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Comparative Morphology and Genealogical Delimitation of Cryptic Species of Sympatric Isolates of *Sphaeroforma* (Ichthyosporea, Opisthokonta)

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Of the ancient clades of unicellular relatives of the multicellular animals, ichthyosporea are among the easiest to collect, cultivate, and analyze at the population level. Once identified, species can be correlated with their animal hosts and geographical ranges. However, the spherical stages common to many ichthyosporea provide little basis for morphological species identification. This study of the genus *Sphaeroforma* is the first to apply patterns of genetic discontinuity to delimit species among any of the unicellular ‘holozoa.’ Sequences of three loci from 148 sympatric isolates, along with type cultures, provided concordant support for new species “*Sphaeroforma nootkatensis*” and “*Sphaeroforma gastrica*,” and for formally describing ‘*Pseudoperkinsus tapetis*,’ as “*Sphaeroforma tapetis*”. We document light and electron microscopic characters that distinguish the genus but not its species. “*S. tapetis*” sometimes had brief amoeboid or plasmodial motile stages and endospore release through pores. Unlike closely related *Creolimax*, *Sphaeroforma* lacked a central vacuole but had multiple peripheral nucleoli. Like distantly related eccrinales, *Sphaeroforma* cell walls had pores and a calyx. Analyses of allele frequencies in “*S. tapetis*” indicated geographical differentiation but no host specificity. Accurate molecular identification of species will increase the feasibility and reliability of further studies of *Sphaeroforma* in its natural habit.

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Introduction

Ichthyosporeans are one of six known unicellular lineages that, together with the multicellular animals and fungi, make up the eukaryote supergroup Opisthokonta (Adl et al. 2005; Cavalier-Smith 1998). Since they represent a divergent sampling

from a common ancestor of unicellular and multicellular life, the ichthyosporea are important in reconstructing the pattern of early animal evolution. For this reason, ichthyosporea are included in genome sequencing initiatives such as the BROAD Institute’s ‘Origin of Multicellular Life’ project (Ruiz-Trillo et al. 2007). We are contributing to knowledge of ichthyosporean biology by isolating new cultures and characterizing them by microscopy, molecular phylogenetics, and by analyzing their population

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genetic structure for clues about their natural history. We anticipate that our studies will complement and provide context for genome sequences.

Class Ichthyosporea was first discovered as a clade by sequencing of ribosomal RNA genes of unicellular parasites of fish and crustaceans, hence “ichthyo-” (Cavalier-Smith 1998; Ragan et al. 1996; Spanggaard et al. 1996). At the level of light microscopy, the simple, walled cells of the ichthyosporea lack diagnostic characters, and phylogenetic analysis of ribosomal RNA sequences remains the basis for adding new members to the class (Cafaro 2005; Jostensen et al. 2002; Lohr et al. 2010; Marshall et al. 2008; Marshall and Berbee 2011; Mendoza et al. 2002). Ichthyosporea are now recognized as parasites or commensals of marine and fresh water vertebrate and invertebrate hosts (Mendoza et al. 2002). Most grow as osmotrophs encased in a cell wall, but some form naked plasmodia (Marshall and Berbee 2011).

We recently cultured 148 organisms from marine invertebrate hosts. Based on habitat, morphology, and ribosomal RNA, the isolates belonged to a clade of spherical ichthyosporeans that also includes *Sphaeroforma arctica* and *Creolimax fragrantissima*. The ribosomal small subunit (*SSU-rDNA*) and internal transcribed spacer regions (*ITS*) sequences of 126 of the isolates most closely matched GenBank sequences of ‘*Pseudoperkinsus tapetis*,’ (referred to here as “*Sphaeroforma tapetis*”). In a multilocus analysis of allelic diversity, we found the 126 isolates to be part of a single species (Marshall and Berbee 2010). Although their complete life cycle is unknown, the species was haploid while in culture. In vitro it undergoes regular rounds of asexual reproduction but it may also have irregular rounds of genetic recombination in nature (Marshall and Berbee 2010). However, whether these or any other of the 148 isolates were truly conspecific with “*S. tapetis*” or other described ichthyosporea was unclear. The level of *ITS* sequence variation among the isolates led us to suspect that they encompassed cryptic species that were not yet described.

Criteria for recognition of species of microorganisms vary (Tibayrenc 2006). For unicellular microbes including algae, fungi and protozoa, biological and molecular phylogenetic species recognition criteria can have better resolving power than morphological methods (e.g. Amato et al. 2007; Kasuga et al. 1999). The ichthyosporea are not notably morphologically charismatic. Their small size and apparent simplicity, combined with within-species plasticity, as we illustrated in another ichthyosporean, *Abeoforma whisleri*

(Marshall and Berbee 2011), makes morphological species difficult to delimit. Previous work detailing morphological characters of either *S. arctica* or “*S. tapetis*” was limited to three micrographs (Figueras et al., 2000; Jostensen et al. 2002). However, based on available information, application of a morphological species concept would likely unite all 148 isolates of spherical unicells, and probably also *S. arctica* and “*S. tapetis*,” into a single species.

An alternate solution to species recognition, the genealogical concordance phylogenetic species recognition (GCPSR) (proposed by Baum and Shaw 1995, and reviewed by Taylor et al. 2000) seemed applicable to our situation. The GCPSR uses multi-locus phylogenies and takes advantage of the genetic recombination that occurs within but not usually among species. Conflict among phylogenies from different loci is assumed, reasonably, to result from recombination within a single species. Alternatively, genealogical concordance among phylogenies indicates genetic isolation among different species. Assumptions underlying application of the GCPSR are that the species are recombining, as opposed to being entirely clonal, and that they are not hybridizing (Taylor et al. 2000). Fortunately, as we earlier showed, recombination occurred among the subset of 126 of the 148 isolates that we had studied in detail (Marshall and Berbee 2010). Therefore, we expected to see a transition from complete concordance between phylogenies from different loci between species to phylogenetic conflict generated by the recombination associated with normal sexual exchange within species according to a GCPSR approach.

Accurate species delineation is important among recombining species with discontinuous genetic variation. Too broad a species definition would make impossible the recognition of correlated biologically important characters, such as cryptic morphological variation, host or substrate specificities, geographical ranges and temperature and nutrient requirements (e.g. Amato et al. 2007; de Vargas et al. 2001). Too narrow a definition would underestimate variation and fail to predict new character combinations that may appear in the next generations. In our case the 148 isolates were collected from a wide variety of hosts and from two geographically separated sites. Accurate species delimitation was a necessary prerequisite to identify host and geographic ranges and would allow for analysis of migration, niche differentiation or other possible causes for divergence among species (Reece et al. 2001).

Our isolates, unlike most other ichthyosporeans, grew readily in culture as healthy cells that

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