be presented elsewhere (B. M. Yashar *et al.*, in preparation).
7. Strain SY1984-RP is SY1984 (*ste11Δ his3Δ FUS1::HIS3*) (*27*) transformed with pADU-Raf and pNC318-P368. pADU-Raf contains c-Raf-1 controlled from the *ADH1* promoter. pNC318-P368 carries the *STE7^{P368}* allele controlled from the *CYC1* promoter. SY1984-RP was transformed with a multicopy (YEp13) yeast genomic library. Of the 6800 transformants screened, 43 colonies were His$^+$. Only six of these had a His$^+$ phenotype that was dependent on the presence of pADU-Raf, pNC318-P368, and the library plasmid.
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11. The *bmh1::LEU2* disruption allele of pG12-14 was constructed by insertion of a 2.7-kb Bgl II fragment of *LEU2* into the Bgl II site of *BMH1*. Strain SY1984-43 is identical to strain SY1984 (*27*) except that the *BMH1* locus is replaced with the *bmh1::LEU2* allele. It was constructed by a gene replacement with the 5.5-kb Xho I–Xba I fragment from pG12-14 (*10*). The resulting disruption was confirmed by Southern (DNA) blot analysis.
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24. Strain SY1984-VP is SY1984 with pADU and pNC318-P368 (*7*).
25. The expression plasmid pMAL-14-3-3, encoding the 14-3-3 protein fused to maltose-binding protein (MBP-14-3-3), was provided by Y. Takai and T. Isobe, and MBP-14-3-3 was purified from overexpressing *E. coli* (*28*).
26. The activity of Raf was measured by its ability to activate *Xenopus* MEK. Recombinant histidine-tagged *Xenopus* MEK and catalytically inactive *Xenopus* glutathione-S-transferase (GST)–MAPK (K57D) were produced in *Escherichia coli* and purified as described (*29*). MEK activity was assayed by its ability to phosphorylate catalytically inactive MAPK as described (*29*).
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31. We thank J. A. Cooper, T. Isobe, S. G. Macdonald, Y. Takai, R. Ruggieri, and A. B. Vojitek for materials and S. Lemmon, F. McCormick, R. Ruggieri, and Y. Takai for helpful discussions and for sharing data before publication. Supported by the Ministry of Education, Science, and Culture of Japan (to K.I., Y.G., E.N., and K.M.) and by grants from the Senri Life Science Foundation, the Takeda Science Foundation, Asahi Glass Foundation, the Kato Memorial Bioscience Foundation, the Human Frontier Science Program Foundation (to K.M.), and the National Institutes of Health (to B.E.).

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Molecular Evidence That the Myxozoan Protists Are Metazoans

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The evolutionary origins of the protistan phylum, Myxozoa, have long been questioned. Although these obligate parasites are like protozoans in many features, several aspects of their ontogeny and morphology have implied a closer relationship to metazoan lineages. Phylogenetic analyses of 18S ribosomal RNA sequences from myxozoans and other eukaryotes, with the use of parsimony, distance, and maximum-likelihood methods, support the hypothesis that myxozoans are closely related to the bilateral animals. These results suggest that the Myxozoa, long considered an assemblage of protozoans, should be considered a metazoan phylum.

The reconstruction of animal phylogeny has engaged scientists for over a century (*1*). With the advent of molecular characters, hypothesized relationships among some taxa have become solidified, whereas others remain contentious (*2*). Most biologists would agree, however, on the set of taxa that constitutes the Metazoa, or multicellular animals. Not since discovery of the Loricifera in the last decade has a phylum been formally added to this taxonomic group (*3*). Here, we present evidence that the Myxozoa, a phylum generally placed in the kingdom Protista (Protoctista) (*4*–*6*), shares its most recent common ancestor with a metazoan, not a protozoan, lineage.

The phylum Myxozoa comprises over 1100 described species of oligocellular, obligate endoparasites and is thus one of the largest protistan assemblages (*4*). Members of the class Myxosporea (the majority of myxozoans) principally infect teleost fishes, and members of the Actinosporea mainly infect aquatic, oligochaete worms (*7*). Myxosporea are either coelozoic within hollow organs or histozoic in solid tissues and cause tissue displacements and destruction and sometimes death of their hosts. For example, infections by the European parasite *Myxobolus cerebralis* in North American salmonid fish can cause serious losses to aquaculture industries. The avirulence of most myxosporeans in their natural hosts, however, implies a long history of association (*8*).

The diagnostic feature of Myxozoa is the infective spore, a dispersal stage composed of distinct gametic (autogamic) cells and polar capsules with coiled, ejectable filaments, enveloped by valve-like somatic cells. Proliferative stages (trophozoites) are often multinuclear and may grow to macroscopic size (*8*). However, the complete life cycle is unknown, because myxozoans have been intractable to

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laboratory culture. Experimental transmission studies of both myxosporeans and actinosporeans suggest that the two parasites are possibly alternative forms of a single, complex life cycle: *M. cerebralis* infections in fish, for example, may be initiated by an actinosporean of tubificid worms (*9*).

