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Monophyly of brachiopods and phoronids: reconciliation of molecular evidence with Linnaean classification (the subphylum Phoroniformea nov.)

Bernard L. Cohen

University of Glasgow, Institute of Biomedical and Life Sciences, Division of Molecular Genetics, Pontecorvo Building, Anderson College Complex, 56 Dumbarton Road, Glasgow G11 6NU, UK (*b.l.cohen@bio.gla.ac.uk*)

Molecular phylogenetic analyses of aligned 18S rDNA gene sequences from articulate and inarticulate brachiopods representing all major extant lineages, an enhanced set of phoronids and several unrelated protostome taxa, confirm previous indications that in such data, brachiopod and phoronids form a well-supported clade that (on previous evidence) is unambiguously affiliated with protostomes rather than deuterostomes. Within the brachiopod–phoronid clade, an association between phoronids and inarticulate brachiopods is moderately well supported, whilst a close relationship between phoronids and craniid inarticulates is weakly indicated. Brachiopod–phoronid monophyly is reconciled with the most recent Linnaean classification of brachiopods by abolition of the phylum Phoronida and rediagnosis of the phylum Brachiopoda to include tubiculous, shell-less forms.

Recognition that brachiopods and phoronids are close genealogical allies of protostome phyla such as molluscs and annelids, but are much more distantly related to deuterostome phyla such as echinoderms and chordates, implies either (or both) that the morphology and ontogeny of blastopore, mesoderm and coelom formation have been widely misreported or misinterpreted, or that these characters have been subject to extensive homoplasy. This inference, if true, undermines virtually all morphology-based reconstructions of phylogeny made during the past century or more.

Keywords: Protostomozoa; Deuterostomozoa; Brachiopoda; Phoronida; 18S rDNA; DNA sequence

1. INTRODUCTION

The Brachiopoda, Ectoprocta, Phoronida and Pterobranchia share a tentacular, ciliated, feeding organ, the lophophore and are sometimes referred to as 'lophophorates' (Emig 1977, 1984, 1997; Hyman 1959). Long-standing controversy over the place of these phyla among Metazoa (Brusca & Brusca 1990; Eernisse *et al.* 1992; Nielsen 1995; Nielsen *et al.* 1996; Schram 1991; Willmer 1990) has been clarified by molecular sequence data, mainly from the 18S rDNA gene. These data demonstrate that pterobranchs are most closely related to deuterostome hemichordates, but that the other three phyla have clear protostome affinities, and they have been assigned to a supraphylum, clade-based, assemblage Lophotrochozoa, along with molluscs and annelids, etc. (Aguinaldo *et al.* 1997; Cohen & Gawthrop 1996, 1997; de Rosa *et al.* 1999; Field *et al.* 1988; Halanych 1995; Halanych *et al.* 1995). Protostome affinities have been confirmed by data from *hox* and keratin genes (de Rosa *et al.* 1999; Erber *et al.* 1998), and genetically independent evidence for the protostome affinity of brachiopods has come from analyses of mitochondrial rDNA sequences (Cohen *et al.* 1998*b*) and the complete mitochondrial genome sequence of *Terebratulina retusa* (Stechmann & Schlegel 1999).

