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Towards testate amoebae genomics and beyond, a wish list…

Anush KOSAKYAN[1, 2]

[1] **Department of Life Sciences, University of Modena and Reggio Emilia, Modena, Italy**

[2] **National Biodiversity Future Center (NBFC), Palermo, Italy**

Abstract. Despite of the rapid advancements in genomic tools over the past decade, genomic data remains limited for many protists, with some taxonomic groups lacking it entirely. One such group is testate amoebae, a group of shelled amoeboid protists known to have a long fossil record. Indeed, for some of them (i.e., arcellinid testate amoebae) the evolutionary roots trace back approximately 730 million years. Beyond their fascinating evolutionary history, these organisms are highly valued for their diverse applications across various research fields, including paleoecology, environmental monitoring, ecotoxicology, microbial community ecology, endosymbiont studies, and more. Consequently, their genomic data harbors significant potential for yielding crucial insights within the contexts of evolution, ecology, and climate change, while also offering opportunities for exploration from both biotechnological and functional perspectives. This short communication gives an overview of possible research directions for testate amoebae based on molecular data and outlining innovative techniques that have the potential to elevate testate amoebae research to a new next level.

Keywords: testate amoebae, single cell genomics, future perspectives, molecular techniques, WGA

INTRODUCTION

The advent of molecular techniques such as genomics, transcriptomics, and proteomics has finally given us the ability to understand the organisms in a new and profound way. Despite the huge diversity and importance of the protists in various fields of research, genomic data for these organisms remains limited (Gao et al. 2024). Protist genome datasets began to emerge in the early 2000s (Gardner et al. 2002; Worthey and Myler 2005). Subsequently, genomic datasets of protists have been made available on different platforms, for example, ciliate genome database https://tet.ciliate.org, the algae genome database DataBase, http://www.fwalagedb.com, the parasitic protozoa genome database VEuPathDB https://veupathdb.org, etc., with approximately 2000 datasets available by 2022 (Gao et al. 2024). The newest and largest portal for protist genome data, the P10K database, aims to collect and disseminate genome and transcriptome sequences and annotations from diverse protist groups (Gao et al. 2024). As of August 2024, it has rapidly expanded to include

Address for correspondence: Anush Kosakyan, Department of Life Sciences, University of Modena and Reggio Emilia, Modena, Italy; e-mail: anush.kosakyan@unimore.it

2977 datasets, covering approximately 45 % of protist orders.

The reason protist datasets are much fewer compared to other groups of organisms (e.g., 5000–10000 genomic datasets for plants, invertebrates, bacteria, etc. (Mukherjee et al. 2023)) is likely due to the lower popularity of protists and the challenges in obtaining sufficient and clean genomic material from single cells. A significant fraction of protists remains non-culturable, likely due to the difficulty of replicating their natural environments in growth media. Additionally, many protists require co-culturing with other microorganisms that serve as prey or provide essential nutrients (Del Campo et al. 2024). This is especially true for symbiotic protists, particularly intracellular symbionts adapted to live within host cells (Del Campo et al. 2024). These culturing limitations make it difficult to extract a sufficient amount of genomic material, as obtaining genomic material from a single cell is highly challenging. Additionally, there is a likelihood of contaminants coexisting with the scant material obtained. These factors are probably among the main reasons that genomic data is limited or absent for many taxonomic groups. One such protist group is the testate amoebae. Testate amoebae are a polyphyletic assemblage of mostly heterotrophic unicellular amoeboid eukaryotes enclosed in a shell or test. Some representatives of testate amoebae are known to have a long fossil record. For example, Lahr et al. (2019) have shown that Neoproterozoic vase-shaped microfossils are most probably the earliest members of the arcellinid testate amoebae (Fig. 1). Having a shell that can be preserved for millions of years makes these organisms useful bioindicators. Thus, the main research on these organisms traditionally focused on environmental monitoring and palaeoecology (Mitchell et al. 2008), but later rapidly expanded to microbial community ecology, forensic sciences, and ecotoxicology (Kosakyan et al. 2016; Kosakyan and Lara 2019). It is difficult to estimate how many species of testate amoebae exist. Although Kosakyan et al. (2016) mentioned about 1000 published testate amoebae species names, this number is not representing the true diversity. Culturing testate amoebae isn't easy, likely due to similar challenges faced with other protists, such as selecting the correct growth media, prey, and materials used for building the shell. Indeed, only a few species of testate amoebae have been successfully cultured (see Table 1), although it is reported (communication with R. Meisterfeld, mentioned in Marcisz et al. 2020) that over 100 testate amoebae taxa can potentially be cultured.

