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Report

Mikrocytids Are a Broadly Distributed and Divergent Radiation of Parasites in Aquatic Invertebrates

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Summary

Microcell parasites have independently evolved in several eukaryotic lineages and are increasingly recognized as important and emerging pathogens of diverse hosts, including species of economic importance subject to international legislation concerning the trading of aquatic animals [1–3]. The microcell *Mikrocytos mackini* causes Denman Island disease of oysters and represents one of the most genetically divergent eukaryotes known. *Mikrocytos* has remained an isolated lineage with a limited distribution. We investigated two emerging diseases of juvenile crabs and oysters from the UK using massively parallel sequencing and targeted primer approaches to reveal that their causative agents are highly divergent lineages related to *M. mackini* (*Paramikrocytos canceri* n. gen. et n. sp. and *M. mimicus* sp. nov., respectively). We demonstrate a major new globally distributed parasite radiation (Mikrocytida ord. nov.) with phylogenetic affinities to the commercially important haplosporidian parasites of invertebrates. Mikrocytids have eluded detection because of their small size, intracellular habit, and extreme sequence divergence. *P. canceri* was frequently detected in a range of shoreline invertebrates, demonstrating that these newly recognized parasites are in fact common, diverse, and widespread and should be considered when assessing the risks of aquaculture activities, invasive species spread, and movements of ballast water and sediments with associated invertebrates.

Results and Discussion

In 2011 a "haplosporidian-like" microcell infection was reported at high prevalence in juvenile European edible crab *Cancer pagurus* in Weymouth, UK [4], and recently a similar parasite was reported in the same host from south Wales, UK [5]. The development of unicellular stages into plasmodia and the presence of cytoplasmic inclusions suggested an affinity with asporous haplosporidians of decapod crustaceans [2, 6] (Figure 1). However, our phylogenetic analyses robustly grouped the parasite as a sister to *Mikrocytos mackini* (Figures 2 and 3), which was previously known as an orphan lineage with one very close relative, *Mikrocytos* sp. BC (HM563061) [7], very recently described as *M. boweri* (KF297352/3) (C.L. Abbott, personal communication). Our finding of a highly divergent relative commonly infecting a commercially significant crustacean was unexpected as *Mikrocytos* was known only from bivalve mollusks.

The novel microcell induces hypertrophy of the antennal gland and bladder of reproductively immature crabs (Figure 1A). Infections comprise unicellular and plasmodial parasite stages within the epithelial cells of infected glandular tubules (Figures 1B–1D). As all attempts to sequence the small subunit (SSU) rDNA from this microcell using existing pathogen assays and an extensive range of PCR primer combinations failed, a high-throughput next-generation sequencing (NGS) approach (Illumina MiSeq) was used to sequence total DNA extracted from a heavily infected juvenile crab antennal gland, from which SSU sequences of only a decapod and a highly divergent eukaryote were recovered. The complete parasite SSU-ITS1-5.8S-ITS2-LSU (LSU, large subunit) rDNA was assembled and verified by Sanger sequencing. The SSU rDNA sequence was only 68% similar to that of *M. mackini* and was more dissimilar to all other eukaryotes, a level of sequence dissimilarity equivalent to phylum-level differences or above in many other eukaryotic lineages. Lineage-specific in situ hybridization (ISH) was then used to demonstrate that this sequence type derived from the observed microcells within crab tissues (Figure 1B). We thus describe a new taxon, *Paramikrocytos canceri* n. gen. et n. sp., on the basis of its phylogenetic affinity to (but with high divergence from) the genus *Mikrocytos*, different host range, ability to form plasmodia, and relative abundance of putative mitochondria-related organelles (MROs) (Figures 1F and 1G; see the Supplemental Results, Section 1, available online). The putative MROs in *P. canceri* are double-membrane-bounded organelles of ~0.5 μm diameter. Their presence contrasts with ultrastructural observations in *M. mackini*, in which obvious MROs were not recorded [8], even though genomic signatures suggest their presence [9]. Further work is required to determine the identity of MRO-like organelles in *P. canceri*.