Molecular phylogenetic data on brachiopod–phoronid relationships developed erratically. The first report with 18S rDNA sequences from both phyla (Halanych *et al.* 1995) presented a tree with 100% bootstrap support for a sister-group relationship between phoronids (represented by *Phoronis 'vancouverensis'* GenBank accession U12648) and articulate brachiopods (represented by *Terebratalia*, U12650) and no support for a close relationship between the phoronid and an inarticulate brachiopod (*Glottidia*, U12647). Like the seminal work in which 18S rDNA sequences were first applied to metazoan molecular phylogeny (Field *et al.* 1988), this study relied on a single sequence from each phylum. It also included sequence errors (Conway Morris *et al.* 1996; Halanych *et al.* 1996). Lacking the test of phylogenetic congruence provided by multiple sequences from each higher taxon, these studies ran the risk that some types of sequencing error might go undetected and indeed, when a wider sample of brachiopod and phoronid sequences was obtained, it became apparent that U12648 from *P. 'vancouverensis'* did not cluster well with the additional phoronid sequences, two of which had been congruently determined in independent laboratories (Cohen & Gawthrop 1996, 1997; Mackey *et al.* 1996). Moreover, the additional phoronid sequences clustered preferentially with those of inarticulate, rather than articulate brachiopods. Thus, the spectre was raised that molecular data might indicate phoronids to be diphyletic (Cohen & Gawthrop 1997). However, this ghost was laid by analyses suggesting that U12648 might be a chimaeric artefact, perhaps combining 5′-data chiefly from *Terebratalia* with 3′-data from the indicated phoronid (Cohen *et al.* 1998*a*). Molecular support for phoronid–brachiopod monophyly was also obtained in analyses of 18S alignments that included large numbers of outgroup taxa and from which the most variable sites had been removed (Cohen & Gawthrop 1996, 1997; Zrzavy *et al.* 1998). Thus, published 18S sequence analyses demonstrate monophyly of brachiopods and phoronids, the closest relationship being between phoronids and inarticulate brachiopods.

This paper reports molecular phylogenetic analyses of an enhanced phoronid 18S rDNA data set, which includes

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Table 1. *Classification, provenance and identification of specimens and sequences*

(Details are given only for previously unreported specimens which, unless otherwise stated, were identified by the collector (*P. australis*) or by A. Williams (University of Glasgow), C. H. C. Brunton or S. Long (Natural History Museum, London). The following sequences were retrieved from GenBank and relevant publications are cited in the text: *Abyssothyris*, AF025928; *Eohemithiris*, AF025936; *Gryphus*, AF025940; *Megerlia*, U08321; *Neorhynchia*, AF025937; *Notosaria*, U08335; *Terebratulina*, U08324; *Discina*, U08333; *Discinisca*, U08327; *Glottidia pyrimidata*; U12647; *Lingula adamsi*, U08329; *Lingula anatina*, U08331; *Lingula* '*lingua*', X81631; *Neocrania anomala*, U08328; *Neocrania huttoni*, U08334; *Phoronis* '*architecta*', U3627; *P. hippocrepia*, U08325; *Acanthopleura*, X70210, *Cristatella*, AF025947, *Glycera*, U19519; *Pedicellina*, U36273; *Plumatella*, U12649, *Clathrina*, U42452; *P. psammophila*, AF025946.)

genus or binomial	Glasgow accession	GenBank accession	collector (locality and depth where known)
Magellania fragilis	D1296	AF202110	T. Brey; 73°4′ S, 2°1′ W
Discinisca sp.	D1330	AF202444	J. Laudien and C. Lüter; Intertidal, Swakopmund, Namibia
Glottidia palmeri	D1345	AF201744	M Kowalewski and K. Flessa; Baja, CA, USA
Phoronis australis	D1269	AF202111	B. R. de Forges; 10 m, 21°30′ S, 166° E
Phoronis hippocrepia	D1257	AF202112	C. C. Emig; Marseilles harbour
Phoronis ijimai	D1328	AF202113	see § 2

resequencing of *P.* '*vancouverensis*' from newly collected material. This new sequence disagrees with U12648, but agrees at 'signature' sites and clusters closely with all other phoronid sequences.

Monophyly of brachiopods and phoronids bears on interpretations of morphology and the reconciliation of molecular and Linnaean systematics. The former casts doubt on much classical work and the latter leads us to propose changes in classification: the phylum Phoronida is reduced to a subphylum within the Brachiopoda. If further data confirm the suggestion that phoronids and craniid inarticulates are monophyletic, it may be necessary to further reduce phoronids to the class grade and a potential class name is proposed.