Recognizing the fundamental importance of precise species identification for ecological application, traditional taxonomy of testate amoebae has been integrated with molecular data. The application of molecular methods has indeed revolutionized our initial understanding of the diversity and systematics of testate amoebae, reshaping numerous evolutionary and ecological hypotheses.

The first studies of testate amoebae based on molecular data were those single gene-based phylogenetic studes (Lara et al. 2008), using ribosomal small subunit (SSU rDNA) as a genetic marker to resolve deep phylogeny of the group (family or genus level). Later on mitochondrial COI and NADH genes were used for fine-level phylogeny and discriminating closely related species (Kosakyan et al. 2012; Blandenier et al. 2017). Single gene based phylogenies were soon challenged by multigene phylogenies, first handful of genes (Lahr et al. 2011) and then multigene supermatrix based analysis retrieved from transcriptomic datasets (250 genes, Lahr et al. 2019). Gene sequences discussed in all these studies mostly were obtained from single cells, and sometimes from cultures whenever it was possible. Among all the sequenced genes, mtCOI remains popular for species barcoding and metabarcoding studies (Ribeiro et al. 2024).

Metabarcoding is another field that become very popular in the recent years, allowing the retrieval of large amounts of molecular data without direct observation of the species, that facilitates the fieldwork (González-Miguéns et al. 2023), or to investigate microbiome of testate amoebae (Gomaa et al. 2022). Other than phylogenetic/omics and metabarcoding, a new developing filed is the gene expression studies focusing on specific genes (Ribeiro et al. 2020; Weiner et al. 2023), holding a great potential for expanding transcriptomic and genomic field to the new directions.

Why genomics of testate amoebae?

Despite that great progress has been made in some directions of testate amoebae research applying molecular data (i.e., phylogenetics, phylogenomics, barcoding, metabarcoding, transcriptomics) (Heger et al. 2010; Kosakyan et al. 2012; Singer et al. 2015; Lahr et al. 2019; Ribeiro et al. 2020; González-Miguéns et al. 2023; Weiner et al. 2023), the exploration of testate amoebae using omic datasets is still in its nascent phase. To move forward in this direction, obtaining reference genomes of the representatives of the major

Table 1. The list of testate amoebae species that were successfully cultured.

Order	Genera	Species	References
Arcellinida	*Arcella*	*A. vulgaris*	Netzel and Heunert 1971
		A. dentata	Hegner 1919
		A. gibbosa	Lahr et al. 2011
		A. polypora	Hegner 1920
		A. discoides	Hegner 1920
	Centropyxis	*C. aculeata*	Root 1918
	Cryptodifflugia	*C. operculata*	Lahr et al. 2011
	Difflugia	*D. geosphaira*	Ogden 1991
		D. louisi	personal communication with F. Siemensma
	Hyalosphenia	*H. minuta*	personal communication with F. Siemensma
	Microchlamys		personal communication with F. Siemensma
	Netzelia	*N. tuberculata*	Anderson 1992
	Phryganella	*P. acropodia*	Dumack et al. 2020
	Pyxidicula	*P. operculata*	Lahr et al. 2011
Euglyphida	*Assulina*	*A. muscorum*	Anderson and Cowling 1994
	Euglypha	*E. acanthophora*	Hedley et al. 1974; Ogden 1981
		E. filifera	Wanner et al. 2016
		E. rotunda	Cowling 1986; Aoki et al. 2007
		E. strigosa	Hedley et al. 1974
	Corythion	*C. dubium*	Cowling 1986
	Lecythium	*L. terrestris*	Dumack et al. 2016
	Tracheleuglypha	*T. dentata*	Coûteaux and Ogden 1988
	Trinema	*T. galeata*	Aoki et al. 2007
		T. enchelys	Aoki et al. 2007
	Trivalvularis		personal communication with F. Siemensma

