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Cladistics

Letter to the Editor

Myxozoa, *Polypodium*, and the origin of the Bilateria: The phylogenetic position of ''Endocnidozoa'' in light of the rediscovery of *Buddenbrockia*

To the Editor:

Since their discovery, endoparasitic Myxozoa have been considered protozoans; however, a century ago, Štolc (1899) remarked that they should be classified as reduced cnidarians because of the nematocyst-like architecture of their spores. Later on, the multicellular nature of Myxozoa and the structural and developmental similarity of their polar capsules to cnidarian nematocysts were recognized repeatedly (recently by Lom and Dyková, 1997; Siddall et al., 1995, and references therein). In the past few years, the topic of myxozoan relationships has become further complicated. A new clade, the ectoproct–parasitic (possibly ectoproct–fish–parasitic) Malacosporea (Canning et al., 2000), was discovered and identified as a sister group of the rest of the Myxozoa. In 2002, one of the most enigmatic metazoan species, *Buddenbrockia plumatellae* Schröder, 1910, was rediscovered and reinterpreted phylogenetically (for its pre-2002 interpretation see Nielsen, 2001, p. 497, and references therein). *Buddenbrockia* is a motile worm-like animal inhabiting body cavities of freshwater ectoprocts. It possesses four nematode-like bands of longitudinal muscular cords, cnidarian-like complement of numerous polar capsules in its ''mural cells'' (=epidermis?), and myxozoan-like infective spores (including polar capsules) that are formed inside the body cavity. Surprisingly, *Buddenbrockia* has been shown to be conspecific with the malacosporean species *Tetracapsula bryozoides* Canning, Okamura & Curry, 1996, a conclusion based on morphology (Canning et al., 2002; Okamura et al., 2002) and small-subunit (=18S) ribosomal RNA (''SSU'' hereinafter; Monteiro et al., 2002).

The molecular data, viz. SSU and Hox genes, clearly indicate a metazoan origin of Myxozoa but those data are as yet unable to reveal their specific relationships. Two conflicting hypotheses concerning the position of the Myxozoa within Metazoa have so far been formulated, one treating the Myxozoa as modified cnidarians and the other placing them within bilaterians. According to the former, more traditional point of view, supported by several SSU analyses, myxozoans probably represent a sister group of *Polypodium hydriforme* Ussov, 1885 (Siddall et al., 1995; Siddall and Whiting, 1999; Zrzavý et al., 1998), an aberrant cnidarian with a life cycle including both free-living medusoid stages and stages adapted to intracellular parasitism in oocytes of fishes (Raikova, 1994). Traditionally, *Polypodium* is regarded a member of the hydrozoan group of Narcomedusae, but Raikova (1994) has suggested (with no cladistic analysis) to elevate *Polypodium* to the level of a new cnidarian class, Polypodiozoa. The comprehensive phylogenetic analysis of the cnidarian SSU sequences (Collins, 2002) indeed retrieves monophyletic Cnidaria and Hydrozoa (=Hydroidolina and Trachylina, the latter including the Narcomedusae) but an uncertain position of *Polypodium* and Myxozoa outside the Hydrozoa (A.G. Collins, pers. comm.).

In most other SSU studies (which usually do not include *Polypodium*'s sequence at all), the Myxozoa group within Bilateria, close to basal clades such as Mesozoa and Nematoda (Hanelt et al., 1996; Pawlowski et al., 1996; Schlegel et al., 1996; Smothers et al., 1994). Application of different tree-building methods shows that the clade consisting of *Polypodium* and Myxozoa (=''Endocnidozoa,'' a name informally proposed by P. Schuchert; see de Meeûs and Renaud, 2002, p. 247, who misspelled the name as ''Endocnydozoa'' and attributed it erroneously to Siddall et al., 1995) is not likely an artifact caused by the ''long-branch attraction'' of aberrant SSU sequences (Siddall and Whiting, 1999). However, most of their experimental trees tend to show the Endocnidozoa as basal bilaterians rather than derived cnidarians. In their distance tree, Kim et al. (1999) found a weakly supported *Polypodium*–Myxozoa clade branching as a sister group of Bilateria; in the maximum-likelihood tree, Myxozoa remained a sister group of bilaterians while *Polypodium* branched off an unresolved base of the tree (with Placozoa and several cnidarian subclades). The analysis of myxozoan Hox genes suggests that Myxozoa is more closely related to bilaterians

