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Molecular phylogeny of brachiopods and phoronids based on nuclear-encoded small subunit ribosomal RNA gene sequences

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Brachiopod and phoronid phylogeny is inferred from SSU rDNA sequences of 28 articulate and nine inarticulate brachiopods, three phoronids, two ectoprocts and various outgroups, using gene trees reconstructed by weighted parsimony, distance and maximum likelihood methods. Of these sequences, 33 from brachiopods, two from phoronids and one each from an ectoproct and a priapulan are newly determined. The brachiopod sequences belong to 31 different genera and thus survey about 10% of extant genus-level diversity. Sequences determined in different laboratories and those from closely related taxa agree well, but evidence is presented suggesting that one published phoronid sequence (GenBank accession U12648) is a brachiopod–phoronid chimaera, and this sequence is excluded from the analyses. The chiton, *Acanthopleura*, is identified as the phenetically proximal outgroup; other selected outgroups were chosen to allow comparison with recent, non-molecular analyses of brachiopod phylogeny. The different outgroups and methods of phylogenetic reconstruction lead to similar results, with differences mainly in the resolution of weakly supported ancient and recent nodes, including the divergence of inarticulate brachiopod sub-phyla, the position of the rhynchonellids in relation to long- and short-looped articulate brachiopod clades and the relationships of some articulate brachiopod genera and species. Attention is drawn to the problem presented by nodes that are strongly supported by non-molecular evidence but receive only low bootstrap resampling support.

Overall, the gene trees agree with morphology-based brachiopod taxonomy, but novel relationships are tentatively suggested for thecideidine and megathyrid brachiopods. Articulate brachiopods are found to be monophyletic in all reconstructions, but monophyly of inarticulate brachiopods and the possible inclusion of phoronids in the inarticulate brachiopod clade are less strongly established. Phoronids are clearly

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excluded from a sister-group relationship with articulate brachiopods, this proposed relationship being due to the rejected, chimaeric sequence (GenBank U12648). Lineage relative rate tests show no heterogeneity of evolutionary rate among articulate brachiopod sequences, but indicate that inarticulate brachiopod plus phoronid sequences evolve somewhat more slowly. Both brachiopods and phoronids evolve slowly by comparison with other invertebrates. A number of palaeontologically dated times of earliest appearance are used to make upper and lower estimates of the global rate of brachiopod SSU rDNA evolution, and these estimates are used to infer the likely divergence times of other nodes in the gene tree. There is reasonable agreement between most inferred molecular and palaeontological ages. The estimated rates of SSU rDNA sequence evolution suggest that the last common ancestor of brachiopods, chitons and other protostome invertebrates (Lophotrochozoa and Ecdysozoa) lived deep in Precambrian time.

Results of this first DNA-based, taxonomically representative analysis of brachiopod phylogeny are in broad agreement with current morphology-based classification and systematics and are largely consistent with the hypothesis that brachiopod shell ontogeny and morphology are a good guide to phylogeny.

Keywords: brachiopod; phoronid; SSU rDNA; molecular phylogeny; lophophorate; lophotrochozoa

1. INTRODUCTION

In this report, we present a molecular phylogeny based on a taxonomically representative selection of brachiopods. Because these organisms share with ectoprocts and phoronids a tentacular, ciliated, feeding organ, the lophophore, they have sometimes been brought together in a phylum Tentaculata (or Lophophorata) but they share few other morphological, ontogenetic or functional traits and current zoological opinion generally treats them as separate phyla, belonging to a loose assemblage or super-phylum of lophophorates. Although some earlier workers recognized affinities of lophophorates with protostomes such as annelids and molluscs, other authors have treated them as deuterostomes or intermediate. Thus, the broad phylogenetic relationships of the lophophorates have been controversial (Brusca & Brusca 1990; Eernisse *et al*. 1992; Emig 1977; Erwin 1991; Hyman 1959; Nielsen 1991, 1994, 1995; Nielsen *et al*. 1996; Rowell 1981; Schram 1991; Willmer 1990).

Recently, phylogeny has been revitalized by the use of gene sequences, the 'documents of evolutionary history' (Zuckerkandl & Pauling 1965). Provided that congruent gene trees are obtained from independent sources such as nuclear and mitochondrial DNAs, and that paralogy is avoided (Buckler *et al*. 1997; Patterson 1985), this new information provides a means of settling long-standing phylogenetic controversies or at least of seeing them in a new, genealogical light (Hillis *et al*. 1996). Such studies, based on sequences of nuclear-encoded small subunit ribosomal RNA genes (SSU rDNA) have demonstrated unambiguously that brachiopods, ectoprocts and phoronids cluster alongside molluscs, annelids and other coelomate protostomes (Banta & Backus 1995; Cohen & Gawthrop 1996, 1997; Conway Morris 1995; Conway Morris *et al*. 1996; Halanych *et al*. 1995, 1996; Ishikawa 1977; Mackey *et al*. 1996) in a clade named Lophotrochozoa (de Querioz & Gauthier 1990; Halanych *et al*. 1995) and that arthropods, priapulans and other phyla that moult a chitinous cuticle form an Ecdysozoan sister clade (Aguinaldo *et al*. 1997). Molecular studies have also suggested that brachiopods and phoronids form a clade which does not include phylactolaemate ectoprocts (Cohen & Gawthrop 1996, 1997; Halanych *et al*. 1995; Mackey *et al*. 1996), and similar evidence indicates that the also-lophophorate pterobranchs are indeed deuterostomes (Halanych 1995). Thus, unless the cenancestor of protostomes and deuterostomes was lophophorate, at least some lophophores must have originated by parallel or convergent evolution (Moore & Willmer 1997), and the original concept of a lophophorate phylum or other assemblage is no longer tenable. The strong molecular evidence based on the nuclear SSU gene tree for the protostome affinities of brachiopods has recently received independent molecular support from mitochondrial DNA (Cohen *et al*. 1998).

It may seem incongruous that a substantial effort should be expended on gene sequencing in a minor phylum like the brachiopods, but this view overlooks the overall importance of the phylum in Phanerozoic history. Brachiopods first appeared in the Lower Cambrian and they were the dominant filter-feeders and reef-builders until the late Ordovician. Following a decline culminating in the Permian mass extinction, brachiopod diversity has remained fairly stable, with about 300 genera divided between over 20 families. As extant forms represent five of 23 orders, a substantial proportion of Phanerozoic brachiopod diversity is available for sampling (A. Williams, personal communication; Williams *et al*. 1996) and 31 genera from all five orders are included in this study. Although globally rare and patchily distributed, brachiopods may locally number hundreds per square metre, and occur in every ocean, generally in low-energy habitats ranging from barely subtidal to abyssal depths (James *et al*. 1992; Peck 1996). During the past several decades brachiopod studies have been both wide-ranging and vigorous (reviewed in Williams (1997)).

Because of their early origin, continuity throughout the Phanerozoic, high diversity and propensity for fossilization, brachiopods are important to stratigraphy. Indeed, the principal reference source on brachiopod biology, both living and fossil, is the *Treatise on invertebrate paleontology* (Williams 1965, 1997) and most students of brachiopods have been palaeontologists. But because brachiopod systematics has been based largely on shell characters, it has been described as 'nothing more than [a] guide to a taxonomic catalogue' (Williams 1956). Although brachiopods have not been completely neglected during the recent development of molecular phylogeny, no previous work on them has investigated an adequate species sample (Field *et al*. 1988; Ghiselin 1988; Halanych *et al*. 1995; Patterson 1989), and the main object of the present study is to provide the first brachiopod molecular phylogeny that is both based

on informational molecules (Cohen 1994; Zuckerkandl & Pauling 1965) and encompasses a representative taxonomic sample. As such a phylogeny is both genealogical and independent of shell morphology, it will provide a test of the extent to which the existing morphology-based systematics is a good guide to phylogeny and will also provide a basis for the assessment of the more controversial brachiopod shell immuno-taxonomy (see references in Cohen (1994); Curry *et al.* 1993).

Traditionally, brachiopods have been divided into two high-level taxa (now subphyla, see Williams (1997); Williams *et al.* 1996) comprising inarticulated and articulated forms, although other arrangements have been proposed (Carlson 1990; Gorjansky & Popov 1986; Popov *et al.* 1993; Valentine 1973; Williams 1997; Wright 1979). The earliest known fossil brachiopods were inarticulated, with paired valves joined only by muscles and ligaments and the archetypal 'living fossil' *Lingula* belongs to this group, which contains three extant lineages: craniids, discinids and lingulids, all of Early Cambrian origin and now placed in two orders, the Lingulida and Craniida. In both discinids and lingulids (order Lingulida) the shell is chitino-phosphatic (chitin reinforced with apatite), whereas craniids have a calcitic shell (Williams 1997; Williams *et al.* 1996). Articulated brachiopods, in which calcitic shell valves join with an interlocking hinge, first appeared in the Early Cambrian (Benton 1993; Williams 1997; Williams *et al.* 1996) and these provide the largest part of both fossil and extant brachiopod diversity. There are three main lineages of articulated brachiopods which are recognized in the present-day fauna. The order Rhynchonellida, in which the shell valves lack punctae and the spiral lophophore is supported only by short rods, is the oldest lineage and was first recognized in Ordovician strata. The other two extant principal lineages, in which the shell is punctate and the lophophore is supported by either a long or a short, calcareous loop, are placed in the order Terebratulida, which probably dates from the Silurian (Benton 1993; D. E. Lee and D. I. MacKinnon, personal communication; Williams 1997; Williams *et al.* 1996). A possible fourth lineage is represented by thecideidines, in which the lophophore is supported by bas-relief ridges in the brachial valve. Various relationships have been proposed for these enigmatic forms (Baker 1990; Williams 1973).

In contrast with brachiopods, phoronids have almost no fossil record (MacKinnon & Biernat 1970). They are small, worm-like creatures, inhabiting a chitinous tube that is often reinforced with mineral grains. Only two genera are recognized, with about ten species (Emig 1979, 1982). Phoronids are generally thought to be the most 'primitive' of the lophophorates (Brusca & Brusca 1990; Hyman 1959; Willmer 1990), perhaps close to an ancestral form. From the SSU rDNA gene sequence of a single phoronid (GenBank accession U12648) it has recently been proposed that they are the sister group of articulated brachiopods (Halanych *et al.* 1995), but this result has been challenged (Cohen & Gawthrop 1996, 1997). Here, we present results which suggest that phoronids may possibly belong among the inarticulate brachiopods and that the proposed sister-group relationship with articulated brachiopods depends on misleading sequence data.

Ectoproct 'individuals' are generally small and their organization is colonial. They appeared first in the Ordovician from unknown, presumably solitary ancestors (Dzik 1991). Diversity has been and remains high, with three main extant classes and many orders and lower taxa, but SSU rDNA sequences are currently available only from an unrepresentative sample (Banta & Backus 1995; Cohen & Gawthrop 1996, 1997). Concordant sequence evidence from two phylactolaemate genera (this paper; Halanych *et al.* 1995) indicates that this group belongs among the Lophotrochozoa but is not closely related to either brachiopods or phoronids. One sequence from a gymnolaemate (Mackey *et al.* 1996) will not be included in our analyses because its tree position is unreliable (Cohen & Gawthrop 1996, 1997). Little can be concluded about ectoproct phylogeny until a taxonomically representative range of sequences is available.

Many previous authors have discussed the use of quasi-complete SSU rDNA sequences for phylogenetic reconstruction (for example, Adoutte & Philippe 1993; Hillis 1996; Hillis *et al.* 1996; Mindell & Honeycutt 1990; Olsen & Woese 1993; Philippe *et al.* 1994; Raff *et al.* 1994). Overall, it is clear that these sequences provide a wide (but not unlimited) range of phylogenetic resolution. Although the resulting trees are gene trees, there are both theoretical and empirical reasons to treat them (with due caution) as honest reporters of organismal phylogeny and to equate them with species trees. This conclusion receives strong support when, as here, the SSU gene tree is largely concordant with a pre-existent, morphology-based phylogeny and congruent with genetically independent molecular data.

Gene trees are normally rooted (polarized) with a sister group, using the outgroup method (Nixon & Carpenter 1993; Smith 1994). But because phyla reflect morphological discontinuities (*Baupläne*, discussed in Raff (1996)), identification of sister groups at this taxonomic level is inherently difficult or impossible and previous explorations of this problem are unhelpful because they treated lophophorates as deuterostomes (Backeljau *et al.* 1993; Brusca & Brusca 1990; Eernisse *et al.* 1992; Nielsen 1995; Nielsen *et al.* 1996; Schram 1991). We have used the phenetically closest outgroup among lophotrochozoan protostomes (a chiton, identified by a molecular, parametric approach), as well as basal ecdysozoa (representing the sister group of the lophotrochozoa). In addition, we have used phoronid, ectoproct and sipunculan outgroups because these are needed to permit comparison of the molecular results with relevant non-molecular cladistic analyses (Carlson 1990, 1995; Holmer *et al.* 1995; Williams *et al.* 1996). Although each of the last three outgroups is problematical, most conclusions about the SSU rDNA phylogeny of brachiopods and phoronids are not affected by alternative outgroup rootings.