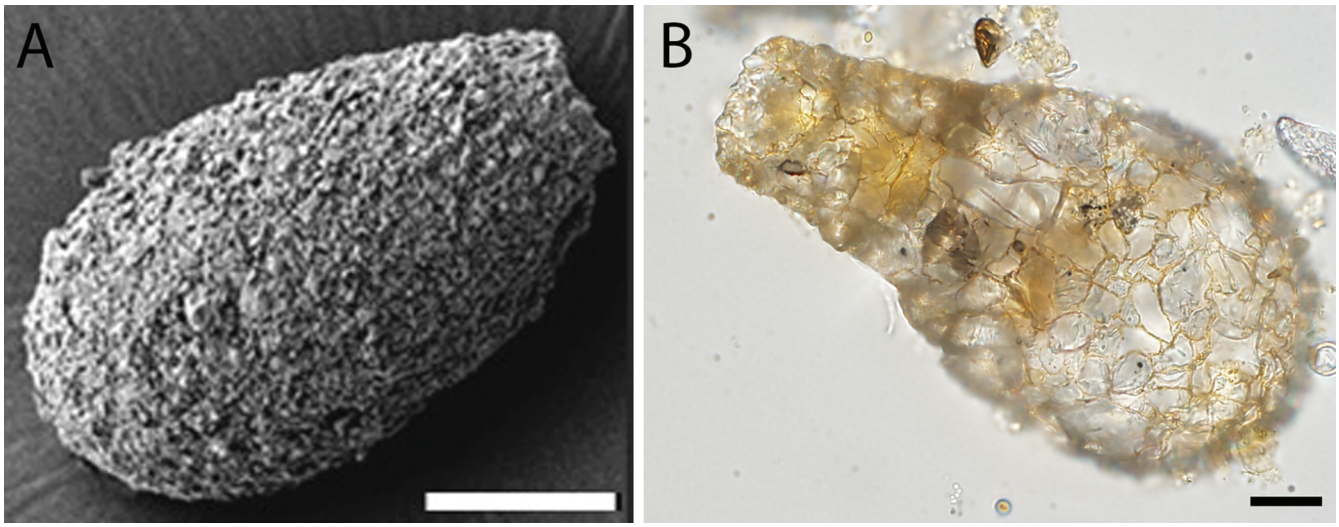

Figure 1. Examples of neoproterozoic vase-shaped microfossil from the Togari Group, Tasmania (A) and modern testate amoebae from genus *Difflugia* (B). Scale bars = 20 µm. A is modified from Porter and Riedman 2019.

linages of testate amoebae is a fundamental first step. Genomic dataset will become a solid background and will contribute of development of at lease four major directions of testate amoebae research (Fig. 2):

1. ***Evolutionary studies***. One of the fascinating aspects of testate amoebae is their long evolutionary history. As discussed by Porter et al. (2003), Lahr et al. (2019), and Porfirio-Sousa et al. (2024), some testate amoebae, namely arcellinids, most probably diversified before the beginning of the Sturtian glaciation, about 720 million years ago, having already distinct functional types related to ecological niches, feeding behaviors, and evolutionary schemes, thus holding intriguing information about the diversification and evolution of their molecular strategies. The availability of genomic data will advance:

(i) Multigene Phylogeny: It has been demonstrated (Lahr et al. 2019; Porfirio-Sousa et al. 2024) that phylogenetic studies based on multiple (hundreds or thousands of) genes allow for better resolution of evolutionary relationships between lineages or the positioning of uncertain taxa, which was not always possible using a single or a handful of genes. Additionally, we should keep in mind that many testate amoebae species still lack molecular data, and many species are *incertae sedis*. There is also a significant number of cryptic species that may have different ecological optima (Kosakyan et al. 2012, 2016; Singer et al. 2018). Beyond general evolutionary and biodiversity interest, many other research fields (e.g., ecology, palaeoecology, biomonitoring, etc.) might rely on robust phylogenetic results.

(ii) Comparative Genomics: This will allow for the determination of lineage-specific genes and the linking of these results with ecological data. One example can be exploring and comparing the genomes of testate amoebae lineages or groups with the ability for silica biosynthesis or mixotrophy.

