BRACHIOPOD MOLECULAR PHYLOGENY

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ABSTRACT- Analysis by parsimony, maximum likelihood and distance methods of newly determined nuclear-encoded SSU rRNA gene sequences from 23 species of articulate brachiopods, six inarticulate brachiopods, two phoronids and an ectoproct, together with other sequences from published and unpublished sources show that lophophorates cluster with protostome, not deuterostome metazoa and that phoronids cluster with inarticulate brachiopods. Phoronids, inarticulate, and articulate brachiopods form a monophyletic assemblage. A chiton is the closest known outgroup of brachiopods plus phoronids. Within articulates, separate rhynchonellid and long- and short-looped terebratulid clades are identified and a thecideidine falls within the short-looped articulate clade. Forms with incomplete loops belong either to the short or long-looped clades, thus, a three-fold division of articulate brachiopods suffices to encompass the range of extant diversity so far examined. A perfect correlation was found between clade rank and lineage age rank for five well-dated brachiopod lineages. The important underpinning role of classical brachiopod taxonomy for molecular phylogeny is stressed.

INTRODUCTION

The information bearing molecules of the genome (DNA and its transcript, RNA) are 'the documents of evolutionary history' (Zuckerkandl & Pauling, 1965) and recent developments in DNA sequencing and polymerase chain reaction (PCR) amplification mean that these documents can now be read with reasonable ease. As a result, genealogical relationships are being clarified at an unprecedented rate (Hillis & Moritz, 1990) and our laboratory has been privileged to extend this approach to brachiopods and other lophophorates, using nuclear-encoded small subunit ribosomal RNA (SSU rRNA) gene sequences. To the extent that the genomes of living brachiopods retain phylogenetically useful information, our results and those of our successors will, for the first time, make it possible to justify classification on genealogical grounds independent of morphology and the fossil record.

Previously it was shown that a brachiopod, a phoronid and an ectoproct had nuclear SSU rRNAs of protostome type (Ishikawa, 1977) and this was confirmed by the partial sequence of the *Lingula* RNA (Adoutte & Philippe, 1993; Field, 1988; Ghiselin, 1988; Patterson, 1989). Since then many more nuclear SSU rRNA gene sequences have been determined (Benson et al., 1994; Van de Peer et al., 1994), making this sequence the most useful for wide-ranging phylogenetic studies. Our work extends the range of complete SSU sequences to include all the major extant brachiopod lineages (Cohen & Gawthrop, 1995; Cohen et al., 1995). Two other brachiopod SSU sequences have also been reported (Halanych et al., 1995).

The sequence differences observed in SSU gene comparisons reflect the accumulated results of many evolutionary processes of which we can observe only the end product. The initial events, mutations leading to a nucleotide substitution are, to a first approximation, randomly distributed within and between genes, but we see only those few substitutions that rise to high frequency (effectively to fixation, frequency = 1.0) amongst the multiple genomic copies of rRNA genes and in the population (Coen et al., 1982). Because substitution events are rare and lineage sorting (extinction) is common, extant taxa have an invariant or almost invariant rRNA gene sequence, making it possible to use sequences from only one or a few individuals to exemplify a taxon (Hillis & Dixon, 1991). Since rRNAs play a central structural and functional (even enzymatic) role in protein synthesis with multitudes of interactions within their own structure as well as with ribosomal proteins, their primary and secondary structures are subject to strong selection for conserved function and the majority of mutations that achieve fixation will be neutral or (rarely) advantageous. In consequence, we expect the number of accumulated changes to be roughly proportional to time (a molecular clock hypothesis), though complicating factors exist. An important practical advantage of rRNA sequences is that they combine blocks that are highly conserved (because functionally constrained) interspersed with more variable regions (Hillis & Dixon, 1991). The highly conserved blocks make it possible to be confident that sequences from very diverse taxa have been correctly aligned, with most homologous nucleotide sites in register. The more variable regions are aligned by using the highly conserved segments to anchor their ends, by nucleating the alignment on phylogenetically close taxa and by use of secondary structure information (Huss & Sogin, 1990). Thus, analysis of divergence in complete SSU sequences is possible, providing a wide (but not unlimited) range of phylogenetic resolution. In the absence of evidence for paralogy, the SSU gene phylogeny is assumed to be an honest reporter of organismal phylogeny. Additionally, since metazoan mitochondrial DNA (mtDNA) generally accumulates base substitutions several-fold faster than nuclear DNA, analysis of mitochondrial rRNA genes further increases the divergence-time range over which useful results may be obtained (Adoutte & Philippe, 1993; Hillis & Dixon, 1991; Phillipe et al., 1994).

