



OPEN Quantifying genus-level divergence using 18S rDNA and its application to heterolobosea with discovery of a novel genus from Mombasa Kenya

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The phylum Heterolobosea comprises a morphologically diverse and ecologically versatile assemblage of free-living microbial eukaryotes, yet genus-level boundaries remain difficult to resolve due to limited diagnostic characters and extensive phenotypic plasticity. Here, we apply a reproducible 18S rDNA divergence framework to quantify genus-level divergence in Heterolobosea and to evaluate taxonomic placement of a newly discovered isolate from coastal sediments of Mombasa, Kenya. Microscopic observations reveal a highly plastic amoeba exhibiting monopodial limax-type locomotion, episodic eruptive activity, and the formation of large multinucleate and polyploid stages that fragment into smaller cells, suggesting an unusual parasexual-like life cycle. Phylogenetic analyses recover the isolate in a strongly supported clade with *Orodruina flavescens* and an uncultured environmental lineage from the Lost City hydrothermal field. Pairwise 18S rDNA distance analyses show a clear bimodal separation between intragenus and intergenus comparisons with divergence values among these lineages consistently exceed empirical intrageneric thresholds across multiple analytical frameworks. Substitution saturation diagnostics confirm that these divergences occur within the phylogenetically informative region of the SSU rDNA gene. Together, the molecular, morphological, and ecological evidence support recognition of the Mombasa lineage as a distinct genus and species, *Mombasina parasexualis* gen. nov. et sp. nov., within Orodruinidae. This study reveals previously underappreciated diversity within Tetramitida and demonstrates the broader utility of quantitative divergence-based frameworks for resolving genus-level boundaries in microbial eukaryotes.

Keywords Tetramitida, 18S rDNA divergence, DNA barcode, Molecular taxonomy, Species delimitation, Marine protists

The phylum Heterolobosea comprises a morphologically and ecologically diverse assemblage of free-living microbial eukaryotes that inhabit terrestrial, freshwater, and marine environments¹. Members of the group exhibit remarkable morphological plasticity, transitioning between amoeboid, amoeboid-flagellate, and/or flagellated stages, although these elements are differentially expressed or secondarily lost across lineages². Amoeboid stages typically possess eruptive limax-type morphology, characterized by monopodial locomotion, anterior hyaline bulging, and rapid directional movement^{2–4}. Discoid mitochondrial cristae, the absence of a stacked Golgi dictyosome, and the ability to assemble the flagellar apparatus de novo further distinguish heteroloboseans from superficially similar amoebozoans². Although many taxa are uninucleate, the class also includes large multinucleate amoebae and species adapted to extreme environments, including halophilic, thermophilic, anaerobic, and halophilic lineages^{5–11}. This morphological and ecological flexibility underscores the evolutionary success of the group.

Ecologically, heteroloboseans function as active bacterivores and predators of microbial eukaryotes, contributing to nutrient cycling and shaping microbial community dynamics⁴. Several species also possess

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medical or veterinary significance. The best-known example is *Naegleria fowleri*, the causative agent of primary amoebic meningoencephalitis (PAM) in humans¹². Additional amphizoic or endobiotic taxa, including *Paravahlkampfia* and several species of *Vahlkampfia*, infect fish, invertebrates, or other hosts, highlighting the broader biological relevance of the group¹³.

Despite this ecological and morphological breadth, heterolobosean taxonomy has long been challenging⁴. Traditional classifications relied heavily on morphological traits such as cyst structure, locomotion type, and presence or absence of a flagellate stage, but these characters are often homoplasious, plastic, or insufficiently diagnostic^{2,14,15}. The advent of molecular systematics, particularly analyses of the small-subunit ribosomal RNA gene (18S rDNA), transformed our understanding of heterolobosean diversity and revealed extensive polyphyly within historically defined genera such as *Vahlkampfia* and *Tetramitus*^{4,9,15–17}. However, most studies still rely almost exclusively on the 18S rDNA locus, sometimes supplemented by secondary-structure information of the 18S rDNA¹⁶, to define taxonomic groups. Only limited effort has been made to incorporate additional markers such as ITS^{18,19}, and only a fraction of heterolobosean diversity is represented by transcriptomic or genomic datasets (e.g.,^{20,21}). As a result, genus-level circumscription remains particularly difficult to apply consistently across the group.

A major advance occurred with the expanded phylogenomic framework of Pánek et al.²², which incorporated dozens of transcriptomes and genomes, resolved deep relationships, and introduced several higher-level taxonomic revisions within Heterolobosea. Yet, for many lineages especially those that are uncultured, environmentally sampled, or morphologically less complex species- and genus-level boundaries continue to rely heavily on 18S rDNA gene. Given its ubiquity in environmental metabarcoding datasets, 18S rDNA remains the most practical and operationally powerful marker for heterolobosean taxonomy. Nonetheless, the absence of standardized, quantitative approaches for interpreting 18S rDNA divergence has limited its consistent application at the genus level and beyond. To address these limitation, the present study quantifies genus-level divergence across Heterolobosea using a custom computational pipeline that evaluates the stability of 18S rDNA divergence patterns across alternative alignment and masking strategies, rather than relying on a single alignment context or a universal divergence threshold. This approach provides a reproducible, data-driven framework for applying 18S rDNA to genus-level taxonomy, complementing morphological and phylogenomic perspectives.

The East African coast, particularly the marine habitats surrounding Mombasa, Kenya, remains largely unexplored with respect to heterolobosean and overall microbial diversity. Although recent culture independent studies have begun to characterize coastal microbial communities, including high throughput surveys of bacterial assemblages in Kenyan marine waters²³ and metagenomic analyses of seawater and sediment microbiomes from nearby coral reef systems²⁴, the broader microbial landscape of the Mombasa coast remains insufficiently characterized and continues to present major gaps in our understanding of regional protistan and microbial diversity. These ecosystems, comprising coral reefs, tidal pools, seagrass beds, mangroves, and anthropogenically impacted zones, represent a mosaic of habitats likely to harbor undescribed microbial eukaryotes. No formally described heterolobosean genera have previously been reported from this region. Here, we apply our genus-level divergence framework to Heterolobosea and describe a new genus and species of free-living marine heterolobosean from the coast of Mombasa. By integrating morphological observations, molecular phylogenetics, and divergence-based evaluation of the 18S rDNA, we demonstrate that this lineage represents a distinct and deeply diverging taxonomic entity. The findings highlight both the utility of 18S rDNA divergence profiling for resolving genus- and species-level boundaries in Heterolobosea and the hidden microbial diversity present in understudied African coastal ecosystems.

Materials and methods

Study site description and culturing

Samples were collected from Bamburi Beach, a coastal stretch of the Indian Ocean situated directly in front of Severin Sea Lodge in Mombasa, Kenya, at 3.98599° S and 39.74195° E. The beach is characterized by fine, white coral-derived sand, shallow near-shore waters, and a fringing lagoon shaped by semi-diurnal tides. The intertidal zone consists of sandy flats, coral rubble, scattered seagrass patches, and accumulations of drift seaweed. Mixed seaweed and sand samples were collected in June 2024 from the upper intertidal zone where decomposing macroalgal mats accumulate. These wrack deposits, formed by wave-driven accumulation, create nutrient-rich microhabitats that support diverse microbial assemblages including free-living amoebae and other protists. Sampling in this area provides a representative snapshot of coastal microbial diversity influenced by both natural shoreline dynamics and tourism activity. A portion of the collected material was cultured in Petri dishes containing 3% artificial seawater solution along with autoclaved rice grains that supported bacterial growth. After several rounds of subculturing, the amoeba appeared in high abundance and was isolated to establish a monoclonal culture that was used for microscopy and molecular analyses.

Light microscopy and immunocytochemistry

General morphology was examined using an ECLIPSE Ti2 inverted microscope (Nikon Corporation, Japan). Still images and time-lapse videos were acquired using the NIS-Elements software package under phase contrast and Differential Interference Contrast settings. Amoeba cells measurements were taken directly in the NIS software. Subcellular structures, including the plasma membrane and nuclear DNA, were visualized using immunocytochemistry and confocal microscopy following the methods described in²⁵.