(iii) Exploration of Taxon-Specific Genes: Also known as taxonomically restricted genes or orphan genes, these are known to play a critical role in the evolution of species, organism responses to environmental changes, and new adaptations (Fakhar et al. 2023).

(iv) Gene Gain-Loss Analysis: This will enable the study of the evolution of separate gene repertoires.

2. ***Functional genomics***. Testate amoebae include examples of species with restricted and large geographical distribution (e.g., *Apodera vas* and *Nebela tincta*) and it was suggested that this distribution range can be correlated with climatic tolerance (Singer et al. 2019). Testate amoebae also include taxa with ability to survive in extreme environments and to encyst (Marcisz et al. 2020; Kuuri-Riutta et al. 2022), thus holding a great potential for investigation of functional gene families or pathways related to survival (e.g., heat shock proteins, antifreeze proteins, encystation related pathways), or environment switching molecular mechanisms.

3. ***Microbiome studies***. Testate amoebae are important players in nutrient cycling and contribute at various

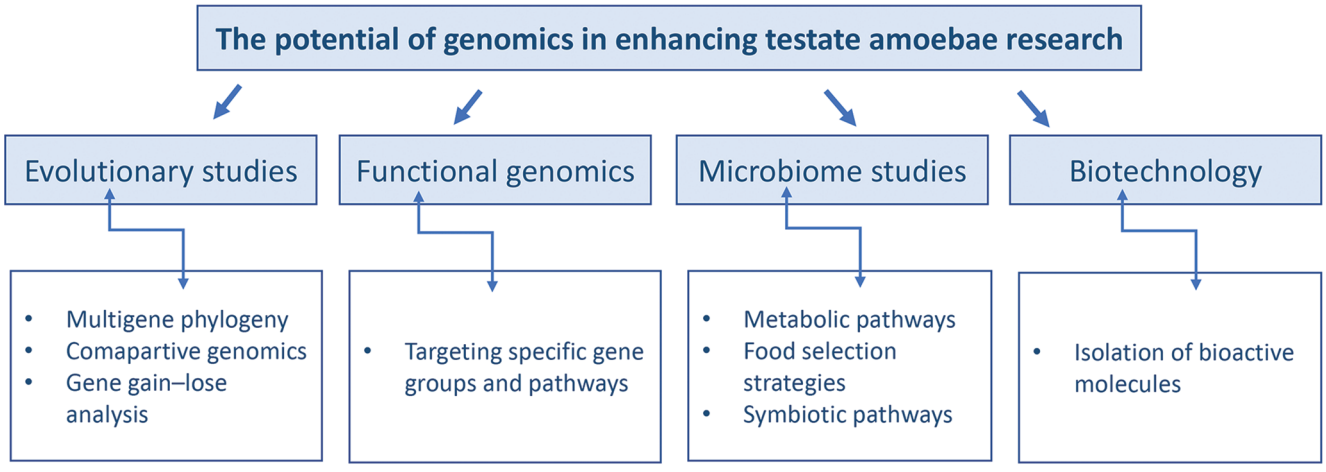

Figure 2. Schematic representation of possible research directions that testate amoebae genomics can contribute.

trophic levels within the microbial food web. While many studies have examined testate amoebae feeding and food selection strategies, most of these studies are based on morphological observations (Gilbert et al. 2003; Jassey et al. 2012, 2013). Genomic datasets will enable us to investigate the testate amoebae microbiome, metabolic pathways, food preferences, and feeding strategies, and link these findings to climate change, thereby predicting the consequences of these changes.