MATERIALS AND METHODS

Full details of materials and methods will be published elsewhere (Cohen et al., 1995). In brief, nuclear-encoded SSU rRNA gene sequences, each ca. 1,790 nucleotides long were obtained by the direct sequencing of DNA amplification products synthesized by PCR using oligonucleotide primers matching highly conserved terminal regions of the gene. Thus, the sequences are complete except for some short regions and the two large sections missing from the *Lingula reevii* sequence (Field, 1988). In addition to new sequences from 23 species of articulate brachiopods, six inarticulate brachiopods, two phoronids and an ectoproct, sequences from one articulate, two inarticulates, a phoronid and an ectoproct were available elsewhere (Field, 1988; Halanych et al., 1995; Winnepenninckx & De Wachter, 1994). Sequences were

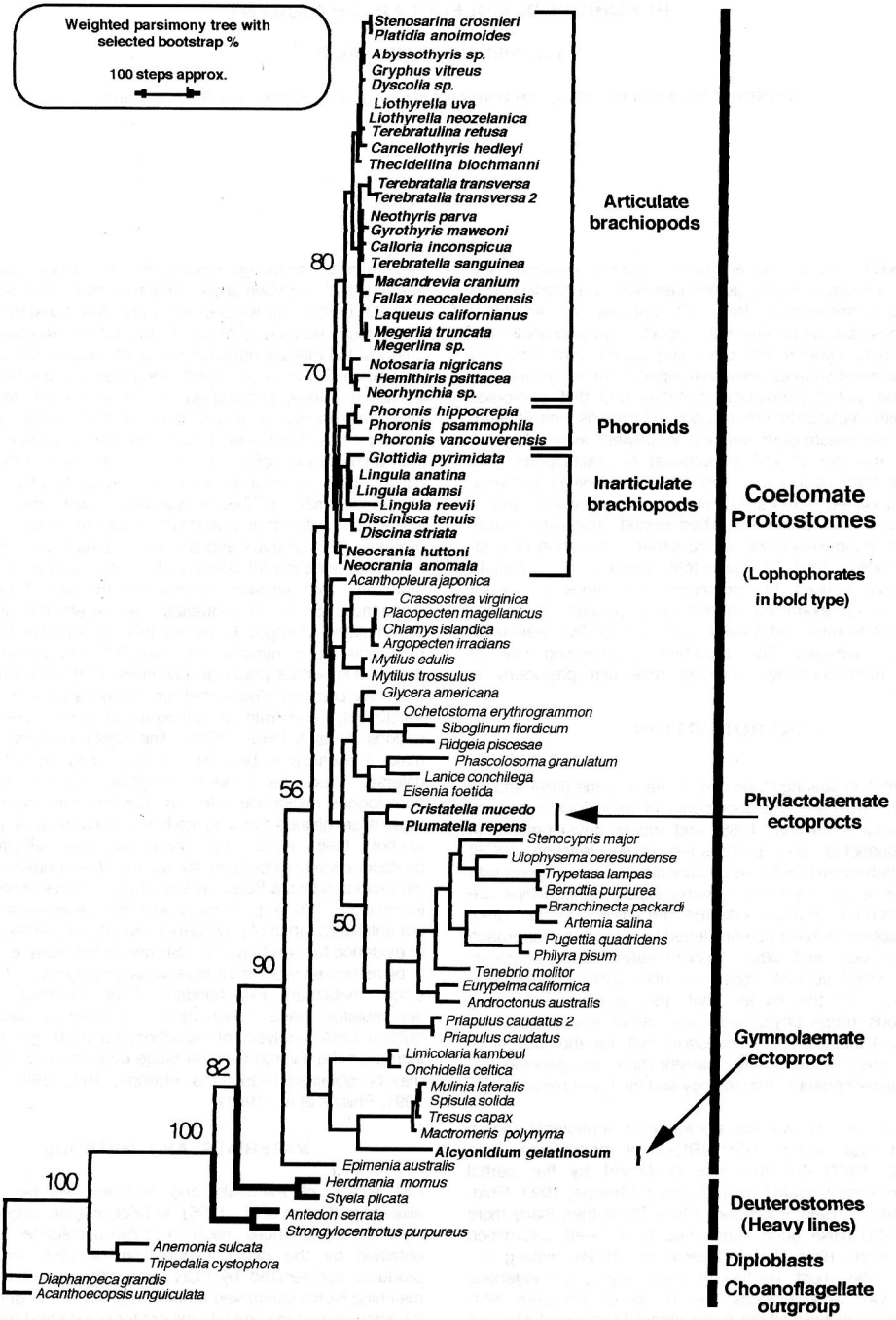

Figure 1. The high-level phylogenetic relationships of brachiopods, phoronids and ectoprocts. Weighted parsimony tree based on an alignment of 80 complete SSU sequences from all available brachiopods, phoronids and ectoprocts together with protostome and other outgroups. The alignment contained 2114 sites of which 804 were parsimony-informative. Heuristic search options (using PAUP, Swofford, 1993) were: collapse zero-length branches, no topological constraints, outgroup rooting, closest addition, no steepest descent, TBR, MULPARS, ACCTRAN. In trial analyses random addition, steepest descent and DELTRAN did not alter tree topology. A search with equally weighted characters found 48 most parsimonious trees of length = 4711 steps, RI = 0.604. After three cycles of character reweighting with the rescaled consistency index (worst fit) followed by heuristic search, the number of trees reduced to 6 of 86565 weighted steps, RI = 0.717. These trees differed only in arrangement of articulate brachiopod terminal taxa on the shortest branches. Bootstrap % are given for key nodes, based on 50 heuristic search replicates with reweighted characters. The limited number of replicates was dictated by computational constraints, which also prevented calculation of support indices.