than to the two cnidarians that were included in the study (Anderson et al., 1998); the same result has tentatively been reached by the analysis of large-sub-unit (= 28S) ribosomal RNA gene (I. Fiala, pers. comm.).

Two questions arise from this survey: (1) Are *Polypodium* and Myxozoa sister taxa? (2) Are *Polypodium* and/or Myxozoa derived cnidarians or basal bilaterians? Unfortunately, no cladistic analysis including Malacosporea, Myxosporea (= the rest of myxozoans), *Polypodium*, true cnidarians (including Narcomedusae), and an array of bilaterian representatives (including putatively basal phyla and nematodes) has yet been published. Here we show that the cnidarian and bilaterian hypotheses can be reconciled.

To reanalyze *Polypodium* and Myxozoa relationships, SSU sequences of 46 metazoan taxa (Ctenophora, Placozoa, 10 Cnidaria incl. 2 Narcomedusae, *Polypodium*, 3 Myxozoa incl. both valid malacosporean species, *B. plumatellae* and *Tetracapsuloides bryosalmonae*, 2 Acoela, 2 Mesozoa, Nemertodermatida, Myzostomida, 3 Deuterostomia, 6 Ecdysozoa, 2 Gnathifera, Gastrotricha, 4 Platyhelminthes, 2 Ectoprocta, 2 Brachiozoa, 3 Trochozoa, and a calcareous sponge as an outgroup; for a list and GenBank accession numbers see Appendix 1)[1] were aligned (MALIGN, options "build randorders 10 alignaddswap alignswap treeaddswap treeswap"). Three different alignments were constructed, with substitution cost to gap cost ratios 3:4, 3:5, and 3:6 (empirically found to exhibit the lowest levels of character incongruence in previous "phylum-level" SSU-morphological analyses; see Zrzavý et al., 1998, 2001). The alignments were analyzed by the maximum parsimony method (NONA; options "hold 10000; mult*100; hold/100"); the Bremer support scores were counted to assess branch support (NONA, option "bsupport 10000").

All complete alignments are combined in a single data matrix so that the more robustly aligned positions present in more than one alignment have higher weights (see Wheeler et al., 1995). The resulting tree (Fig. 1), based solely on substitution characters (tv:ts = 1:1, gaps treated as missing data), contains a *Polypodium*–Myxozoa–Bilateria clade, with *Polypodium* as the sister group of Myxozoa–Bilateria (i.e., paraphyletic "Endocnidozoa").

To extract variable and ambiguously aligned positions, the sequences were realigned according to two slightly different options (the option "contig" was used in one but not in the other, the "contig" tends to create alignments with fewer gap locations but contiguous gaps that include more positions; all other options remain