Finally, the unparalleled quality of the articulate brachiopod fossil record suggests that our data should prove useful for the correlation of molecular evolution with time. We show by relative rate tests that the principal articulate brachiopod lineages do not depart significantly from the molecular clock hypothesis and we use selected well-established lineage times of origin to estimate the rate of SSU rDNA sequence evolution. This leads to the conclusion that (unless Precambrian rates of evolution

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

(DNA accession numbers, GenBank accession numbers and, where a taxonomic voucher was available, the Natural History Museum, London, accession numbers are given, together with the collector's initials and locality information. Collectors' names are given in full in the acknowledgements. The majority of newly collected brachiopod specimens were identified by brachiopod taxonomic specialists including C. H. C. Brunton, Natural History Museum; B. Laurin, University of Bourgogne; D. E. Lee, University of Otago and D. I. MacKinnon, University of Canterbury. *Fallax neocaledonensis*, *Stenosarina crosnieri*, and reasons for naming *Eohemithyris grayi* have been described (Laurin 1997). For sequences retrieved from databases, GenBank accession numbers and publication references are given. Outgroup sequences not listed in the table, and their GenBank accession numbers included: *Atrina* (X90961) and *Arca* (X90960) (Steiner & Muller 1996); *Argopecten* (L11265) (Rice *et al*. 1993); *Lepidochitona* (X91975), *Lineus* (X79878) and *Eisenia* (X79872) (Winnepenninckx *et al*. 1995); *Alcyonidium* (X91430) (Mackey *et al*. 1996). Sequences from taxa marked * were used for outgroup selection. Abbreviations: AB, articulate brachiopods; IB, inarticulate brachiopods; lophophore support (loop) types: L, long; M, megathyrid; O, other; R, rhynchonellid; S, short.)

classification	binomial	Glasgow accession number	GenBank (GB) and Natural History Museum (NHM) accession numbers	collector (locality and depth where known)
AB, S	*Abyssothyris* sp.*	D1181	GB: AF025928; NHM: ZB4456	BRdeF (23°05.00′ S, 166°47.81′ E, 830 m)
AB, L	*Calloria inconspicua*	DNZ378	GB: AF025938; NHM: ZB4457	IS (36°24′ S, 174°4.0 E)
AB, S	*Cancellothyris hedleyi**	D1150	GB: AF025929; NHM: ZB4458	LAM (Meelup, Geographe Bay, Western Australia)
AB, S	*Chlidonophora* sp.	D1221	GB: AF025930; NHM: ZB4459	RT (Agulhas Bank, S. E. Africa)
AB, S	*Dyscolia* sp.*	D1219	GB: AF025931; NHM: ZB4460	ET (41°34.7′ S, 148°44.6 E, 1090–1150 m)
AB, R	*Eohemithyris grayi**	D1185	GB: AF025936; NHM: ZB4461	BRdeF (23°02.34′ S, 166°59.14′ E, 295–306 m)
AB. L	*Fallax neocaledonensis**	D1148	GB: AF025939; NHM: ZB4462	BRdeF (23°43.89′ S, 169°16.32′ E, 394–401 m)
AB, S	*Gryphus vitreus**	D525	GB: AF025932; NHM: ZB4463	CCE (off Calvi, Corsica, Mediterranean Sea)
AB, O	*Gwynia capsula**	D1238	GB: AF025940; NHM: ZB4484	AL (Menai Strait, Anglesea, UK)
AB, L	*Gyrothyris mawsoni**	DNZ45	GB: AF025941; NHM: ZB4464	Portobello Marine Laboratory (45°41′ S, 171°05′ E, 100–200 m)
AB, R	*Hemithiris psittacea**	D836	GB: U08322; NHM: ZB4465	SAP (Godthaabsfjord, W. Greenland)
AB, M	*Kraussina rubra*	D1213	—	RT (Agulhas Bank, S. E. Africa)
AB, L	*Laqueus californianus**	D1065	GB: U08323; NHM: ZB4466	Bamfield Marine Laboratory (off Bamfield, BC)
AB, S	*Liothyrella neozelanica**	DNZ289	GB: U08332; NHM: ZB4467	LS (Doubtful Sound 45°20′ S, 167°02′ E)
AB, S	*Liothyrella uva*	D930	GB: U08330	LP (Signy Island, Antarctica)
AB, L	*Macandrevia cranium**	D1224	GB: AF025942; NHM: ZB4468	HGH (Skagerrak 59°0.7′ N, 11°7′ E, 80 m)
AB, M	*Megerlia truncata**	D986	GB: U08321; NHM: ZB4469	GBC (Mediterranean Sea)
AB, M	*Megerlina* sp.	D1218	GB: AF025943; NHM: ZB4470	RT (Agulhas Bank, S. E. Africa)
AB, R	*Neorhynchia* cf. *profunda**	D1090	GB: AF025937; NHM: ZB4471	LL (31°36.00′ N, 120°07.24′ W, 3706–3806 m)
AB, L	*Neothyris parva**	DNZ53	GB: AF025944; NHM: ZB4472	Portobello Marine Laboratory (45°41′S, 171°05′E, 100–200 m)
AB, R	*Notosaria ngricans**	DNZ100	GB: U08335; NHM: ZB4473	CWT (Stewart Island, 46°58′ S, 168°09′ E, 10 m)
AB, O	*Platidia anomioides*	D1216	GB: AF025933	NH (Marion Island, Indian Ocean)
AB, S	*Stenosarina crosnieri**	D1163	GB: AF025934; NHM: ZB4474	BRdeF (23°47.5′ S, 169°48.75′ E, 731–751 m)
AB, L	*Terebratalia transversa**	D1055	GB: AF025945; NHM: ZB4475	CWT (Friday Harbor, San Juan, WA, USA)
AB, L	*Terebratalia transversa* 2	—	GB: U12650	(Halanych *et al*, 1995, 1996)
AB, L	*Terebratella sanguinea**	DNZ150	GB: U08326; NHM: ZB4476	CWT (Stewart Island, 46°58′ S, 168°09′ E, 15 m)
AB, S	*Terebratulina retusa**	D679	GB: U08324; NHM: ZB4477	ASGC (Sound of Jura, Scotland, 30 m)
AB, O	*Thecidellina blochmanii*	D1168	GB: AF025935; NHM: ZB4478	GP and CB (Pago Bay fore-reef, Guam, Pacific Ocean)
IB	*Discina striata**	D1067	GB: U08333; NHM: ZB4479	AW (Solifar Point, Gambia)
IB	*Discinisca* cf. *tenuis**	D1109	GB: U08327; NHM: ZB4480	AW (Walvis Bay, Namibia)
IB	*Glottidia pyrimidata*	—	GB: U12647	(Halanych *et al*. 1995, 1996)
IB	*Lingula adamsi**	D1117	GB: U08329	BRdeF (Touhou, New Caledonia)
IB	*Lingula anatina**	D1139	GB: U08331; NHM: ZB4481	BRdeF (Baie de Dumbea, New Caledonia)
IB	*Lingula* '*lingua*'	—	GB: X81631	(Mackey *et al*. 1996)
IB	*Lingula reevii*	—	GB: M20086–M20088	(Field *et al*. 1988)
IB	*Neocrania anomala**	D320	GB: U08328; NHM: ZB4482	SMBA, Oban (56°29.9′ N, 06°48.2′ W, 85–135 m)
IB	*Neocrania huttoni**	DNZ418	GB: U08334; NHM: ZB4483	GL (North Cape 34°24′ S, 173°01′ E, 15 m)
phoronid	*Phoronis* '*architecta*'*	—	GB: U36271	(Mackey *et al*. 1996)
phoronid	*Phoronis hippocrepia**	D932	GB: U08325	CCE (Marseilles harbour)
phoronid	*Phoronis psammophila**	D1205	GB: AF025946	Gulf Specimen Supply Inc., Panacea, FL, USA
phoronid	*Phoronis* '*vancouverensis*'	—	GB: U12648	(Halanych *et al*. 1995, 1996)
arthropod	*Eurypelma californica*	—	GB: X13457	(Hendriks *et al*. 1988)
chiton	*Acanthopleura japonica*	—	GB: X70210	(Winnepenninckx *et al*. 1993)
phylacto-laemate	*Cristatella mucedo*	D1187	GB: AF025947	DNA provided by BO
phylacto-laemate	*Plumatella repens*	—	GB: U12649	(Halanych *et al*. 1995, 1996)
polychaete	*Lanice conchilega*	—	GB: X79873	(Winnepenninckx *et al*. 1995)
priapulan	*Priapulus caudatus*	D1100	GB: AF025927	Marine Biological Laboratory, Millport, Firth of Clyde
sipunculan	*Golfingia gouldii*	—	M20109–20111	(Field *et al*. 1988)
sipunculan	*Phascolosoma granulatum*	—	GB: X79874	(Winnepenninckx *et al*. 1995)

Table 2. *Oligonucleotide primers used for amplification and sequencing*

(The position of the 3′ nucleotide of each primer is referred to the human SSU rDNA sequence (McCallum & Maden 1985). Primers F20 and H1842 include cloning polylinkers. Primers R1023, R1839 and R954 were newly designed or modified from existing primers (Ellwood *et al*. 1985). Primers F172, F875, R149 and R865 were suggested by J. M. Turbeville (personal communication).)

forward (F) or reverse (R)	position	primer sequence (5′>3′)
F	20	CCGAATTCGTCGACAACTGGTTGATCCTGCCAG
F	38	GATCCTGCCAGTAGTCATATGCTTGTCTC
F	172	TAATTCTAGAGCTAATA
F	433	AGGGTTCGATTCCGGAG
F	638	CGGTAATTCCAGCTCC
F	875	GAATAATGGAATAGGA
F	1037	ATCAAGAACGAAAGT
F	1202	GAAACTTAAA(G/T)GAATTG
F	1337	GGTGGTGCATGGCCG
F	1502	CAGGTCTGTGATGC(C/T)C
F	1706	TGTACACACCGCCCGT
R	149	CGAGATCTTAATGATGTCA
R	427	AGTCCGAGGGAGAGGCC
R	614	(A/T)ATTACCGCGGC(GT)GCTG
R	865	CCGAGGTCCTATTCCA
R	954	AGAATTTCACCTCT
R	1023	ACTTTCGTTCTTGAT
R	1186	ATTCCTTT(G/A)AGTTTC
R	1320	CGGCCATGCACCACC
R	1487	GGGCATCACAGACCTG
R	1839	TGATCCTTCTGCAGGTTCACCTACGG
R	1842	CCCGGGATCCAAGCTTGATCCTTCTGCAGGTTCACCTA

were much higher) a long period of metazoan evolution preceded the first appearance of shelly fossils, as others have also suggested (see, for example, Fortey *et al*. 1997; Guigo *et al*. 1996; Wray *et al*. 1996).

2. MATERIALS AND METHODS

(a) *Specimens*

Provenance, identification and taxonomy of the animals studied are given in table 1. Taxonomic vouchers (where available) have been deposited in the Natural History Museum, London, and DNA sequences have been submitted to GenBank (Benson *et al*. 1997), details in table 1.

(b) *Isolation of DNA*

Total genomic DNA was isolated from the soft tissues of single individuals or, for very small specimens, from pooled whole animals after careful removal of epifauna. Acid-washed tools were used for dissections. Most DNA preparations were from specimens that had been preserved in the field by immersion in alcohol. To remove excess alcohol the tissues of these specimens were first soaked briefly in digestion buffer without detergent or enzymes and then blotted dry by squeezing between clean paper. Tissues were digested in proteinase K/RNAase (20 μg ml^{-1} each) in the presence of 0.1% sodium dodecyl sulphate at 55–60 °C, followed by phenol–chloroform and chloroform extractions, after which DNA was recovered by alcohol precipitation (Sambrook *et al*. 1989) or by absorption on a silica-based spin-column (Qiagen GmBH). Purified DNA was dissolved in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and stored at −20 °C.

(c) *Polymerase chain reaction amplification, purification and sequencing of SSU rRNA*

Double-stranded PCR (DS-PCR) amplification, primer removal and preparation of single-stranded sequencing template by asymmetric PCR (SS-PCR) generally followed the procedures outlined by Allard, Ellsworth & Honeycutt (Allard *et al*. 1991), using oligonucleotide primers listed in table 2.