The phylogenetic affiliations of Myxozoa have remained unclear since their discovery and recognition as a distinct taxonomic group in the 19th century (*10*). Though protistan in habits and size, myxozoans exhibit a degree of multicellularity and cell differentiation found in no other protozoans (*11*). On the basis of the common possession of spores with ejectable filaments and amoeboid cells, Myxozoa and Microsporea once shared the class or subphylum Cnidosporidia in the old phylum Protozoa. After discoveries about their morphology and life history, Microsporea were assigned to their own protistan phylum; molecular evidence confirmed that these minute, unicellular organisms are extremely ancient eukaryotes (*12*). The cellular complexities of myxozoans, in contrast, have provoked speculation that these parasites share a most recent common ancestor with metazoan taxa, not protists (*13*). For example, the structural and developmental similarities of polar capsules and cnidarian nematocysts, and the striking parallels between sporoblasts and larval Narcomedusae, have engendered hypotheses that myxozoans share a most recent common ancestor with the Cnidaria (*14*). Other workers have interpreted their cellular differentiation, and features such as the desmosome-like structures between valve cells, to suggest that myxozoans are a primitive (non-Cnidarian) animal lineage (*15*).

Given such uncertainty regarding myxozoan origins, we determined the sequences of small-subunit (18S) ribosomal RNAs (rRNAs) for five myxosporean species in three different genera (*16*) to resolve their phylogenetic position according to molecular evidence. We used parsimony and neighbor-joining methods to find the best supported tree for myxozoans and a selection of other eukaryotes (*17*) and then used parsimony and maximum-likelihood methods to test three alternative hypotheses: that myxozoans share their most recent common ancestor with (i) an alveolate or rhizopod protistan lineage, (ii) a cnidarian lineage, or (iii) a bilateral metazoan lineage.

The position of the myxozoans as a metazoan lineage was supported with 100% bootstrap confidence in both parsimony (*18*) and neighbor-joining analyses (*19*) (Fig. 1). The topology recovered in bootstrap parsimony analysis was the same as one of four shortest length trees inferred with maximum parsimony, all sites and transformations weighted equally [the four trees (length = 1586) differed only in the positions of the placozoan and ctenophoran]. The inclusion of myxozoans in the bilateral animal clade also held when transversions were weighted 10 to 1 over transitions. The relationships of nonmyxozoan taxa that were well supported in our analyses (bootstrap values >80%) generally corroborate previous morphological and molecular studies that included the same groups (*20–24*), whereas branching events with lower bootstrap support (<80%) involve taxa that show conflicting arrangements in other studies (*1*, *2*, *23*, *24*, *25*). It is possible that the set of taxa included, or the presence of long, unbranched lineages can affect, patterns recovered in phylogenetic analyses (*26*). The myxozoans are comparatively long-branched taxa, as are all the bilateral animals relative to other taxa in this study. For this reason, we analyzed subsets of the taxa shown here, as well as different alignments including other taxa. In all of these analyses, the status of the Myxozoa as a metazoan lineage did not change.

With both parsimony and maximum-likelihood methods (*27*), we evaluated different tree topologies that corresponded to the alternative hypotheses of myxozoan origins, as stated above. Under topologically constrained parsimony searches, 35 to 46 extra steps were required if myxozoans were forced to be a sister group to different protistan lineages, 22 extra steps were added if myxozoans were constrained as a sister group to the Cnidaria, and 16 to 29 extra steps were necessary if myxozoans were sisters to different bilateral animal lineages. Only five extra steps were added if myxozoans were a sister group to all bilateral animals. When similar topologies were evaluated with maximum likelihood, the tree in which myxo-

