Gromochytrium mamkaevae gen. & sp. nov. and two new orders: Gromochytriales and Mesochytriales (Chytridiomycetes)

S.A. Karpov1,2, A.A. Kobseva2, M.A. Mamkaeva2, K.A. Mamkaeva2, K.V. Mikhailov3,5, G.S. Mirzaeva4, V.V. Aleoshin3,5,6

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Abstract
During the last decade several new orders were established in the class Chytridiomycetes on the basis of zoospore ultrastructure and molecular phylogeny. Here we present the ultrastructure and molecular phylogeny of strain x-51 CALU – a parasite of the alga Tribonema gayanum, originally described as Rhizophydia sp. based on light microscopy. Detailed investigation revealed that the zoospore ultrastructure of this strain has unique characters not found in any order of Chytridiomycetes: posterior ribosomal core unbounded by the endoplasmic reticulum and detached from the nucleus or microbody-lipid complex, and kinetosome composed of microtubular doublets. An isolated phylogenetic position of x-51 is further confirmed by the analysis of 18S and 28S rRNA sequences, and motivates the description of a new genus and species Gromochytrium mamkaevae. The sister position of G. mamkaevae branch relative to Mesochytrium and a cluster of environmental sequences, as well as the ultrastructural differences between Gromochytrium and Mesochytrium zoospores prompted us to establish two new orders: Gromochytriales and Mesochytriales.

INTRODUCTION
Molecular phylogeny has dramatically changed chytrid taxonomy. Investigation of gene sequences of nearly any species or strain initiates a revision of neighbour taxa and often permits authors to establish new taxa of higher rank, e.g. family, order and class, divisions normally supported by zoospore ultrastructure. In the past few years we have seen several big changes in chytrid taxonomy: Letcher et al. (2006) described the Rhizophydia clade (James et al. 2000, 2006) as the order Rhizophydia; Mozley-Standridge et al. (2009) established the order Cladochytriales from the Cladochytrium clade (James et al. 2006) and Simmons et al. (2009) described the clade formerly represented in phylogenetic trees (James et al. 2006) by Chytriomycetes angularis as the order Lobulomyctes. “This removal of clades from the polyphyletic Chytridiomycota and began the corrective process of classifying the Chytridiomycota (chytrids) into phylogenetic groups according to the best tools available.” – wrote Longcore and Simmons in the introduction to the new order Polychytriales (Longcore & Simmons 2012: 276). This conclusion highlights the fact that we need molecular data for each traditionally described species of Chytridiomycota to construct a meaningful and comprehensive classification of Chytridiomycota.

Rhizophydia is one of the largest genera of Chytridiomycetes known from the middle of the 19th century (Rabenhorst 1868). It accounts for more than 225 species, which were described from freshwater, primarily as parasites of algae, and from soil as saprotrophs (Longcore 1996, Letcher et al. 2004). The data on this genus were significantly expanded in recent investigations (Letcher et al. 2006, 2008) and reviewed in a comprehensive taxonomic summary and revision of the genus (Letcher & Powell 2012).

Nevertheless, the list of species investigated with modern methods is still far from being complete, and new data on the ultrastructure and molecular phylogeny of other strains are always important for understanding the huge morphological and genetic diversity of this genus. Moreover, the transmission electron microscopy (TEM) sometimes reveals peculiarities that can be used as new taxonomic characters, or may show the unimportance of some commonly accepted ultrastructural characters.

Here we present the ultrastructure and molecular phylogeny of an algal parasite, strain x-51 CALU, which was described in a preliminary study as ‘Rhizophydia sp.’. We show that zoospore ultrastructure of this strain differs from that of other described species, and includes characters not described in any orders of Chytridiomycetes. These morphological data confirm an isolated phylogenetic position of x-51 obtained from the analysis of 18S and 28S rRNA sequences, and serve as the basis for the description of a new species and genus. Sister position of the x-51 branch relative to a cluster of environmental sequences, which includes Mesochytrium penetrans, and the ultrastructural differences of x-51 and Mesochytrium zoospores prompt us to establish two new orders: Gromochytriales and Mesochytriales.

MATERIALS AND METHODS

Strain CALU x-51 was isolated from a water sample collected from a ditch by the highway near town Kirovsk, Leningrad

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Table 1  List of rRNA genes used in phylogenetic analysis.