Occasionally, when difficulty was experienced with the production of a particular single-stranded sequencing template, the DS-PCR was repeated with one biotin-labelled primer, the product captured on streptavidin-coated paramagnetic particles and the captured strands sequenced following the manufacturer's recommendations (Dynal, UK). In all cases a single DS-PCR product of *ca*. 1.8 kb was observed after electrophoresis in 1% agarose gel followed by staining with ethidium bromide. Sequencing reactions were performed with Sequenase 2.0, used according to the manufacturer's recommendations (USB/Amersham Ltd). Sequencing products were generally labelled by ^{35}S dATP incorporation, occasionally with ^{32}P or ^{33}P end-labelled primers. Sequencing products were separated in 6% acrylamide/7 M urea/1 ×TBE gels with a salt gradient formed by addition of 1.5 M sodium acetate to the anode compartment (Sheen & Seed 1988). Gels were dried unfixed and exposed to film before photographic processing. Except for a few nucleotide sites in highly conserved regions where data from a single DNA strand were accepted, both strands were fully sequenced with four to six independent readings obtained for most regions. Sequence ladders were read visually and the sequence files recorded and edited, using SeqApp 1.9a (Gilbert 1993) or GDE 2.2 (Smith *et al*. 1994). Nucleotides corresponding to

the terminal PCR primers were excluded from the sequence alignments, which therefore run from positions 21 or 39 to 1838 or 1841 in the corresponding human SSU rRNA sequence (McCallum & Maden 1985).

(d) *Sequence alignment and masking*

Newly determined sequences were aligned manually, following which, selected outgroup sequences were added. Because of the existence of highly conserved motifs, alignment was unambiguous in all except two regions corresponding to parts of helices E10 and E10-1 in the *Onchidella* secondary structure model (Winnepenninckx *et al.* 1994) and these regions were aligned on the basis of functional homology in predicted secondary structure. For this purpose variable regions were extracted at highly conserved boundaries corresponding to the immediately adjacent 5′ and 3′ helix start-points. To the extracted segments a terminal 6-bp G:C clamp sequence was added, and the program MULFOLD was used to find the minimum-energy folded structure of the corresponding RNA strand (Jaeger *et al.* 1989*a*,*b*; Zuker 1989; Zuker & Jacobson 1995; Zuker *et al.* 1991). The terminal 'tetraloops' (Woese & Pace 1993) so identified were then aligned with one another taking acccount of base similarity, after which the remaining nucleotides were aligned to parsimoniously maximize base similarity with minimum alignment gaps. The connect (ct) files obtained from MULFOLD were converted to graphics (pict) files using loopDloop (Gilbert 1992). After alignment, the most variable sites in E10 and E10-1 were identified using a 50% sequence consensus mask implemented in GDE (Smith *et al.* 1994) and excluded from phylogenetic analyses. Excluded sites corresponded to regions probably involved in slippage-replication or other processes that lead to helix length variation (Hancock 1995; Vogler *et al.* 1996). More stringent masks were also used on an experimental basis. The alignment is available on request from the corresponding author or from http://www.ibls.gla.ac.uk/IBLS/staff/bl-cohen or, as a NEXUS file, from ftp://ftp.ebi.ac.uk/pub/databases/embl/align/ (accession number DS 31426).

(e) *Parametric outgroup selection*

For this computation ten ingroup sequences were discarded from the alignment because they differed only slightly from one or more of 30 retained sequences identified in table 1. Each candidate outgroup sequence was added in turn to the 30 retained sequences and three parameters were calculated: (i) reweighted parsimony tree length; (ii) retention index, an inverse measure of similarity explained by homoplasy in parsimony trees; and (iii) Kimura-corrected nucleotide distance between the tested outgroup and the ingroup node. A fourth parameter, tree log-likelihood gave concordant results and was not used.

(f) *Phylogenetic analyses*

Similarity was calculated with the GCG program Plotsimilarity (Devereaux *et al.* 1984), using a one-base window and the distribution of variable sites was displayed using MacClade (Maddison & Maddison 1992). Parsimony analyses, both equally weighted (maximum parsimony, MP) and with *a posteriori* weighting (weighted parsimony, WP) were made using Paup* (pre-release version d55) (Swofford 1997). Pairwise transition and transversion differences, Kimura two-parameter and LogDet (paralinear) nucleotide distances (Kimura 1980; Lake 1994; Lockhart *et al.* 1994) and neighbour-joining (NJ) distance trees (Saitou & Nei 1987) were obtained using Paup* (Swofford 1997) or PHYLIP 3.5 (Felsenstein 1993). Maximum likelihood (ML) analyses were made with fastDNAml 1.0 (Olsen *et al.* 1994) and PUZZLE 3.1 (Strimmer & von Haeseler 1996); only the latter are reported. The shape parameter of the gamma distribution used to model the distribution of substitution rates at variable sites in ML and distance analyses was estimated from the data with both PUZZLE and Paup*.

The alignment was tested for phylogenetic information-content using Paup* by plotting the distribution of 10 000 random trees, with calculation of g_1 (Hillis & Huelsenbeck 1992) and by a permutation tail probability test (PTP) with 100 replicates (Faith & Cranston 1991). Because the data set was too large for any exact tree-finding algorithm, MP and WP trees were recovered by heuristic search (HS) using closest addition order and tree bisection–reconnection (TBR) branch exchange with MULPARS and ACCTRAN options. Use of this combination was validated by finding that 10–100 cycles of random addition with TBR branch exchange, with or without steepest descent, never led to a shorter tree and that other combinations of addition order and branch swapping routine sometimes failed to recover the shortest trees. For WP, following an HS with equal weighting, characters were reweighted using the rescaled consistency index (RCI) with a base-weight of 1. A total of three cycles of reweighting and HS generally led to stable tree lengths. This character-weighting procedure, which is equivalent to successive approximation (Farris 1969), tends to correct maximum parsimony for site-to-site variation in rate of evolution and greatly reduces the number of equally most parsimonious trees. Bootstrap consensus trees based on 100–1000 replications were obtained by HS with simple addition or by fast HS, with character weights applied. Parsimony jackknifing with 1/*e* exclusion (Farris *et al.* 1996) was performed in Paup* with the default sampling procedure. Jackknife support frequencies led to the same conclusions as the bootstrap and are not presented.

In parsimony analyses, a wide variety of analytical options was explored, including differential weighting of transversions and transitions (2:1 to 10:1), but these weights were without effect on tree topology and equal weights were therefore used. Differential weighting of helices and loops using empirically determined relative rates was also explored, but found to be of minor significance and is not reported. Decay analysis (Bremer 1988) was reported in a preliminary account of this work (Cohen & Gawthrop 1997), but results are not presented here because computational problems prevented complete analysis and the results only confirmed that nodes with low bootstrap support also have low Bremer support.

(g) *Estimation of evolutionary distances and rates*

A total of four methods of correcting raw distances for multiple substitutions and site-to-site variation were tested: (i) an empirically derived correction (Van de Peer *et al.* 1993, 1996) of '*p*' distances; (ii) maximum likelihood

distance under the discrete gamma-distribution model with four to eight rate categories, with invariant site frequency and gamma-distribution shape parameter estimated from the data; (iii) Kimura two-parameter distance similarly transformed; and (iv) untransformed Kimura distances per variable site. Only results obtained with the latter are reported. For comparison with times of earliest appearance of brachiopod lineages, branch lengths from each node were calculated as half the average pairwise distance between all taxa in the descendant pair of lineages and the standard deviations (s.d.) were taken as half the s.d. of the lineage average pairwise distance. Where two sequences were available from the same species, the one giving the longer distances was omitted. Earliest and latest geological periods for the appearance of brachiopod lineages were modified from Harper *et al.* (1993) on the basis of personal communications from several contributors (see acknowledgements) to the *Treatise on invertebrate paleontology* (Brachiopoda, revised) (Williams 1997). Absolute ages for geological periods were taken from Harland *et al.* (1989). Lineage relative rate tests (Li & Bousquet 1992) were implemented in a spreadsheet provided by Dr J. Laroche, Université de Laval, Canada.

3. RESULTS

(a) *Sequence reliability and alignment parameters*

The phylogenetic reconstructions reported here are based on comparisons of newly determined SSU rDNA sequences from 33 brachiopods, two phoronids and one ectoproct, together with rDNA and rRNA sequences from four brachiopods, two phoronids, one ectoproct and other outgroups obtained from public databases (table 1). Reliability of the new sequences is indicated by agreement with previously reported congeneric or conspecific sequences from *Lingula reevii* (Field *et al.* 1988), *Terebratalia transversa* (Halanych *et al.* 1995), *Priapulus caudatus* (Winnepenninckx *et al.* 1995) and *Phoronis 'architecta'* (Mackey *et al.* 1996) (a junior synonym of *P. psammophila* (Emig 1979, 1982)). Of the new sequences, two will be treated with reserve: *Platidia anomioides* because the specimens were recovered from a broken transit vial (Cohen & Gawthrop 1996, 1997) and its tree position is unexpected; and *Kraussina rubra* because it is incomplete, contains ambiguities and is prone to long branch attraction artefacts (not shown). Where appropriate, the incomplete sequences from *L. reevii* and *K. rubra*, were omitted from analyses.

Before insertion of alignment gaps, median length of the newly determined sequences (excluding *K. rubra*) was 1768 bp (range 1723–1781 bp), most of the variation owing to *ca.* 30 bp of undetermined 5′ terminal sequence in *Thecidellina* and *Platidia*. Total length of the alignment (new sequences plus outgroups) was 1878 sites and the average similarity (ingroup only) was 90%. In helices E10 and E10-1 (Winnepenninckx *et al.* 1994), ingroup sequences showed evidence of short (up to three nucleotide) changes in helix length and some outgroups showed greater length variation. As these sites are prone to misalignment they were excluded from all analyses, forming a 'minimal exclusion set' comprising 12 sites. In addition, an unalignable, autapomorphic insertion in helix E10-1 of the sipunculan outgroup *Phascolosoma* was excised. Figure 1 shows the distribution of base substitutions along the ingroup alignment and gives a visual impression of the extent of rate heterogeneity across sites. The minimal exclusion set is identified in the figure legend.

Other insertions or deletions (indels) in ingroup sequences consisted of one or two nucleotides only and some of these (but probably none in our sequences) could have resulted from misreading the number of bases in conserved homopolymeric runs. Alignment gaps at indel sites were treated as missing data rather than as a fifth character state because these autapomorphic features make no contribution to parsimony analyses and, being short, contribute trivially to distance and maximum likelihood branch lengths.

Mean (range) base composition of the SSU genes from 35 brachiopods, three phoronids (*Phoronis vancouverensis* excluded) and the closest outgroup, *Acanthopleura*, was A: 0.229 (0.220–0.236); C: 0.208 (0.198–0.218); G: 0.252 (0.247–0.256); T: 0.234 (0.223–0.240). Heterogeneity χ^2 tests found no significant differences ($p=0.05$) in the base composition of variable sites among brachiopod and phoronid sequences, neither overall, nor between morphological groups, nor between representative single sequences from each morphological group. Thus, base composition differences are unlikely to generate misleading phylogenetic reconstructions. The presence of substantial phylogenetic structure in the alignment of 40 brachiopod plus phoronid sequences was indicated by: (i) skewness parameter $g_1=-0.52$, corresponding to $p<<0.01$ for the number of characters and taxa involved (Hillis & Huelsenbeck 1992); (ii) PTP=0.01 (Faith & Cranston 1991); and (iii) the low frequency of unresolved quartets (Strimmer & von Haeseler 1996) in the analysis for figure 6 (see legend).

As absolute numbers of pairwise transition and transversion differences between all ingroup sequences (figure 2) show only slight saturation, pairwise distances and transition–transversion ratios were corrected for unseen multiple events by the Kimura two-parameter method (Kimura 1980). The mean Ti:Tv ratio was 2.1 ± 0.03(s.e.m.):1 at parsimony-informative sites and 1.95 ± 0.02:1 at variable sites. When empirical and gamma-distribution-based corrections were applied (Van de Peer *et al.* 1996; Yang 1994), the 'corrected' pairwise distances led to implausible ingroup tree topologies (see § 3b (iii)). When absolute numbers of pairwise transition and transversion differences between all ingroup and outgroup sequences were plotted, considerable saturation of transitions was evident (not shown).

(b) *Phylogenetic reconstructions*

(i) *Outgroup selection*

Tables 3 and 4, and figure 3, present details relating to parametric outgroup selection and the weighted parsimony bootstrap consensus trees obtained with a variety of outgroups. The outgroups, and considerations leading to their selection, were as follows.