DNA extraction, PCR amplification, and RNA-seq sequencing

Clean monoclonal cultures of the isolate, designated YT1_Mombasa, were used to extract total genomic DNA with the Quick-DNA MicroPrep Kit (Zymo Research, Irvine, California, cat. no. D3020) according to the manufacturer's instructions. The 18S rDNA gene was amplified using primers from Medlin et al.²⁶ and the

barcode primers described in Tekle et al.²⁷. Phusion DNA polymerase, a high-fidelity proofreading enzyme, was used with PCR conditions described in the above study. Amplicons from multiple reactions were purified using the QIAquick PCR Purification Kit (Cat. no. 28106) and pooled to achieve sufficient concentration for Oxford Nanopore sequencing.

Single-cell RNA sequencing followed Tekle et al.²⁸. Between one and thirty clean single cells were collected by mouth pipetting into sterile 0.2 mL PCR tubes and processed using the SMART-Seq v4 Ultra Low Input RNA Kit (Takara Bio USA). Sequencing libraries were prepared from one nanogram of cDNA with the Nextera XT DNA Library Preparation Kit (Illumina Inc., San Diego, California) according to the manufacturer's protocol. Library concentration was measured using a Qubit Fluorometer and the DNA High Sensitivity assay. All libraries were sequenced at Azenta Life Sciences (Burlington, MA) using high-output 150 bp reads. Read processing and assembly followed Tekle et al.²⁸, resulting in a full-length 18S rDNA sequence used for phylogenetic and divergence analyses. Oxford Nanopore sequencing was also attempted on PCR amplicons obtained with both full-length and barcode SSU primers following Tekle et al.²⁷. Due to a high error rate in the resulting reads, Nanopore SSU sequences were not included in the final dataset.

Genus-level divergence and phylogenetic analysis

Genus-level genetic divergence was quantified using an automated Python pipeline (Supplementary Data 1) that takes as input a single FASTA file containing unaligned 18S rDNA sequences grouped by genus. The pipeline was intentionally designed to be simple and easy to use, requiring minimal user input while automating all major analytical steps. The primary objective of this pipeline was to quantify empirical genus-level divergence using 18S rDNA and to evaluate whether divergence thresholds were stable across alternative alignment and masking schemes. Sequences included in the analyses were carefully selected to ensure high quality and broadly comparable length and coverage, such that extreme sequence truncation was not a defining feature of the dataset. The sequences were grouped by genus and aligned using MAFFT v7 with the L-INS-i algorithm (localpair and maxiterate 1000)²⁹. Three complementary alignment contexts were generated and compared throughout the pipeline: (i) a Global-Default alignment containing all taxa for intragenus and intergenus comparisons, (ii) a Global-Strict masked alignment derived from the global dataset using stringent gap- and entropy-based filtering to retain only unambiguous nucleotide positions; and (iii) Local-Strict per-genus alignments, generated for each genus represented by two or more sequences, to assess intragenus divergence without a global alignment context. All alignments were manually inspected in AliView³⁰ to confirm positional homology.

Each alignment underwent a two-step masking procedure based on gap fraction and Shannon entropy, following standard practice for identifying ambiguously aligned and fast-evolving sites in phylogenetic analyses to reduce alignment uncertainty and substitutional saturation prior to distance-based inference^{31,32}. Threshold values were selected to span a conservative to liberal range of alignment stringency, consistent with previous SSU rDNA-based phylogenetic and taxonomic studies. Rather than defining a single optimal cutoff, we evaluated whether genus-level inferences were stable across alternative masking schemes. The default mask retained positions with a gap fraction of 0.5 or lower and entropy between 0.1 and 1.8, explicitly excluding positions with entropy < 0.1 (nearly invariant sites) and > 1.8 (near-random, highly variable sites), while retaining intermediate-entropy positions that preserve phylogenetically informative signal at the genus level. The strict mask retained only positions containing unambiguous nucleotides across all sequences. Gaps and ambiguous characters were therefore treated conservatively and were not considered informative in downstream analyses. This multi-scheme masking strategy allowed explicit evaluation of the sensitivity of distance estimates to alignment stringency and ensured that downstream taxonomic conclusions were not driven by any single filtering choice. Masked alignments were exported as FASTA files for downstream analyses.

Pairwise uncorrected proportional genetic distance (p-distances) was calculated from masked alignments, excluding gaps and ambiguous characters. Distance calculations were based exclusively on positions where both sequences contained unambiguous nucleotides, ensuring that any residual variation in sequence length did not bias divergence estimates. For every genus, distributions of intragenus distances were summarized, including the number of comparisons and the minimum, maximum, median, mean, standard deviation, and interquartile range. Equivalent summaries were produced for intergenus comparisons. A genus-level cutoff was estimated by maximizing Youden's J statistic, which optimized the separation between intragenus and intergenus distances. We note that this cutoff is not interpreted as a universal taxonomic threshold, but rather as a lineage-specific quantitative reference used to facilitate objective comparison between intragenus and intergenus divergence. Minimum intragenus distances and maximum intergenus distances were examined to assess robustness of the threshold and to identify outlier taxa. Species that exceeded the genus-level cutoff or showed unusually low distances to other genera were flagged as candidates for reassignment or possible establishment of new genera.

Phylogenetic trees were reconstructed in IQ-TREE^{33–35} using alignments generated by the pipeline. The best-fitting evolutionary model was selected automatically with the -m AUTO (TEST) option, and node support was assessed using 1000 ultrafast bootstrap replicates as well as 1000 non-parametric (standard) bootstrap replicates. To further assess the robustness of inferred relationships, Bayesian phylogenetic analyses were performed in MrBayes v3.2.7 under a GTR+I+Γ model, using two independent runs with four Markov chains each for 2 million generations^{36,37}. Bayesian analyses recovered the same overall topology and major clade structure as the maximum-likelihood analyses, with comparable support values, indicating that phylogenetic inferences were robust to alignment masking, sequence length variation, and analytical framework.

18S rDNA saturation diagnostics

Mutational saturation in the 18S rDNA dataset was assessed using the global masked alignment. Pairwise uncorrected p-distances were calculated for all sequence pairs, and Jukes Cantor corrected distances were calculated for comparisons with p-values below 0.75. Here, p-values refer to uncorrected p-distance values,

defined as the proportion of nucleotide differences between two sequences; because DNA consists of four nucleotides, the theoretical maximum uncorrected p-distance approaches 0.75, corresponding to complete substitutional saturation where multiple substitutions obscure phylogenetic signal. A p-distance versus Jukes Cantor scatterplot was examined for curvature or flattening, which would indicate loss of linearity and saturation. The number of comparisons for which the Jukes Cantor correction was undefined, specifically those with *p*-values of 0.75 or higher, was recorded as an indicator of extreme divergence. These analyses serve as a diagnostic benchmark confirming that 18S rDNA divergence values fall within a phylogenetically informative range and are not intended as an independent line of taxonomic evidence.

Results

Morphology of the isolate YT1_mombasa

The trophic amoebae of the new isolate exhibit substantial morphological plasticity, with cells ranging from 12.63 to 57.01 μm in length (mean 24.93 μm , $n = 133$) and 3.57 to 11.47 μm in breadth (mean 7.3 μm , $n = 150$) (Figs. 1, 2, S1). Cell shape varies considerably depending on culture age and physiological condition, including resting, active locomotion, and non-directional movement (Figs. 1, 2, S1). During active locomotion, cells display a characteristic monopodial limax form with a tapered posterior end (Fig. 1A,C). Cytoplasmic streaming is smooth and continuous during this state and lacks eruptive hyaline expansions (Supplementary Video). The posterior region is generally rounded and slightly darker than the remainder of the cell and may occasionally possess delicate uroidal filaments, although these are not prominent and consistently visible (Supplementary Video).