In this era of global warming and increasing environmental pollution, it is critical to understand and evaluate how organisms and their interactions are changing, and how these changes will affect ecosystem health. Some members of testate amoebae are known to establish different types of symbiotic associations. For example, the myxotrophic relationship between *Hyalosphenia papilio* and its green photobionts (Jassey et al. 2015; Lara and Gomaa 2017), or the symbiotic relationship of some *Arcella* species with diverse bacteria, including pathogenic bacteria (Gomaa et al. 2022). However, we know little about symbiotic associations in testate amoebae, especially regarding environmental stressors that influence these associations. One known example is the disruption of the mutualistic relationship between the testate amoeba *Hyalosphenia papilio* and its green photobionts with increasing temperature, leading to reduced carbon uptake efficiency in boreal peatlands (Jassey et al. 2015). Genomic datasets, coupled with gene expression studies, will help us understand how a presumably stable symbiotic association responds to environmental changes by identifying and measuring modifications at molecular level and may help discover important bacterial and fungal pathogens associated with testate amoebae, thus opening new horizons for further research in the frames of one health program.

4. ***Bioactive molecules and biotechnology***. Biotechnology of protists is still young but rapidly developing field of research. In 2019, a symposium entitled "Bioactive molecules from protists: perspectives in biotechnology" was organized during the VIII European Congress of Protistology – ISOP joint meeting in Rome, which discussed recent advances in this field showing that "*Protists are a gold mine for the discovery of new bioactive molecules which are still almost unknown*" (Valessi et al. 2020). The bioactive molecules (e.g., ice binding proteins, defence proteins, immune modulators, etc.) isolated from diatoms, ciliates and dinoflagellates were suggested as potential chemotherapeutic agents and suitable industrial candidates. The resilience of testate amoebae in cold environments, their capacity to construct intricately complex shells, engage in predation, and more, presents a promising avenue for uncovering novel bioactive molecules with significant potential. This in turn will open new possibilities for developing gene editing programs for discovering target molecules and developing their applications in health and industry.

METHODOLOGICAL CHALLENGES AND PERSPECTIVES

In the beginning, two methods based on cloning approach were popular to sequence the entire genome of protists: the Hierarchical sequencing strategy or Clone-by-Clone (CBC) approach, and the Whole Genome Shotgun (WGS) approach (Worthey and Myler 2005; Loftus et al. 2005), which prove to be less time and cost effective. Nowadays, the sequencing of entire genome (especially for the organisms with a small genome) became comparably easy. Especially, with the development of long read sequencing (Oxford Nanopore, PacBio), it is possible to reach to chromosome level assembly. Thus, combining illumine short but low error rate reads together with long high error rate reads, it is possible to obtained complete and good quality assemblies.

This task becomes more challenging when the organism is not culturable, as for example in case of testate amoebae, when only few species have been successfully cultured (Table 1). However, with the development of new technologies, unculturability may no longer be a limitation. Some kits (e.g., REPLI-g Single Cell Kit, Qiagen) allow for the acquisition of sufficient material from a single cell for successful whole genome sequencing with complete genome coverage.

For example, the Whole Genome Amplification (WGA) pipeline using the REPLI-g Single Cell Kit and the Blobology filtering pipeline has proven to be very efficient in the amplification and analysis of protist mitochondrial genomes (Serra et al. 2020; Gammuto et al. 2024). Another comprehensive pipeline for WGA, assembly, filtration, and annotation is presented by Gao et al. 2024, which is used for the PK10 protist genomics large project. While these pipelines can likely be applied successfully to testate amoebae, the main challenge remains the absence of well-curated reference datasets for

structural and functional annotations. To date, no testate amoeba genome or genome of closely related species is available. However, the 34 available transcriptomic datasets of testate amoebae (Porfirio-Sousa et al. 2024) and a few well-annotated genomes of amoebozoans, such as *Dictyostelium discoideum* and *Entamoeba histolytica* (GenBank accession ADBJ01000025, RefSeq GCF_00004695.1, GCF_000208925.1; Eichinger et al. 2005; Loftus et al. 2005), which are phylogenetically distinct, can still be used for orthology analysis in Arcellinid testate amoebae datasets. These resources can serve as a valuable starting point for genome assembly and annotations first for arcellinid testate amoebae, and then to expand for euglyphids and other groups of testate amoebae.

And as Dacks and Ginger (2023) made a prediction of next 20 years for protists "*We confidently expect that surprising and paradigm-shifting discoveries will be made. We cannot anticipate what many of these might be, but given the field's powerful tools, and even more powerful curiosity, it should be an exciting period*," it is undeniable that this outlook holds true for testate amoebae as well, with their true potential awaiting further exploration and revelation…

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