2. MATERIAL AND METHODS

(a) *Specimens*

P. '*vancouverensis*' was collected by Sea Life Supply, Inc. (Sand City, CA, USA) from pilings of Monterey pier 2, opposite the west end of Figueroa Street, about 800 ft (250 m) offshore and about 20 ft (7 m) below the surface. Collected material was fixed in ethyl alcohol. Since *P.* '*vancouverensis*' is a junior synonym of *Phoronis ijimai* (Emig 1982), the latter name will be used henceforth. A portion of this sample was prepared histologically and confirmed as *P. ijimai* by C. C. Emig, Station Marine d'Endoume, Marseilles, France. When a bulk sample of this dense 'turf' of interwoven phoronid tubes was teased apart with DNA-free tools under a dissecting microscope, no other macroscopic organisms were seen, and DNA was therefore prepared from a portion using standard methods (Sambrook *et al.* 1989). Other brachiopod and phoronid specimens are described in table 1.

(b) *Polymerase chain reaction (PCR) amplification and DNA sequencing*

Methods were as previously described (Cohen *et al.* 1998*a*) except for changes associated with automated sequencing by the departmental service, performed with the manufacturer's recommended protocols for ABI (Warrington, UK) dye-terminator chemistry, equipment and software. A monodisperse PCR product of the expected size (*ca.* 1.8 kb) was used to produce a double-stranded sequencing template: after de-oiling and deproteinizing with chloroform, DNA was ethanol-precipitated, redissolved and gel-purified by electrophoresis (1.0% agarose, in 1 × Tris–borate–EDTA buffer, pH 8.3). After ethidium bromide post-staining, the template DNA fragment was recovered from a gel slice using Qiagen (Crawley, UK) spin-columns from which it was eluted with buffered water. Templates were sequenced on both strands with three- to fourfold redundancy using selected terminal and internal primers. Base calls were checked and edited manually using ABI software. Alignment and phylogenetic analyses were as previously described, except that PAUP*4b2 (Swofford 1997) was used. New sequences have been submitted to GenBank (Benson *et al.* 1998); accession numbers are given in table 1.

(c) *Outgroup and ingroup selection*

Since brachiopod–phoronid monophyly had been previously found using different, large alignments (Cohen & Gawthrop 1996, 1997; Zrzavy *et al.* 1998), a smaller one was constructed for demonstration purposes. This contained 31 sequences including all available phoronid and inarticulate brachiopods except U12648, omitted for reasons given earlier, and the sequence from *Lingula reevi* (M20086–20088), omitted because incomplete. Since the focus of interest is at phylum level, the selected outgroup must be more distant than the parametrically closest lophotrochozoan (a chiton) used earlier. Exploratory analyses of a large alignment and examination of published data suggested that the sponge *Clathrina*, U42452 (Cavalier-Smith *et al.* 1996) was a suitable, distant outgroup. To demonstrate that phoronids do not cluster with non-brachiopod protostomes, two ectoprocts, one entoproct, one annelid and one mollusc were also included (Cohen & Gawthrop 1997; Cohen *et al.* 1998*a*; Halanych *et al.* 1995; Mackey *et al.* 1996; Winnepenninckx *et al.* 1993). Where a choice was available, taxa whose sequences fell on short branches were preferred. Four or more sequences were available from the three main articulate brachiopod clades and three were retained in each.