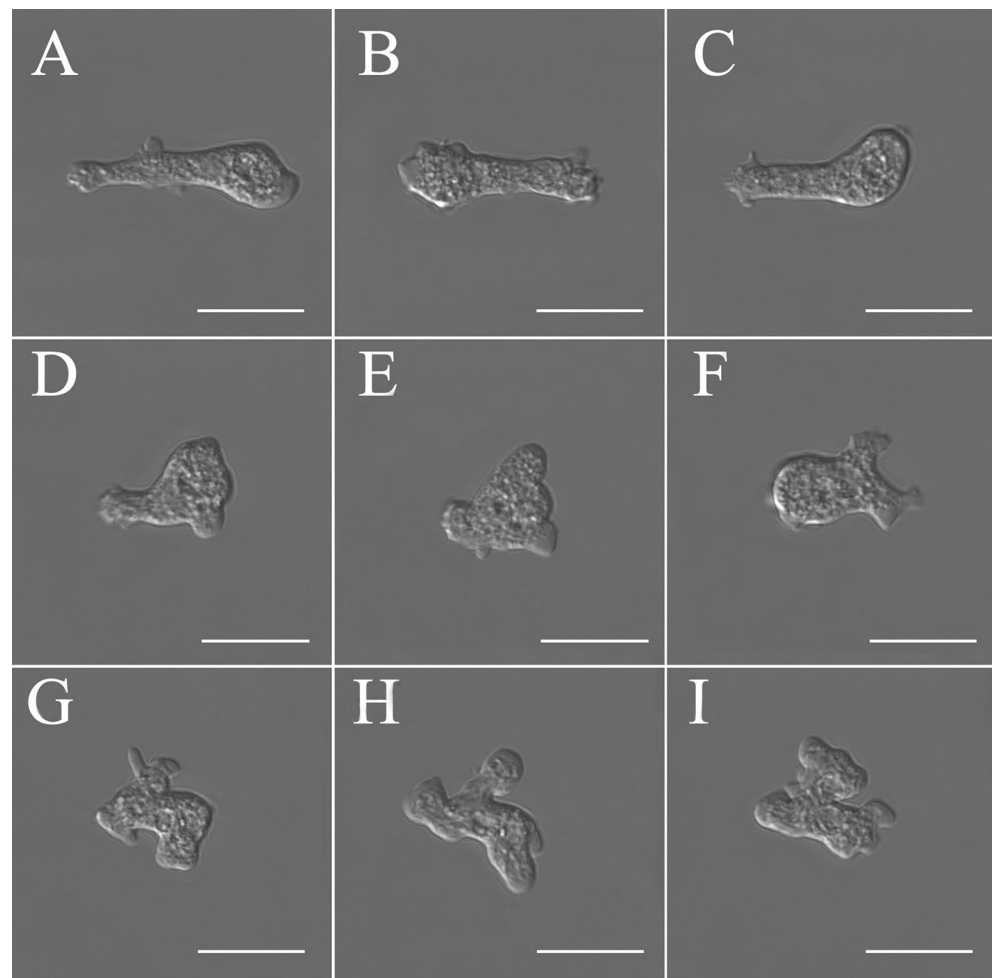


Fig. 1. General morphology and locomotive behavior of *Mombasina parasexualis* gen et sp. nov. Representative Differential Interference Contrast (DIC) micrographs showing a series of frames of a single amoeba during active movement and non-directional behavior. (A–C) Limax-type monopodial forms with a prominent anterior hyaline cap and a tapered posterior region. Note in (B) During non-directional movement, the cell transiently reverses polarity, switching anterior and posterior positions; the posterior hyaloplasm briefly forms a leading cap before the cell resumes anterior-directed movement. (D–I) Eruptive lateral expansions produced during directional changes, including transient bulges that facilitate redirection of movement. Scale bars = 10 μm .

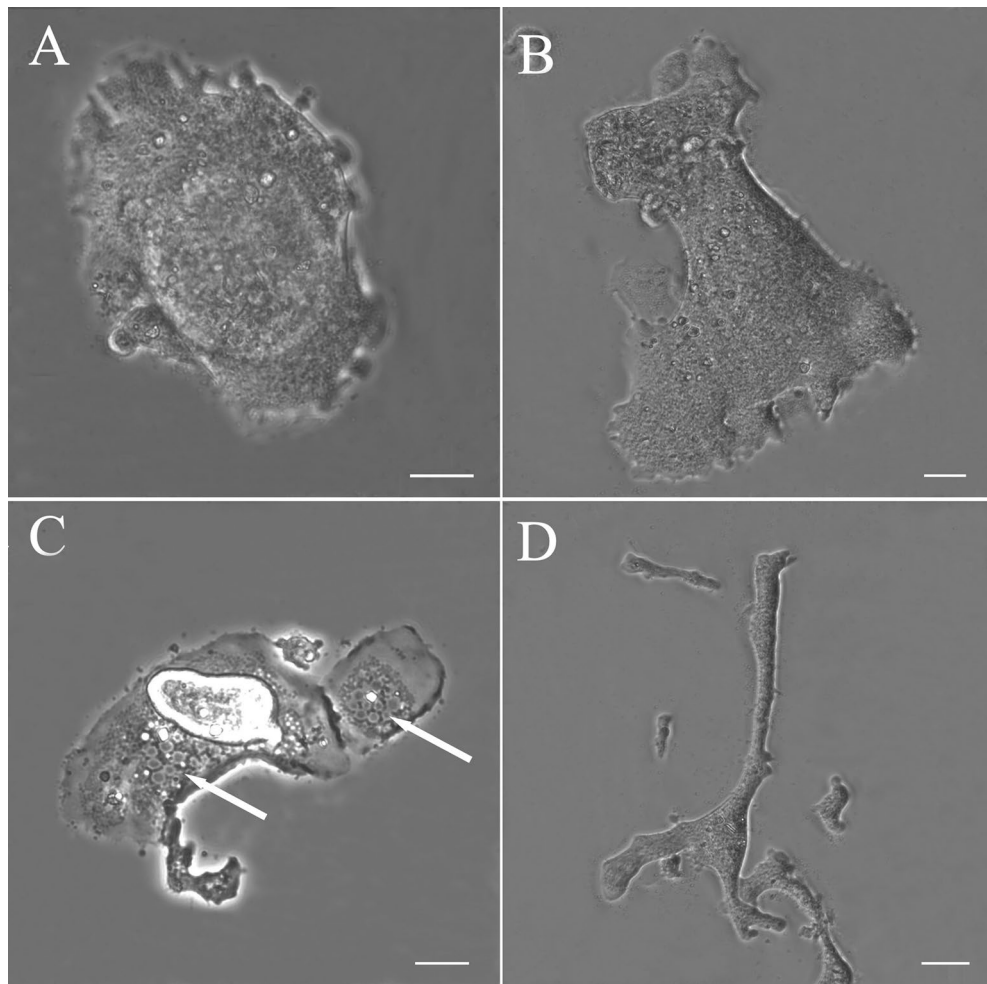


Fig. 2. Large multinucleate amoebae and morphological variation in older cultures of *Mombasina parasexualis* gen. nov. et sp. nov. Phase contrast micrographs showing the size and shape variability of enlarged trophic cells. Cells exhibit diverse morphotypes, including round (A), irregular (B), flattened vannellid-like forms with a distinct hyaloplasm (C), and elongated or branched forms (D). These multinucleate amoebae display the same locomotive and eruptive behaviors observed in smaller cells and frequently fragment to produce smaller daughter amoebae. In (C), an oval-shaped multinucleate cell is moving over a larger flattened multinucleated form. Arrows indicate nuclei. Scale bars = 20 μm .

In addition to the limax form, cells frequently display circular, elongate, irregular, or branched morphologies (Figs. 2, S1). Floating forms appear rounded, and once they settle, they develop a pronounced monopodial hyaline cap that produces a snail-like appearance (Supplementary Video). Non-directional movement is characterized by conspicuous eruptive activity, especially during changes in movement direction. Amoebae may move in a zigzag pattern, and the leading hyaline cap often forms one or more lateral eruptive bulges (Fig. 1F–I, Supplementary Video). These transient expansions can redirect movement briefly, and at times the cell reverses direction from the posterior end before resuming forward migration. Cells may even switch anterior and posterior positions, displaying what appears to be bidirectional locomotion, where they are capable of moving from either the posterior or anterior region without turning (Supplementary Video).