Paramikrocytos canceri Is Frequently Detected in a Wide Range of Invertebrates

After the discovery that *M. mackini* and *P. canceri* are sister lineages, we designed a mikrocytid PCR assay and screened 511 marine, freshwater, and soil environmental DNA/cDNA (eDNA) samples from the UK, continental Europe, South Africa, Panama, and Borneo and 425 organismal samples from eight invertebrate phyla for mikrocytid sequences. The most intensive sampling was conducted at two sites, one with high *P. canceri* prevalence in *C. pagurus* (a rocky shore at Newton's Cove in Dorset, UK [35 ppt salinity; 50°34′N, 2°22′W]), and a nearby brackish site with few *C. pagurus*

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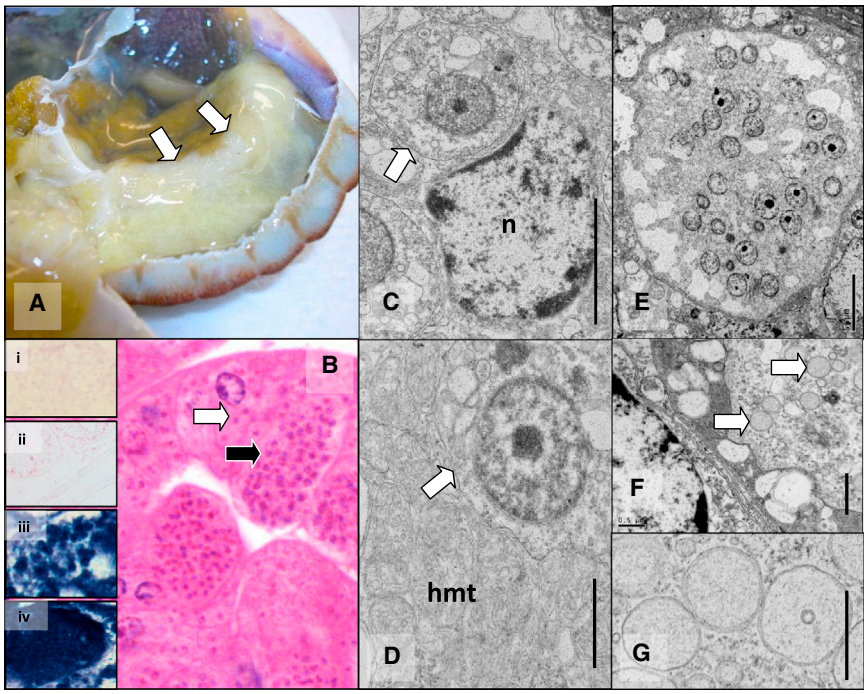

Figure 1. *Paramikrocytos canceri* Infection of the Edible Crab, *Cancer pagurus*

Hypertrophy of the antennal gland (A, arrows) is associated with colonization of the epithelium by masses of uninucleate (B, white arrow) and plasmodial (B, black arrow) life stages of the parasite. Both life stages are detected using in situ hybridization (B, inset: i, no probe; ii, uninfected tissue with probe; iii, uninucleate form with probe; iii, plasmodium with probe). Uninucleate life stages occur in direct contact with the host cell cytoplasm and can displace organelles (see nucleus, n, in C) and are often closely opposed to host mitochondria (hmt in D, opposed to parasite, arrow). Plasmodial life stages (E) containing many nuclei and an abundance of putative mitochondria-related organelles are shown (arrows in F and higher power in G). Scale bars represent C 3 μm (C), 1 μm (D), 5 μm (E), 1 μm (F) and 500 nm (G). Additional figures are provided in the Supplemental Results, Section 1, Figure S1.

(Fleet Lagoon [<10–30 ppt salinity; 50°35′N, 2°28′W]). DNA was also extracted from 0.45-μm-filtered sterile artificial seawater (ASW), in which candidate hosts had been incubated.

A major finding was that *P. canceri* occurs in association with many invertebrates other than *C. pagurus*. *P. canceri* was found frequently in mollusks, decapods, and annelids from three sites on the UK south coast. Fifteen of 56 gastropod samples (*Gibbula* and *Littorina* spp.) from two sites in Weymouth were PCR positive, as were incubation trials using the shore crab *Carcinus maenas*, the mussel *Mytilus edulis*, and tubificid worms. Detection of *P. canceri* in invertebrates varied, but was frequent in *Carcinus maenas* (46%; n = 24). An aeolid nudibranch mollusk (n = 2) also tested positive for *P. canceri* (Fleet, Weymouth). No *P. canceri* was detected in zoeae and megalopae of *C. pagurus* or in other decapod larvae in a plankton sample from the eastern English Channel coast, suggesting that infection of *C. pagurus* by *P. canceri* may occur after the larvae have settled in the littoral zone. The presence of *P. canceri* in littoral habitats close to the plankton tow site has not yet been confirmed.