| Taxon                     | Isolate number | GenBank accession no. | Cumulative length (%) |
|---------------------------|----------------|-----------------------|-----------------------|
|                           |                | 18S       | 5.8S       | 28S length |                   |
| **Outgroup: aphelids and rozellids** |                |           |            |            |                   |
| Amoeboaphelidium protococcarum | CALU X-5       | JX507298  | JX507298  | JX507298   | 99                  |
| Rozella allomycis         | UCB 47-054 (AFTOL-ID 297) | AY635838  | AY997087  | DQ273803   | 99                  |
| Rozella sp.               | JEL347 (AFTOL-ID 16)     | AY601707  | AY997086  | DQ273766   | 98                  |
| **Blastocladiomycota**    |                |           |            |            |                   |
| Blastocladiella emersonii |                | M54937    | AY997032  | X90411     | 98                  |
| Allomyces arbuscula       | AFTOL-ID 300     | AY552524  | AY997028  | AY552525   | 98                  |
| Physoderma maydis         | AFTOL-ID 19       | AY601708  | AY997072  | DQ273768   | 98                  |
| **Neocallistigmomyctota** |                |           |            |            |                   |
| Neocallimastix sp.        | GE13 (AFTOL-ID 638) | DQ322625  | AY997064  | DQ273822   | 97                  |
| Orpinomyces sp.           | OUS1            | AJ864616  | AJ864475  | AJ864475   | 98                  |
| Cyllamyces aberensis      | EO14 (AFTOL-ID 846) | DQ356481  | AY997042  | DQ273829   | 100                 |
| D3                       | uncultured       | EU910609  |            |            | 36                  |
| **Monoblepharidomycetes** |                |           |            |            |                   |
| Monoblepharella mexicana  | BK 78-1 (AFTOL-ID 33) | AF164337  | AY997061  | DQ273777   | 98                  |
| Gonapodya prolifera       | JEL478          | JGI v. 1.0 | JGI v. 1.0 | JGI v. 1.0 | 98                  |
| Oedogoniomycetes sp.      | CR84 (AFTOL-ID 298) | AY635839  | AY997066  | DQ273804   | 99                  |
| Hyaloraphidium curvatum   | SAG 235-1 (AFTOL-ID 26) | Y17504    | AY997055  | DQ273771   | 91                  |
| PFE7AU2004                | uncultured       | DQ244008  |            |            | 36                  |
| L73_ML_156                | uncultured       | FJ354068  |            |            | 22                  |
| Elev_18S_563              | uncultured       | EF024210  |            |            | 36                  |
| **Gromochytriales, ord. nov.** |                |           |            |            |                   |
| Gromochytrium mamkaevae   | CALU X-51        | KF586842  | KF586842  | KF586842   | 99                  |
| kor_110904_17             | uncultured       | FJ157331  |            |            | 33                  |
| IIN1-34                   | uncultured       | EU516964  | EU516964  |            | 15                  |
| **Mesochytriales, ord. nov.** |                |           |            |            |                   |
| Mesochytrium penetrans    | CALU X-10         | FJ804149  | FJ804153  |            | 37                  |
| WS 10-E02                 | uncultured       | AJ867629  |            |            | 34                  |
| WS 10-E14                 | uncultured       | AJ867630  |            |            | 36                  |
| WS 10-E15                 | uncultured       | AJ867631  |            |            | 36                  |
| Spring_08                 | uncultured       | JX069031  |            |            | 11                  |
| Spring_37                 | uncultured       | JX069054  |            |            | 11                  |
| Spring_57                 | uncultured       | JX069067  |            |            | 11                  |
| Spring_71                 | uncultured       | JX069077  |            |            | 11                  |
| T2P1AeB05                 | uncultured       | GQ995415  |            |            | 36                  |
| T2P1AeF04                 | uncultured       | GQ995412  |            |            | 36                  |
| T3P1AeC03                 | uncultured       | GQ995413  |            |            | 36                  |
| TSP2AeC07                 | uncultured       | GQ995414  |            |            | 36                  |
| SAPA5_E7                 | uncultured       | FJ483310  |            |            | 15                  |
| P60E-9                    | uncultured       | DQ104060  |            |            | 13                  |
| P60E-29                   | uncultured       | DQ104068  |            |            | 14                  |
| Clones from a lake in China | uncultured | JX426910, JX426918, JX426923, JX426998, JX427002, JX427011 | | | 7 |
| Clones from Lake Bourget (B74, B1, B43, B44, B46-138, B49, B52, B56, B78, B88, B100, B104, B107, B1121, B123, B15, B172, B176, B186-161, B15) | uncultured | EF196711, EF196713, EF196728, EF196729, EF196731, EF196734, EF196735, EF196738, EF196745, EF196749, EF196751, EF196753, EF196755, EF196762, EF196763, EF196765, EF196775, EF196776, EF196778, EF196779 | | | 20 |
| PFF5SP2005                | uncultured       | EU162641  |            |            | 36                  |
| PFD6SP2005                | uncultured       | EU162637  | 3'-end    |            | 30                  |
| PFA12SP2005               | uncultured       | EU162643  |            |            | 36                  |
| PA2007C10                 | uncultured       | JQ689425  |            |            | 35                  |
| F08_SE1B                  | uncultured       | FJ592495  | 3'-end    |            | 17                  |
| ThJAR2B-48                | uncultured       | JF972676  |            |            | 33                  |
| 528-025                   | uncultured       | EF586095  |            |            | 17                  |
| GA089                     | uncultured       | HM489688  |            |            | 28                  |
| GF29312                   | uncultured       | JX417945  |            |            | 16                  |
| PFG9SP2005                | uncultured       | EU162638  |            |            | 36                  |
| PA2009C3                  | uncultured       | HQ191369  | HQ191369  |            | 40                  |
| PA2009B6                  | uncultured       | HQ191400  | HQ191400  |            | 40                  |
| PA2009D8                  | uncultured       | HQ191406  | HQ191406  |            | 40                  |
| PA2009E7                  | uncultured       | HQ191286  | HQ191286  |            | 40                  |
| Va2007BB6                 | uncultured       | JQ689445  |            |            | 35                  |
| FY23_1H5                  | uncultured       | DQ310332  |            |            | 29                  |
Table 1 (cont.)