Most trophic cells are uninucleate, with nuclei measuring 2.28 to 5.88 μm (mean 3.61 μm , $n = 53$) based on DAPI-stained material (Fig. 3). Nucleoli are centrally located, as revealed by DAPI-stained material (Fig. 3D). Rare elongated binucleate cells were observed and likely represent stages of binary division. In older cultures (> 3 weeks), much larger amoebae ranging from 50 to > 160 μm appear and show the same locomotive and eruptive behaviors as smaller forms (Fig. 2, Supplementary Video). These large cells adopt diverse shapes, including irregular, round, elongated, and occasionally flattened vannellid-like forms with a distinct hyaloplasm (Fig. 2C). Large amoebae are frequently multinucleate, with DAPI staining revealing dozens to > 100 nuclei (Figs. 2C and 3). Nuclear size also varies, with some nuclei more than twice the diameter of the typical uninucleate stage, suggesting polyploidy (Fig. 3D). No evidence of cell fusion was observed. Instead, smaller amoebae appear to arise through fragmentation of the larger branched multinucleate forms. Although infrequent, these events have been repeatedly observed and may represent part of the life cycle as observed in other amoebae²⁵.

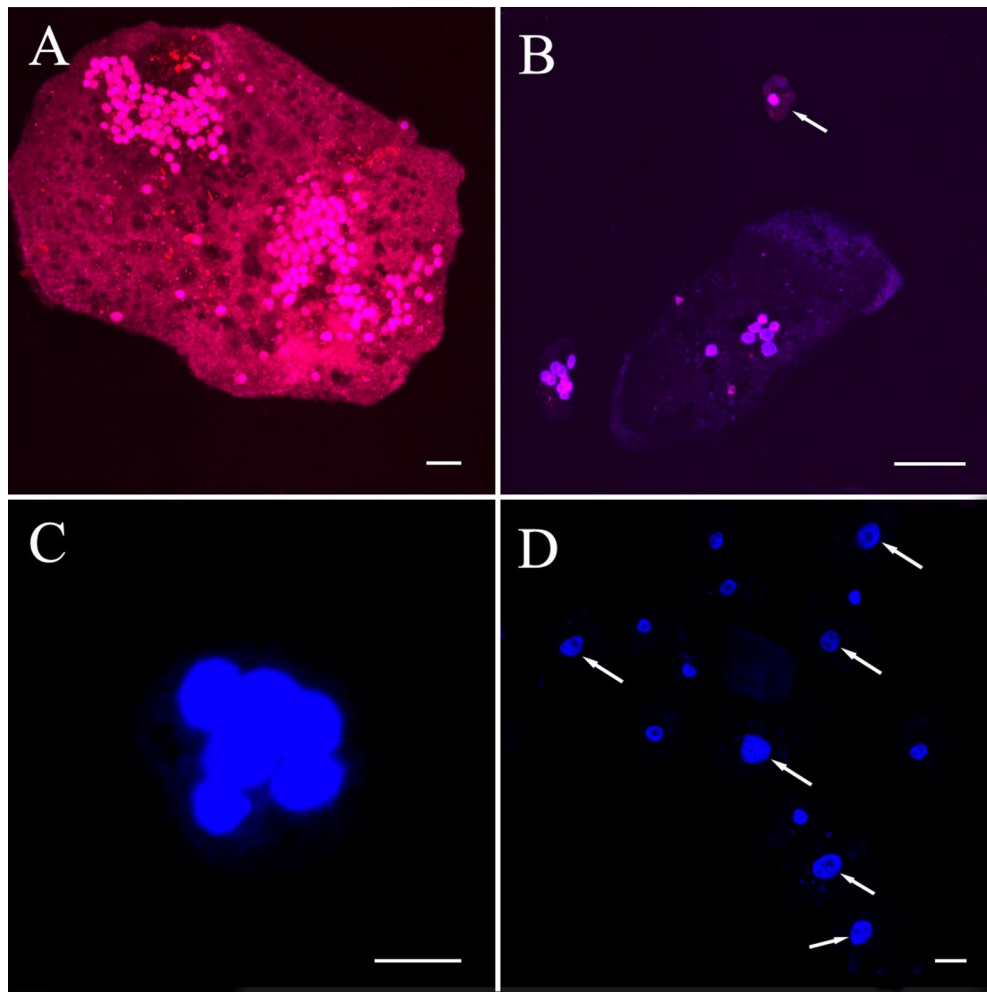


Fig. 3. Confocal micrographs of *Mombasina parasexualis* gen. nov. et sp. nov. stained using immunocytochemistry (ICC) with DAPI (blue) and a cell-membrane dyes (red). **(A)** A large multinucleate amoeba containing over 100 nuclei. **(B)** Three amoebae, including a uninucleate cell (arrow) and both large and small multinucleate cells (bottom). **(C–D)** DAPI staining of smaller multinucleate amoebae showing numerous nuclei with varying degrees of polyploidy (arrows) in addition to regularly sized nuclei. Note in **D** the centrally located nucleoli. Scale bars are 20 μ m in **(A, B)**; 10 μ m in **(C, D)**.

Under standard culture conditions in Petri dishes, no persistent cysts were observed. In aging cultures or on glass slides under stressful conditions such as strong illumination, heat, or desiccation, cells occasionally formed rounded cyst-like stages (Fig. S1D–F). These structures persisted only a few days and were often degraded by bacteria, and they likely represent short-term stress responses rather than true cysts. The isolate proliferates rapidly and frequently occupying an entire Petri dish overnight and was easy to maintain in culture. A flagellate stage was never observed despite repeated attempts under various conditions including nutrient scarcity, temperature stress, or osmotic changes (data not shown).

Phylogenetic placement of new isolate within the Heterolobosea

Phylogenetic analyses using broad heterolobosean taxon sampling recovered a topology congruent with previously published studies (Fig. S2^{22,38}). The dataset contained 96 taxa, including 92 ingroup heteroloboseans representing all major families and Tetramitia subclades, and four outgroup taxa (Fig. S2). Within this framework, the new isolate YT1_mombasa consistently formed a strongly supported clade with *Orodruina flavescens* and the uncultured clone DQ504339.1. This three-taxon lineage was recovered in all analyses including Bayesian inference (Fig. S2) and appeared as a robust sister clade to Neovahlkampfiidae, including *Neovahlkampfia damariscottae* and *N. nana*.

A reduced phylogeny restricted to taxa used in the pairwise divergence analyses (Fig. 4) produced the same placement for the YT1_mombasa, *Orodruina*, and DQ504339.1 group with maximal support. The placement of the new isolate and its close relationship to the genus *Neovahlkampfia* were stable across alignment strategies, algorithm choice and taxon sampling schemes, demonstrating the robustness of its phylogenetic position.