Two hypervariable regions corresponding to helices 10 and 10–1 in a well-fitting secondary structure model (Winnepenninckx

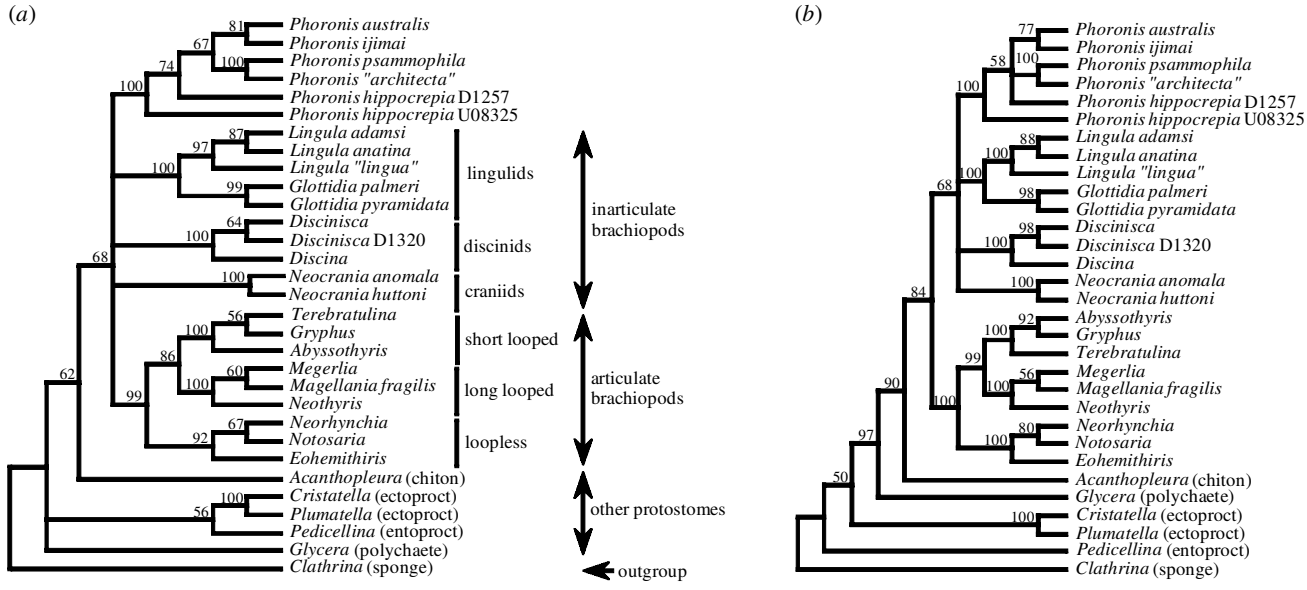

Figure 1. Phylogenetic analyses of 18S rDNA sequences. (*a*) Maximum parsimony bootstrap 50% consensus of 100 heuristic searches with ten random addition replicates and tree bisection–reconnection branch exchange, without steepest descent. Zero-length branches and nodes with less than 50% support were collapsed. Acctran optimization enforced. The single most parsimonious tree obtained under the same search conditions (288 informative sites) was 862 steps total length, CI = 0.52, RI = 0.69 (not shown). (*b*) Minimum evolution, neighbour-joining, bootstrap 50% consensus tree based on Kimura pairwise distances calculated from 1000 pseudoreplicates based on all substitutions, assuming no sites invariant, gap sites ignored, ties (if any) broken arbitrarily and negative branch lengths set to zero.

et al. 1994) were retained in the alignment. To align these regions functional homology of the loops was assumed. A secondary structure model for each taxon was obtained as described (Cohen *et al.* 1998*b*) and the terminal loop and loop-closing bases so identified were aligned, maintaining base-sequence similarity of the largely canonical loop sequences. If the secondary structure model revealed length variation in the base-paired stem, this was accommodated by gaps placed between the loop-closing base-pair and the remainder of the helical stem. Since gap sites were ignored for pairwise distance calculations, length variation will exert little effect and also be largely parsimony uninformative. Moreover, the amount of length variation was small. In a third hypervariable region (h47), length variation was exceptional and secondary structure was used only to confirm the alignment of terminal loops whose base sequences diverge. Phylogenetic analyses of 5′- and 3′-moiety partitions and of the whole alignment with and without the hypervariable regions showed that these regions did not control the observed phoronid–brachiopod clustering.