In contrast to the invertebrate tissue and incubation water samples, *P. canceri* was not detected in any environmental sample (eDNAs). This was surprising, given (1) the high prevalence of parasite infection in crabs at the sampling site, (2) the large volume of the planktonic samples (200 l), and (3) that the uninucleate and plasmodial forms of *P. canceri* are excreted copiously in the urine via the crabs' antennal glands [4]. The reasons for this nondetection are unknown; perhaps the parasite cells rely on trophic transmission and degrade soon after excretion from the antennal gland. The wide range of invertebrates in which *P. canceri* was detected would certainly support such a trophic transmission route via incidental consumption during grazing and detritus feeding. In any case, this pattern is in clear contrast to haplosporidian invertebrate parasite SSU types from the same sites, which amplify from many of the same eDNA samples using very similar methods [10].

Mikrocytid Diversity Revealed by PCR Screening

The mikrocytid PCR assay also revealed a divergent radiation of other novel mikrocytid lineages. One SSU type from filtered water from the brackish Fleet Lagoon (Weymouth, UK; marked "a" in Figure 2) was highly distinct from other known and novel mikrocytids. Two further related SSU types originated from the slightly brackish lagoon of Rondevlei Nature Reserve (Cape Town, South Africa) and from sediment associated with mangroves near Sandakan, Borneo. These are sister to two further novel lineages from eDNA samples, one from a freshwater forest stream in Panama and the other from a freshwater lake near Reading, UK. This clade (marked "b" in Figure 2) therefore comprises separate lineages from freshwater and brackish environments, providing the first evidence that mikrocytids have radiated in a wide diversity of aquatic habitats. Other novel SSU types were detected only in DNA extracted from organisms, one from the trochid gastropod *Gibbula umbilicalis*, another from *Cancer pagurus* incubation trials, and another from several individuals of tidepool shrimp from the Pembrokeshire coast, Wales (marked "c" in Figure 2).

During the course of our study, a novel pathogen was received for investigation by the UK Centre for Environment, Fisheries and Aquaculture Science from an intertidal Pacific oyster (*Crassostrea gigas*) farm on the North Norfolk coast (Brancaster), UK. The causative agent was initially suspected to be the first UK record of *M. mackini* due to its superficially similar gross pathology (green pustules within the adductor muscles) and morphology. However, histology and transmission electron microscopy (TEM) analyses revealed distinct pathological responses and ultrastructure (Supplemental Results, Section 2). SSU phylogenies robustly grouped this parasite in the same clade as *M. mackini* and *M. boweri*, but the sequence was only 79% similar to *M. mackini* (Figure 2). The standard assays for *M. mackini* listed by the World Organization for Animal Health (OIE) did not detect it in infected oyster tissue. This pathogen is the first incidence of a *Mikrocytos* infection in Pacific oysters cultured in Europe and represents a new potential threat to the commercial mollusk industry. We describe it here as *Microcytos mimicus* n. sp. (Supplemental Results, Section 2).

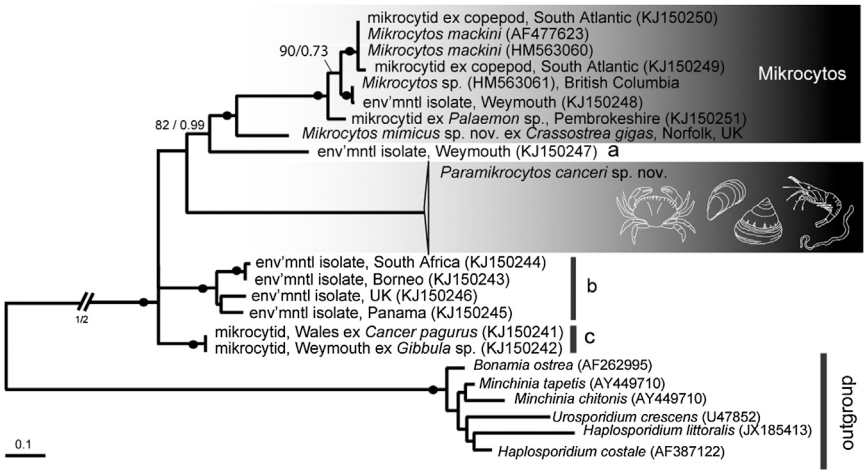

Figure 2. Novel Hosts and Geographic Range of Microcytids Revealed by Targeted Lineage-Specific PCR of the Approximately 480 bp Variable Regions V5 to V7 of the SSU rDNA