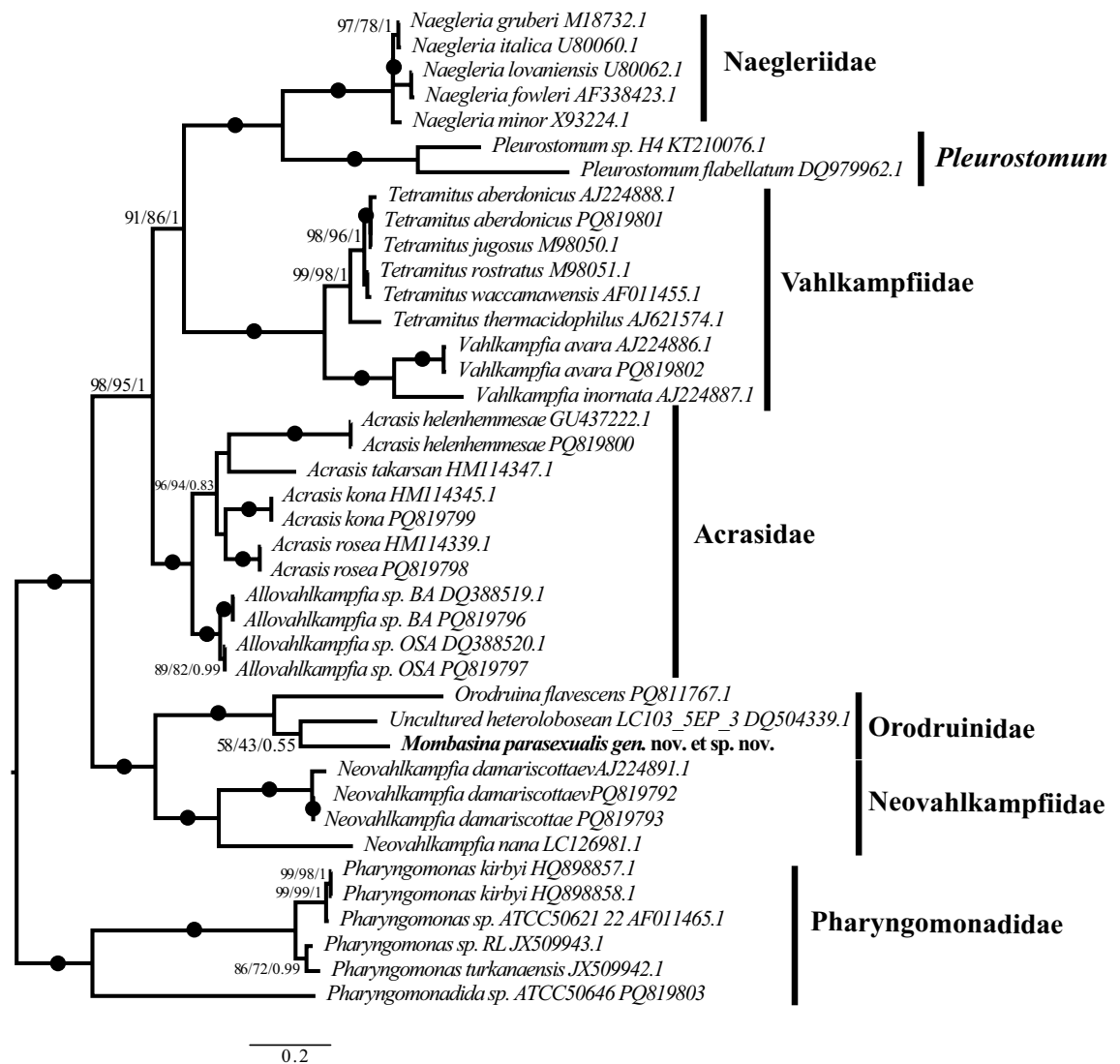


Fig. 4. Phylogenetic placement of *Mombasina parasexualis* gen. nov. et sp. nov. within Heterolobosea. Maximum-likelihood tree inferred from the strictly masked 18S rDNA alignment, including species selected for pairwise comparisons. The new isolate forms a strongly supported clade with *Orodruina flavescens* and the uncultured clone DQ504339.1, together representing a distinct lineage sister to the Neovahlkampfiidae. Node support values represent ultrafast/regular IQtree bootstrap percentages and posterior probability, respectively. Scale bar indicates the expected number of substitutions per site.

18S rDNA divergence analysis

Pairwise 18S rDNA p-distance analyses using both the Global-Default alignment (2014 base pairs) and the Global-Strict alignment (1,130 base pairs) revealed a clear bimodal separation between intragenus and intergenus comparisons, as shown by kernel density plot (Fig. 5). In the Global-Default alignment, intragenus divergence values ($n = 78$) had a mean of 10.3% and ranged from 0.8 to 28.9%, while intergenus values ($n = 792$) averaged 34.9% and ranged from 15.3 to 44.7% (Table S1). In this Global-Default alignment, the optimal genus-level divergence cutoff was formally identified by maximizing Youden's J statistic, which balances sensitivity and specificity between intragenus and intergenus comparisons; this threshold corresponds approximately to the 95th percentile of intragenus pairwise distances (24.5%) (Fig. 5). The 95th percentile intragenus cutoff was 24.5% (Fig. 5). In the global strict masked alignment, intragenus values had a mean of 8.6% and ranged from 0.6 to 23.2%, with a 95th percentile cutoff of 17.3% (Table S1, Fig. 5).

Accordingly, intragenus divergence estimates were interpreted primarily using strictly masked alignments (Global-Strict and Local-Strict) retaining only unambiguous nucleotide positions, which preserve conserved SSU rDNA signal expected among closely related taxa. Global-Default masking removed a larger proportion of sites in some intragenic comparisons due to the prevalence of low-entropy positions and was therefore used as a sensitivity check. Divergence patterns and relative relationships were consistent across masking schemes, indicating that conclusions were not driven by alignment filtering choice.

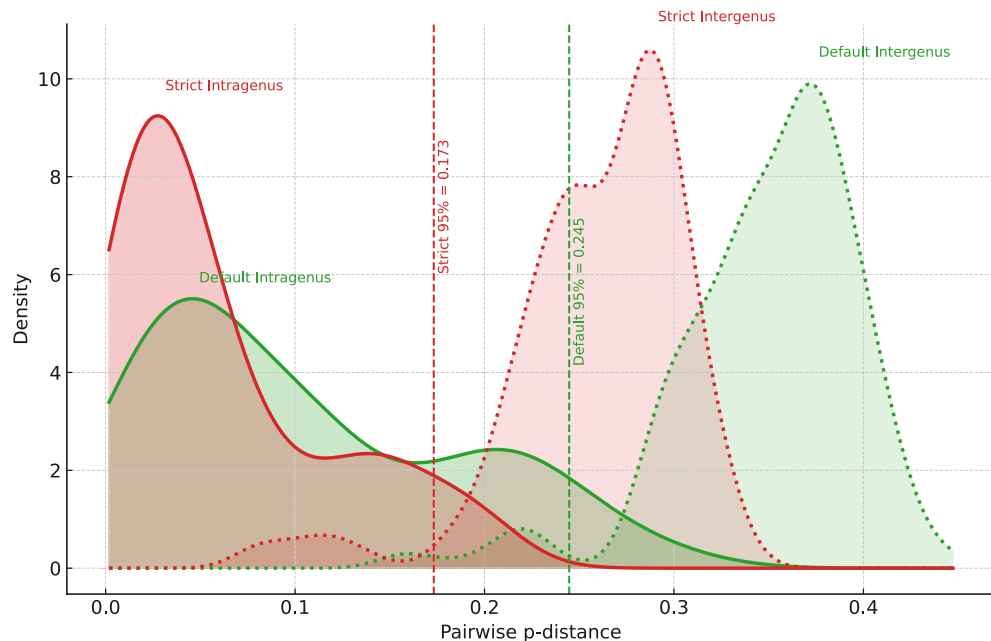


Fig. 5. Global divergence patterns of 18S rDNA across Heterolobosea. Kernel density distributions showing intragenus and intergenus uncorrected p-distance values from the global default (2,014 bp) and global strict-masked (1,130 bp) alignments. In both alignments, intragenus and intergenus distributions form a clear bimodal pattern with limited overlap. Dashed vertical lines indicate empirically derived 95th-percentile intragenus cutoffs for each alignment. These patterns demonstrate strong separation between intragenus and intergenus divergence classes.

Consistent with this interpretation, Local-Strict per-genus alignments produced intragenus distributions highly consistent with both global approaches (Fig. 6). These analyses generated narrow intragenus divergence ranges and reproduced the relative rankings observed under global alignments, confirming that neither alignment scale nor masking strategy significantly influenced intragenus patterns. Genera falling below empirical cutoffs in the global analyses also remained below these thresholds in the Local-Strict alignment, demonstrating that divergence estimates were robust and not inflated by highly variable positions shared across genera. Kernel density plots for both global alignments (Fig. 5) showed limited overlap between intra-genus and inter-genus classes, with overlap approximately 20 to 25% in the Global-Default alignment and 15 to 18% in the Global-Strict alignment. Genera retained similar relative divergence rankings across alignment approaches, highlighting the stability of the intragenus versus intergenus separation.

To evaluate divergence consistency across methods, maximum intragenus p-distances were compared across three analytical frameworks: the Global-Default, Global-Strict, and Local-Strict alignments (Table 1). Across all three approaches, seven genera, including *Acrasis*, *Allovahlkampfia*, *Naegleria*, *Pharyngomonas*, *Pleurostomum*, *Tetramitus*, and *Vahlkampfia*, had maximum intragenus values lower than both empirical cutoffs, supporting current genus boundaries (Table 1). *Neovahlkampfia* exceeded the global default cutoff but remained below both strict thresholds, indicating that its elevated divergence is driven by rapidly evolving sites removed during masking (Tables 1, S1).