1. Protostome phyla form two sister clades, the Lophotrochozoa and Ecdysozoa (Aguinaldo *et al.* 1997). Basal ecdysozoans were therefore used as the most distant outgroup (figure 3*a*).
2. When the protostome tree is rooted with deuterostome, diploblast or non-metazoan outgroups, phylactolaemate

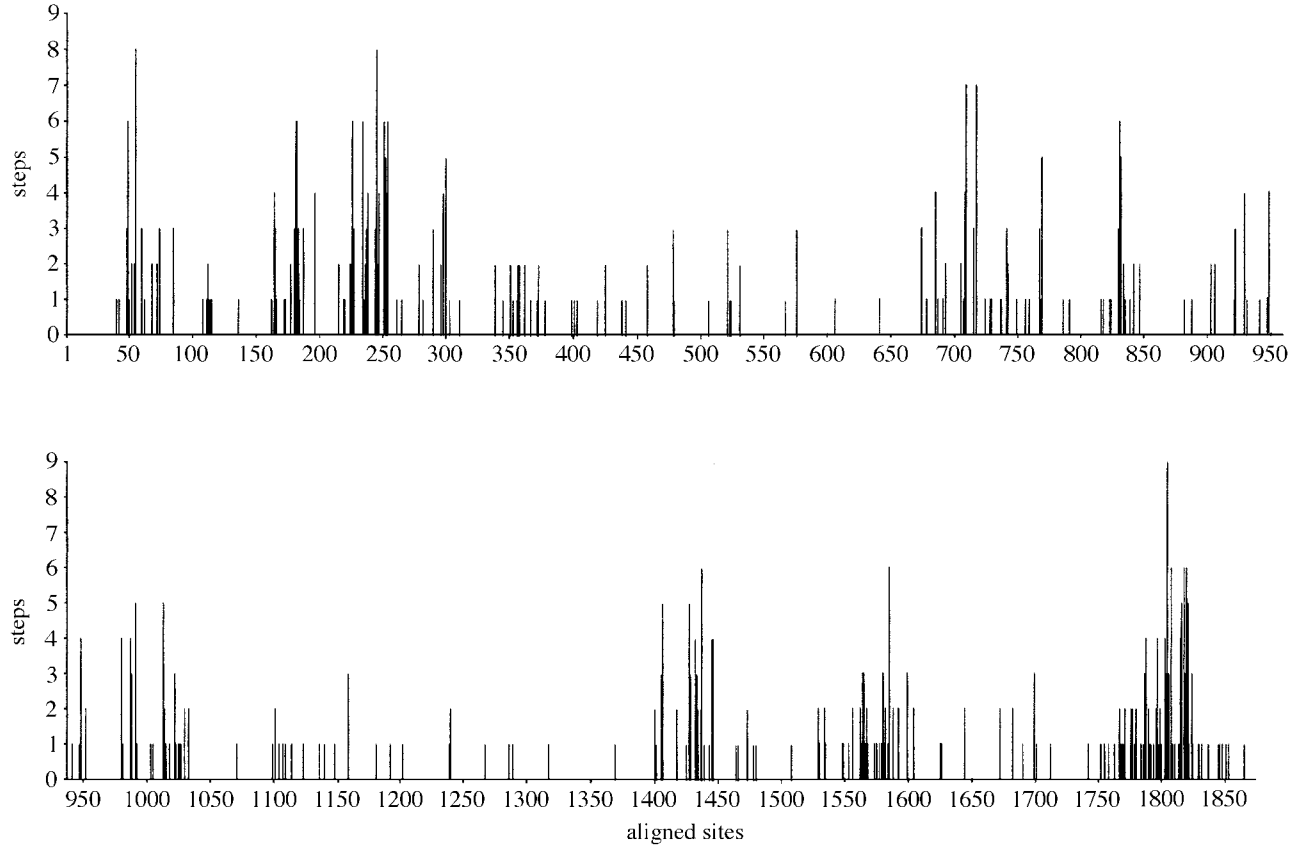

Figure 1. Distribution of parsimony-informative differences in the ingroup alignment. (Based on tree 1 of 18 equally most parsimonious trees of 209.03 reweighted steps, chiton outgroup. The vertical lines indicate the number of differences occurring at each of the 1878 aligned sites. The minimal exclusion set comprised sites 176–178, 183, 225–228 and 233–236.)

ectoprocts branch near the base of the Lophotrochozoa. They are therefore rather distant from the brachiopod plus phoronid ingroup (this work; Cohen & Gawthrop 1996, 1997; Halanych *et al.* 1995) and introduce avoidable homoplasy. Furthermore, available phylactolaemate sequences are unrepresentative of all ectoprocts (Banta & Backus 1995; Conway Morris *et al.* 1996). Nevertheless, ectoproct outgroups are needed to permit comparison with morphology-based analyses (Carlson 1990, 1995; Williams *et al.* 1996) (figure 3*b*).

3. Like ectoprocts, sipunculans (figure 3*c*) are distantly related to brachiopods (Cohen & Gawthrop 1996, 1997; Halanych *et al.* 1995; Winnepenninckx *et al.* 1995) but are required for comparison with other studies (Carlson 1990, 1995; Williams *et al.* 1996). Of two available sequences, one is incomplete (Field *et al.* 1988) and the available complete sequence (*Phascolosoma*, Winnepenninckx *et al.* 1995) is prone to long-branch attraction effects, e.g. the gymnolaemate ectoproct *Alcyonidium* may behave as its sister group, but trees elsewhere in its absence (not shown).
4. The closest available polychaete (table 3, *Lanice*) was chosen as representative of that diverse lophotrochozoan phylum, consistent with suggestions that brachiopods, phoronids, molluscs and annelids (*s.l.*) are sister groups (Aguinaldo *et al.* 1997; Cohen & Gawthrop 1996, 1997; Field *et al.* 1988; Halanych *et al.* 1995; Patterson 1989) (figure 3*d*).
5. Using a multi-parametric approach (table 3), the polyplacophoran mollusc *Acanthopleura*, a chiton, was identified as the proximal (lophotrochozoan) outgroup (figure 3*e*). In addition, the parametrically closest three outgroups used together gave similar results (not shown).
6. A phoronid outgroup (figure 3*f*) is required for comparison with morphological studies (Carlson 1990, 1995; Williams *et al.* 1996). However, phoronids sometimes cluster within the inarticulate brachiopod clade (this work; Cohen & Gawthrop 1996, 1997), and if this is a true relationship they cannot be a valid outgroup.

(ii) *Weighted parsimony analyses*

Figure 3*a–f* and table 4 show the results of WP analyses with different outgroups; the main differences are in resolution of weakly supported nodes. The data in table 4 do not show any outgroup to be unambiguously superior: ectoprocts yield the smallest number of unweighted trees but also lead to a low retention index (RI) and likelihood. Sipunculans give the least resolution when unweighted, but a marginally superior likelihood. Of the undoubted outgroups (allowing that phoronids might be an ingroup), the chiton gives the highest RI and likelihood, but also yields more most parsimonious trees.

Among the articulate brachiopods in figure 3, three main clades corresponding to the rhynchonellid, and (broadly interpreted) long- and short-looped forms are always recognized, but rhynchonellids appear either as the basal clade (figure 3*d*) or, more often (and unconformably with the fossil record) as the sister clade

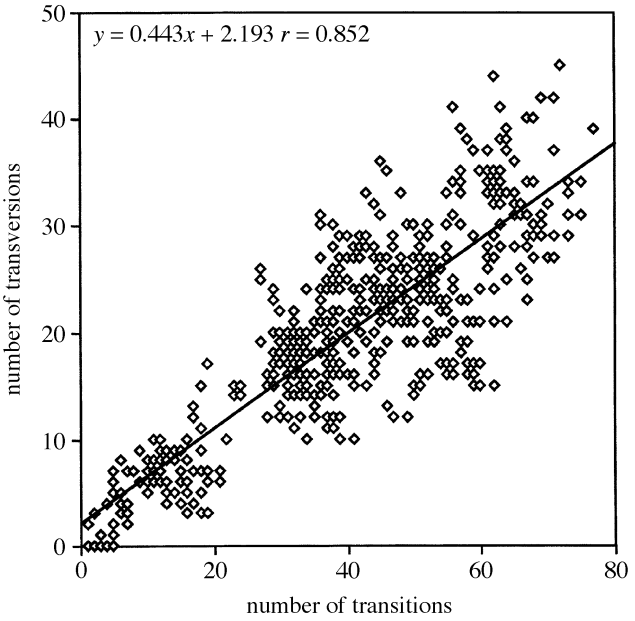

Figure 2. Correlation of pairwise transition and transversion differences beween ingroup sequences. (The data points represent 630 non-self pairwise comparisons among the brachiopod and phoronid ingroup.The straight line through the data points is a linear correlation function. Gapped or ambiguous sites were ignored in the pairwise distance calculations.)

of long-looped forms. Within the short-looped clade, support for a cancellothyrid subclade rarely exceeds 50%. This result, and a similar one involving the inarticulate lingulids, raises an issue concerning the interpretation of resampling support indices that will be discussed below (and see 'note added in proof', at end). Also in the short-looped clade, a *Dyscolia–Liothyrella* clade is consistently found, though the divergence between the two *Liothyrella* species is somewhat unexpected. In the other short-looped clade the position of *Platidia* is suspect for reasons given in this paper and because of its great similarity (but not identity) with the undoubted short-looped form *Stenosarina*. Classically, *Platidia* has been placed close to other megathyrids (Williams 1965). The basal position of *Gryphus* in this clade may reflect its post-Miocene isolation in the Mediterranean. The thecideidine *Thecidellina* invariably clusters with short-looped forms, whereas, on morphology, it might have been expected to branch at the base of all articulate brachiopods (Baker 1990; Williams 1973). However, its internal position in these WP trees appears to be strongly supported. *Kraussina*, like *Platidia*, might have been expected to join the megathyrids. Its position close to or within the cancellothyrids appears to be robust, but caution is needed on account of its imperfection.

Resolution within the long-looped clade is fairly consistent: the minute, infaunal form *Gwynia* is uniformly basal and *Macandrevia*, which is thought to be the sole extant representative of a Triassic lineage (MacKinnon & Gaspard 1995), is on an appropriately long branch. A subclade unexpectedly contains both two 'megathyrids' (*Megerlia* and *Megerlina*—with distinctive loops) and two morphologically very different long-looped forms (*Laqueus* and *Fallax*) whose sequences are so similar that they are generally unresolved. Given the great ontogenetic differences between the latter genera, this result needs confirmation. The other subclade contains the north-eastern Pacific form *Terebratalia* basally, and a terminal cluster comprising several genera endemic to New Zealand, Australian and Antarctic waters. Alone among the outgroups, ectoprocts (figure 3*b*) identify a previously reported (Cohen & Gawthrop 1996, 1997) phoronid plus inarticulate brachiopod clade; with other outgroups the phoronids are either excluded from the brachiopods (figure 3*c*,*d*,*e*,*f*) or join an unresolved polytomy with the three inarticulate brachiopod lineages (figure 3*a*). No outgroup yields a tree in which phoronids are most closely related to articulate brachiopods (see §3d below concerning *Phoronis vancouverensis*; Halanych *et al.* 1995).

In figure 4, the results obtained with the six outgroups were combined into single trees by using two approaches (provisionally treating phoronids as an outgroup). These are a majority-rule consensus (figure 4*a*) of all the bootstrap trees (i.e. those underlying the consensus trees in figure 3) and a similar consensus (figure 4*b*) of the 48 WP trees listed in table 4. Weaknesses of this majority-rule 'ballot-box' approach are: (i) that the sample of outgroups used was neither unlimited nor necessarily optimal; (ii) that because they are based on sampled data, bootstrap trees are not necessarily reliable as phylogenies; and (iii) that trees built with different outgroups have been amalgamated and the outgroups removed. Nevertheless, this approach provides a practical summary of multiple trees and the differences between the resulting consensus trees give an indication of the loss of resolution owing to bootstrap resampling: evidently trivial in this case. So that weakly supported clades can be seen, groups with less than 50% bootstrap support are also shown (figure 4*a*) and this is further discussed.

Another approach to conflicting or alternative trees rejects those that contradict strong, independent evidence. The fossil record shows that rhynchonellids are the basal extant articulate brachiopod lineage (Williams 1997; Williams *et al.* 1996), a result given only by the polychaete outgroup (figure 3*d*). In addition, the morphologically supported cancellothyrid clade (Cooper 1973) received marginally significant resampling support only from the ectoproct outgroup (figure 3*b*) and in the consensus of bootstrap trees (figure 4*a*) (see 'note added in proof', at end). Thus, in these WP bootstrap consensus trees, no one outgroup gives results in full accord with the fossil record.