Letters a–c indicate novel mikrocytid lineages detected in this study, and images indicate the range of hosts in which *P. canceri* was detected by PCR. The full range of samples tested is provided in the Supplemental Results, Section 3, Table S1. The maximum-likelihood topology is shown. Dark circles indicate bootstrap support values >90 and Bayesian posterior probabilities >99, and intermediate support values are mapped on the tree and branches with bootstrap support values <80 and/or posterior probabilities <90 were collapsed.

M. mackini itself, which causes disease and mortalities in several economically important oysters [11], has so far only been found along the west coast of North America (C.L. Abbott, personal communication). A very closely related lineage referred to as "*Mikrocytos* sp. BC" (HM563061), originally found in Pacific oysters (*Crassostrea gigas*) [7], has now also been found causing pathology in Olympia oysters (*Ostrea lurida*) in British Columbia, Canada, from which it is described as *M. boweri* (C.L. Abbott, personal communication). Mikrocytids with an identical SSU variable region 4 (V4) sequence to *M. boweri* have been detected in edible oysters (*O. edulis*) in Nova Scotia, Canada (after transport and quarantine in France) [12]; in *C. gigas* from the north coast of the Yellow Sea, China [13]; and in *Donax trunculus* in France [14]. Other *Mikrocytos*-like parasites have been detected in *Donax trunculus* in France [14] and *O. lurida* in San Francisco Bay, California [15]. We detected the *M. mackini* SSU type (AF477623, HM563060) only once in our study, from copepods sampled from a gyre in the southern Atlantic Ocean (Supplemental Results, Section 3, Table S1) and a sequence type very similar to *M. boweri* from filtered water samples from Newton's Cove (Figure 2). It is therefore clear that environmental and organismal samples provide complementary information, suggesting that wider and more diverse sampling would reveal higher diversity.

Mikrocytids Are Not Represented in Public Sequence Databases

Environmental sequencing approaches are facilitating a massive increase in knowledge of microbial eukaryote diversity. Despite this, no sequences similar to any mikrocytid lineage were found in any publicly available NGS data sets on Camera [16] or on NCBI GenBank portals. Their absence from even large-scale amplicon data sets can be explained by their extremely divergent SSU rDNA, eluding "universal" eukaryotic PCR primers. Therefore the use of targeted primers on host-associated material clearly has huge potential for expanding our knowledge of exceptional and enigmatic groups. In this study, PCR-free metagenomic shotgun sequencing was initially required to determine the SSU rDNA sequences of *P. canceri* from infected crab tissue. Mikrocytids have been overlooked because of their very small size, intracellular habit, lack of spore stages (all conspiring toward faint staining via histology), and extreme sequence divergence [9, 12]. Therefore, highly targeted PCR and PCR-free approaches are likely to become powerful tools offering insights into the ecology of divergent lineages currently not afforded by traditional parasitological methods.

The Phylogenetic Position of Mikrocytids

The extreme divergence of mikrocytids precluded molecular phylogenetic placement until a recent 119-gene phylogenomic analysis [9] showed that *M. mackini* most likely groups within the nonfilosan Cercozoa, possibly within a clade including *Gromia* and *Filoreta* (incorrectly "*Corallomyxa*" [17]). However, *M. mackini* was very long branched on the multigene tree, and its specific placement within nonfilosan Cercozoa was unresolved [9]. We judged that a similar analysis including *Paramikrocytos* would not be more informative and that a better way to increase phylogenetic resolution was to increase taxon sampling in this region of the tree. We chose four genes (SSU and LSU rDNA, *hsp90* and *β-tubulin*, assembled from MiSeq parasite-host metagenomes or generated using lineage-specific primers), which allowed us to add new gene data for some related lineages: *Filoreta marina* (LSU rDNA), the plasmodiophorid *Spongospora* sp. (SSU and LSU rDNA), and *Haplosporidium littoralis* (SSU and LSU rDNA, *hsp90*, and *β-tubulin*).