aligned manually with one another and with protostome and other outgroup sequences (Benson et al., 1994; Maidak et al., 1994; Runnegar et al., 1995; Winnepenninckx et al., 1995; Winnepenninckx & De Wachter, 1994).

DNA was purified, using standard procedures (Sambrook et al., 1989), generally from specimens preserved in alcohol. Sequencing was by the dideoxy termination method using Sequenase 2.0 (USB/Amersham plc) on single-stranded template DNA prepared by asymmetric PCR Allard et al., 1991). Occasionally, a magnetic bead capture method (Dynal, plc) was used (Hultman et al., 1989). Sequence gels and autoradiographs were prepared by standard methods (Sambrook et al., 1989) and sequences were read and recorded manually. With trivial exceptions, every sequence was fully determined from both DNA strands with multiple redundancy. Sequences were aligned by hand Gilbert, 1993; Smith et al., 1994and phylogenetic reconstructions were performed with various methods. The parsimony program PAUP (Swofford, 1993) was used with either equally weighted characters (EP) or with characters reweighted a posteriori on their rescaled consistency index values (WP). Because of the large dataset, parsimony analyses used heuristic searches. Support indices were calculated from the strict consensus trees of non-minimal reconstructions Bremer, 1988; Källersjö et al., 1992). The maximum likelihood method (ML) program fastDNAml Olsen et al., 1994) was used with global branch exchange. Nucleotide divergence was also estimated with PHYLIP's DNAdist with the Kimura 2-parameter correction and bootstrap samples were prepared with SeqBoot; distance and bootstrap trees were constructed with Neighbor for neighbor-joining trees and Consense for bootstrap trees (Felsenstein, 1993).