(d) *Phylogenetic analyses*

Phylogenetic analyses were performed in PAUP*4b2 (Swofford 1997) with maximum parsimony and minimum evolution optimizations; details given in figure legends. In earlier analyses, in which much of the alignment was identical, phylogenetic signal was adequate, base composition did not vary significantly and essentially the same topology of brachiopod relationships was recovered by parsimony, distance and maximum-likelihood analyses using a variety of outgroups. Parameter-rich distance and maximum-likelihood models of molecular evolution, including those which attempt to compensate for variable rates of change at different sites, gave biologically implausible topologies (Cohen & Gawthrop 1997; Cohen *et al.* 1998*a*), perhaps reflecting weaknesses of model-based approaches (e.g. Farris 1999). As here, higher bootstrap support frequencies were obtained at some nodes with minimum evolution than with parsimony, consistent with use by the former of substitutions that would not be parsimony informative. Rates of change were not significantly different among principal clades (Cohen *et al.* 1998*a*) and were not retested.

3. RESULTS

Parsimony and minimum evolution bootstrap consensus trees in which nodes with less than 50% support were collapsed are shown in figure 1. In these analyses a clade of brachiopods + phoronids occurs in 68 and 84% of bootstrap replicates, respectively, indicating that this clade is supported by substantial phylogenetic signal. In the parsimony bootstrap tree (figure 1*a*) and in the topologically identical parsimony jackknife tree (not shown), the clade of articulate brachiopods is well resolved, but there is no basal resolution of the three inarticulate and phoronid clades although each is internally well resolved. In the minimum evolution tree (figure 1*b*), an inarticulate brachiopod + phoronid clade is well supported (68%). In exploratory trees using many additional outgroup and ingroup combinations, phoronids were never separated from articulate + inarticulate brachiopods by sequences belonging to taxa from any other phylum and never joined the clade of articulate brachiopods (data not shown). The relationships in figure 1 are consistent with most previous molecular analyses (Cohen *et al.* 1998*a*,*b*; Zrzavy *et al.* 1998) and with brachiopod phylogeny as inferred from their excellent fossil record (Williams 1997; Williams *et al.* 1996). Thus, these data strongly support brachiopod–phoronid monophyly. In one previous report (Mackey *et al.* 1996) the brachiopod–phoronid clade was reported to be unstable, but this was probably due to inclusion of