Our phylogenetic analyses group mikrocytids as sister to Haplosporida (represented by *H. littoralis* and *Bonamia ostreae*) within a clade also including *Gromia* and *Filoreta* (Figure 3). This is consistent with morphological analyses, which show more affinities with haplosporidia than any other microcell taxa [2–4].

Bayesian posterior probabilities for these groupings are maximal, but bootstrap support is low (64% and 53%, respectively) and only marginally higher when the relatively divergent sequence of the foraminiferan is removed (75% and 83%, respectively). Given the high sequence divergence of mikrocytids (and to some extent haplosporidia), our main concern was to ascertain that the tree topology we obtained was not the result of long-branch attraction artifacts. The four genes analyzed (plus polyubiquitin) were therefore screened for known and novel clade-specific genetic signatures, which can provide robust phylogenetic signal without calculating molecular phylogenies. Such signatures are considered highly unlikely to occur several times independently in unrelated lineages and are therefore a powerful means of confirming evolutionary relationships when statistical support from molecular phylogenies is weak or long-branch attraction

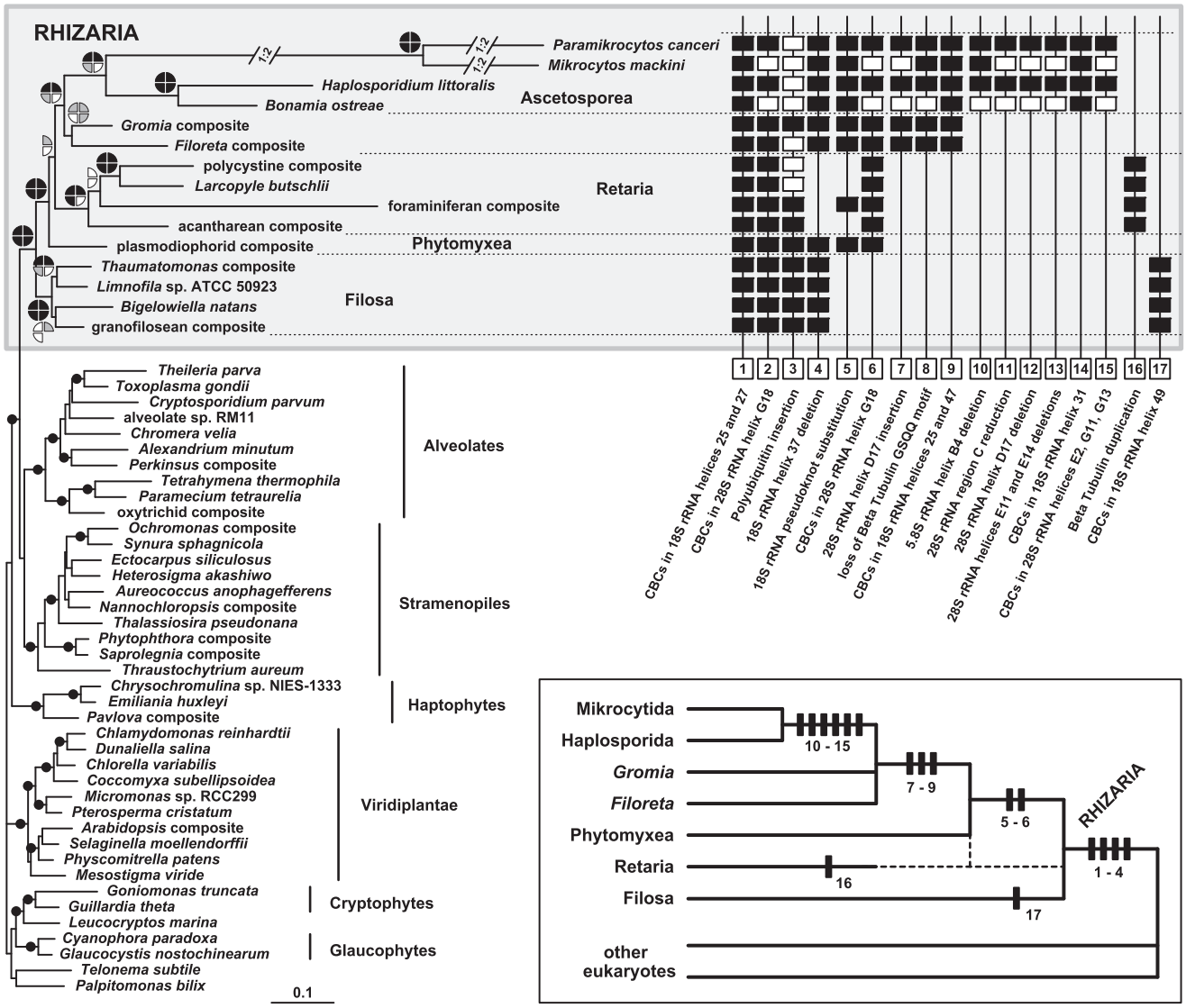

Figure 3. Phylogenetic Placement of Mikrocytids within Rhizaria, Based on Concatenated Sequences of SSU and LSU rDNA, *Hsp90*, and *β-tubulin*, Highlighting Known and Newly Identified clade-Specific Genetic Signatures that Corroborate the Tree Topology

The maximum-likelihood tree is shown. All branch lengths are drawn to scale, except in Mikrocytida, where they were reduced to half of their actual size. Within Rhizaria, circles at internal nodes indicate Bayesian posterior probabilities (upper half) and ML bootstrap support values after 1,000 replicates (lower half) for analyses performed without (left half) and with (right half) the foraminiferan composite sequence. The following color code is used: black, ≥0.99 or 95%; gray, ≥0.90 or 75%; and white, ≥0.75 or 50%, respectively. Outside Rhizaria, only nodes with support values ≥0.99 or 95% are highlighted (black dots). Seventeen genetic signatures supporting internal relationships in Rhizaria are listed on the left (CBCs, complementary base changes; SSU and LSU rDNA helix numbering are explained in the Supplemental Results, Section 4). Black rectangles highlight taxa in which the signature has been