| Taxon | Isolate number | GenBank accession no | Cumulative length (%) |
|-------|----------------|----------------------|-----------------------|
|       |                | 18S                  | 5.8S                  | 28S                  |
| **Order Lobulomycetales** |                |                      |                       |                      |
| Lobulomyces angularis | JEL45 (AFTOL-ID 630) | AF164253             | AY997036              | DQ273815             | 100                  |
| Lobulomyces angularis | PL70 | EF443138              | EU525774              | EF443143              | 53                   |
| Gen. sp. | AF011 | EF342819              | EF342819              | EF342819              | 57                   |
| Maunachytrium keaense | AF021 | EF342822              | EF342822              | EF342822              | 54                   |
| CCW64 | uncultured | AY180029              |                       |                      | 35                   |
| RSC-CHU-20 | uncultured | AJ506002              |                       |                      | 32                   |
| D2P03D7 | uncultured | EF100268              |                       |                      | 29                   |
| AV2009B4 | uncultured | HQ219419              | HQ219419              |                      | 40                   |
| IIS1-20 | uncultured | EU517013              | EU517013              |                      | 14                   |
| **Family Synchytriaceae** |                |                      |                       |                      |
| Synchytrium decipiens | DUH0009363 (AFTOL-ID 635) | DQ354675              | AY997094              | DQ273819              | 100                  |
| Synchytrium macrosporum | JEL325 | AY349046              | AY349081              |                      | 33                   |
| Neokarlingia chitinophila | JEL93 | AY997056              | DQ273814              |                       | 100                  |
| Karlingiomyces asterocystis | JEL572 | HQ901769              | HQ901708              |                      | 52                   |
| **Order Polychytriales** |                |                      |                       |                      |
| Polychytrium aggregatum | JEL109 (AFTOL-ID 24) | AY601711              | AY997074              | AY546686              | 100                  |
| Lacotromyces hiemalis | JEL31 | AY997009              | HQ51700               |                       | 49                   |
| arkaya lepida | JEL93 (AFTOL-ID 629) | AY997074              | DQ273814              |                       | 100                  |
| Neokarlingia chitinophila | JEL510 | HQ901766              | HQ901703              |                       | 52                   |
| Karlingiomyces asterocystis | JEL572 | HQ901769              | HQ901708              |                       | 52                   |
| **Order Cladochytriales** |                |                      |                       |                      |
| Cladochytrium replicatum | JEL180 (AFTOL-ID 27) | AY546683              | AY997037              | AY546688              | 98                   |
| Endochytrium sp. | JEL125 | AH009049              | EU825811              |                       | 41                   |
| Neokarlingia chitinophila | JEL510 | HQ901766              | HQ901703              |                       | 52                   |
| Karlingiomyces asterocystis | JEL572 | HQ901769              | HQ901708              |                       | 52                   |
| **Order Chytridiales** |                |                      |                       |                      |
| Podochytrium dentatum | JEL30 (AFTOL-ID 1539) | AY997055              | DQ543650              | DQ273938              | 95                   |
| Chytridiomycota sp. | JEL378 (AFTOL-ID 1532) | DQ543683              | DQ273932              |                       | 73                   |
| Rhizochloamasium sp. | JEL347 (AFTOL-ID 20) | AY601709              | AY997076              | DQ273816              | 97                   |
| Chytridiomycota sp. | JEL59 (AFTOL-ID 1540) | AY997063              | DQ273839              |                       | 86                   |
| Chytridiomycota sp. | JEL187 (AFTOL-ID 39) | AY635825              | AY997035              | DQ273873              | 98                   |
| Chytridiomycota sp. | JEL221 (AFTOL-ID 1534) | AY546683              | AY997037              | AY546688              | 98                   |
| Diplochytridium lagenarium | JEL72 | AY997056              | DQ273809              |                       | 100                  |
| Nowakowskia sp. | JEL572 (AFTOL-ID 146) | AY635835              | AY997065              | DQ273798              | 98                   |
| **Order Spizellomycetales** |                |                      |                       |                      |
| Spizellomyces punctatus | JEL318 (AFTOL-ID 1532) | AY997055              | DQ543650              | DQ273938              | 95                   |
| Powellomyces sp. | JEL95 (AFTOL-ID 32) | AF164245              | AY997075              | DQ273776              | 98                   |
| Triparticalcar arcticum | JEL342 (AFTOL-ID 1532) | DQ543680              | AY997089              | DQ273826              | 100                  |
| Gaertneriomyces semiglobiferus | JEL342 | AY997051              | DQ273778              |                       | 99                   |
| **Order Rhizophlyctidales** |                |                      |                       |                      |
| Rhizophlyctis rosea | JEL171 (AFTOL-ID 31) | AY635829              | AY997078              | DQ273787              | 99                   |
| Catenomyces sp. | JEL342 (AFTOL-ID 47) | AY635830              | AY997033              | DQ273789              | 99                   |
| Rhizophlyctis harderi | JEL171 (AFTOL-ID 31) | AY635829              | AY997078              | DQ273787              | 99                   |
| Rhizophlyctis sphaerotheca | JEL151 (AFTOL-ID 31) | AY635829              | AY997082              | DQ273781              | 97                   |
| Entophlyctis helioformis | JEL326 (AFTOL-ID 40) | AY635829              | AY997094              | DQ273774              | 96                   |
| Homaloaphlyctis polyrhiza | JEL142 | DQ543649              |                       |                       | 99                   |
| Batrachochytrium dendrobatidis | JEL197 (AFTOL-ID 21) | AY997031              | AY546693              |                       | 96                   |
| incertae sedis | 18s1-47 | uncultured | EU733554 | 21 | 18s3 24 | uncultured | EU733608 | 21 | LLSG10-1 PML-2011t | uncultured | JN049552 | 13 |
Fig. 1  Stages of the life cycle of Gromochytrium mamkaevae (x-51 CALU) on the host Tribonema gayanum. — a–c: LM images of living parasite on filament of host Tribonema, phase contrast. — a. Two cysts with a lipid globule; b. young sporangium with 3 lipid globules; c. mature sporangium contains zoospores. — d. Rhizoid in the host cell in TEM. — e. Drawing of the life cycle. — Abbreviations: cy = cyst; I = lipid globule; msp = mature sporangium; rh = rhizoid; sp = sporangium; spw = sporangium wall; ysp = young sporangium; zs = zoospores. — Scale bars: a–c = 10 µm; d = 2 µm.
Fig. 2 Bayesian phylogenetic tree based on concatenated rDNA sequences (18S, 5.8S, 28S). Node support values are given by Bayesian posterior probability (left of the vertical line) and Maximum Likelihood bootstrap support (right of the vertical line). Support values are omitted for nodes that score above 95% in both analyses (edges drawn with thick lines) and nodes that score less than 50% in both analyses (edges drawn with striated lines). The strain x-51 - Gromochytrium mamkaevae is highlighted with red. Two groups of nearly identical clones in the Mesochytriales clade are collapsed into single branches (represented by triangles).
verification step that involved breaking the sequence into two or more non-overlapping fragments that were then used as independent OTUs for preliminary phylogenetic analysis (data not shown). This method identified seven sequences (accession numbers: EU162637, EF196798, EF196785, EF196773, EF196750, FJ592495, HQ191339) from three independent environmental samples as potentially chimaeric. The parts of sequences EU162637 and FJ592495 that presumably have fungal source were retained; the remainder and the other four sequences were excluded from the phylogenetic analysis. To minimise missing data a small number of sequences was assembled by fusing or constructing a consensus of sequences from different isolates of the same species or by fusing partial sequences that have a 98–100 % overlap identity. The full list of consensuses and chimaeric sequences constructed for the purpose of phylogenetic analysis is presented in Table 1 and Fig. 2. The sequences of early-branching fungal taxa – *Rozella allomyces* and *Amoeboaphelidium protococcarum* were chosen