identical). The resulting "contig" and non-"contig" alignments were then culled to include only positions shared by both ("culled" dataset hereinafter). The dataset combined from all three culled alignments (3:4 + 3:5 + 3:6; tv:ts = 1:1, gaps treated as missing data) provides a tree (Fig. 2) with monophyletic Endocnidozoa as a sister group of the Bilateria. When the gaps that have survived the culling procedure are analyzed together with substitutions (gap:substitution = 1:1; tv:ts = 1:1), the branching pattern is more symmetrical (Fig. 3): the clade consisting of Acoela, Myzostomida, Mesozoa, and Endocnidozoa appears as a sister group of the Nemertodermatida–"higher" Bilateria clade, a topology similar to that recently published by Giribet (2002) who unfortunately studied neither *Polypodium* nor myxozoans. The strict consensus of all three topologies (not shown) is not well resolved; nevertheless it contains a few clades important for the *Polypodium*–Myxozoa problem: there are monophyletic Cnidaria less *Polypodium* (incl. monophyletic Hydrozoa), monophyletic *Polypodium*–Myxozoa–Bilateria (the traditional Bilateria might in fact include both *Polypodium* and Myxozoa as a derived ex-group or ex-groups), and monophyletic "higher" Bilateria (= Nephrozoa) less Myzostomida. In all three trees, the *Polypodium*–Myxozoa–Bilateria clade is one of the most strongly supported groups (see Bremer supports in Figs. 1–3). In addition, when all nine alignments (three "complete," three "culled," three "culled + gaps") were combined, the resulting tree (not shown) includes a monophyletic Endocnidozoa as sister to Bilateria, with the latter group consisting of Acoela–Mesozoa and Myzostomida–Nemertodermatida–Nephrozoa subclades.