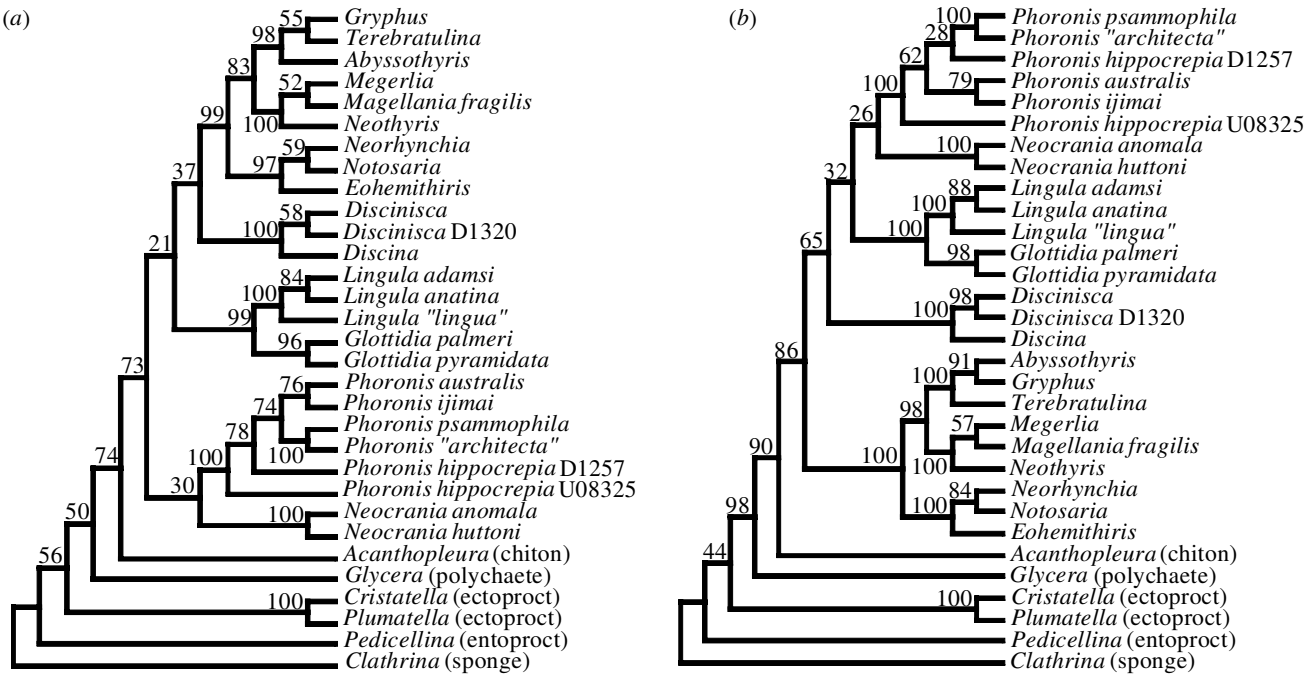

Figure 2. Phylogenetic analyses of 18S rDNA sequences. (*a*, *b*) As figure 1*a*,*b* except that nodes with less than 50% support were not collapsed.

sequence U12648 from *P.'vancouverensis'*. Outgroup relationships are unremarkable, but confirm that ectoprocts (an inadequate taxon sample is available) do not join the brachiopod + phoronid clade. The parsimony bootstrap tree alone indicates stronger support for an association between ectoprocts and the entoproct than was previously reported (Mackey *et al.* 1996), but the significance of this is uncertain, especially as the clade does not also occur in the minimum evolution tree.

Figure 2 shows parsimony and minimum evolution bootstrap consensus analyses identical to those in figure 1, except that nodes with support values below 50% were not collapsed. The parsimony tree (figure 2*a*) has increased support for the brachiopod + phoronid clade (73%—probably a stochastic fluctuation), and both trees show about 30% support for a craniid–phoronid clade. Neither tree shows clear support for a discinid–lingulid clade, which is slightly surprising considering the morphological and ontogenetic characters that these taxa share; similar findings have been discussed elsewhere (Cohen *et al.* 1998*a*). In the light of this result the weak support for a sister-group relationship between craniids and phoronids, which has been noted before (Cohen & Gawthrop 1996, 1997), can be no more than a provisional working hypothesis.

Within the phoronid clade, results are unsurprising. Sequences obtained by different methods from individuals of *Phoronis hippocrepia* collected simultaneously at the same location agree reasonably well. Similarly *Phoronis 'architecta'* and *Phoronis psammophila* agree closely; expected since the former is a junior synonym of the latter (Emig 1982) and the two samples, obtained from the same supplier (Cohen *et al.* 1998*a*; Mackey *et al.* 1996), may be from the same population. The two Pacific species (*P. ijimai* and *Phoronis australis*) cluster together, separate from Mediterranean and Atlantic species. Otherwise, there are only weak grounds for expecting particular relationships among the tested phoronids; morphological features are few and there is no obvious basis on which to polarize a cladistic analysis (Emig 1979, 1982).