Table 2  List of environmental clones of the Mesochytriales and Gromochytriales.

| Name                        | GenBank accession no. | Habitat / Geographic location | Characterisation/Season | Reference |
|-----------------------------|-----------------------|-------------------------------|-------------------------|-----------|
| *Gromochytrium mamkaevae*   | KF586842              | Ditch near town Kirovsk, Leningrad Region | parasite of yellow-green alga Tribonema sayaram | This paper |
| *Mesochytrium penetrans*    | FJ804149, FJ804153    | Small lake in Karelia (Northern Europe) | parasite of green alga Chlorococcum minutum | Karpov et al. (2010) |
| CALU x-51                  |                       |                               |                         | Dopheide et al. (2008) |
| CALU x-10                  | EF586095              | Opanuku Stream biofilm, Auckland, New Zealand |                         | Lefèvre et al. (2008) |
| PFDSSP2005, PFGSSP2005, PFFSSP2005, PFA125SP2005 | EU162637, EU162638, EU162641, EU162643 | Oligo-mesotrophic mountain Lake Pavin, France | May – June | Lefèvre et al. (2008) |
| BI74, B1, B43, B44, B46-138, B49, B52, B56, B17, B87, B108, B100, B104, B117, B121, B123, B15, B172, B76, B68-161, B15 | EF196711, EF196713, EF196728, EF196729, EF196734, EF196735, EF196738, EF196745, EF196749, EF196751, EF196754, EF196756, EF196765, EF196775, EF196776, EF196786, EF196788 | Large mesotrophic alpine Lake Bourget, France | May – August | Lepère et al. (2008) |
| F08_SE1B                  | FJ592495              | Cold-fumarole soil, Socompa Volcano, Andes (elev. 5624 m) |                         | Costello et al. (2009) |
| P60E-9, P60E-29            | DQ104060, DQ104068    | Glacial ice from Tibetan plateau | 150-yr-old ice | Zhang et al. (2009) |
| T2P1AeB50, T2P1AeF04, T3P1AeC03, TSP2AeC07 | GQ995415, GQ995412, GQ995413, GQ995414 | High-elevation soil not far from ice and snow | July – October | Freeman et al. (2009) |
| PA2009C3, PA2009B6, PA2009D8, PA2009E7 | HQ191369, HQ191400, HQ191406, HQ191286 | Oligo-mesotrophic mountain Lake Pavin, France | May | Monchy et al. (2011) |
| SAPAS_E7                  | FJ483310              | Salt marsh, USA: RI | Summer | Mohamed & Martiny (2011) |
| ThJARBZ-48                | JF972676              | Air sample, Greece | October | Genitsaris (2011) |
| GA069                     | HM486988              | Feces from a detritus-feeding crustacean Gammarus tigrinus; water | September – October | Sridhar et al. (2011) |
| Spring_08, Spring_37, Spring_57, Spring_71 | JX069031, JX069054, JX069067, JX069077 | River site, Southern Alberta, Canada |                         | Thomas et al. (2012) |
| Pa2007C10                 | JQ689425              | Oligo-mesotrophic mountain Lake Pavin, France | April | Jobard et al. (2012) |
| Va2007BB6                 | JQ689445              | Large brown-coloured humic and mesotrophic Lake Vassivière, France | May | Jobard et al. (2012) |
| WS 10-E02, WS 10-E14, WS 10-E15 | AJ867629, AJ867630, AJ867631 | Melted white snow water, alpine Lake Joerli XIII, Switzerland | – | Unpubl. data |
| GS239312                  | JX417945              | Greenhouse soil, China | – | Unpubl. data |
| Seven clones from a freshwater lake in China | JX426910, JX426918, JX426923, JX426937, JX426998, JX427002, JX427011 | Freshwater lake, China | – | Unpubl. data |
| kor_110904_17            | FJ157331              | Lake Koronia water column, Greece | Nov. | Genitsaris et al. (2009) |
| INF1-34                   | EU516964              | Alpine snow-covered soil, Alpes, Austria | – | Unpubl. data |
| Nineteen clones: E109_XXX, E107_XXX | KC561936–KC561954 | High mountain soil Nepal | October | Naff et al. (2013) |
| Five clones: R11a_XX       | KC561955–KC561959 | Rocky Mountain talus snow, Colorado, USA | July – August | Naff et al. (2013) |
| Sixteen clones: 31a_XX, 31b_XX | KC561960–KC561975 | Rocky Mountain talus snow, Colorado, USA | July – August | Naff et al. (2013) |
| NKS146                    | JX296576              | Hyposaline soda lake Nakuru, Kenya, East Africa | November | Luo et al. (2013) |
as outgroup (James et al. 2006, Karpov et al. 2013). Alignments were generated with MUSCLE (Edgar 2004) and refined manually using BioEdit (Hall 1999). After discarding ambiguously aligned nucleotide positions and concatenating the alignments of 18S, 5.8S and 28S rRNA genes, the alignment consisted of 4 850 positions. Tree search for the concatenated alignment was performed using the Bayesian method implemented by MrBayes v. 3.1.2 (Ronquist & Huelsenbeck 2003). The tree reconstruction used GTR+G12+I model and partition by genes (18S, 5.8S, and 28S) with all parameters unlinked, except the topology and branch lengths. Four independent runs of eight Markov Chain Monte Carlo (MCMC) were performed to evaluate the convergence. Chains were run for 10 million generations sampling trees every 1 000 generations after discarding the first 8 million as burn-in. Sampled trees were used to generate a majority rule consensus tree with Bayesian posterior probabilities. Bootstrap support values for the consensus tree reconstructed by MrBayes were generated using RAxML v. 7.2.6 (Stamatakis 2006) on the basis of 1 000 replicates under the GTR+G+I model.