(iii) *Distance and maximum likelihood analyses*

For comparison with the WP trees, figure 5 shows bootstrap consensus trees built from the same alignment by the neighbour-joining (NJ) method using two different distance measures, and rooted with the chiton outgroup. The two trees illustrated, which differ trivially in topology, were based on Kimura two-parameter (figure 5*a*) and LogDet (paralinear) distances (figure 5*b*). The agreement between them confirms the unimportance of base-frequency differences between taxa. Generally high concordance between these NJ trees and the corresponding WP trees (figures 3 and 4) is evident, with two main exceptions in the NJ trees: (i) the rhynchonellids are in their expected, basal position with respect to other articulate brachiopods; and (ii) phoronids are a moderately

Table 3. *Parametric outgroup selection*

(Parsimony tree parameters resulting from HS of an alignment of 30 ingroup sequences (see § 2*e*) with characters equally weighted. Neighbour-joining (NJ) tree branch lengths were based on Kimura distances (Kimura 1980) with equal rates assumed. The outgroup branch length represents the distance from the outgroup to the first ingroup node. RI, retention index.)

	ingroup tree parameters and ranks							
	parsimony				neighbour-joining			
outgroup	tree length	tree length rank	RI (×1000)	RI rank	outgroup branch length	branch length rank	sum of ranks	rank of ranks
Acanthopleura	452	1	801	1	0.1626	2	4	1
Atrina	462	3=	788	6=	0.1399	1	10	2
Arca	462	3=	793	5=	0.1717	3	11	3
Chlamys	465	5	797	2=	0.1868	5	12	4=
Lepidochiton	456	2	797	2=	0.2447	8	12	4=
Argopecten	467	7	794	4	0.1928	6	17	6
Cristatella	468	8=	783	11=	0.2437	7	26	7=
Lanice	466	6	786	9	0.3751	11	26	7=
Crassostrea	472	11	788	6=	0.3590	10	27	9
Glycera	477	12=	781	14	0.1768	4	30	10=
Lineus	470	10	783	11=	0.2812	9	30	10=
Eisenia	477	12=	788	6=	0.4050	13	31	12
Plumatella	468	8=	784	10	0.4254	14	32	13
Priapulus	489	14=	783	11=	0.4006	12	37	14
Eurypelma	489	14=	777	15	0.5253	15	44	15
Phascolosoma	507	16	774	16	0.7436	16	48	16

Table 4. *Reconstructions using different outgroups*

(The alignment comprised 37 ingroup taxa and one, two, or three outgroups, listed in descending order of proximity to the ingroup. The unweighted HS employed TBR branch exchange on an NJ Kimura distance starting tree; the same end-point was reached more slowly from random or closest addition sequence starting trees. RCI-reweighted trees were obtained by three (occasionally four) cycles of reweighting on the best fit of the character RCI with HS on all trees in memory using closest addition sequence and TBR branch exchange. Log likelihoods were calculated using identical maximum likelihood option settings. Likelihoods of each group of equally most parsimonious trees agreed to five significant figures. CI, consistency index; RI, retention index.)

		equally weighted			RCI reweighted			
outgroup (no. of taxa)	no. of informative sites	no. of trees (length)	CI ×1000	RI ×1000	no. of trees (length)	CI ×1000	RI ×1000	−ln likelihood
ecdysozoa (2)	228	216 (621)	533	792	6 (240)	714	891	3268.81
ectoprocts (2)	229	72 (588)	537	805	6 (238)	723	896	3174.04
sipunculans (2)	220	1548 (611)	538	794	6 (239)	709	890	3163.27
polychaete (1)	197	108 (521)	524	810	6 (207)	705	900	2782.54
chiton (1)	197	396 (505)	537	819	18 (208)	719	908	2969.34
phoronids (3)	195	108 (468)	556	833	6 (205)	741	918	2592.31

well-supported sister group of inarticulates, as previously reported (Cohen & Gawthrop 1996, 1997). These distance trees were obtained without any attempt to 'correct' for site-to-site rate variation, although this appears to be present in the data. As has been noted previously in SSU rDNA sequences, there are many more constant sites than would be predicted from base frequencies (see, for example, Aguinaldo *et al.* 1997), and the numbers of sites with 0, 1, 2, etc. changes approximately fits a gamma distribution with strong rate heterogeneity (see, for example, Kumar & Rzhetsky 1996). However, when maximum likelihood estimates of the gamma-distribution parameters were used to 'correct' Kimura distances, the resulting NJ trees contained nonsensical relationships such as phoronids as sister group of *Lingula* spp. to the exclusion of *Glottidia* or ectoprocts and ecdysozoans as sister groups of different lingulids (details not shown). Thus, this method for 'correcting' distance analyses for site-to-site rate variation appeared problematical and was not used. Similarly, 'correction' of raw ('*p*') distances with an empirical transformation (Van de Peer *et al.* 1996) led to an unacceptable NJ tree topology and is not reported.

Broadly speaking, figures 4 and 5 establish that, except for the phoronid and rhynchonellid clades, the topologies of WP and NJ trees (based on untransformed Kimura distances) are largely robust and congruent. But

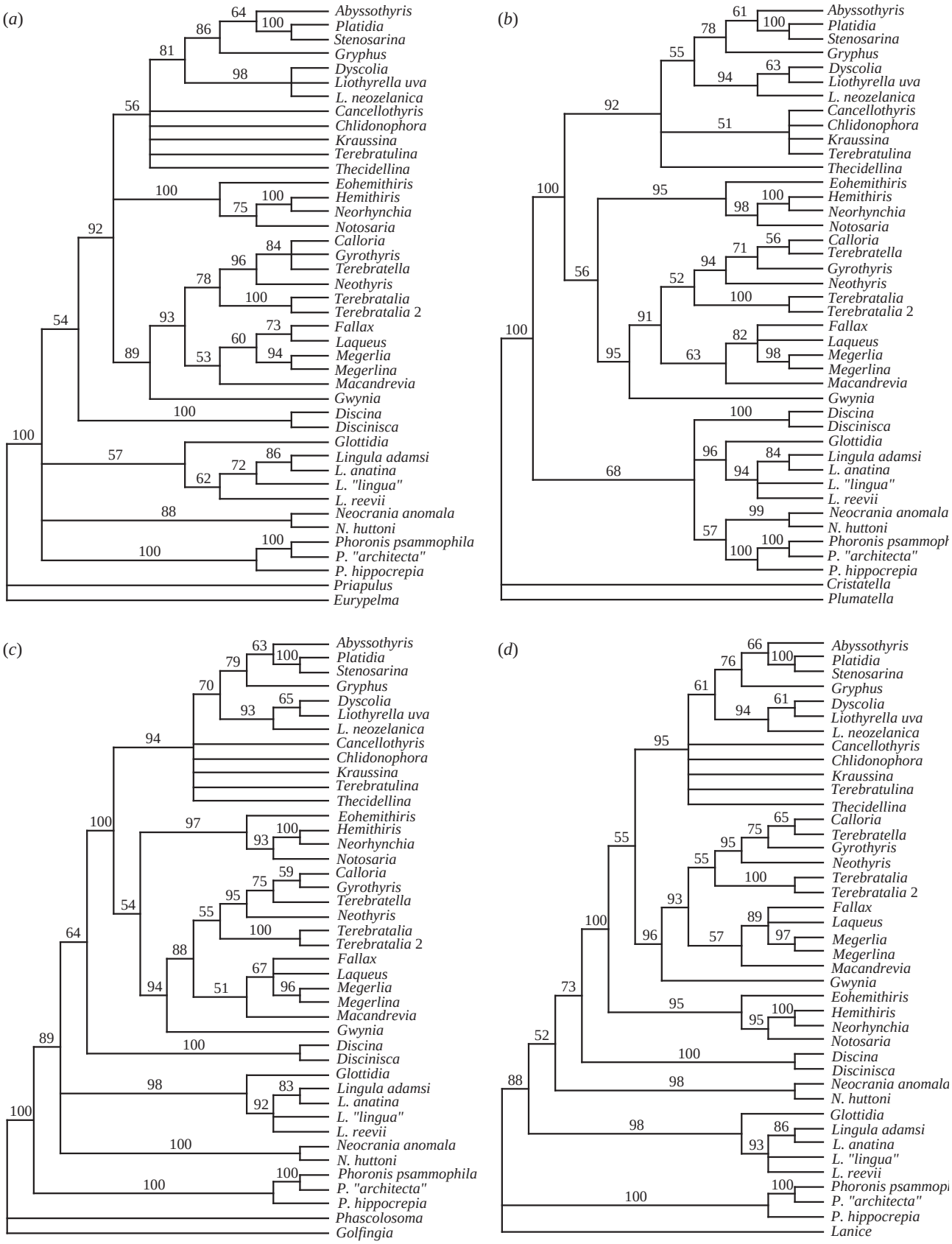

Figure 3. Bootstrap 50% majority-rule consensus trees obtained with different outgroups. (These trees resulted from 500 fast random addition bootstrap replicates using RCI-reweighted characters. Nodes receiving less than 50% support were collapsed. In tests, bootstrapping with full HSs gave similar support values to those obtained by fast addition. Support frequencies are given as per cent. For tree-drawing, outgroups were designated as monophyletic sister groups of the ingroup. The outgroups were as shown: (*a*) ecdysozoans; (*b*) phylactolaemate ectoprocts; (*c*) sipunculans; (*d*) polychaete; (*e*) chiton (parametrically closest outgroup); (*f*) phoronids.) (*Cont.*)

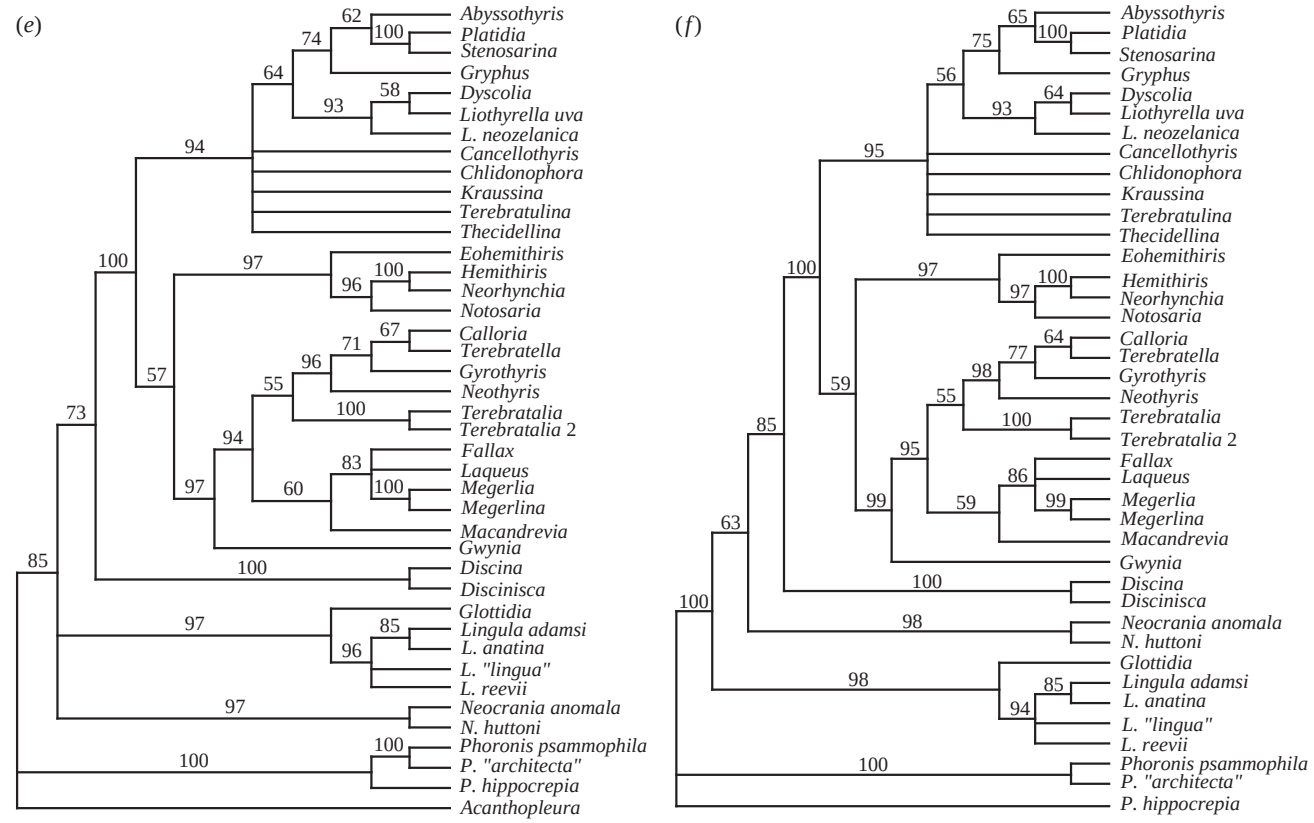

Figure 3. (*Cont.*)

these trees may not be acceptable phylogenies because they are either based on sampled data or represent a consensus; furthermore none shows branch lengths. These deficiencies are remedied by the WP, NJ and ML trees in figure 6, all of which were constructed using the proximal outgroup. As expected, since they are based on all variable sites (minus the minimal exclusion set) rather than only parsimony-informative sites, the NJ and ML trees (figure 6*b*,*c*) show generally higher bootstrap support levels than the WP tree (figure 6*a*) and, as a result, some morphologically validated nodes that had less than 50% support in the WP tree are better supported. The main differences between the trees in figure 6 affect the deepest nodes, i.e. the rhynchonellid, craniid, discinid, lingulid and phoronid lineages. None of these trees unites all three inarticulate lineages into a single clade, but the NJ tree contains a craniid, lingulid and phoronid clade with moderate support. The expected basal position of the rhynchonellids among articulate brachiopods is well supported in the NJ and ML trees. Interestingly, figures 3–6 confirm the usefulness of the partial SSU rRNA sequences dating from the first SSU sequence analysis of metazoan phylogeny (Field *et al.* 1988); both sipunculan and lingulid partial sequences cluster closely with cognate quasi-complete sequences. To our knowledge this has not previously been noted.