However, the tree topology can be suspected of deteriorating due to the influence of the "long-branch attraction" artifact as numerous extremely "long-branch" sequences (e.g., *Polypodium*, *Buddenbrockia*, *Tetracapsuloides*, *Kudoa*, *Rhopalura*, *Dicyema*, *Myzostoma*) are placed at the base of the bilaterian stem. One approach to identify and neutralize "long-branch" artifacts is the "long-branch extraction" technique applied by Siddall and Whiting (1999): if two "long branches" are attracting each other, the absence of one of the branches should allow the remaining branch to locate elsewhere in the pruned tree. In the absence of all problematic "long-branch" SSU sequences, Placozoa groups with Cnidaria, and Bilateria is split into two traditional subclades, Deuterostomia and monophyletic Protostomia (not shown). The "long-branch" sequences—appended in one-by-one manner—group at positions identical with the topology of the all-species trees (*Polypodium*, Myxozoa, Acoela, Nemertodermatida, Mesozoa, and Myzostomida as basal Bilateria) or at positions more comparable with the previously published SSU trees (Gastrotricha and Gnathifera with flatworms, Ectoprocta with the Brachiopoda–Phoronida clade).
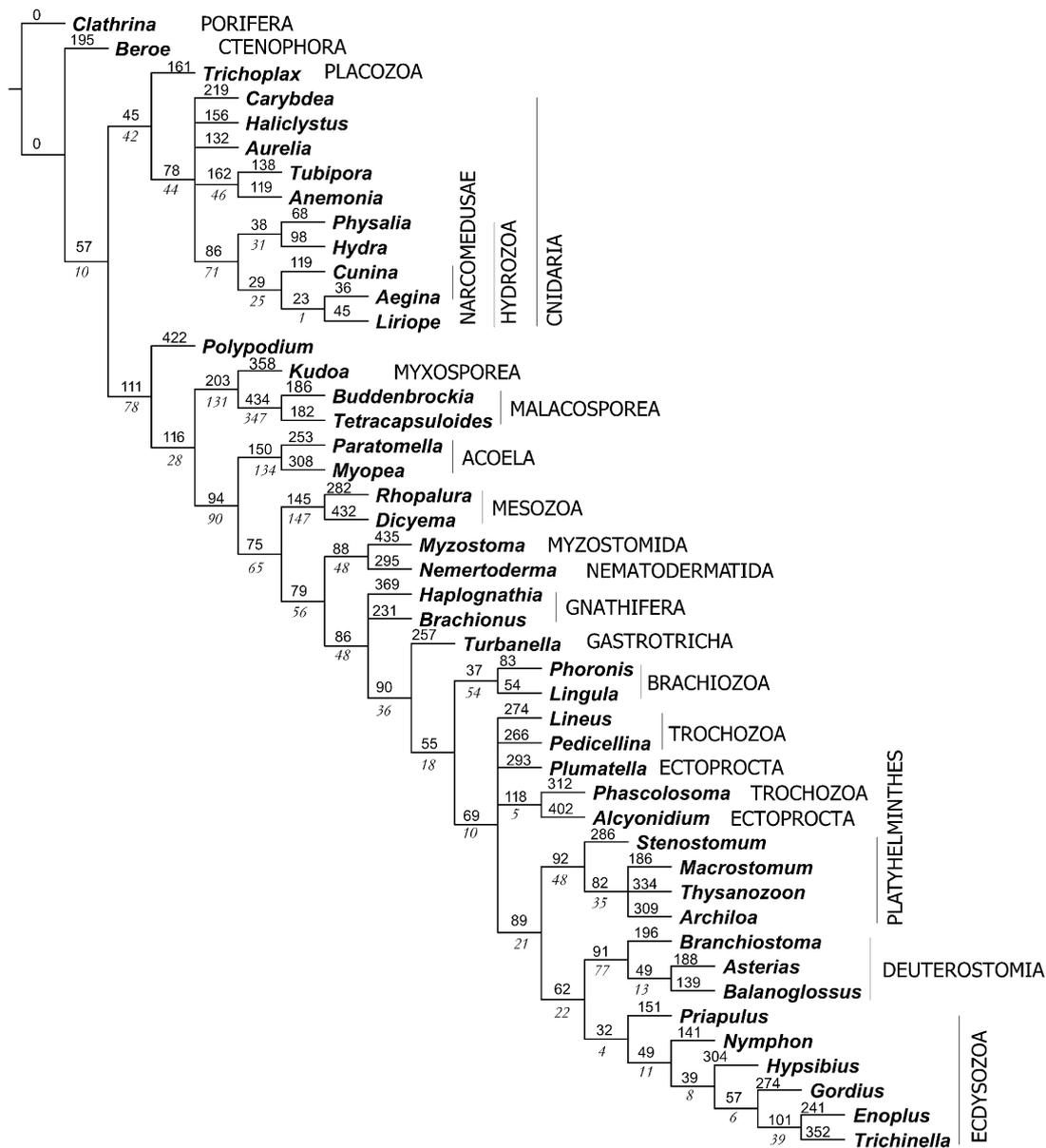
Fig. 1. Phylogenetic hypothesis of Metazoa based on combined complete alignments (gap:substitution ratio = 4:3, 5:3, 6:3); tree derived from substitutions only (gaps as missing data, tv:ts = 1:1; strict consensus of six trees, 20,718 steps, CI 0.29, RI 0.49). Branch lengths are indicated above branches, Bremer support scores are indicated below branches (in italics).

