

COEVOLUTIONARY RELATIONSHIPS IN A NEW TRIPARTITE MARINE SYMBIOSIS

Phylogenetic affinities of the symbiotic protist *Nephromyces*

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Abstract

All molgulid ascidian tunicates (Urochordata, phylum Chordata) contain, in the lumen of the peculiar, ductless “renal sac,” an equally peculiar symbiotic protist, *Nephromyces*. From the first description of *Nephromyces* in the 1870’s, the phylogenetic affinities, even existence, of this taxon has been a matter of debate. Though classified by Alfred Giard in 1888 as a chytridiomycete, *Nephromyces* has resisted definitive identification with any particular fungal or protistan taxa. While its chitinous walls and hyphal-like cells support the notion of fungal affinities for *Nephromyces*, many other features, including the divergent phylogenetic implications of its morphologically eclectic life cycle, do not. Sequencing of ssu rDNA, indicates, however, that *Nephromyces* is an apicomplexan; these molecular data are supported by apicomplexan-like characters in the morphology *Nephromyces*’ host-transfer and infective stages. Other morphological and biochemical features of *Nephromyces*, some of them unusual among apicomplexa, or even protists generally, challenge us to consider the significance of certain phenotypic characters as diagnostic tools in inferring deep phylogenies. *Nephromyces*’ several unusual features are consistent with the suggestions of ssu rDNA data that *Nephromyces* is a distinct apicomplexan clade. Some of these features might also be fruitfully interpreted not just in light of their phylogenetic significance, but also as reflections of *Nephromyces*’ dual symbiotic life: that is, as adaptations to endosymbiotic life in the renal sac, and/or as an evolutionary or physiological consequence of the presence of endosymbiotic bacteria within *Nephromyces*.

Introduction After some years documenting physiological and morphological aspects of interactions in the three-partner symbiosis among molgulid ascidians, their protistan symbiont, *Nephromyces*, and endosymbiotic bacteria within *Nephromyces*, I am now turning to a focus on two explicitly evolutionary questions:

What are the phylogenetic affinities of the microbial partners? How did this symbiosis originate, and how have these three partners coevolved?

In this SGER grant, I am developing morphological, biological and molecular tools for phylogenetic analyses of the microbial partners, and for studies of coevolutionary patterns among all three symbiotic partners.. Here I present what we have learned of the phylogenetic affinities of *Nephromyces*. Recent success in amplifying and sequencing ssu rDNA from *Nephromyces*, in collaboration with Adam McCoy, has yielded especially provocative data, laying a foundation for future molecular phylogenetic studies of this system.

Background: the symbiosis Molgulids are diverse, abundant and biologically surprising inhabitants of world oceans. One of their most puzzling features is the presence of a large, ductless organ (the “renal sac”) containing urate and calcium oxalate concretions in its lumen. In all adults of all species of molgulids, these concretions are surrounded by a dense microbial community with high urate oxidase activity: the protist *Nephromyces* (Saffo, 1982, 1988, 1991) and *Nephromyces*’ endosymbiotic bacteria (Saffo 1990). A ductless, deeply situated organ seems an unlikely site for colonization by horizontally-transmitted symbionts such as *Nephromyces*, but adult molgulids in nature are always infected with these microbes (the bacteria are transmitted vertically, along with *Nephromyces*). *Nephromyces* is itself an obligate symbiont, found exclusively in molgulids; the bacterial partners also appear to be obligately associated with *Nephromyces*. This distribution, along with experimental studies, suggest that this symbiosis is a **mutualism**, and that molgulids have coevolved with *Nephromyces*. Which of the several unique features of each of these clades might be explained as correlates of that coevolution?

What are the phylogenetic affinities of *Nephromyces*?

THE DIVERSE MORPHOLOGIES OF NEPHROMYCES CELLS SUGGEST DIVERGENT PHYLOGENETIC ANSWERS.

We have shown (Saffo and Nelson, 1983), that, despite their resemblance to multiple protistan clades, the morphologically eclectic cells of *Nephromyces* are nevertheless life-history stages of a single taxon (fig. 2). But where does this peculiar belong in the Tree of Life?

1. FUNGUS-LIKE (or chytrid-like) PHENOTYPIC FEATURES OF *Nephromyces*:

- hyphal-like vegetative stages (figs 2, 3)
 - chitinous walls (however, chitin is a taxonomically widespread character, and not, in itself proof of fungal affinities)
 - apparently osmotrophic nutrition in most stages
 - no evidence for autotrophy, or (with one possible exception) holotrophic nutrition
- .. But the life-history of *Nephromyces* does not closely resemble that of chytridiomycota, or of Eumycota.

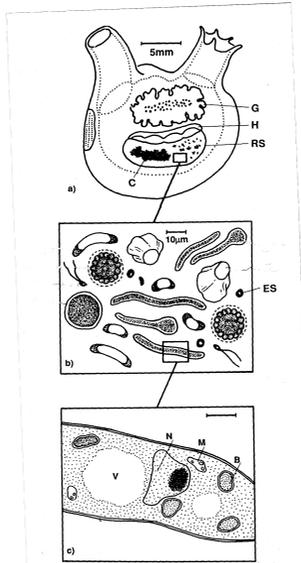


Fig1.
b) *Molgula manhattensis*
RS, renal sac
b) *Nephromyces* cell types
c) *Nephromyces* TEM. B, bacteria;
M, mitochondria; N, nucleus



Fig. 5 *Nephromyces*(TEM)
Note bacteria, tubular
Mitochondrial cristae, and outer
membranes