4. DISCUSSION

(a) *Reconciling molecular and classical systematics*

Although taxonomic revision should rarely be based solely upon data from gene sequences, it seems right to adapt the classification of brachiopods and phoronids to recognize their genealogical unity. Different approaches are possible. In a purely cladistic approach a new, clade-based structure is created, independent of the existing Linnaean hierarchy, for example, the 'Phoronozoa' (Zrzavy *et al.* 1998). This approach is of limited use to taxonomists, especially of groups with important fossil taxa from which soft tissue and molecular data are unavailable. Alternatively, a new clade structure may be superimposed upon the existing Linnaean hierarchy without associating it with any particular grade, e.g. the Lophotrochozoa (Halanych *et al.* 1995). This approach can lead to novel insights or stimulate new work and is convenient since the new taxon can be abandoned easily. The third approach, to be followed here, alters the existing Linnaean hierarchy, but may be controversial.

(b) *Taxonomy*

The many taxonomic arrangements that have been used to represent phylum-level relationships of brachiopods, phoronids, ectoprocts and other metazoans have been well reviewed (Emig 1997), but since none was genealogical, and most assume deuterostomy of brachiopods and/or phoronids, little detailed discussion is necessary. However, after Hyman (1959) united brachiopods,

phoronids and ectoprocts in the supraphylum assemblage 'Lophophorates' and recognized that they did not fit readily into the deuterostome strait-jacket, Emig (1977) defined a phylum 'Lophophorata' to contain Brachiopoda, Phoronida and Bryozoa as classes. This attractive arrangement will not be adopted here, in part because inclusion of Bryozoa (Ectoprocta) is currently unjustified.

Another arrangement is provided by a recent supraordinal classification of the Brachiopoda (Williams *et al.* 1996), coordinated with the ongoing revision of the brachiopod volumes of the *Treatise on invertebrate paleontology* (Williams 1997). Since this edition will influence present and future brachiopod workers, both neo- and palaeo-, it seems best to follow this classification, especially as the authors considered it to be flexible enough to accommodate the phoronids, although they did not suggest how this might be accomplished (Williams *et al.* 1996). In this classification, articulate brachiopods, and the two major subdivisions of extant inarticulate brachiopods (lingulids + discinids with chitinophosphatic shells, and craniids, with calcitic shells) are each subphyla of the phylum Brachiopoda, but no taxon unites the three inarticulate lineages. Phoronid–brachiopod monophyly can readily be accommodated in this system by designating Phoronida as a fourth subphylum ('Phoroniformea') in the phylum Brachiopoda, consistent with the topology shown in figure 1*a*. However, in the absence of any all-embracing taxon of inarticulate brachiopods, the more specific monophyly of phoronids with inarticulate brachiopods seen in figure 2 cannot be so readily incorporated. But since the available molecular evidence for this clade is weak, we propose that this relationship should be left without classificatory expression for the time being; if it later becomes clear that phoronids and inarticulate brachiopods (or more specifically craniids) really are closely related, this could be recognized by designating phoronids as a class (e.g. 'Phoronata') in the subphylum Linguliformea, with corresponding amendment of diagnoses.

The proposed reclassification requires that the diagnosis of the phylum Brachiopoda (Williams *et al.* 2000) be amended to include the subphylum Phoroniformea, comprising lophophorates with a tube in place of a bivalved shell. This can be achieved simply by generalizing: instead of the common body plan feature being formation of a bivalve shell, it now becomes the ectodermal secretion of a multi-layered, successional extracellular armour. This is lineage-specific: three radically different forms of mineralized bivalved shell in (extant) brachiopods; a polymeric mucopolysaccharide tube in phoronids. Incidentally, polymeric mucopolysaccharides also occur within the layered chitinophosphatic shells of discinid and lingulid inarticulates (Williams *et al.* 1994, 1998), but potential homologies of the underlying molecular and cellular mechanisms of shell and tube formation in these brachiopods and in phoronids remain unexplored. If phoronid–craniid monophyly were to be confirmed, the absence of a shell in phoronids could perhaps be reinterpreted as a character loss.