found, and white rectangles indicate that the signature is expected to be present but the gene sequence is missing for that taxon. The insert shows a schematic summary of relationships within Rhizaria as evidenced by our phylogenetic analyses and the seventeen genetic signatures. The exact position of Retaria remains uncertain due to the absence of some Rhizaria and nonfilosan signatures in radiolarians and foraminiferans (dashed lines), but it does not pertain to the position of Mikrocytida as a sister taxon to Haplosporida. The sequence signature details are provided in the Supplemental Results, Section 4.

artifacts are suspected [18]. Not all individual substitutions were considered for SSU and LSU rDNA (but note that these contribute statistical support to the relationships shown in Figure 3). We mostly focused on sequence signatures affecting a whole stem or hairpin or on indels that could not be included in the sequence mask used for the phylogenetic analyses. These genetic signatures are summarized in Figure 3 and detailed in the Supplemental Results, Section 4; they are concordant with the concatenated gene phylogeny. In particular, they support the existence of a clade containing *Filoreta*, *Gromia*, mikrocytids and haplosporidia, with the latter two as sisters.

We group *M. mackini*, *M. mimicus*, and *P. canceri* in a novel family (Mikrocytiidae) and order (Mikrocytida), and our phylogenetic analyses support their inclusion in class Ascetosporea together with Haplosporida.

Conclusions

Evidence of ten distinct mikrocytid lineages including at least two genera associated with three invertebrate phyla (Arthropoda, Annelida, and Mollusca) on four continents and in both hemispheres greatly increases the known diversity of mikrocytids. Although the mikrocytids are mostly marine, we reveal a

new radiation in freshwater and brackish habitats. A more extensive and diverse sampling, particularly by PCR-based methods, is likely to reveal even greater diversity and distribution of these parasites. To facilitate future reference, we define a novel order (Mikrocytida) and family (Mikrocytiidae) to include the lineages discovered here. Highly targeted PCR primer approaches for disease monitoring and risk assessment are areas of growing interest [10, 19, 20], and we show here that this approach can also reveal novel diversity and distribution patterns of endoparasites, including the identification of putative hosts and vectors.