(c) *Correlation of genetic distance with classification*

Table 5 presents an analysis of the relationship between genetic distance and taxonomic grade. This analysis is provisional, pending availability of the revised *Treatise* taxonomy (Williams 1997), but in its present state indicates that there is a satisfactory correspondence between mean pairwise distance and taxonomic grade.

(d) *Exclusion of the sequence from* Phoronis vancouverensis

About 10 species of phoronids have been described, in two genera separated by relatively minor morphological characters (Emig 1979). The four available phoronid sequences all come from one genus and three have been included in the alignment analysed here. The fourth sequence, GenBank accession U12648, from *Phoronis vancouverensis* (a junior synonym of *P. ijimai* (Emig 1982)), has been excluded for reasons detailed in this paper. Of the three included sequences, two derive from animals purchased from the same supplier (this work; Cohen & Gawthrop 1996, 1997; Mackey *et al.* 1996) and are probably samples from the same population, but as noted here the suppliers' species name is a junior synonym which has been corrected for our isolate. These two sequences are closely concordant and cluster with the somewhat more divergent sequence from *P. hippocrepia*. The *P. vancouverensis* sequence differs markedly from the other three phoronid sequences and was used to claim a sister-group relationship between phoronids and articulate brachiopods (Halanych *et al.* 1995). However, when first published it was associated with another sequence that contained obvious errors (Conway Morris *et al.* 1996; Halanych *et al.* 1996) and the proposed sister-group relationship has been criticized

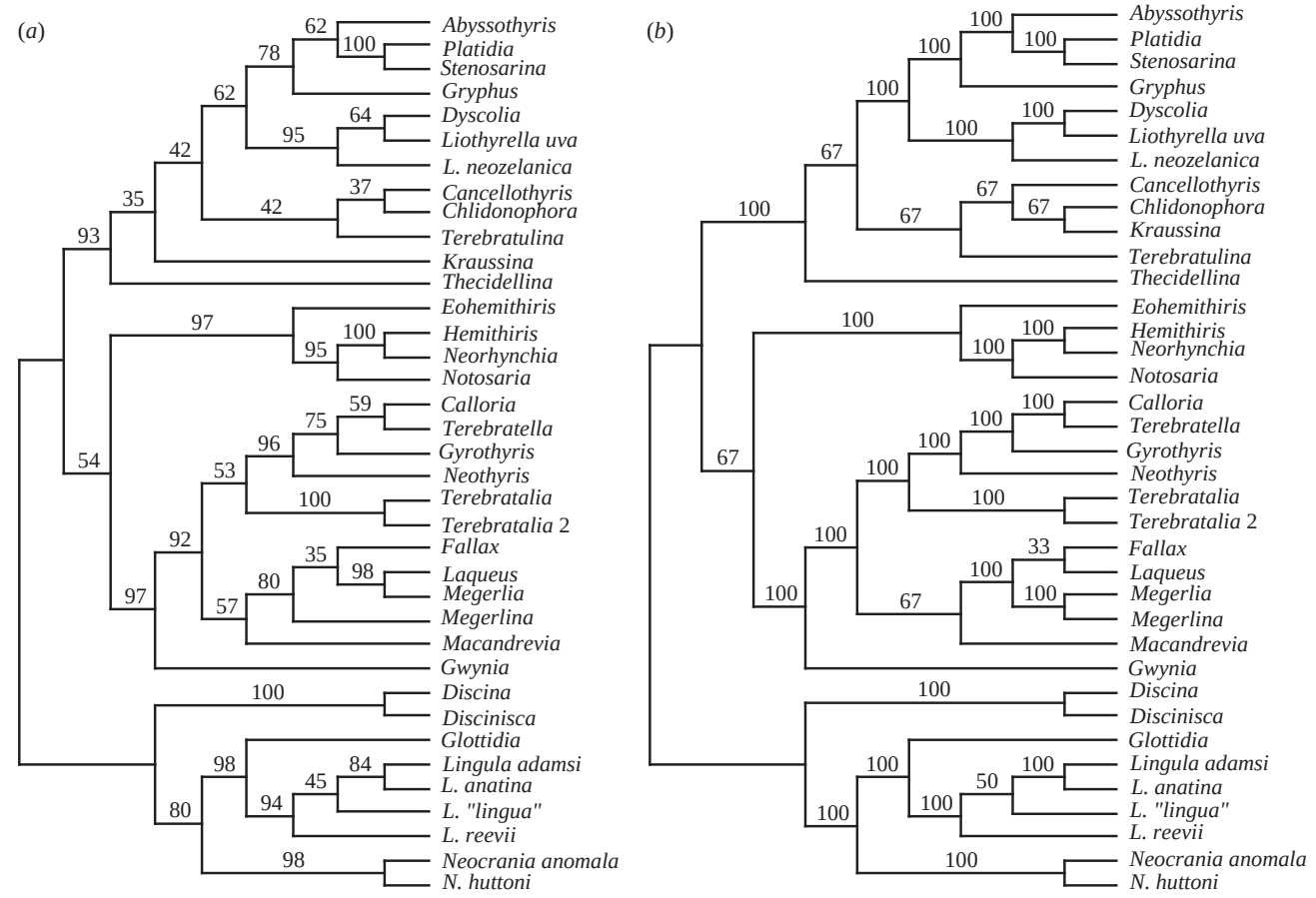

Figure 4. Majority-rule consensus bootstrap and WP trees. (*a*) Bootstrap consensus. After pruning to remove all non-brachiopods, the 500 bootstrap trees generated with each of the six outgroups identified in figure 3 were combined into one file of 3000 trees *and* condensed to 2989 different trees. The 50% majority-rule consensus (with other compatible groupings) derived from these 2989 bootstrap trees is shown, with midpoint rooting. The frequencies (%) at each node indicate the proportion of the 2989 underlying bootstrap trees which contained that node. (*b*) WP consensus. The 48 WP trees obtained using all six outgroups (as detailed in table 4) were combined into one file and all non-brachiopod taxa were pruned out. These 48 trees were condensed to give 18 different trees, and the 50% majority-rule consensus (with other compatible groupings) of these 18 trees is shown, with midpoint rooting. The frequencies (%) at each node indicate the proportion of the underlying trees which contained that node.)

(Cohen & Gawthrop 1996, 1997). By counting 'splits' (informative synapomorphies) separately in the 5′ and 3′ halves of relevant sequences, we have now found evidence suggesting that the *P. vancouverensis* sequence (GenBank U12648) may be a brachiopod–phoronid chimera.

When the 5′ and 3′ moieties of the sequences from *Phoronis 'architecta'*, *P. hippocrepia* and *P. psammophila* were compared with six representative articulate brachiopod sequences, two from each main clade, these three phoronids were unambiguously divided from brachiopods by 12 splits in the 5′ half and 19 splits in the 3′ half. Thus, the distribution of splits along these three phoronid sequences did not differ significantly from uniform expectation ($\chi^2_1=1.58$, $0.3>p>0.2$). When the *P. vancouverensis* sequence was compared with the same brachiopods, there was a highly significant difference, with five splits in the 5′ half and 26 splits in the 3′ half ($\chi^2_1=14.2$, $p<0.001$). In a similar analysis including all four phoronids, the 5′ half contained ten splits which united *P. vancouverensis* with the articulate brachiopods but only one split which united it with the other three phoronids, whereas in the 3′ half, six splits united *P. vancouverensis* with the brachiopods and 13 united it with the other three phoronids ($\chi^2_1=9.7$, $p<0.01$). Overall, these comparisons revealed that about 1100 sites (but not every site) towards the 5′ end of the *P. vancouverensis* sequence were unaccountably similar to the corresponding region of articulate brachiopods, whereas most of the remainder showed the expected similarity to other phoronids. This result was confirmed by comparison of nucleotide distances calculated separately from the 5′ and 3′ halves (not shown) and by experimental phylogenetic reconstructions which revealed that the similarity was diagnostically taxon-specific. When articulate brachiopods were represented by any of nine sequences from long-looped forms, *P. vancouverensis* became their sister group and separated from the other three phoronids, but when sequences from long-looped brachiopods were replaced by sequences from rhynchonellid and/or short-looped taxa, the four phoronids remained together in a strongly supported clade (not shown). As the sequences of *P. vancouverensis* and the long-looped articulate brachiopod *Terebratalia transversa* 2 were cloned and sequenced in the same laboratory (Halanych

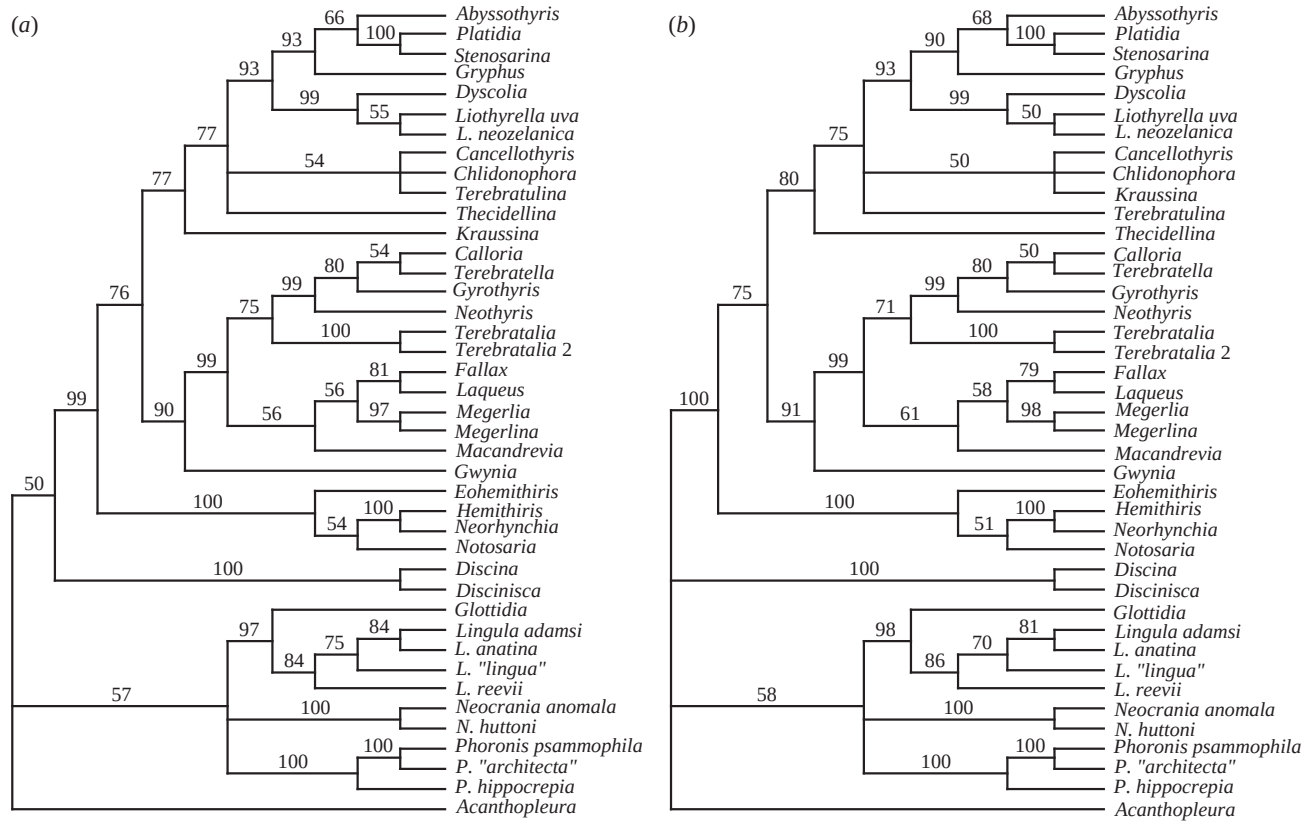

Figure 5. NJ bootstrap consensus trees. (Consensus of 500 fast-heuristic bootstrap replicates showing frequencies (50% or over) with which each node was found. For the distance calculations it was assumed that there were no invariant sites and that rates of change were equal at each site. Ties were broken systematically. (*a*) Kimura two-parameter distance, (*b*) LogDet distance.)

et al. 1995), we infer that much data derived from the 5′ half of the *T. transversa* 2 sequence and perhaps some from the 3′ half too, were misattributed to *P. vancouverensis* and that the published phoronid sequence is a chimeric artefact; its database entry (GenBank accession U12648) should therefore be annotated or withdrawn. The *Terebratalia* 2 sequence is largely concordant with our sequence from the same species.