It has been suggested that too-distant, "diploblast" outgroups may cause a rooting problem for the Bilateria by distorting ingroup relationships (Giribet et al., 2000). However, none of the unrooted bilaterian and cnidarian trees displayed important topological changes that would suggest misplacement of some ingroup taxa by the remotely related outgroups. In all unrooted bilaterian trees (either including or excluding the gaps), there are two convex (i.e., nonpolyphyletic) groups, one including *Polypodium*–Myxozoa, Acoela, Mesozoa, and Myzostomida and the other the "higher" bilaterian groups. The Nemertodermatida is the only taxon dis-

playing an unstable position: when gaps are included and all diploblast outgroups removed, *Nemertoderma* is nested deeply within Nephrozoa.

In conclusion, the basal branching pattern (Cnidaria vs *Polypodium*–Myxozoa–Bilateria) is supported by the SSU evidence and does not seem to be affected by "long-branch" and "distant-outgroup" artifacts. Under no analytical parameters does *Polypodium* group with cnidarians. Even if no bilaterians are included in the analysis, *Polypodium* is still placed as a sister group of Placozoa–Cnidaria, i.e., in the same position where the bilaterian sequences would be placed. Forcing
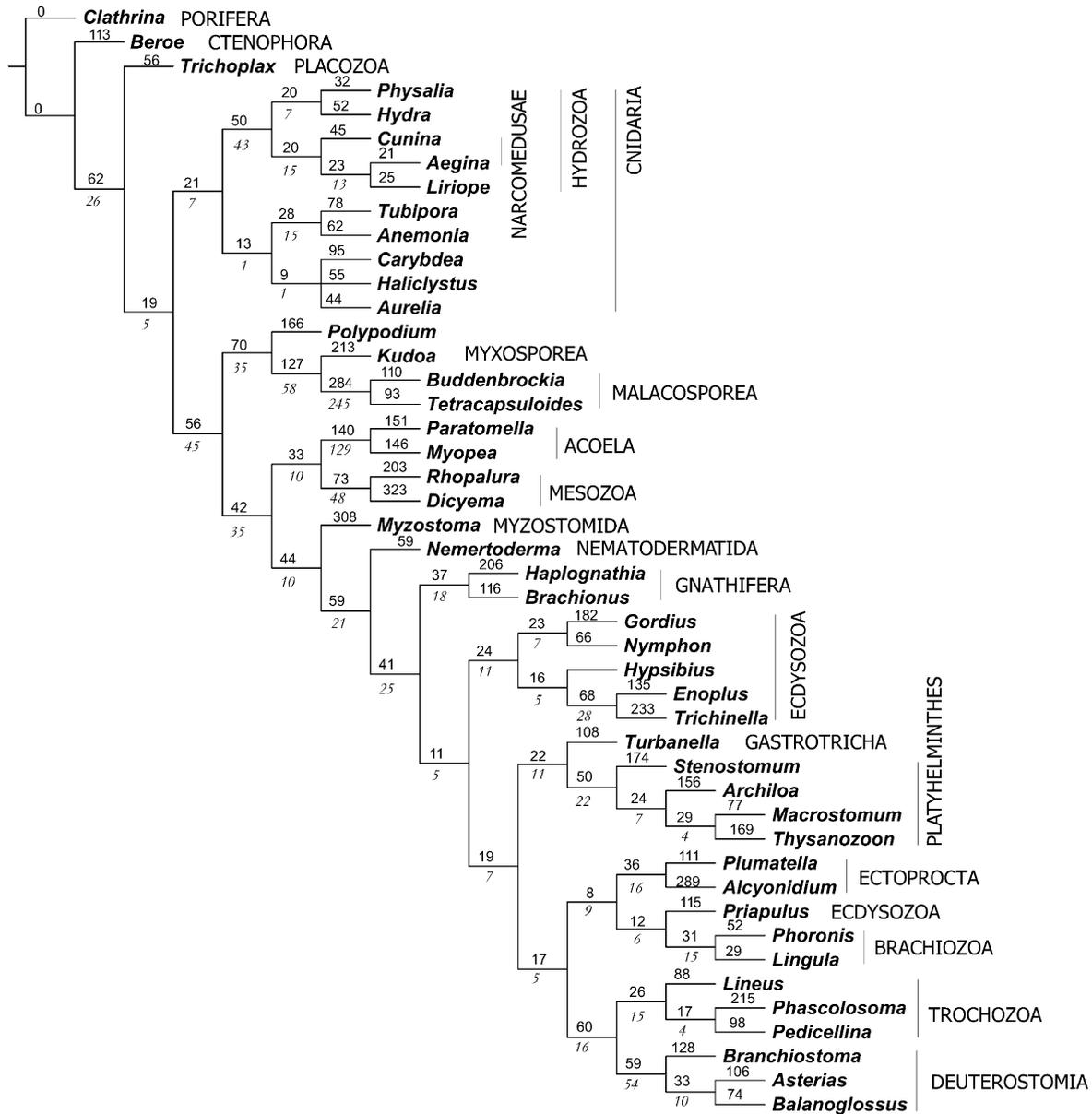
Fig. 2. Phylogenetic hypothesis of Metazoa based on combined "culled" alignments (gap:substitution ratio = 4:3, 5:3, 6:3); tree (cf. Fig. 2) derived from substitutions only (gaps as missing data, tv:ts = 1:1; strict consensus of two trees, 11,278 steps, CI 0.30, RI 0.48). Branch lengths are indicated above branches, Bremer support scores are indicated below branches (in italics).