RESULTS

Light microscopy

The parasite has a typical chytrid endogenous life cycle with tiny (2 µm diam) zoospores that attach to the host cell surface, retract the flagellum and encyst. After the germ tube enters (~ 2 µm diam) zoospores that attach to the host cell surface, retract the flagellum and encyst. After the germ tube enters the host the zoospore cyst enlarges; a prominent lipid globule is clearly visible at this early stage (Fig. 1a). The young sporangium has homogenous contents with few lipid globules of different size (Fig. 1b), and the mature sporangium contains zoospores, which are released through an apical pore. The inopercular sporangium is long ovoid (~ 18 x 10 µm diam) without a differentiated apical papilla (Fig. 1c). The apical pore varies in its dimensions: from narrow to as broad as the diameter of the sporangium or even broader (Fig. 1e). The delicate rhizoidal system is poorly visible, but can be estimated as weakly branched with short rhizoids emerging from a slender main axis (Fig. 1d, e). According to this description the fungus could be identified as Rhizophyllum mammillatum (A. Braun) A. Fish. (1892) or, less likely, R. melosirae (Sparrow 1960, Letcher & Powell 2012), and therefore it was identified as R. mammillatum (Mamkaeva et al. 2006).

Molecular phylogeny

The rDNA sequences of strain x-51 occupy an isolated position in the tree (Fig. 2); its closest relatives are three uncultured clones: one from Lake Koronia in Greece (clone kor-110904_17), another from snow-covered soil in alpine Austria (clone lin1-34), and one more from a hyposaline soda lake in Kenya, East Africa (Genitsaris et al. 2009, Kuhnert et al. 2012, Luo et al. 2013). Together these sequences form a new phylogenetic group. Among the described organisms, the closest relative of this group is Mesochytrium penetrans, which was classified in the Chytridiomycetes incertae sedis (Karpov et al. 2010). Mesochytrium penetrans is the only described species of a diverse group of uncultured fungi from soil, freshwater and hydrobiont gut samples collected from temperate zone of Eurasia and North America (Table 2). This group was recognised earlier as an order-level ‘Novel clade I’ within the Chytridiomycetes (Lefèvre et al. 2008, Jobard et al. 2012). Another name for ‘clade I’ is ‘snow chytrids’ (‘Snow Clade I’ or SC1) according to Naff et al. (2013). The rDNA data places the clade uniting x-51 and the ‘clade I’ (Lefèvre et al. 2008) sister to Lobulomyctales (Simmons et al. 2009), albeit with relatively low support (Fig. 2). The distances inside the clusters of OTUs that contain x-51 and M. penetrans on the rDNA tree are comparable to the distances inside the established orders of Chytridiomycota, and distances between the OTUs in these clusters and the members of Lobulomyctales are no less than the distances between different orders of Chytridiomycota (Fig. 2).