(e) *Rate and time-course of molecular evolution*

(i) *Relative rate tests*

Relative rates of change were compared using sequences grouped to represent four lineages. Lineage 1 comprised the inarticulate brachiopods *Discina*, *Discinisca*, *Lingula adamsi*, *L. anatina*, *Neocrania anomala*, and *N. huttoni*, together with *Phoronis hippocrepia* and *P. psammophila*. Lineage 2 comprised the rhynchonellid articulate brachiopods *Eohemithyris*, *Hemithiris*, *Neorhynchia* and *Notosaria*. Lineage 3 comprised the short-looped articulate brachiopods *Abyssothyris*, *Cancellothyris*, *Chlidonophora*, *Dyscolia*, *Gryphus*, *Liothyrella neozelanica*, *Stenosarina*, *Terebratulina* and *Thecidellina* and lineage 4 comprised the long-looped articulate brachiopods *Calloria*, *Gwynia*, *Gyrothyris*, *Laqueus*, *Macandrevia*, *Megerlia*, *Megerlina*, *Neothyris*, *Terebratalia* and *Terebratella*. Lineage 1 was significantly different from all other lineages (test statistic including phoronids, 2.4, $p<0.05$, excluding phoronids, 2.77, $p<0.01$), but lineages 2, 3 and 4 showed no significant differences (test statistics from -0.47 to 0.91, $p>0.05$). The ratio of relative rates was lineage 1 including phoronids : lineages 2–4, 0.84 : 1.0; lineage 1 excluding phoronids : lineages 2–4, 0.82 : 1.0. Thus, as is evident in figure 6, inarticulate brachiopods (and phoronids) have evolved more slowly than articulates. All brachiopods are slow-evolving when compared with other invertebrates (see figures in Cohen & Gawthrop (1996, 1997)), although formal relative rate tests have not been performed.

(ii) *Correlations with palaeontology*

We have shown in a preliminary communication that there is a perfect non-parametric correlation between the order of appearance of the principal brachiopod lineages in the fossil record and their ordering in the SSU rDNA gene tree (Cohen & Gawthrop 1996; Norell & Novacek 1992), and here we extend the analysis to investigate the correlation between pairwise nucleotide distances and apparent times of origin of brachiopod lineages in the fossil record (table 6 and figure 7). This analysis is confined to articulate brachiopods on account of the greater richness and definition of their fossil record.

The rate of base substitution in articulate brachiopod SSU rDNA sequences was estimated from figure 7 to be between 0.52 and 0.70 substitutions per 100 variable sites per 100 Ma. This estimate is directly based on uncertainties around a single palaeontologically dated node and is supported by fair agreement with five other calibration nodes and these rates have been used to infer approximate times of divergence for other nodes (table 6 and figure 7).

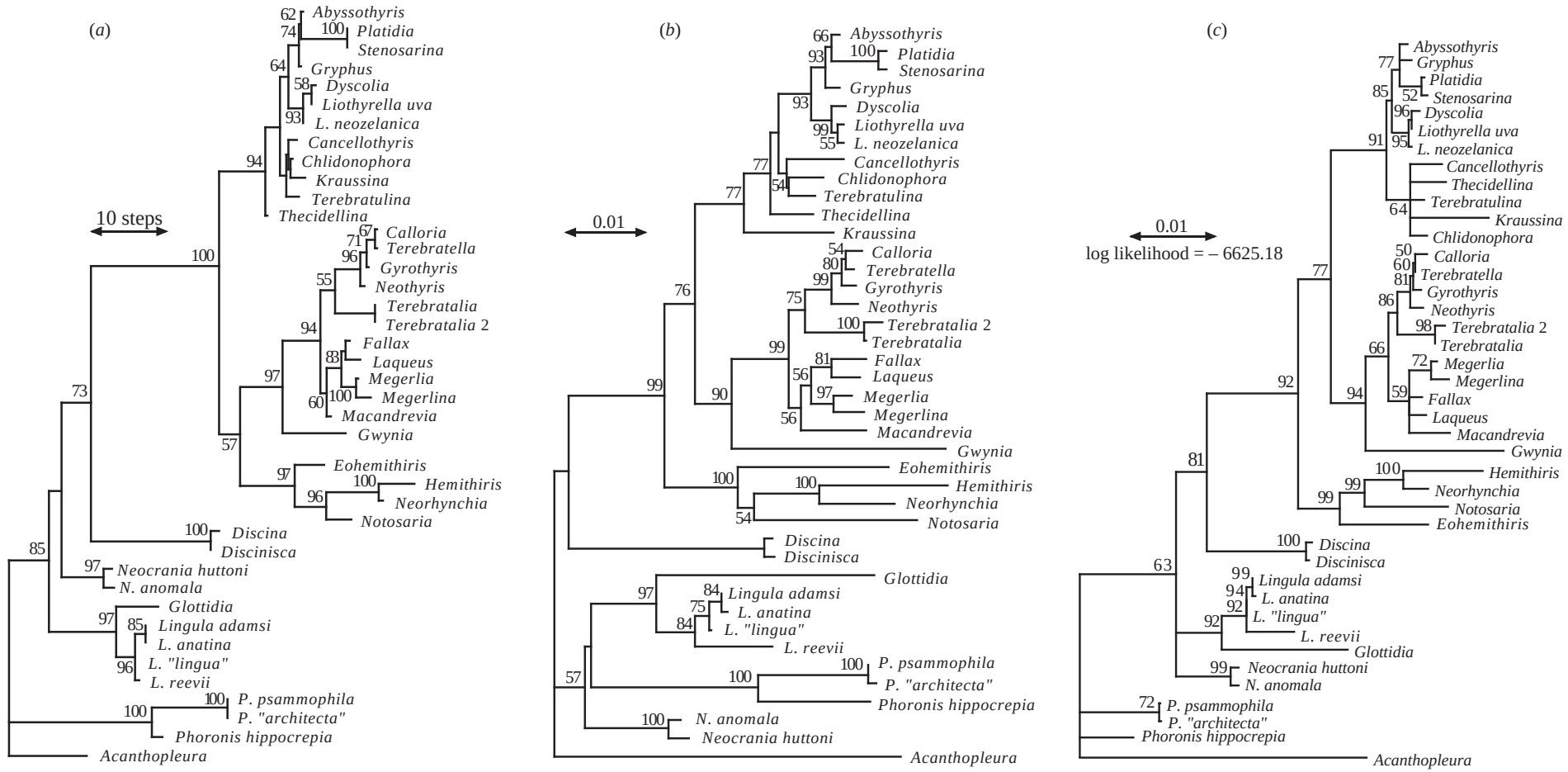

Figure 6. WP, NJ and ML reconstructions rooted with the proximal outgroup, the chiton *Acanthopleura*. (Resampling support frequencies above 50% were taken from the corresponding WP and NJ bootstrap consensus trees. The WP tree in (*a*) was one of 18 equally most parsimonious trees (length = 209.03 reweighted steps, CI = 0.719, RI = 0.908). The 18 trees differed mainly in the distribution of tree length over branches and represented four agreement subtrees in which *Fallax*, *Laqueus*, *Lingula reevii* and *L.* '*lingua*' were identified as unresolved taxa. The NJ tree (*b*) was built using Kimura two-parameter distances, assuming no invariant sites and that rates of change were equal at each site. Ties were broken systematically. Trees (*a*) and (*b*) were constructed using Paup* (Swofford 1997). The ML tree (*c*) was constructed using 1000 quartet puzzling steps (Strimmer & von Haeseler 1996) with the Tamura–Nei model (Tamura & Nei 1993). Parameters estimated from the data were: transition–transversion parameter = 1.81, purine–pyrimidine transition parameter = 1.91, constant sites = 27.7%, gamma-distribution shape parameter $\alpha = 0.11$, rate categories = 4. Unresolved quartets numbered 2602 (2.6%) of 101 270 analysed, confirming the presence of strong phylogenetic structure. A virtually identical tree was obtained using the HKY model (Hasegawa *et al*. 1985) and with equal rates rather than the gamma distribution, but the tree shown had a slightly higher likelihood. The ML tree support values (%) have a meaning similar to bootstrap frequencies. As discussed in the text, some nodes with less than 50% support frequency in WP bootstrap analyses are supported by morphological evidence and these receive more than 50% support in the NJ and ML trees, which are based on more informative sites.)

Table 5. *Correlation of genetic distance between articulate brachiopods and their place in the taxonomic hierarchy*

(Average Kimura distances and standard deviations per 100 variable sites were calculated for pairs of brachiopod sequences in taxa at the levels indicated.)

taxonomic level	number of comparisons	average distance ± s.d.
species within genera	5	0.22 ± 0.13
genera within families	10	0.70 ± 0.29
families and superfamilies within orders	30	1.52 ± 0.46

What these nodes represent, and the palaeontological periods currently associated with them, are detailed in table 6. No allowance has been made for the fact that relative rate tests indicated that inarticulate brachiopods plus phoronids evolved more slowly than articulate brachiopods, nor for obvious heterogeneity in rate among the former group, with craniids notably slow-evolving and phoronids relatively fast-evolving. Many simplifying assumptions (including a single, constant rate molecular clock) are involved in these extrapolations and care should be taken not to over-interpret them.

4. DISCUSSION

(a) *Molecular phylogenetic analyses*

Given an approximately constant rate of nucleotide substitution, the resolution of molecular phylogenetic analysis is mainly limited by two factors: (i) the most recently diverged sequences differ little; and (ii) informative changes marking the most ancient divergences may be erased by subsequent events. Within boundaries set by these limitations, the results described here are quite satisfactory, but resolution would need to be increased through the addition of more sequence data (either longer sequences or sequences from more taxa, or both), before some important questions could be satisfactorily answered. Despite these limitations, both this and other SSU rDNA analyses (see, for example, Field *et al.* 1988; Halanych *et al.* 1995) unambiguously show that brachiopods, ectoprocts and phoronids are much more closely related to other protostomes than they are to deuterostomes. Indeed, because in SSU rDNA analyses both protostomes and deuterostomes generally form monophyletic groups, one may justifiably conclude that these assemblages are real, but misleadingly named. Brachiopods, ectoprocts and phoronids are protostomes, in this operational sense. Furthermore, as is evident by simple inspection of an alignment of many SSU rDNA sequences, the clear dichotomy between protostomes and deuterostomes is no artefact of tree-building. Thus, as previously noted (for examples, see Carlson 1995; Cohen & Gawthrop 1996, 1997; Willmer 1990), this conclusion runs strongly counter to most interpretations of morphological and embryological characters (see, for example, Nielsen 1995; Nielsen *et al.* 1996), the significance of which must therefore be reappraised. The possible importance of convergent and parallel evolution in this context has been noted (Moore & Willmer 1997; Raff 1996), as has the possibility that the methods so far used to define the morphological and embryological characters are insufficiently precise or too subjective (Cohen & Gawthrop 1997).

The phylogenetic resolution attained in this study is clearly sufficient to demonstrate the strongly expected monophyly of articulate brachiopods, but support is less strong for monophyly of the inarticulate brachiopods, i.e. the craniid, discinid and lingulid lineages, and for the inclusion within this clade of phoronids. So far as the inarticulates are concerned, this result is consistent with the most recent and comprehensive cladistic analysis of high-level relationships, in which these inarticulate brachiopod lineages are assigned subphylum status (Williams *et al.* 1996). Slightly stronger support for monophyly of brachiopods plus phoronids has been reported in preliminary accounts of the data reported here (Cohen & Gawthrop 1996, 1997), but clearly these phylum- and subphylum-level relationships are close to or at the resolution limit of SSU rDNA sequences (Adoutte & Philippe 1993; Philippe *et al.* 1994). More sequence data are required.

Our approach to analysis of these data has been bedevilled by the outgroup problem. Unrooted trees most simply represent phylogenetic relationships, but do not reveal the direction of evolution, which is usually defined through rooting the tree by the midpoint or outgroup methods. Midpoint rooting may be used where it is desirable to avoid problems of outgroup selection, provided that evolutionary rates are similar in disparate lineages (Swofford *et al.* 1996). But rates often differ, so that the midpoint method is unsuitable for general use. With outgroup rooting, the outgroup is normally selected on independent evidence such as morphology, and may be either the sister group of the ingroup or a taxon in the next more-inclusive clade. However, as it was impossible to select outgroups on independent, morphological criteria, we devised a molecular, parametric basis for outgroup selection and coupled this with use of those other outgroups necessary to allow our results to be compared with pre-existing studies. Whether it is best to use single or multiple outgroups is debatable: a single outgroup may give rise to false results arising from chance similarities, but multiple outgroups (especially if remote) increase the frequency of homoplastic character-change and thus may decrease analytical sensitivity (Donoghue & Cantino 1984; Farris 1972; Maddison *et al.* 1984; Nixon & Carpenter 1993; Wheeler 1990). The outcome of our analyses with a variety of outgroups is both comforting and unsettling: resolution of brachiopod relationships is not much affected by changing outgroups, and no one outgroup is demonstrably superior. On the other hand, resolution is affected to some extent and somewhat unpredictably. However, as would be expected, varying the outgroup mainly affects resolution of the most weakly supported nodes and does not compromise resolution of the principal brachiopod relationships.