Doubt has been cast on the use of 18S rDNA sequences for the resolution of deep metazoan branches (e.g. Abouheif *et al.* 1998), and we agree that caution is required and that this gene has its limitations, demonstrated here by, for example, the failure of discinids and lingulids to cluster together despite morphological and life-history similarities. However, the good overall agreement between molecular phylogenetics and the palaeontological history of the extant Brachiopoda suggests that the conclusions drawn here are not unreasonable. Many doubts about molecular systematics may reflect reliance on scanty taxon samples and the necessary exclusion of variable regions when analysing sequences from distantly related organisms; operations that lower reliability (Källersjö *et al.* 1999; Hillis 1996).

As previously noted (Cohen & Gawthrop 1997; Cohen *et al.* 1998*a*,*b*; Emig 1982, 1997), recognition that phoronids and brachiopods are Protostomozoa rather than Deuterostomozoa (Cohen *et al.* 1998*b*) implies either (or both) that the morphology and ontogeny of blastopore, mesoderm and coelom formation on which the original assignments were based have been misrecorded or misinterpreted, or that these characters have been subject to extensive homoplasy. This implication undermines virtually all morphology-based reconstructions of metazoan high-level phylogeny that have accumulated over the past century or more.

(c) *Classification*

Phylum Brachiopoda (Duméril 1806)
Subphylum Rhynchonelliformea (Williams *et al.* 1996)
Subphylum Linguliformea (Williams *et al.* 1996)
Subphylum Craniiformea (Popov *et al.* 1993)
Subphylum Phoroniformea nov.

Diagnosis: Phylum Brachiopoda (adapted from Williams *et al.* (1999), and from Emig (1977); translated in Emig (1982)).

Solitary, marine, coelomate invertebrates enclosed in ectodermally secreted, successional, multilayered, generally bivalved, alternatively tubiculous, armour, bilaterally symmetrical about a medial plane normal to the lophophore or, where present, the surface of separation between valves; shell (where present) organophosphatic or organocarbonatic, attached to substrate by muscular stalk (pedicle) or cuticular pad or secondarily cemented or free and composed of larger ventral (pedicle) valve and dorsal (brachial) valve lined by folded extensions (mantle) of body wall pervaded by canaliferous extensions of coelom; each normally with marginal fringe of chitinous setae. Tube, where present, cylindrical, three-layered, of mucopolysaccharide, possibly chitin, with adherent particles of substratum, burrowing or encrusting. Epithelia monolayered; feeding organ (lophophore) as tentacular, ciliated tubular extensions of coelom, variably disposed and suspended between mantles or protruberant; alimentary canal with or without anus, often U-shaped; nervous system subepithelial; in adults, secretory system comprising one or two pairs of metanephridia, also acting as gonoducts in main body cavity (metacoel); circulatory (haemal) system open or closed; coelom schizo- or enterocoelic; reproduction dioecious or hermaphrodite, possibly protandrous; larvae planktotrophic or commonly lecithotrophic, generally metamorphosing after settlement. *Lower Cambrian–Holocene.*

Diagnosis: subphylum Phoroniformea nov. Diagnosis as given by Emig (Emig 1977, p. 343, 'Phoronidiens'; translated in Emig (1982)).

Note added in proof. The mitochondrial genetic code used by the articulate brachiopod *Laqueus rubellus* provides further evidence for protostome affinities (Saito *et al*. 2000).

The new name for the class Phoronata was suggested by Alwyn Williams. I am grateful to Carsten Lüter and Alwyn Williams for critical reading of a draft. Foundations of the work described were provided by research grants GR3/8708 and GR9/1995 from the UK Natural Environment Research Council. No progress would have been possible without the help of those who collected and identified the specimens, including Tom Brey (Alfred Wegener Institute, Bremerhaven), Bertrand Richer de Forges (Institute for Research and Development, Nouméa, New Caledonia), C. C. Emig (Station Marine d'Endoume, Marseille), Sarah Long and Howard Brunton (Natural History Museum, London).

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As this paper exceeds the maximum length normally permitted, the author has agreed to contribute to production costs.