When the three *Orodruina*-associated sequences, *O. flavescens*, the uncultured clone DQ504339.1, and the Mombasa isolate, were analyzed as a single genus, maximum values exceeded the Global-Default and Strict-Local thresholds and approached but did not exceed the Global-Strict threshold (17.05% versus 17.3%) (Table 1). Pairwise divergences ranged from 24.4 to 28.87% between *O. flavescens* and DQ504339.1, 22.9 to 24.4% between *O. flavescens* and the new isolate, and 18.77 to 20.1% between DQ504339.1 and the new isolate. These results, combined with the phylogeny albeit poor support (Fig. 4), indicate that the new isolate is more closely related to DQ504339.1 than to *O. flavescens*, but all values exceed intragenetic thresholds, supporting non-congeneric placement.

To further assess the broader applicability and robustness of the divergence pipeline beyond the curated dataset used for primary analyses, we conducted exploratory analyses incorporating additional heterolobosean genera with heterogeneous sequence architecture, variable taxon sampling, and incomplete SSU rDNA coverage, including *Pocheina*, *Percolomonas*, *Pleurostomum*, and related lineages. These analyses required substantial manual curation due to differences in sequence length, inclusion of adjacent ribosomal regions (e.g., ITS or LSU fragments), and elevated levels of ambiguous or truncated positions. Despite these limitations, the resulting divergence patterns were broadly consistent with those observed in the main dataset, with intragenus and intergenus comparisons remaining largely separable and falling within comparable divergence ranges. Interpretation of these results is therefore cautious, and they are referenced here to demonstrate pipeline generality rather than presented as primary results (data not shown).

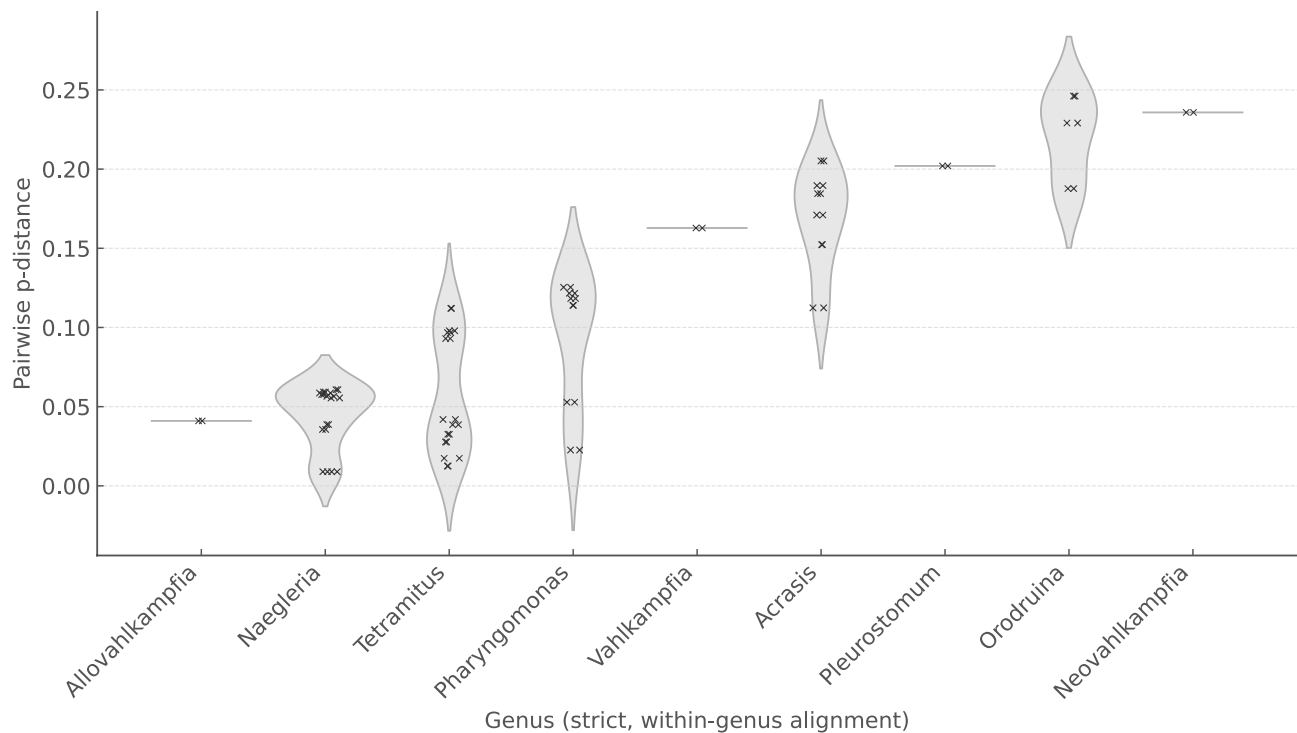


Fig. 6. Violin plots showing maximum intragenus p-distance values based on strict local alignments for each genus.

Genus	No. pairwise	Max default (%)	Max strict (%)	Default status (< 24.5%)	Strict status (< 17.3%)	Max local (%)	Local status (< 17.3%/ < 24.5%)
<i>Allovahlkampfia</i>	1	4.29	1.08	Below	Below	4.10	Below/Below
<i>Naegleria</i>	10	5.63	3.00	Below	Below	6.07	Below/Below
<i>Pharyngomonas</i>	6	11.00	3.60	Below	Below	12.54	Below/Below
<i>Tetramitus</i>	10	11.93	3.60	Below	Below	11.21	Below/Below
<i>Vahlkampfia</i>	1	18.22	7.20	Below	Below	16.28	Below/Below
<i>Pleurostomum</i>	1	21.82	14.05	Below	Below	20.20	Above/Below
<i>Acrasis</i>	6	22.45	10.92	Below	Below	20.52	Above/Below
<i>Neovahlkampfia</i>	1	24.86	14.41	Above	Below	23.58	Above/Below
*‘ <i>Orodruina</i> ’	3	28.87	17.05	Above	Border	24.61	Above/Above

Table 1. Maximum intragenus 18S rDNA divergences used to assess genus-level boundaries under global default/strict and strict-local alignment strategies. *‘*Orodruina*’ = *Orodruina*-associated sequences (*O. flavescens*, uncultured DQ504339.1 and Mombasa isolate).

Assessment of substitution saturation across default and strict 18S rDNA alignments

Substitution saturation analyses using Jukes-Cantor-corrected distances (JC69³⁹) distances showed that both the default and strict 18S rDNA alignments exhibited strong monotonic increases between uncorrected p-distance and corrected distance without evidence of flattening or saturation (Fig. 7). For low to moderate divergence values at or below 0.25, points clustered near the line of expected linearity, indicating minimal influence of multiple substitutions. Higher divergence values near 0.30 to 0.40 showed the expected upward deviation but no plateauing. The two datasets produced nearly identical scatter patterns, and removal of highly variable sites in the strict alignment did not alter the relationship between observed and corrected distances. Because empirically derived intragenus divergence values fall within the linear portion of the curve, these results confirm that genus-level divergence thresholds were estimated from sequence space free from saturation effects.

Discussion

Taxonomic interpretation of genetic divergences in the Heterolobosea

Defining species and genus boundaries in microbial eukaryotes remains difficult because morphological characters are limited, phenotypic plasticity is extensive, and cryptic diversity is widespread^{40–43}. This challenge is especially pronounced in the Heterolobosea, where taxonomic frameworks have historically relied on

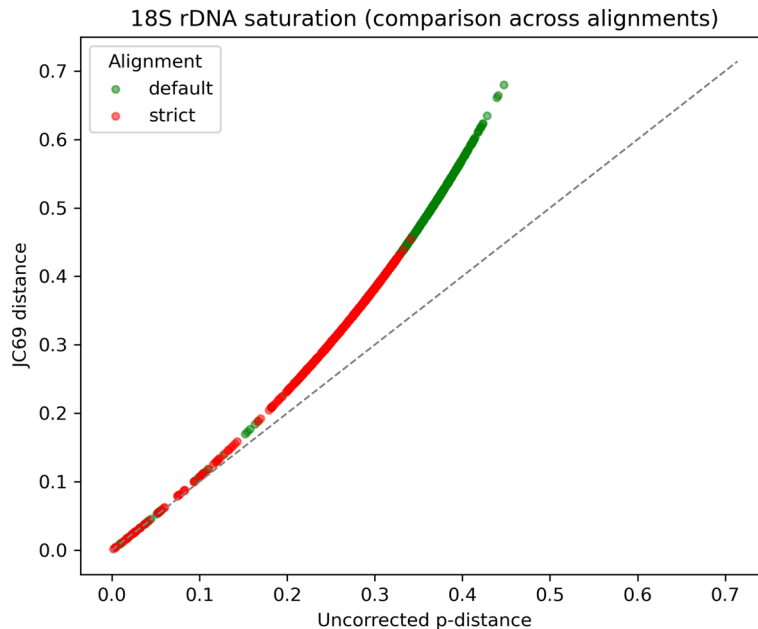


Fig. 7. Substitution saturation analysis of the 18S rDNA dataset. Scatterplots of uncorrected versus JC69-corrected percent distances (showing as decimals) for default and strict alignments show a consistent monotonic increase with no flattening, indicating no substitution saturation. Both alignments behave similarly, and intragenic divergences fall within the linear range, confirming that genus-level thresholds are unaffected.