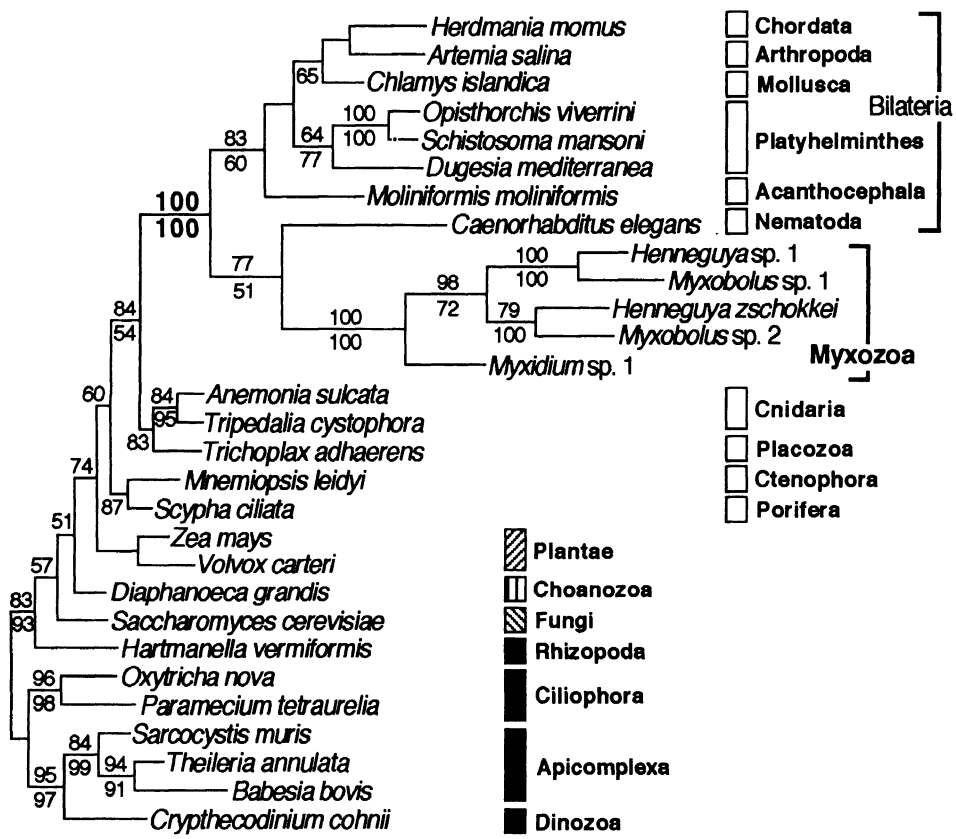

Fig. 1. Phylogenetic position of Myxosporea (Myxozoa) among representative eukaryote groups (*17*), as inferred from 18S ribosomal RNA sequences. Open bars indicate metazoan taxa; closed bars indicate protistan taxa. Four of the five Myxosporea (numbered) were previously uncharacterized species. The structure of the tree is the consensus of 500 parsimony bootstrap replicates (*18*). The percentage of parsimony bootstrap resamplings supporting a given branching event is shown above the branch, and the percentage of neighbor-joining (*19*) replicates supporting a branching event is shown below the branch (confidence levels greater than 50% are indicated). Branch lengths are proportional to the number of substitutions. The branch uniting the myxozoans and all bilateral animals has a decay index (*31*) of >5. Only sites that could be aligned without ambiguity were included (*32*). All analyses were performed as unrooted; here, the root is placed between the alveolates and the rhizopod (*24*). Maximum-likelihood methods (*27*) also supported the position of the myxozoans shown here.

zoans were a sister group to the Nematoda (Fig. 1) had the highest log-likelihood value and was significantly more likely than other topologies that placed the myxozoans as a sister group to individual protistan, cnidarian, or other animal lineages. However, the topology of Fig. 1 was not significantly different from an alternative topology that placed the myxozoans as a sister group to all bilateral animals.

These molecular data provide evidence, as others have suggested on the basis of morphological criteria, that myxozoans constitute a metazoan, not a protozoan, lineage. Further refinement of myxozoan relationships to other metazoan phyla may emerge when complete 18S rRNA sequences are available for additional myxozoan and metazoan taxa, or when information from other molecules is gathered. We found no support, however, for the hypothesis that myxozoans and cnidarians share a recent, common evolutionary history. Rather, myxozoan origins appear to date later in metazoan phylogeny, to the appearance of the bilateral animals. At present, we cannot distinguish whether myxozoans are members of the bilateral animal clade or a sister group to them. Under either scenario, the ancestors of extant myxozoans must have undergone extensive reductions in morphology and development. Unlike cnidarians and higher metazoans, myxozoans are not known to develop multiple differentiated tissues, eggs, and sperm or form a blastula (*15*, *28*), but the common ancestor of myxozoans, bilateral animals, and cnidarians most likely possessed these features. Myxozoans may be an extreme example of the pattern of degeneracy that is characteristic of parasite evolution.

Our results also have implications for myxozoan systematics. Classification of these parasites traditionally has been based on spore morphology (*29*), now known to be a plastic trait in some species (*30*). Uncertainty concerning the closest relatives of Myxozoa has precluded the use of outgroups to determine primitive versus derived traits; thus, hypotheses of relationships within the phylum necessarily have relied on host phylogeny. Our study shows that rRNA sequences contained sufficient information to resolve even intraclass relationships. In fact, the data imply that two genera (*Henneguya* and *Myxobolus*) are paraphyletic (Fig. 1). Further sequence data will be instrumental in the systematics of these widespread but little recognized organisms. Potential hosts of myxozoan parasites are vastly undersampled, and given the abundance of teleost fishes (more than 40,000 species), there are likely many undescribed species. Perhaps our results will lead to future studies of myxozoan life history, development, and evolution, as well as additional molecular work to further refine the systematic position of these organisms.

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17. Metazoan taxa were chosen to represent the breadth of phyla for which complete 18S rRNA sequences were available in GenEMBL. Protozoan taxa were chosen to represent the more recently derived groups, which have been proposed as close relatives of Myxozoa (*6*). Plants and the fungus were taxa that have been included in other published molecular phylogenies.
18. Parsimony analyses were conducted with the computer program PAUP (*PAUP: Phylogenetic Analysis Using Parsimony*, 3.1; D. L. Swofford, Illinois Natural History Survey, Champaign, IL). We performed 500 bootstrap replicates using the heuristic search strategy, with random addition and all sites and transformations weighted equally. Searches were performed with no outgroups specified, to avoid influencing the position of myxozoans.
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