RESULTS

Relationships between brachiopods and other phyla-- The WP tree is reconstructed from an alignment of 80 sequences from a wide range of metazoa, rooted on choanoflagellates (Figure 1).This tree and others (Cohen & Gawthrop, 1995) clearly separate diploblasts and deuterostomes from protostomes and the position of the lophophorates within the protostome assemblage is strongly supported. Phoronids clearly tree within the brachiopod clade; they do not represent an independent phylum. These two inferences have been reported elsewhere (Halanych et al., 1995). The ectoprocts appear diphyletic and generally remote from the clade of brachiopods plus phoronids (Cohen & Gawthrop, 1995), but the conclusions which can legitimately be drawn from a result based on so few species are a matter of debate (Conway Morris et al., 1995); additional data are needed. Nodes uniting brachiopods and phoronids in this tree are strongly supported, but the same is not true of nodes connecting many other protostomes: bootstrap values around 50% are common because the SSU

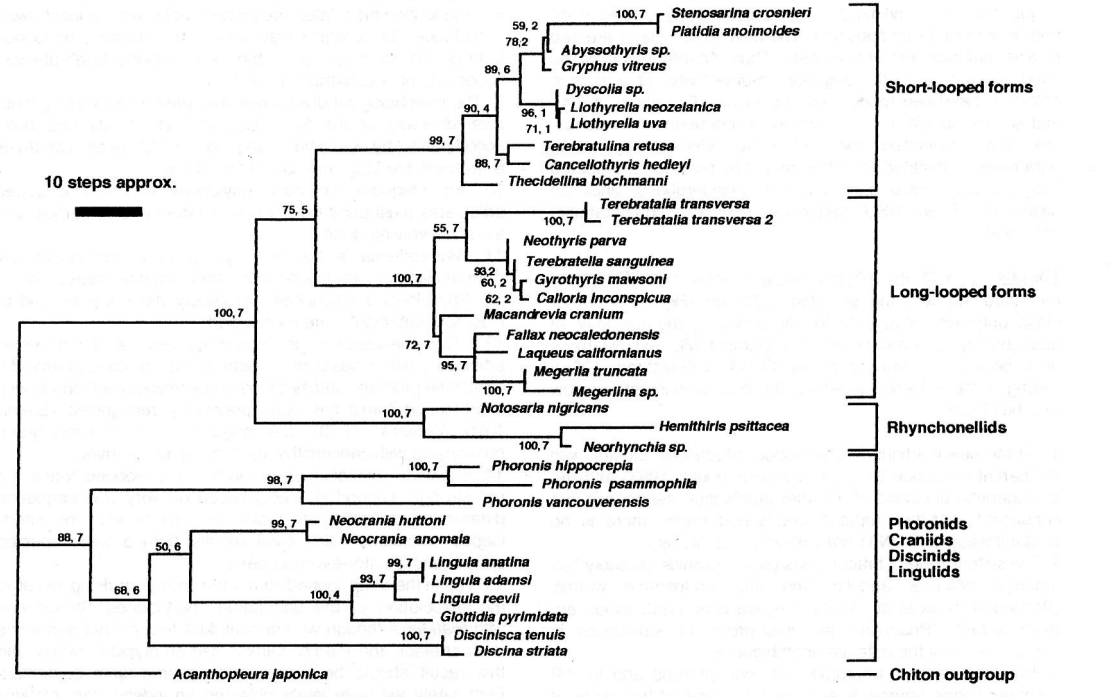

Figure 2. Phylogeny of brachiopods and phoronids based on nuclear-encoded SSU rRNA gene sequences. The alignment was as used for Figure 1 but with unused taxa removed. It contained 1813 sites of which 198 were parsimony-informative. The skewness index of 10,000 random trees was g_1 = -0.504, indicating that WP has a high probability of finding the true tree (Hillis et al., 1994). Heuristic search found 36 minimal trees of 495 steps, RI = 0.801. After 3 cycles of reweighting these reduced to 3 of 20355 weighted steps, RI = 0.892, differing in topology only at the unresolved *Laqueus*, *Fallax*, *Megerlia* plus *Megerlina* node and one of these trees is shown. See caption, Figure 1 for other search details. The numbers adjacent to each node are first, the frequency with which that node appeared amongst 100 bootstrap replicates and second, the support index for that node (Bremer, 1988; Källersjö et al., 1992) on a 7-point scale (Cohen & Gawthrop, 1995).