Polypodium to group as a basal cnidarian, basal hydrozoan, or basal trachyline hydrozoan requires 81, 87, and 136 extra steps, respectively.

The basal-bilaterian position of the possibly monophyletic Endocnidozoa is not morphologically "absurd." First, the systematic position of *Polypodium* within the hydrozoan Narcomedusae is improbable and is based exclusively on parasitism and similarities in early development. Although its body plan is undeniably that of a cnidarian, homology of *Polypodium*'s life cycle stages to the cnidarian life cycle is uncertain and *Polypodium* evidently lacks some hydrozoan characters (e.g., epidermal gonads). Moreover, several characters of

Polypodium (e.g., body symmetry of parasitic stages, presence of gonoducts, and complete separation of epidermal and muscle cells; Raikova, 1994) could be assessed as bilaterian-like features.

Second, the most primitive Myxozoa exhibit a mixture of cnidarian-, *Polypodium*-, and bilaterian-like features. Both Myxozoa and *Polypodium* share the "cell-in-a-cell" early development, a single type of polar capsule/nematocyst, mode of attachment of the infective stages to the host tissue by using the nematocysts, fish parasitism, tubulocristate mitochondria, and loss of the epidermal ciliation (see Lom and Dyková, 1997; Raikova, 1994; Siddall and Whiting, 1999). The bilaterian

Fig. 3. Phylogenetic hypothesis of Metazoa based on combined "culled" alignments (gap:substitution ratio = 4:3, 5:3, 6:3); tree derived from substitutions and gaps (1:1) (tv:ts = 1:1; strict consensus of two trees, 13,374 steps, CI 0.29, RI 0.50). Branch lengths are indicated above branches, Bremer support scores are indicated below branches (in italics).

body architecture of *Buddenbrockia* and the bilaterian complex of Hox genes in both classes of the Myxozoa (Anderson et al., 1998) corroborate the placement of Myxozoa in the proximity of (or within) the Bilateria. The longitudinal muscles of *Buddenbrockia* are strikingly nematode-like, and Okamura et al. (2002) proposed a close relationship between Myxozoa and Nematoda on that basis. However, it has been acknowledged that the basal position of the Nematoda in early SSU analyses (e.g., Smothers et al., 1994) was an artifact caused by depauperate species sampling that had included exclusively the rapidly evolved species of nematodes. In the presence of "short-branch" nematode

sequences (e.g., SSU sequence of *Trichinella*), all Nematoda are deeply nested within the Ecdysozoa but unable to "carry" myxozoans into the intra-ecdysozoan position (e.g., see Monteiro et al., 2002, p. 970). Moreover, it should be emphasized that *Buddenbrockia* bears numerous polar capsules in its epidermis, not exclusively in the infective spores, and hence is the *most* cnidarian-like species of Myxozoa. In fact, the presence of the polar capsules both in the integument and in the spores of a single animal is the first evidence that cnidarian and myxozoan polar capsules might indeed be homologous.

This study supports the bilaterian (or near-bilaterian, instead of cnidarian) relationships of Myxozoa, while

allowing at the same time for a homology of myxozoan polar capsules to the cnidarian nematocysts (secondarily suppressed in Bilateria *s. str.* above the Endocnidozoa level). If so, the successive clades of nematocyst-bearing animals (Cnidaria, *Polypodium*, Myxozoa) seem to form a grade bridging the deep structural and developmental gaps between the bilaterians and the more primitive "diploblast" animals. Myxozoa were rarely included in the morphological analyses of the phylogeny of Metazoa; now, the ultrastructural and developmental research on *Buddenbrockia* promises to provide key information on the origin of Myxozoa (and/or Endocnidozoa).

Acknowledgments

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