Zoospore ultrastructure

The spherical zoospore has a posterior flagellum and sometimes produces short anterior filopodia (Fig. 3c). A core of aggregated ribosomes is located in the posterior part of the cell. The ribosomal aggregation is relatively small and does not have surrounding endoplasmic reticulum (Fig. 3, 4). The ribosomes fill the space between the flagellar base and the nucleus and have no connection with nucleus, mitochondria or other membrane bounded organelles.

Several mitochondria with flat cristae reside at the cell periphery. A nearly central nucleus associates with anteriorly adpressed narrow microbody and a single large lipid globule anteriorly attached to the microbody (Fig. 3a). The anterior flat side of the lipid globule is bounded by a prominent fenestrated cisterna (rumposome) oriented to the cell exterior. Thus, the microbody-lipid globule complex (MLC) contains a single microbody enveloping a large anterior lipid globule with fenestrated cisterna.

Endoplasmic reticulum cisternae are rare and are normally found at the cell periphery. A vesicle rich zone occupies an area from one side of the ribosomal core extending from the nucleus to the centriole (Fig. 3a, c). Several small vesicles with electron-opaque contents (dense bodies) are present in the cytoplasm of the anterior part of the cell.

Kinetid structure

The structure of the flagellar apparatus was investigated with serial sections of six released zoospores. The kinetosome and centriole are embedded in the ribosomal core (Fig. 3d, e, 4). The kinetosome is c. 400 nm long and composed of microtubular doublets (not triplets) with developed transitional fibers (props) (Fig. 4b–d). The flagellar transition zone is simple without transversal plate, but with a slightly inward curved diaphragm at the distal end of kinetosome (Fig. 4g). Two thin lines parallel to the peripheral microtubular doublets are present above the diaphragm, and seem to correspond to the spiral fiber, or cylinder (Fig 4g). The centriole is about 100 nm long and lies at an angle of c. 30° to the kinetosome (Fig. 4e, f). The kinetosome is connected to the centriole by a broad fibrillar bridge composed of at least three thick connectors (Fig. 4d). The longest middle connector passes through the bottom of kinetosome to the side of centriole. The structure of interconnecting bridge seems to be an unstable character. The bridge looks rather broad and prominent, connecting the sides of kinetosome and centriole at the longitudinal sections (Fig. 4e, f), but it is not visible at the corresponding transverse sections (Fig. 4b–d). Approximately 1/3 of all serial sections had the broad bridge connecting the sides of kinetosome and centriole and in 2/3 of the series the bridge connects the bottom of kinetosome to the side of centriole. The diagram (Fig. 5a) shows the more common state.

The kinetosome produces at least two microtubular roots. The anterior root consists of two microtubules and passes laterally in the direction of the lipid globule crossing the surface of fenestrated cisterna (Fig. 3a, 5). The posterior root is much shorter, composed of one or two microtubules and is directed right about the anterior root (Fig. 4a–d). Their origin is not clear: anterior root emerges in the vicinity of kinetosome, and posterior root appears somewhere in between the kinetosome and the centriole.

One more kinetosomal derivate, a spur, lies close to the outer surface of the kinetosome on the side opposite the centriole.
Fig. 3 General ultrastructure of *Gromochytrium mamkaevae* (x-51 CALU) zoospore. — a. General disposition of nucleus and other organelles at LS; b. tangential section of fenestrated cisterna crossed by anterior microtubular root; c. pseudopodia at cell anterior; d, e. two consecutive sections of the kinetid. — Abbreviations: ar = anterior microtubular root; c = centriole; d = kinetosome diaphragm; db = dense bodies; fc = fenestrated cisterna; k = kinetosome; l = lipid globule; m = mitochondrion; mb = microbody; n = nucleus; ps = pseudopodia; rc = ribosomal core; tf = transitional fibers (prope); vz = vesicular zone. — Scale bar on E: a = 300 nm; b, c = 400 nm; d, e = 200 nm.

The spur is thin and short, projecting about 70–100 nm from the kinetosome into the ribosomal core (Fig. 4f).

A general scheme of zoospore ultrastructure is illustrated in Fig. 5a.

**DISCUSSION**

According to the morphology of strain x-51 at different life cycle stages it belongs to the genus *Rhizophydium* sensu Sparrow (1960). It has a simple thallus composed of inoperculate monocentric epibiotic elongated sporangium. It bears a single slightly branching rhizoidal axis. Judging by the shape of the sporangium and its dimensions this strain could be *Rh. mammillatum*, however, contrary to *Rh. mammillatum*, the sporangium of x-51 has no papilla. Our study has shown that zoospore ultrastructure of x-51 differs cardinally from that of *Rhizophydium* and other members of *Rhizophydiales* (Letcher et al. 2006, 2008). The order *Rhizophydiales* has 18 zoospore types that are rather different from each other, but none have a posterior ribosomal core without delimiting ER and mitochondrion separated from MLC as in x-51. The MLC structure in the zoospore of x-51 has similarities with that of the recently established *Gorgonomyces*, which unlike other rhizophydialas has a close association of nucleus with microbody and lipid globule (Letcher et al. 2008), but in all other respects the zoospore of *Gorgonomyces* is different.

Molecular phylogeny places the strain x-51 far from *Rhizophydiales*, as a sister to ‘clade I’ – a cluster containing many environmental sequences of the *Chytridiomycetes* (Lefèvre et al. 2008, Jobard et al. 2012) besides a formally described species *Mesochytrium penetrans*, which was earlier shown to have a rather isolated position among the *Chytridiomycetes* (Karpov et al. 2010). The features that distinguish *Mesochytrium* are the partial penetration of the host cell by the sporangium and a zoospore with a unique ultrastructural organization.