gene alone cannot resolve the protostome radiation (Adoutte & Philippe, 1993; Phillipe et al., 1994).

The outgroup problem.--Outgroups play a crucial role in phylogenetics (Donoghue & Cantino, 1984; Farris, 1972; Hennig, 1966; Maddison et al., 1984). What is the most appropriate outgroup for the analysis of brachiopod phylogeny? Recent analyses of outgroup rooting (Nixon and Carpenter, 1993; Smith, 1994) stress the dangers of remote outgroups (like choanoflagellates); the closest outgroup is preferred, ideally the ingroup's sister-group. This will minimise homoplasy, facilitate alignment of variable regions and minimise the need to exclude data { ADDIN }(Smith, 1994), in contrast with using evolutionarily remote outgroups (Halanych et al., 1995). The sister-group of the ingroup (brachiopods plus phoronids) would normally be identified by reference to independent evidence such as comparative morphology, but this has led to the lophophorates being regarded as deuterostomes (Brusca & Brusca, 1990; Eernisse et al., 1992), contrary to the molecular results. Since no other, independent evidence exists we must make our outgroup choice recursively, by phylogenetic analysis of the only substantial data-source - nuclear SSU rRNA gene sequences - seeking the protostome sequence phenetically closest to brachiopods plus phoronids. Comparison of branch lengths in unrooted WP, ML and NJ trees and other analyses indicate that the chiton *Acanthopleura* is narrowly the closest outgroup (Cohen & Gawthrop, 1995). This should not be taken to mean that chitons are literally the sister-group of brachiopods plus phoronids; they are simply the phenetically closest outgroup on present evidence, reflecting a combination of available sequences, true phyletic position and similarity of sequence and of nucleotide composition. The next closest taxa are two bivalve molluscs and a polychaete. Thus, *Acanthopleura* will be used as the heuristic outgroup. Subjectivity of outgroup selection has been recognized (Donoghue & Cantino, 1984), and is inescapable in the absence of independent evidence. We have minimised the subjective element by using parameters estimated from the data. The need for a selected outgroup also arises from practical considerations since an alignment of 80 SSU sequences causes computational difficulties.

The phylogeny of brachiopods and phoronids --The WP tree is reconstructed with the selected outgroup (Figure 2). Other close outgroups lead only to alterations in the topology of ambiguously resolved nodes. Comparable ML and NJ trees have been presented elsewhere (Cohen & Gawthrop, 1995). Taking all the evidence together, the conclusions listed below may be drawn:

1. All the new inarticulate brachiopod sequences cluster with the partial sequence from *Lingula reevii* (Field, 1988) and the phylogenetic positions of all other brachiopod sequences are consistent with their reputed brachiopod origin: there is no contamination with DNA from irrelevant organisms.
2. The sister-group of brachiopods plus phoronids probably lies amongst molluscs, annelids and other protostome 'worms' (Winnepenninckx et al., 1995); priapulans and arthropods are more distant. (Priapulans as sister-group of arthropods is unexpected, but the data are unambiguous.)
3. Brachiopods plus phoronids are monophyletic and in WP reconstructions phoronids are basal members of the clade of inarticulate brachiopods. However, in other reconstructions phoronids are apparently diphyletic, with *Phoronis vancouverensis* joining the articulate brachiopods (ML and NJ with low bootstrap support in our trees) as has been reported elsewhere (Halanych et al., 1995). Diphyly of phoronids is biologically implausible and can be explained away by study of the three phoronid sequences. Those of *Phoronis hippocrepia* and *P. psammophila* show no unusual features when compared with other protostomes, but the *P. vancouverensis* sequence (Halanych et al., 1995) lacks at least 9 nucleotides in otherwise highly conserved sites, suggestive of mis-reading. More importantly, all three phoronid sequences share at least two variable-region motifs that are clear synapomorphies of phoronids alone, and the support index for the node uniting all 3 phoronids is relatively high (Figure 3). Moreover, in a reconstruction based only on the most conserved and hence most reliably aligned nucleotides, phoronids are again a monophyletic sister-group of *inarticulate* brachiopods (Cohen & Gawthrop, 1995). Thus, the suggestion that phoronids are most closely related to *articulate* brachiopods (Halanych et al., 1995) must be erroneous.
4. One reconstruction (Figure 3) joins the craniids and lingulids in a clade that has low bootstrap support. In other reconstructions a clade of phoronids plus craniids occurs and is the sister-group of discinids plus lingulids. Thus, whilst the association of phoronids with inarticulate brachiopods is certain, there is insufficient information in the SSU sequences for unambiguous resolution of inarticulates + phoronids. Other, independent evidence is required.
5. Most reconstructions, but not all, place the origin of discinids before that of lingulids. Also, the long branch and basal position of *Glottidia* amongst lingulids are uncertain because this sequence (Halanych et al., 1995) lacks ca. 14 nucleotides in otherwise highly conserved positions.
6. Rhynchonellids are the sister-group of all other articulate brachiopods.
7. Long-looped and short-looped articulates are sister-groups.
8. Long-looped articulates are monophyletic with at least two sub-clades. *Terebratalia* may be either a basal long-looped form or the sister-group of the New Zealand terebratellids, depending on reconstruction method.
9. The morphological divergence that gave rise to the genus-level diversity of the New Zealand terebratellids has been accompanied by very little change in the SSU gene. *Neothyris* is probably the basal member of this clade.
10. An adequate molecular phylogeny of the long-looped articulates must await results from a wider species sample and a faster-evolving gene.
11. *Macandrevia* is the sister-group of a morphologically diverse clade of long-looped forms that includes kraussinids.
12. Short-looped articulates are clearly monophyletic and at least four sub-clades are recognised.
13. The thecideidine is unambiguously a short-looped articulate, either basal or a sister-group of cancellothyrids. Whilst the probable affinity of these enigmatic brachiopods with short-looped forms has been previously recognized (Baker, 1990; Williams, 1973), the suggestion of a sister-group relationship with cancellothyrids appears to be novel.
14. Subclade relationships within the short-looped forms are not strongly supported, being based on very few sequence differences. An adequate molecular phylogeny of the short-looped articulates must await results from a wider species sample and a faster-evolving gene.
15. Within the short-looped forms the most surprising result is the association of the undoubted short-looped *Stenosarina* with *Platidia*. Although we are confident that neither sequence is an artefact, the *Platidia* sample had an atypical history and this result should be treated with reserve until confirmed. Fortunately we have lately obtained an independent platidiid sample.

Rates of molecular and morphological evolution.--The molecular phylogeny is largely congruent with classical brachiopod systematics represented by the classification used in the Treatise (Williams, 1965). Does congruence extend to

lineage times of origin? Space allows only one preliminary result: Figure 3 shows an analysis of clade rank versus age rank (Norell and Novacek, 1992) for brachiopod lineages with well-established times of origin. In this non-parametric analysis, which depends only on relative age and distance, there is complete agreement between the molecular and stratigraphic rankings.

