fig. 1BD and 1BF). Thus, LGT has contributed to

gene family expansions, at least to some extent, in the *Dictyostelium* genome, as has been suggested for prokaryotes [Lerat et al., 2005].

There are no indications that the patchily distributed proteins were acquired in a single or a few events; they are found on all six *D. discoideum* chromosomes and are not clustered, no specific donor lineage among the genes could be identified, and there are no indications that they are functionally related. This suggests that genes have been from various sources over evolutionary time in the lineage leading to *Dictyostelium*. A much denser taxon sampling would be needed to determine the rate of this process. However, the facts that the gene sequences have ameliorated to the *D. discoideum* genome (see above) and that 96% of the gene families are present in *D. purpureum* indicate that the process is considerably slower than observed in some bacterial genera [Kettler et al., 2007; Welch et al., 2002]. Interestingly, a recent comparison of three sequenced isolates of the human intestinal parasite *Giardia intestinalis* species complex also indicated ongoing gene acquisitions. Dozens of lineage-specific genes were identified, some of which clearly have very recent origins from bacteria inhabiting the intestine [Franzén et al., 2009; Jerlström-Hultqvist et al., 2010].

Co-Occurrence of Proteins in Organisms within Similar Environments

The phylogenetic distributions of the datasets were analyzed on the genus level (fig. 1 and online suppl. table 1). By far the most represented genus is *Naegleria* (heterolobosea), amoeboflagellates found in soil and fresh water [De Jonckheere, 2002; Fritz-Laylin et al., 2010], which is present in 25 of the 49 datasets. *Naegleria* and *Dictyostelium* belong to different super-groups, Excavata and Amoebozoa, respectively (fig. 1). This strongly suggests that the co-occurrence of genes in *Dictyostelium* and *Naegleria* is not explained by a recent common ancestry of the two eukaryotes. A more reasonable explanation for this observation is that they have similar lifestyles and therefore share these genes which probably were acquired via gene transfers; the habitats of both genera include soil and they have amoeboid life stages where they feed on bacteria. Interestingly, fungi, another group with many soil organisms, are also frequently represented within these 49 datasets (fig. 1).

In general, free-living heterotrophic aerobic microbial eukaryotic groups (i.e. *Monosiga*, fungi, *Dictyostelium*, ciliates, *Naegleria*) are more frequently found among the datasets than parasites (i.e. *Entamoeba*), autotrophs (i.e. *Phaeodactylum*), and multicellular eukaryotes (fig. 1).

This is unlikely only due to large differences in rates of gene transfers between eukaryotes because both *Entamoeba* and *Phaeodactylum* have been reported to have acquired a substantial number of bacterial genes [Bowler et al., 2008; Loftus et al., 2005]. Similarly, the ten bacterial genera represented in seven or more datasets are all mesophilic heterotrophs (online suppl. table 1), many of which have free-living members that are frequently found in soil (i.e. *Burkholderia*, *Bacillus*, *Mycobacterium*, *Myxococcus*, *Stigmatella* and *Streptomyces* – online suppl. table 1). Interestingly, 9.2% of the top-ten hits in similarity searches against the environmental sample database at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>) are from a Minnesota farm soil dataset [Tringe et al., 2005], which represent 2.5% of the database. These observations are in agreement with a scenario where these genes tend to be shared within a soil environment.

This kind of co-occurrence of proteins in organisms found in similar environments has been observed in prokaryotes, which has been suggested to be important in the diversification process [Beiko et al., 2005; Boucher et al., 2003]. More recent data have appeared indicating that microbial eukaryote may adapt via gene acquisitions from prokaryotes [Andersson, 2009a; Keeling, 2009]. For example, protists have adapted to anaerobic lifestyle via gene acquisitions from anaerobic prokaryotes [Andersson et al., 2007; Carlton et al., 2007; Loftus et al., 2005; Ricard et al., 2006], and uptake of bacterial genes has likely led to early diversification of plants [Huang and Gogarten, 2008]. Such gene sharing may also happen between microbial eukaryotes; plant pathogens belonging to the distantly related groups oomycetes and fungi have exchanged a dozen genes which are related to their similar lifestyles [Richards et al., 2006]. Gene exchanges may indeed lead to direct changes of the phenotype of the recipient [Andersson, 2009a]. For example, transfer of virulence factors between different lineages of fungi has led to emergences of new plant pathogens within historical time [Friesen et al., 2006; Temporini and VanEtten, 2004]. The results presented in this study indicate that gene exchange occurs between soil organisms from all domains. These gene transfer events have most likely changed the phenotypes of the recipients, although the impact of every single gene is impossible to determine.