Thus, we have to compare the zoospore structure of strain x-51 with that of *M. penetrans*. Two strains of *M. penetrans* (x-10 and x-46 CALU) were studied by electron microscopy, and 18S and 28S rRNA genes were sequenced for x-10 (Gromov et al. 2000, Karpov et al. 2010). Their general organization differs from that of x-51; unlike x-51 the *M. penetrans* has no ribosomal aggregation, its mitochondrion with MLC is enclosed by ER, a fenestrated cisterna faces the posterior of the cell, and a vacuole is present (Fig. 5b). At the same time, some morphological characters are similar in x-51 and x-10; both have small dense vesicles in the cytoplasm, which are common for the *Chytridiomycetes*; the kinetosomes lie at the same angle to each other and the flagellar transition zones contain a spiral...
The kinetid structure also has some differences; x-51 has two microtubular roots which are absent in *M. penetrans*, a bridge in x-51 connects the bottom of kinetosome to the lateral surface of the centriole, not the lateral surfaces of kinetosome and centriole as in *M. penetrans* and the kinetosome of x-51 is composed of microtubular doublets. The spur structure and shape are also different; in x-51 the spur is inconspicuous and straight and in *M. penetrans* it is long and curved enclosing both the kinetosome and the centriole (Fig. 5).

We conclude, that the overall organization and kinetid structure of the zoospores of *M. penetrans* and x-51 differ considerably. According to the modern paradigm stemming from D. Barr’s studies (e.g. Barr 1978, Barr & Hadland-Hartmann 1978, Powell 1978, Longcore 1995, 1996, Letcher et al. 2006, 2008, Simmons 2009), their zoospores certainly have enough peculiarities to separate them at the taxonomic level of order. Moreover, their zoospores can be regarded as having a unique organization among the chytridiomycetes. We have already shown this for *M. penetrans* (Karpov et al. 2010). For the strain x-51 the unique characters are: the posterior core of ribosomes is not bounded by ER membranes, mitochondria are not associated with MLC, and a bridge connects the bottom of kinetosome to centriole.

The nearest branch to the x-51/*Meshochytrium* cluster is the order Lobulomycetales (Fig. 2), a group that was recently established on the basis of SSU and partial LSU gene phylogeny and ultrastructural analysis of zoospores (Simmons et al. 2009). In the previous study, the 18S and 28S sequences of *M. penetrans* (strain x-10 CALU) also placed this strain as a sister lineage to Lobulomycetales but with a rather low support (Karpov et al. 2010). ‘Snow chytrids’ were also suggested as a deep divergent branch sister to Lobulomycetales (Naff et al. 2013). In the present study, the increased taxon sampling through the addition of environmental sequences results in better support for the sister group position of the x-51/*Meshochytrium* cluster relative to Lobulomycetales (Fig. 2).

Zoospores of Lobulomycetales (*Lobulomyces angularis*, *Clydaea vesicula* and *Maunachytrium keaense*) differ from those of x-51 and *Meshochytrium* in a number of ways: kinetids of lobulomyces have parallel centrioles, an electron-opaque plug is present in the flagellar transition zone, and no spur or flagellar roots are found; the ribosomal core in Lobulomyces is bounded by the ER, and the vacuole and 1–2 lipid globules lie posteriorly (Simmons et al. 2009). The presence of a rumposome (fenestrated cisterna) was noted in the text, but not shown in the pictures of the above cited article, therefore its precise position is unknown for Lobulomycetales.

Thus, our morphological data strongly support an isolated position of x-51/*Meshochytrium* cluster on the phylogenetic tree.

**Taxonomy**

An isolated position of *Mesochytrium* was shown by 18S+28S rRNA gene phylogeny and zoospore morphology of two strains: x-46 CALU (Gromov et al. 2000) and x-10 (Karpov et al. 2010), and recapitulated by molecular phylogenetic analysis in the present paper. The sequence of *M. penetrans* clusters with a large number of environmental sequences forming a clear monophyletic branch with good statistical support (Fig. 2). Molecular phylogenetic analysis of this genus does not reveal family or ordinal level affinity of *M. penetrans*, consequently in the previous paper we referred to it as *incertae sedis* (Karpov et al. 2010). Here we have a better resolved tree with a number of environmental sequences and a new neighbour of this branch that includes isolate x-51. Because of the molecular phylogeny of *M. penetrans* and CALU x-51, together with each having a unique organisation of zoospores, we establish new orders and families for both, plus a new genus and species for CALU x-51.

**Gromochytriales** Karpov & Aleoshin, ord. nov. — MycoBank MB805305

Zoospore with posterior ribosomal aggregation not bounded by endoplasmic reticulum. Microbody-lipid complex adpressed to the nucleus and containing a single microbody enveloping a large anterior lipid globule with anteriorly oriented fenestrated cisterna. Several mitochondria are separated from MLC. Small dense bodies present in peripheral cytoplasm. Kinetosome and centriole embedded in posterior side of the ribosomal core. Flagellar transition zone contains a spiral fiber, or a cylinder. Centriole at an angle of c. 30° to kinetosome; bottom of kinetosome connected by a broad fibrillar bridge to centriole. Anterior and posterior microtubular roots and a short straight spur associated with kinetosome.
Gromochytriaceae Karpov & Aleoshin, fam. nov. — MycoBank MB805306

Type genus. Gromochytrium Karpov & Aleoshin.