DISCUSSION

Taking into account both published (Cohen & Gawthrop, 1995) and unpublished analyses, the topology of articulate brachiopod lineages has been stable despite various outgroup(s) and reconstructions, suggesting that the phylogeny is broadly reliable. The topology of the inarticulate plus phoronid clade is less stable, but monophyly of craniids, discinids, lingulids and phoronids is assured, as is monophyly of articulate and inarticulate brachiopods. Thus, our primary aim, to provide a secure, molecular basis for the high-level phylogeny of brachiopods, has been substantially achieved. Remaining uncertain high-level relationships will probably be resolved only by discovery of rare, qualitative evolutionary events such as gene order rearrangements in mtDNA. Such new data will also be needed to complete reconstruction of the protostome radiation (e.g. Boore et al., 1995). And a more detailed molecular phylogeny of articulate brachiopods below the superfamily or family level will require sequence data from genes that evolve more rapidly than the nuclear-encoded SSU rRNA.

The most important points to emerge from the molecular analysis are:

1) on the evidence of the SSU genes, brachiopods, phoronids and ectoprocts certainly belong in the clade that contains all undoubted protostomes, not in the deuterostomes (Backeljau et al., 1993; Brusca & Brusca, 1990; Eernisse et al., 1992; Field, 1988; Halanych et al., 1995; Irwin, 1991; Nielsen, 1991; Nielsen, 1994; Nielsen, 1995; Schram, 1991). To escape from this conclusion (allowing the lophophorates to continue as deuterostomes) it must be proposed that an ancestor (or ancestors) of all three lophophorate phyla, originally a member of the deuterostome assemblage, received its SSU gene family by horizontal gene transfer from a mollusc-like protostome. There is no precedent for such a hypothetical event, but further