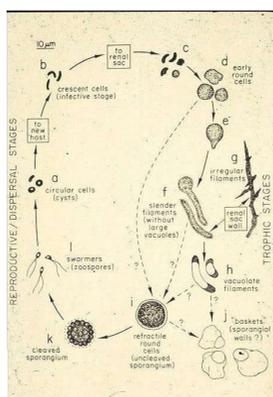


Fig. 2 Life cycle of *Nephromyces*
Saffo & Nelson, 1983



Figure 3: vegetative stages
Vacuolate filaments

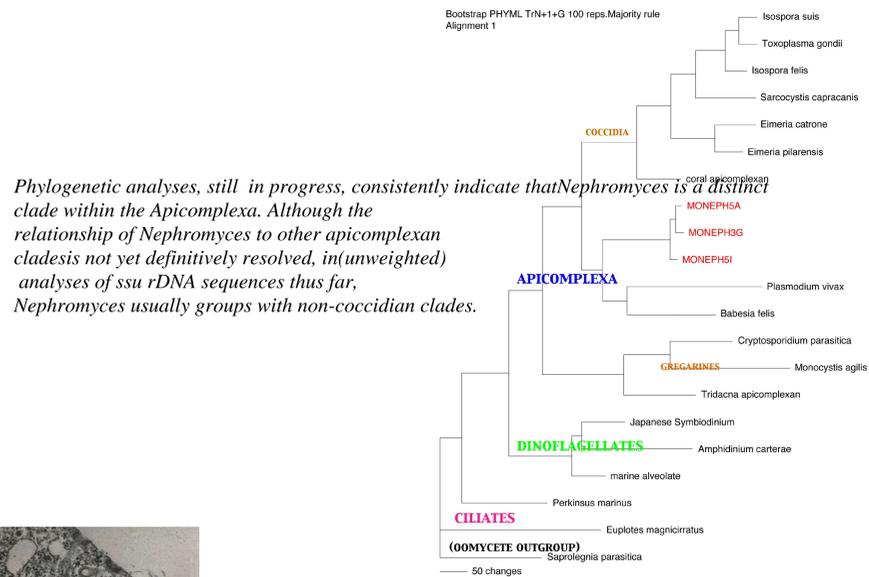


Fig 4. Vacuolate filaments & sporangia

2. Phenotypic features suggestive of general protistan affinities:

- tubular mitochondrial cristae (fig. 5,8)
- thraustochytrid-like sporangia (fig. 4); other developmental stages bear vague resemblances to various other protistan taxa.

3. Ssu rDNA sequences indicate that *Nephromyces* is an apicomplexan clade (preliminary phylogenetic analysis: a sample Maximum likelihood trees shown)



Phylogenetic analyses, still in progress, consistently indicate that *Nephromyces* is a distinct clade within the Apicomplexa. Although the relationship of *Nephromyces* to other apicomplexan clades is not yet definitively resolved, in (unweighted) analyses of ssu rDNA sequences thus far, *Nephromyces* usually groups with non-coccidian clades.

4. Phenotypic features supportive of apicomplexan/alveolate affinities for *Nephromyces*

- morphology of infective stage (fig. 6), the only stage to penetrate host cells
- rhoptry-like inclusions in host-transfer stage, precursor to Infective stage (fig. 7)
- is there a parallel between urate oxidase activity of *Nephromyces* and recycling of host purines by apicomplexans?

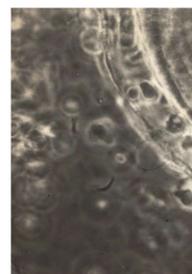


Fig. 6: infective stages in *Molgula*
Blood, before penetration of renal sac wall



Fig. 7: TEM of encysted host-transfer stage, showing rhoptry-like inclusions, bacterial cell and mitochondrion

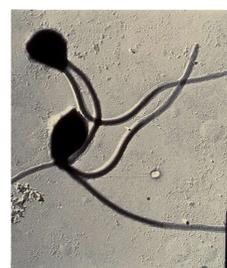


Fig. 8 *Nephromyces* (TEM):
Flagellated cells

5. The phenotypic differences between *Nephromyces* and other apicomplexa raise several questions:

- flagellated cells, though occasionally present (as microgametes) are rare among apicomplexa; flagellate cells are consistently present in *Nephromyces*, and show unusual flagellar morphology (2 posteriorly directed whiplash flagella :fig. 8)
- Although reproductive structures are often considered evolutionarily conservative features, might the unusual flagellar arrangement in *Nephromyces* instead reflect recent adaptation to life in the (viscous) renal sac fluid, rather than deep phylogenetic history?
- There is so far no evidence of an apicoplast in *Nephromyces*, though it does contain bacterial endosymbionts.
- Although all other apicomplexans are parasites, pathogens (and, occasionally commensals), *Nephromyces* is a **mutualist**. Might the presence of bacteria and the absence of an apical complex in *Nephromyces* be correlated with its apparently mutualistic lifestyle, and its apparently distinct evolutionary path?

ONGOING /FUTURE WORK:

- FISH confirmation of *Nephromyces* sequences
- further ultrastructural studies to document presence (or not) of an apical complex and other apicomplexan characters in *Nephromyces*. Integration of morphological with sequence data in formal phylogenetic analysis.
- biological tests of host specificity (cross-infectivity) among *Nephromyces* from different host species, as biological complement to studies of morphological and genetic (initially ssu rDNA) variance among *Molgula* spp and their respective symbionts
- sequencing of ssu rDNA from endosymbiotic bacteria, as first step in probing their phylogenetic relationships, including possible similarities (or not) with apicoplast ssu rDNA.
- Localization of urate oxidase activity in *Nephromyces*, to test possibility that bacteria are locus of urate oxidase activity, possibly acting as peroxisomal analogues (*Nephromyces* has no peroxisomes)