Experimental Procedures

Histological Examination

Thirty juvenile edible crabs (*Cancer pagurus,* carapace width <70 mm) were collected each month, between May 2008 and April 2009, from the shoreline at Newton's Cove, Weymouth, UK (50°34′N, 2°22′W). All sampled crabs appeared externally normal. Crabs were anesthetized by chilling on ice prior to dissection of the hepatopancreas, gills, heart, midgut, antennal gland, gonad, and skeletal muscles from the abdomen, cephalothorax, and claw for histological examination. Thirty-five cultured Pacific oysters (*Crassostrea gigas*) (collected in May 2013) were prepared for histopathology by fixing a steak through the animal that encompassed the main tissue in Davidson's sea water fixative. Samples for molecular biology were preserved in molecular-grade ethanol, while samples for ultrastructural studies were preserved in gluteraldehyde. Details of methods are provided in the Supplemental Experimental Procedures.

Identification of *P. canceri* via NGS Sequencing and ISH

As PCR consistently failed to amplify parasite from the heavily infected *C. pagurus* antennal gland (~80% of the tissue estimated to be parasite), 50 ng of genomic DNA was prepared using Nextera method (Illumina) and sequenced for 250 cycles in each direction on a MiSeq platform (Illumina) at the Natural History Museum, London. The same approach was used for a genomic DNA extraction from a *Haplosporidium littoralis*-infected *Carcinus maenas* hepatopancreas, tagged and sequenced on 50% of a MiSeq run. Blastn searches against GenBank were used to identify SSU rDNA sequences, allowing identification of both the parasite species.

Blastx searches using ~500 bp previously sequenced seeds of target genes from *M. mackini* or haplosporidian representatives were used to identify *P. canceri* and *H. littoralis* gene orthologs for phylogenetic analyses. The blast results were assembled into contigs where possible and used as search seeds that were extended in mira 3.4 [21, 22]. For confirmation of the assemblies, open reading frames were identified and alignments were assessed manually. In addition, the SSU and LSU rDNA sequences of *P. canceri* assembled from the MiSeq data were confirmed via PCR amplification and Sanger sequencing. A lineage-specific ISH assay was designed to confirm that the SSU sequence obtained corresponded with the histology results (see the Supplemental Experimental Procedures).

Collection of Invertebrates and Environmental Samples

Intertidal invertebrates and associated sediment were collected from a wide variety of sites and tissues fixed in ethanol. Size fractions of planktonic material from the water column were collected by filtering large volumes of water (200 l) sequentially through a series of mesh sizes (55, 20, and 0.45 μm). Some invertebrates were roughly surface cleaned and incubated in small volumes of ASW for 1–2 hr, after which 50–100 ml of the incubation ASW was filtered onto 0.45 μm filters. A full list of samples screened is given in the Supplemental Results, Section 3, Table S1.

Environmental and Host Screens by PCR

A nested primer set mik451F/mik15111R in the first round and mik868F/mik1340R was used to screen environmental and host DNA for mikrocytid infections. The expected fragment size was 480 bp (based on *P. canceri* sequence) from the second-round PCR and spanned the variable V5 to V7 regions of the SSU rDNA. Sequences generated with the second-round primers were used in the analyses of mikrocytid diversity (Figure 2). Details of DNA extraction methods, primers, and PCR assays to detect mikrocytids are given in the Supplemental Experimental Procedures.

Taxon and Gene Choice for Phylogenetic Analyses

Alignments of SSU and LSU rDNA, *Hsp90*, and *β-tubulin* genes were constructed for available Rhizaria and suitable outgroups based on the phylogenetic position of Rhizaria. Gene choice was based on good phylogenetic signal for eukaryote phylogeny, maximal taxon sampling within nonfilosan Cercozoa, and presence of known clade-specific genetic signatures within Rhizaria. *β-tubulin* has undergone a clade-specific duplication in Retaria [23, 24], and we checked whether this duplication may be present in *P. canceri* and/or *H. littoralis.* No evidence of more than one version of *β-tubulin* was found in either taxon, but a sequence signature that supports the clade containing *Filoreta*, *Gromia*, Haplosporida, and Mikrocytida was detected. Hsp90 and SSU and LSU rDNA are generally informative genes for eukaryote phylogeny. Phylogenetic methods are detailed in the Supplemental Experimental Procedures.

Accession Numbers

Sequences generated in this study were submitted to GenBank under accession numbers KJ150289–KJ150293, KJ150241–KJ150251, and KJ572223–KJ572226.

Supplemental Information

Supplemental Information includes Supplemental Results, Supplemental Experimental Procedures, two figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2014.02.033.

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