Patchily Distributed Genes Have Been Shared by a Variety of Mechanisms

Many of the most common eukaryotes in the datasets are phagotrophs both on prokaryotic and eukaryotic cells

(e.g. *Naegleria* and *Dictyostelium*). This mode of feeding has been suggested to be a mechanism by which DNA are introduced into the eukaryotic cell [Doolittle, 1998]. However, there are also a number of fungi (non-phagotrophs) present in the datasets (fig. 1 and online suppl. fig. 1, and online suppl. table 1). In many cases the fungal sequences group with prokaryotic sequences (fig. 2f and online suppl. fig. 1), strongly indicating that they have participated in inter-domain transfers. This indicates that there are additional mechanisms besides phagotrophy by which foreign genetic material may enter the eukaryotic cell. Interestingly, viral sequences were present in ten of the datasets (online suppl. fig. 1) hinting at transduction as a mechanism. Eukaryotic viruses may indeed harbor a large fraction of prokaryotic-like genes [Filée et al., 2007; Moreira and Brochier-Armanet, 2008]. Intriguingly, Mimivirus also harbors genes closely related to eukaryotic lineages such as *Dictyostelium* and Heterolobosea [Moreira and Brochier-Armanet, 2008]. These observations hint at a role for viruses both in inter- and intra-domain gene transfer.

Concluding Remarks

The case study presented here suggests that proteins shared between *Dictyostelium* and bacteria, but only a minority of other taxa available in the databases, belong to protein families which undergo frequent gene transfer. This has most likely contributed significantly to the adaptation process of this model eukaryote. The filter criteria used in this study were developed to avoid false positives and to get a suitable number of datasets. Less strict cut-offs would have included additional families, whereas more strict cut-offs would have resulted in a subset of the families. There does not seem to be any strong correlation between unexpected topologies and the size of the datasets. Both small and large datasets show anomalous topologies (fig. 2 and online suppl. fig. 1, table 1). Using different filtering criteria would include a slightly larger or slightly smaller number of protein families. For example, allowing half the number of species reduces the number of datasets to 21 (online suppl. fig. 1). Taken together, this indicates that the general patterns observed are not critically dependent on the selection criteria, although the details would change with different settings. Future similar studies would need to optimize their own selection criteria based on the specific question asked and the expected phylogenetic and environmental biases among the genomes available in the public databases.

Interestingly, the number of genes in this study is slightly higher than the 36 orthologs found suitable to reconstruct the universal tree of life [Ciccarelli et al., 2006], later coined as ‘the tree of one percent’ [Dagan and Martin, 2006]. 31 of the 36 identified universal gene families were reported to likely have been distributed via vertical descent in the three domains of life throughout evolutionary time [Ciccarelli et al., 2006]. Thus, the rate of LGT is extremely different for the universally conserved proteins compared to the patchily distributed proteins in this study. It is impossible to predict the relative contribution of vertical descent, gene loss and gene transfer for the remaining >98% of the proteins in microbial eukaryotes in the absence of systematic studies.

Nevertheless, genomes of microbial eukaryotes contain a subset of genes that are exchangeable over evolutionary time, as has been observed in prokaryotes [Abby and Daubin, 2007; Lapierre and Gogarten, 2009]. The size and turnover rates of this subset of eukaryotic genes remain to be determined for various lineages and evolutionary time scales. Functional characterizations of the genes identified in this case study are expected to provide further insights into the biology of *D. discoideum*. Intriguingly, the proteins with the least widespread distributions are expected to provide most to the characteristic phenotype of any organism. Additional studies of the function and evolution of patchily distributed proteins will deepen the understanding of the diversification process of microbes from all domains of life.

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