Description as for Gromochytriales: simple thallus with inoperculate, monocentric, epibiotic sporangium having endogenous development and single slightly branching rhizoidal axis.

Gromochytrium Karpov & Aleoshin, gen. nov. — MycoBank MB805307

Type species. Gromochytrium mamkaevae Karpov & Aleoshin.

Simple thallus with inoperculate, monocentric, epibiotic sporangium having endogenous development and single slightly branching rhizoidal axis. Zoospore with posterior ribosomal aggregation unbounded by endoplasmic reticulum. Microbody-lipid-complex adpressed to the nucleus and contains a single microbody enveloping a large anterior lipid globule with anteriorly oriented fenestrated cisterna. Several mitochondria are separated from MLC. Small dense bodies present in peripheral cytoplasm. Kinetosome and centriole embedded in posterior side of the ribosomal core. Flagellar transition zone contains a spiral fiber, or a cylinder. Centriole at an angle of c. 30° to kinetosome; ribosomes dispersed through the cytoplasm; mitochondrion and MLC surrounded by rough endoplasmic reticulum.

Gromochytrium mamkaevae Karpov & Aleoshin, sp. nov. — MycoBank MB805308, GenBank KF868842; Fig. 1–5a

Etymology. Genus named in honour of Boris V. Gromov, a prominent Russian microbiologist, and species named in honour of his spouse, colleague and co-author, Kira A. Mamkaeva.

Mature inoperculate epibiotic sporangium long ovoid (18 × 10 µm) without papillae. Zoospores released through apical pore. Delicate, weakly branched rhizoidal system with short rhizoids emerging from a slender main axis. Zoospores 2 µm diam with single lipid globule.

Mesochytriales Karpov & Aleoshin, ord. nov. — MycoBank MB805303

Zoospores with unique ultrastructural organisation; centriole at an angle of c. 30° to kinetosome; ribosomes dispersed through the cytoplasm; mitochondrion and MLC surrounded by rough endoplasmic reticulum.

Mesochytriales Karpov & Aleoshin, fam. nov. — MycoBank MB805304

Description as for Mesochytriales. Sporangium inoperculate, monocentric, epibiotic, endogenous, semi absorbed by host cell.

Mesochytrium B.V. Gromov, Mamkaeva & Pljusch. Nova Hedwigia 71: 159. 2000, emend. Karpov

Type species. Mesochytrium penetrans B.V. Gromov, Mamkaeva & Pljusch.

Sporangium sessile, partially penetrating host cell. Delicate branched rhizoids emerge near the sporangial base. Zoospores spherical to oval with single lipid globule and dispersed ribosomes. Microbody-lipid-complex composed of a single mitochondrion and a single lipid globule partially covered with microbody and posterior fenestrated cisterna; centriole with veil at an angle of c. 30° to kinetosome, the two being connected by a broad, dense fibrillar bridge. Flagellar transition zone contains a spiral fiber. Resting spore endobiotic, spherical with smooth thick wall.

Mesochytrium penetrans B.V. Gromov, Mamkaeva & Pljusch. Nova Hedwigia 71: 159. 2000, emend. Karpov

Sporangium pyriform 10–14 × 6–7.5 µm with thin smooth wall and apical papilla. Zoospores spherical 2–2.5 µm diam with...
a 5–14 µm long flagellum. Parasite of green alga Chlorococcum minutum.

Specimen examined. Small lake Pryazha in Karelia, parasite of Chlorococcum minutum. Holotype CALU x-46.

**Diversity and abundance of Mesochytriales and Gromochytriales in nature**

The fact that *Mesochytrium penetrand* and *Gromochytrium mamkaevae* have thus far not been found during environmental DNA studies indicates that these species are not prevalent in the sampled ecosystems, at least not during the time of sampling. This fact emphasizes the incompleteness of our current knowledge of chytrid diversity and the importance of collecting new samples for exhaustive description of fungal diversity. At the same time, some of the underscribed species from the *Mesochytriales* clade that are represented by almost identical clones were repeatedly recovered in several environmental samples. Such clusters are formed by clones shown on Fig. 2 as small black triangles: one is presented by PFF5SP2005, PA2009C3, PA2009B6, PA2009D8 (Lefèvre et al. 2008, Monchy et al. 2011), another by PFF5SP2005, PFDF6SP2005 (3’-end), Pa2007C10 and 20 clones are from Lake Bourget (Lefèvre et al. 2008, Lepère et al. 2008, Jobard et al. 2012), collected during the course of several years from Mont-Cenis in France. Moreover, the clones of *Mesochytriales* from Lake Bourget form a substantial fraction of all fungal clones in the sample, which implies that their zoospores were ubiquitous during the time of sampling. It is likely that the abundance of *Mesochytriales* may vary by season. Ribosomal DNA clones of *Mesochytriales* accounted for about 50 % of the number of fungal rDNA clones from Lake Pavin (France) in spring and summer seasons (Lefèvre et al. 2008, Jobard et al. 2012), but they were not detected there in autumn (Lefèvre et al. 2007). Similarly to *M. penetrand* and *G. mamkaevae*, these clones probably can be attributed to parasites of algae. The diversity and abundance of rDNA clones from undescribed members in these environmental samples suggest that members of the *Mesochytriales* may play an important role as regulators of phytoplankton populations (Lefèvre et al. 2008, Lepère et al. 2008, Genitsaris et al. 2009, Monchy et al. 2011).

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