amoeboid morphology, the presence or absence of a flagellate stage, cyst morphology, cytoplasmic features, ecological attributes, and locomotive behavior^{2,4,15}. Although informative, these traits are few in number, can vary within individuals or cultures, and often fail to capture deeper evolutionary relationships. As a result, molecular phylogenetics, first through 18S rDNA and later through multigene and phylogenomic datasets, has become essential for resolving heterolobosean systematics^{15,16,18,22}.

The 18S rDNA marker remains especially valuable because of its broad representation in public databases and relatively consistent amplification across diverse heterolobosean lineages. Working within the constraints of limited morphological characters, we evaluated the utility of 18S rDNA divergence for genus-level delimitation using a reproducible pipeline and applied these methods to the placement of a newly isolated heterolobosean from the coast of Mombasa, Kenya.

Divergence patterns, methodological robustness and its broader applicability

The analyses consistently revealed a bimodal distribution of 18S rDNA p-distances that clearly separates intra- from inter-genus comparisons (Fig. 5). Global-Default and Global-Strict alignments produced highly similar empirical intragenus thresholds, approximately 24.5% and 17.3%, respectively, and a third analysis using Local-Strict per genus alignments reproduced the same divergence structure (Fig. 6). The relative ordering of genera remained stable across all approaches, indicating that divergence estimates were not affected by alignment scale, masking stringency, or the inclusion or exclusion of highly variable sites. Consistent divergence patterns were also recovered across all three alignment contexts (Global-Default, Global-Strict, and Local-Strict) when the pipeline was applied to additional heterolobosean genera with more heterogeneous SSU rDNA datasets, supporting the broader applicability of the framework beyond the curated dataset used here (data not shown). Saturation analyses further supported this conclusion (Fig. 7). The strong linear relationship between uncorrected and corrected distances across the divergence range relevant to within-genus comparisons shows that 18S rDNA retains sufficient phylogenetic signal in these lineages and is not compromised by substitution saturation.

Together, these results demonstrate that 18S rDNA provides a reliable indicator of genus-level divergence in Heterolobosea when thresholds are empirically derived and evaluated in context. Because substitution rates vary among microbial eukaryotes and even among heterolobosean genera, such thresholds cannot be applied uniformly across lineages. The pipeline developed here offers a standardized and reproducible framework for quantifying 18S rDNA divergence and determining lineage-specific cutoffs across multiple alignment contexts, enabling its application to newly sequenced isolates, environmental lineages, and under-sampled clades. Although demonstrated here using Heterolobosea, this framework is broadly applicable to microbial eukaryote taxonomy, providing a transparent and reproducible approach for evaluating genus-level divergence using 18S rDNA across diverse protist lineages. Nevertheless, divergence thresholds derived from Global-Strict and Local-Strict alignments should complement, rather than replace, other taxonomic evidence. Integrative evaluation using morphology, life history, ecological traits, ultrastructure, and when possible phylogenomics, remains essential for producing stable and defensible taxonomic decisions in groups with limited diagnostic morphological characters.

Taxonomic placement of the Mombasa isolate

Application of the divergence framework to *Orodruina* and related lineages demonstrated that the three sequences (*Orodruina flavescens*, the uncultured clone DQ504339.1, and the Mombasa isolate) exceed intragenus divergence thresholds under nearly all alignment contexts, including Global-Default, Global-Strict, and Local-Strict per-genus analyses (Figs. 5, 6, Table 1). Although the Mombasa isolate is more closely related to the uncultured DQ504339 sequence than to *O. flavescens*, all pairwise distances exceed empirical bounds (Table 1), and this relationship is either poorly supported (Fig. 1) or not supported in the phylogenetic trees (Fig. S2). Assignment of the Mombasa isolate to *Orodruina* would therefore produce a non-monophyletic and internally heterogeneous genus. In contrast, both the molecular divergence estimates derived from strictly masked alignment contexts and the phylogenetic structure support recognition of the Mombasa isolate, together with the uncultured clone, as belonging to a distinct genus. The morphological traits described below further corroborate this interpretation.

Evolutionary, ecological, and taxonomic significance of the Mombasa–*Orodruina*–uncultured DQ504339.1 clade

The lineage comprising the Mombasa isolate, *Orodruina flavescens*, and the Lost City clone (DQ504339.1) represents a deeply diverging assemblage of Heterolobosea sampled from taxa with distinct, previously described ecological contexts and geographic origins. Although these lineages group together with full support, their substantial genetic divergence, highly distinct morphologies, and broad ecological separation indicate a long evolutionary history with limited recent diversification. The clade highlights the well-known taxonomic difficulties in Heterolobosea, where morphological plasticity and ecological breadth often obscure deep phylogenetic relationships^{4,14,15}.

Orodruina flavescens, formerly described as *Gruberella flavescens* (see⁴⁴), is a predominantly multinucleate coastal marine amoeba with complex cytoplasmic organization and conspicuous eruptive behavior. The Mombasa isolate shares its limax-type locomotion but differs markedly by forming large multinucleate and polyploid stages, along with fragmentation-based proliferation that suggests parasexual-like processes similar to those reported in some amoebozoans²⁵. In addition, nuclear organization differs between the two lineages: nuclei in Mombasa isolate show pronounced size variability across trophic and multinucleate stages, whereas *O. flavescens* exhibits more uniform nuclear morphology. Examined nucleoli were centrally positioned (Fig. 3D), consistent with placement within Neovahlkampfiida, but differ in relative prominence and nuclear context between the two taxa. The life cycle of the new isolate, which involves complex cellular and nuclear transformations, will be described separately. These multinucleate stages often exceed one hundred nuclei and exhibit substantial variation in nuclear size, indicating a degree of cellular flexibility not previously documented in Heterolobosea. In contrast, the Lost City clone originates from a serpentinization-driven hydrothermal system characterized by high alkalinity, methane-rich fluids, and temperatures between 40 and 90 °C⁸. Although known only from sequence data, its extreme habitat and deep divergence from both *Orodruina* and the Mombasa lineage suggest an evolutionarily distinct organism with specialized ecological adaptations.

Although the three lineages form a well-supported monophyletic group, the genetic distances among them are large and similar in magnitude to divergences separating distinct genera in other heterolobosean clades. This pattern parallels that found in *Neovahlkampfiida*, the sister lineage to the clade (Fig. 4), where the two described species (*N. damariscottae* and *N. nana*) differ substantially in ultrastructure, nuclear morphology, habitat (freshwater vs marine), and behavior despite forming a robust clade^{15,45,46}. Such examples underscore that deeply diverging and morphologically disparate lineages are common within the Tetramitida and that the region of the tree containing Neovahlkampfiida and Orodruinida likely harbors extensive hidden diversity.

The molecular divergences in the Mombasa–*Orodruina*–Lost City assemblage consistently exceed genus-level thresholds, and saturation analyses confirm that these values fall within the linear, non-saturated region of 18S rDNA (Table 1, Fig. 7). Combined with marked ecological and morphological differences, these results support the recognition of the Mombasa isolate, *Orodruina flavescens*, and possibly the undescribed Lost City lineage as three independent genera. Rather than forming a single heterogeneous unit, they illuminate the depth of evolutionary diversity within Family Orodruinidae and demonstrate the remarkable capacity of heteroloboseans to colonize and adapt to a wide variety of ecological niches.