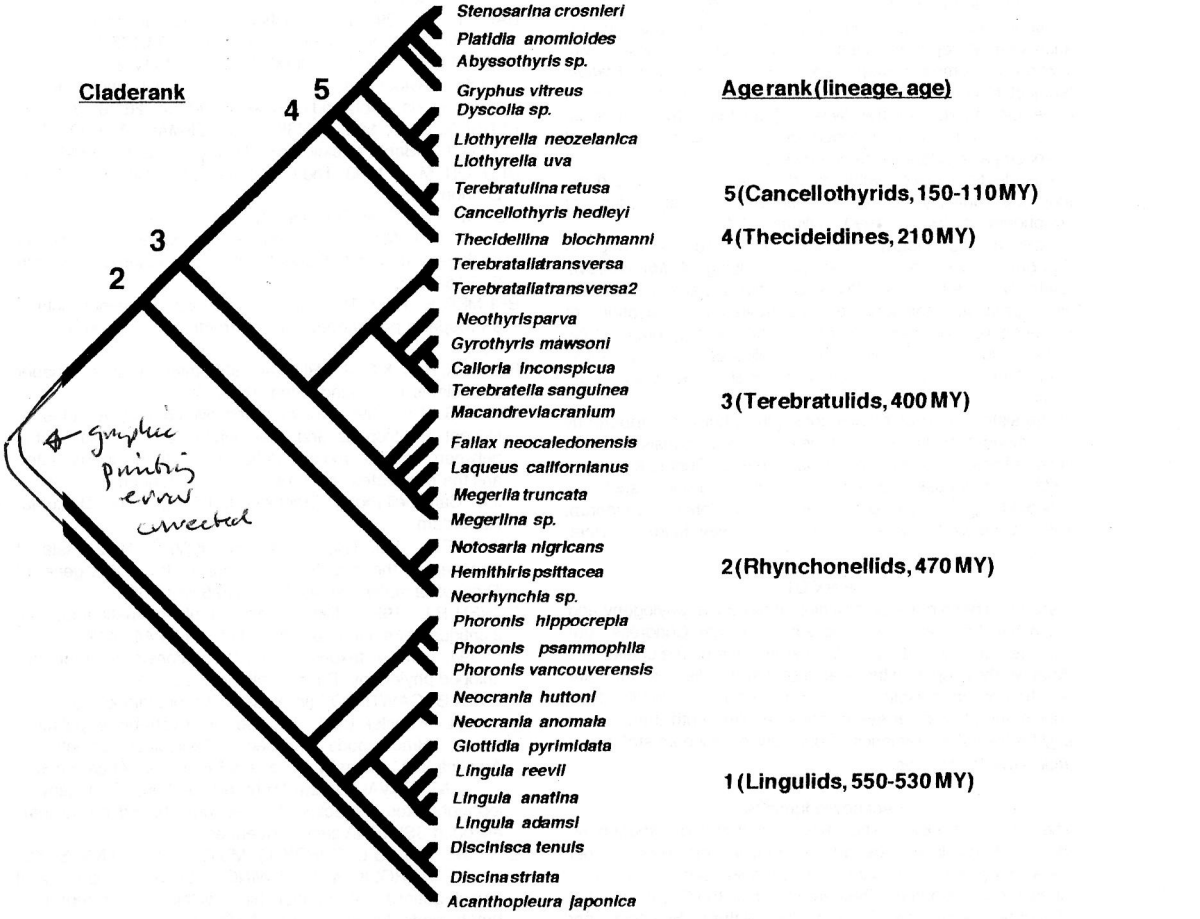

Figure 3. Clade rank compared with lineage age rank. Times of origin of clades are bsed on the origins of the lineages indicated in Benton (1993) and on personal communications (P. Copper, L. Holmer, D. E. Lee, D. I. McKinnon, E. Owen, A. Wlliams).

genetic studies are capable of testing predictions that follow from it. In our view, however, this conflict between molecules and morphology (Conway Morris, 1995; Gee, 1995; Patterson, 1985) arises either because traditional histological methods offer too blunt a tool for the proper recognition of homology in dynamic developmental processes or because such processes are more variable than has been appreciated. For example if three distinct methods of embryonic coelom formation occur in brachiopods, and congeneric species differ references in Chuang, 1990), then coelom formation must be a highly plastic character, unsuited to provide evidence of high-level phyletic relationships.

2) articulate and inarticulate brachiopods do form a monophyletic group within which articulates and inarticulates belong to separate clades. Thus, the traditional system of two brachiopod classes is valid, except that separate phylum status of phoronids is excluded; they should be included with articulate and inarticulate brachiopods (but probably not ectoprocts) in a new phylum. If the possible sister-group relationship of phoronids and craniids is verified, a new taxon (craniids + phoronids) may be called for. Alternatively phoronids should be placed as one of three classes within a new phylum;

3) the results exclude the proposed arrangement uniting craniids with articulate brachiopods (Basset et al., 1993; Gorjansky & Popov, 1986; Popov et al., 1993);

4) short-looped and long-looped articulates (as so far analysed) do represent distinct clades, but articulates with atypical, incomplete loops such as *Megerlia* and *Platidia* (subject to confirmation) may belong to either clade. Thus, a three-fold division of the living articulates (rhynchonellids, short-looped forms, long-looped forms) is presently sufficient to encompass extant sequence diversity;

5) thecideidines belong within short-looped articulates and are therefore unlikely to be descendants of spiriferids or strophomenids (Baker, 1990; Williams, 1973);

6) our results clearly exclude the close clustering of *Terebratalia* and *Laqueus* and the grouping of *Macandrevia* relatively close to *Abyssothyris*, *Gryphus* and *Liothyrella* which were proposed following the controversial application of immunological methods to brachiopod taxonomy (Cohen, 1992; Cohen, 1994; Collins et al., 1991a; Collins et al., 1988; Collins et al., 1991b; Curry et al., 1991; Curry et al., 1993; Endo et al., 1994);

7) the sister-group of brachiopods plus phoronids apparently lies amongst molluscs and annelids. This is consistent with various lines of morphological evidence (e.g. Gustus & Cloney, 1972) and focuses attention on fossil groups such as halkieriids as potential common ancestors (Cohen & Gawthrop, 1995; Conway Morris et al., 1995; Conway Morris & Peel, 1995).

ENVOI

A start has been made on brachiopod molecular phylogeny and much has been accomplished since the last Congress, but much remains to be done. Will someone take up the challenge? What will they report in the year 2000? And if, like us, they are not trained in classical taxonomy, who will identify their specimens? Molecular systematics requires both the genome and traditional systematics. Continuity of museum staffing is imperative for progress.

Acknowledgments

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