In this context, the assignment of the Mombasa isolate as a new genus and species is well supported by its molecular divergence, combination of limax-type and eruptive locomotion, formation of multinucleate polyploid stages, and coastal ecology. Similarly, the sustained distinctiveness of *O. flavescens* and the extreme habitat specialization of the Lost City lineage indicate that each represents a coherent and independent evolutionary lineage. Recognition of these taxa as separate genera reflects the true evolutionary structure of this clade and emphasizes the broader underappreciated diversity within Orodruinidae.

Taxonomic note on the Orodruinidae + Neovahlkampfiidae clade

Phylogenetic analyses highlight both the deep hidden diversity and the close evolutionary relationship between the families Orodruinidae and Neovahlkampfiidae. Across all 18S rDNA divergence analyses and phylogenetic reconstructions, representatives of these two families consistently form a strongly supported monophyletic group, reaffirming their close affinity. This pattern is also broadly consistent with the phylogenomic framework of Pánek et al.²², which recovered a robust Selenionia clade that includes Neovahlkampfiidae and Selenionidae, and further suggested that Orodruinidae may also be closely affiliated with this broader lineage, while refraining from formal taxonomic reassignment.

The taxonomic interpretation of Orodruinidae is complicated by the uncertain placement of *Stachyamoeba* sp. ATCC-50324. Although this strain has historically been assigned to Orodruinidae (former Gruberellidae)⁴⁷, the phylogenomic analysis of Pánek et al.²² showed that ATCC-50324 does not cluster with *Orodruina flavescens*

but instead branches within Eutetramitea, near *Oramoeba* and Psalteriomonadidae. The true identity of ATCC-50324 remains unresolved because the type strain of *Stachyamoeba lipophora* was lost before sequencing, leaving its taxonomic placement ambiguous.

In light of this uncertainty, and without proposing a formal emendation or taxonomic reclassification, the repeated recovery of a coherent clade linking *Orodruina flavescens*, Neovahlkampfiidae, and the Mombasa isolate indicate that Orodruinidae should not be regarded as *incertae sedis* within Tetramitia, as tentatively noted by Pánek et al.²². Rather, our results provide independent molecular support for the interpretation advanced by Pánek et al.²² that Orodruinidae is closely allied with Neovahlkampfiidae. This relationship is consistent with the revised higher-level framework of Pánek et al.²², which places Neovahlkampfiidae within the class Seleniaonea. Accordingly, the placement of *Orodruina* and its associates within this broader evolutionary context is well supported by the available data, while acknowledging that resolution of remaining uncertainties, particularly the phylogenetic position of *Stachyamoeba*, will be required before any formal higher-level taxonomic changes can be justified. The phylogenetic position of the Mombasa isolate reinforces this interpretation. Its consistent grouping with *Orodruina flavescens* and Neovahlkampfiidae, together with 18S rDNA divergence values that fall within the expected range for closely related heterolobosean genera, further supports the view that these lineages represent a coherent evolutionary assemblage within Seleniaonea.

Description and taxonomic treatment of *Mombasina parasexualis* gen. nov. et sp. nov.

Morphological summary of the isolate

The isolate *Mombasina parasexualis* gen. nov. et sp. nov. displays highly plastic amoeboid morphology. Trophic cells range from 12.6 to 57.0 µm in length and exhibit a characteristic monopodial limax-type form during active locomotion, with smooth cytoplasmic streaming and occasional faint uroidal filaments. Non-directional movement produces eruptive hyaline expansions that generate zig-zag trajectories. Floating cells are rounded and develop a prominent hyaline cap upon settling. Most trophic cells are uninucleate, although rare binucleate forms occur. Older cultures commonly contain large multinucleate amoebae (50–160 µm), sometimes branched, with dozens to more than 100 nuclei displaying variable sizes, including enlarged nuclei consistent with polyploidy. No flagellate stage was observed, and stress-induced cyst-like forms were short-lived and unstable. Molecular data place the isolate as a deeply diverging lineage distinct from *Orodruina flavescens*, with 18S rDNA divergences exceeding 22–24%, supporting recognition as a new genus.

Taxonomic summary

Kingdom: Eukaryota

Supergroup: Excavata

Phylum: Heterolobosea Page and Blanton, 1985 sensu Hanousková et al. (2019)

Subphylum: Tetramitia Cavalier-Smith, 1993 sensu Hanousková et al. (2019)

Class: Class Seleniaonea Pánek and Cepicka 2025

Order: Neovahlkampfiida Cavalier-Smith, 2022, as emended by Pánek et al. (2025)

Family: Family Orodruinidae Shishkin, 2021

Genus: *Mombasina* gen. nov. Tekle 2025

Species: *Mombasina parasexualis* sp. nov. Tekle 2025

Diagnosis of the genus Mombasina gen. nov.

A heterolobosean amoeba exhibiting monopodial limax-type locomotion with smooth, non-eruptive cytoplasmic streaming during directional movement and pronounced eruptive hyaline activity during non-directional locomotion. Floating forms are rounded and develop a hyaline cap upon settling. No flagellate stage has been observed. Large multinucleate stages appear frequently in older cultures. 18S rDNA sequences form a distinct, strongly supported lineage separate from *Orodruina* and other tetramitiid genera, with divergence values exceeding established intrageneric thresholds.

Diagnosis of the species Mombasina parasexualis sp. nov.

Cells measuring 12.6–57.0 µm in length during the trophic stage. Typically, uninucleate, but multinucleate and polyploid giant forms are common in older cultures. Locomotion characterized by monopodial limax-type movement with smooth streaming; non-directional movement accompanied by eruptive hyaline caps and occasional faint uroidal filaments. Short-lived cyst-like forms appear under stress. No flagellate stage observed. The 18S rDNA sequence diverges from *Orodruina flavescens* by more than 22–24%, supporting its recognition as a separate species and genus.

Formal description

Trophic amoebae are variable in size and shape, with characteristic limax-type forms during active locomotion. Cytoplasmic flow is smooth and directed anteriorly. The posterior end may bear inconspicuous uroidal filaments. Non-directional movement is marked by eruptive hyaline protrusions that generate irregular, zig-zag trajectories. Floating cells are rounded and develop a hyaline cap immediately upon attachment to the substrate. Nuclei in typical trophic cells measure 2.3–5.9 µm in diameter. Older cultures contain large multinucleate amoebae (50–160 µm), sometimes branched, with more than 100 nuclei exhibiting variable sizes, including markedly enlarged nuclei suggestive of polyploidization. No persistent cysts or flagellate forms were observed in culture.

Type locality

Shallow coastal sediment, Bamburi Beach, Mombasa, Kenya.

Type material

Holotype: Type strain preserved in cryogenic storage in the Tekle Laboratory culture collection. Additional material available upon request.

Etymology

The species epithet *parasexualis* (Latin adjective) refers to the organism's unusual multinucleate and polyploid stages and fragmentation behavior reminiscent of parasexual processes described in other microbial eukaryotes.

Molecular diagnosis

The 18S rDNA sequence (GenBank accession PX620421) diverges by 22.9–24.4% from *Orodruina flavescens* and by 18.8–20.1% from the environmental clone DQ504339.1. These values exceed empirically derived intragenetic divergence thresholds for Heterolobosea and support recognition of *Mombasina parasexualis* gen. nov. et sp. nov. as a distinct genus and species.

Data availability

The 18S rDNA sequence generated in this study is deposited in GenBank under an accession number PX620421. All alignments, divergence-analysis scripts, and phylogenetic datasets used in this study are available from the corresponding author upon request. Additional materials, including raw microscopy files and ICC image stacks, will be made available through a public data repository upon request. Video documentation of cellular behavior is available through the principal investigator's YouTube channel.

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Author contributions

Y.I.T. designed the study, performed field sampling, conducted molecular and microscopy analyses, developed the divergence framework, supervised data interpretation, and wrote the manuscript. V.W.W. contributed to project design, assisted in organizing the field expedition, and edited the manuscript. S.G. assisted with generating molecular and morphological data and contributed to manuscript editing. K.S.C. assisted in the generation of morphological and molecular data, and contributed to manuscript editing. All authors reviewed, edited, and approved the final version of the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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