The bootstrap frequencies in figure 3 and 4*a* raise a technical problem that we have not seen addressed elsewhere. A conservative interpretation of the bootstrap is usually recommended, such that only quite high values (e.g. greater than 60%) are considered to reflect phylogenetic signal; all nodes with values below 50% are generally treated as unsupported and collapsed. However,

Table 6. *Correlation of genetic divergence with the fossil record*

(Nodes identified in figure 7*a* were chosen for use as calibration points (role, C) or for interpolation (role, I) independently of their position in figure 7*b*. First fossil appearances are based on current palaeontological advice. Branch lengths (and s.d.) are half the mean Kimura distance per 100 variable sites and its s.d. between all pairs of taxa in the two lineages descending from the indicated node. The 'molecular' age-range for each node was calculated as the branch length divided by the minimum or maximum rates of molecular evolution estimated from the dashed lines in figure 7*b*. These rates were: minimum, 0.52, maximum, 0.70 substitutions per 100 variable sites per 100 Ma. For simplicity, no account was taken of the indicated branch-length errors. Abbreviations: L, Lower; U, Upper.)

			first fossil appearance			
node	role	rationale (taxonomic level and indicator taxon where relevant)	earliest	latest	branch length ±s.d.	inferred 'molecular' age-range (Ma)
B	C	earliest non-Cancellothyrid short-loop (genus)	Jurassic	Cretaceous	0.67±0.16	(95–129)
E	C	earliest Cancellothyridoidea (superfamily)	—	L. Jurassic	0.59±0.13	(84–113)
G	C	earliest *Pachymagas* lineage member (*Neothyris*, genus)	—	L. Oligocene	0.33±0.07	(47–63)
K	C	earliest Zeillerioidea (superfamily) (*Macandrevia*)	L. Triassic	U. Triassic	0.83±0.07	(119–160)
N	C	earliest Terebratulida (order)	—	L. Devonian	2.15±0.17	(307–413)
R	C	earliest Rhynchonelliformea (subphylum); earliest Rhynchonellida (order)	Cambrian	L. Silurian	3.01±0.35	(430–579)
A	I	Mediterranean recolonization (genus, *Gryphus*)	—	Miocene	0.36±0.21	51–69
C	I	earliest Dyscolioidea (superfamily)	—	L. Jurassic	0.17±0.06	24–32
D	I	earliest short-looped genus	—	?Triassic	0.83±0.19	118–160
F	I	earliest thecideidine	—	Triassic	0.81±0.17	115–155
H	I	North and South Pacific terebratelloids isolated	U. Cretaceous	Palaeocene	0.79±0.04	113–152
J	I	earliest Kraussinoidea (superfamily)	—	L. Cretaceous	0.70±0.09	100–135
L	I	earliest Terebratellidina (suborder)	—	Triassic	0.99±0.07	141–190
M	I	earliest Gwynioidea (superfamily)	L. Triassic	mid-Jurassic	2.59±0.21	370–498
Q	I	?earliest Hemithyrididae and Cyclothyridinae (family)	U. Cretaceous	Palaeocene	2.42±0.45	345–465
S	I	earliest discinids	—	L. Ordovician	3.59±0.33	512–690
T	I	lingulid divergence (genus, *Glottidia*)	—	? Eocene	2.14±0.69	306–411
U	I	lineage divergence (subphylum)	—	L. Cambrian	2.69±0.88	384–517
V	I	inarticulate and articulate brachiopods diverge (subphylum)	—	L. Cambrian	3.65±0.60	521–702
W	I	inter-hemispheric isolation (species, *Neocrania*)	—	—	0.21	30–40
X	I	phoronid divergence (species)	—	—	1.54±0.02	220–296
Y	I	proto-brachiopod lineage diverges from other Lophotrochozoa	—	—	4.48±0.45	640–861

our data provide two examples of taxa that are undoubtedly related on morphological criteria yet form unsupported clades by bootstrapping: support is less than 50% for the nodes joining: (i) three closely similar *Lingula* sequences; and (ii) the three cancellothyrids *Chlidonophora*, *Cancellothyris* and *Terebratulina*. Thus, some clades with low support values may nevertheless be real. Evidently these clades comprise relatively recently diverged taxa, united by few molecular synapomorphies which are readily destroyed by resampling. Therefore, to collapse all nodes with less than some arbitrary support frequency is potentially misleading. The danger of retaining such nodes is that resampling may, by repeatedly picking certain characters, create clades where none truly exist. However, as no clades appear in our resampled consensus trees that are not also present in trees derived from unsampled data, this is not a real and present danger.

(b) *Correlation of molecular and morphological phylogenies*

The existence of a good correspondence between nucleotide distance and taxonomic grade (table 5) suggests that classical brachiopod taxonomists have been broadly successful in recognizing heirarchical structure and translating it into practical taxonomy. This conclusion, if accepted, is one of the most important to come out of the molecular work. It is doubly important when, as here, much of the older taxonomy has inevitably been based on relatively gross analyses of fossil shell structure and ontogeny, unlike more recent studies of both fossil and Recent shell ontogeny and morphology, which resolve exquisite detail (MacKinnon 1993; MacKinnon & Gaspard 1995; MacKinnon & Smirnova 1995; Williams 1956, 1965, 1973; Williams & Brunton 1993; Williams *et al.* 1994; Williams & Holmer 1992). Some recent studies have made a substantial effort to include a wider range of characters (Carlson 1995; Holmer *et al.* 1995; Williams *et al.* 1996), but many of these belong to categories that the molecular results (i.e. the strong association with protostomes) lead us to reject as potentially homoplastic or affected by imprecise and/or subjective methodology. Thus, this molecular study can be seen as the first independent source of evidence to test the hypothesis that brachiopod shell ontogeny and morphology is a reliable guide to phylogeny. The large measure of agreement between our molecular reconstructions and the morphological classification (this paper;

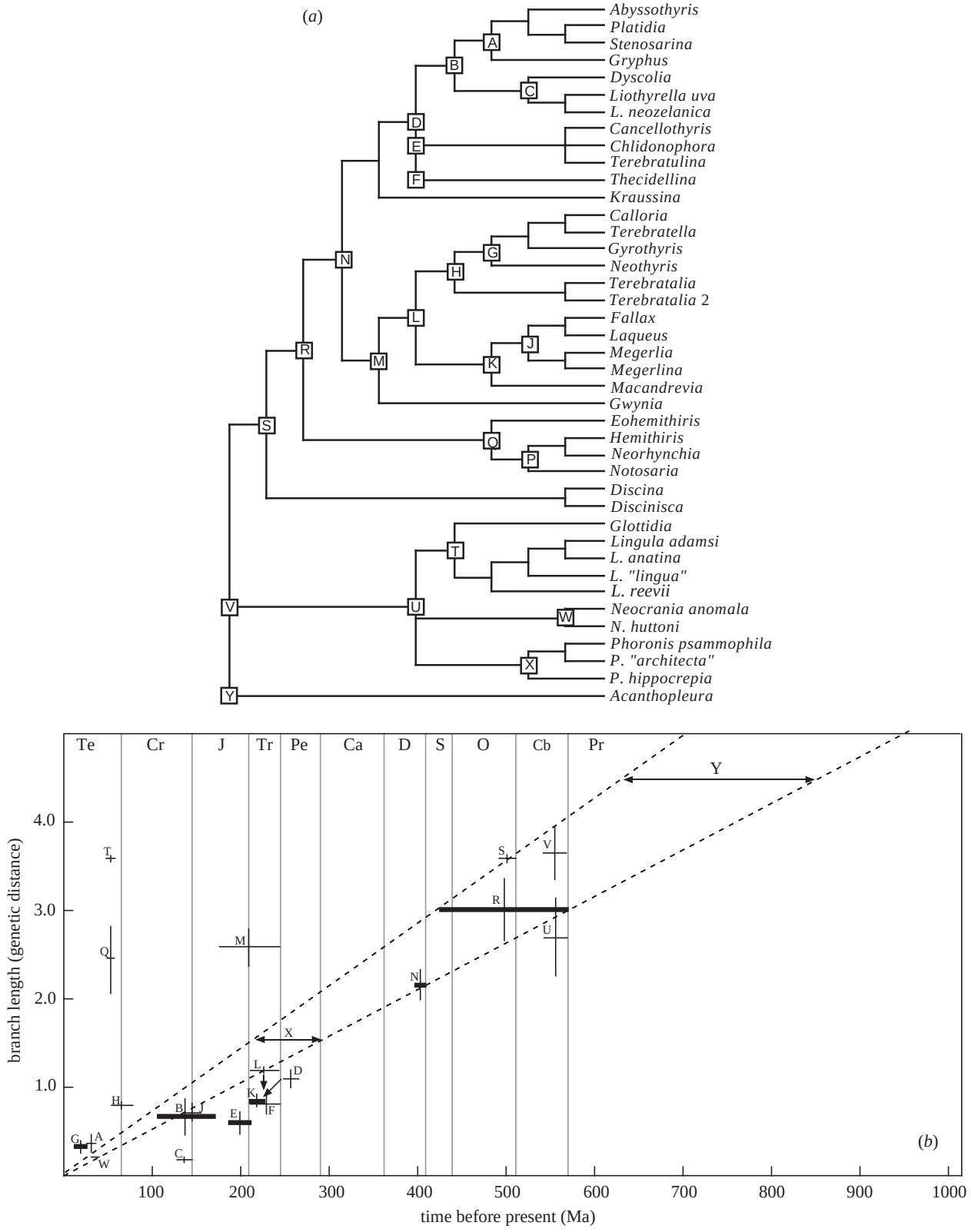

Figure 7. Correlation of molecular evolution with the fossil record. (*a*) Identification of nodes on the NJ bootstrap consensus topology of figure 5*a*. For the meaning of the lettered nodes see table 6. (*b*) Lineage branch lengths in relation to time of earliest appearance of fossils. Horizontal lines are placed at the mean NJ branch length (abscissa: Kimura distance per 100 variable sites) descending from the indicated node with vertical lines scaled to indicate ± 1 s.d. Lengths of the horizontal lines reflect current advice (see acknowledgements) as to the earliest time of first appearance of the lineages concerned. Nodes drawn with thick horizontal lines were selected (before the diagram was constructed) to serve as calibration-points. Node lines with arrowheads and node W are interpolated from genetic distances alone. Upper and lower estimates of the rate of molecular evolution were obtained from the dashed lines, based primarily on node R, because it did not disagree strongly with the other selected calibration nodes whilst having the largest error range (and see § 3e(ii)). Te, Tertiary; Cr, Cretaceous; J, Jurassic; Tr, Triassic; Pe, Permian; Ca, Carboniferous; D, Devonian; S, Silurian; O, Ordovician; Cb, Cambrian; Pr, Precambrian.

Carlson 1995; Cohen & Gawthrop 1996, 1997; Holmer *et al*. 1995; Williams 1965; Williams *et al*. 1996; Williams & Rowell 1965) broadly support this hypothesis, and this is gratifying. Thus for example the rhynchonellids, in which the shell lacks both punctae and complex lophophore supports, clearly form (in NJ and ML analyses) a clade separate from the terebratulids and thecideids, in which punctae and complex lophophore supports are present. The rhynchonellid and terebratulid clades currently form orders (Williams *et al*. 1996). Within the terebratulids, the molecular results recognize two principal clades which correspond broadly to the short-looped (terebratulacean) and long-looped (terebratellacean) morphological subdivisions, forming suborders. There are also particularly satisfying examples of more specific congruence such as *Macandrevia*, where new morphological work concurrent with this study led to recognition of this genus as an isolated relic of an otherwise extinct family of Triassic origin (MacKinnon & Gaspard 1995), thus predicting its observed tree position, isolated on a long branch. This morphological study also leads to the prediction that if sequence could be obtained from *Ecnomiosa*, it would join the tree as another Mesozoic relict taxon. Again, within the short-looped, terebratulacean brachiopods, a weakly supported clade of cancellothyrids emerged, consistent with the recognition that these deserve separate taxonomic status (Cooper 1973).

The position of thecideidine brachiopods is also striking. The one sequence obtained is believed to be reliable and its tree position is unambiguously within the short-looped clade, usually in a basal position although in some analyses it joins the cancellothyrid subclade. The morphological relationships of thecideidine brachiopods are enigmatic; they have been proposed to be either the sole extant descendants of the extinct spiriferids, or more closely related to terebratulids (Baker 1990; Williams 1973). However, spiculation, which first appeared in spiriferids, also occurs in thecideidines (Williams 1973) and is characteristic of cancellothyrids, so that a sister-group relationship of thecidedines and cancellothyrids is perhaps not impossible. Clearly, additional molecular evidence is required.

Some potentially important incongruities do exist between the molecular and morphological articulate brachiopod phylogenies, but in each case the molecular data are compromised by possible contamination or by want of multiple, independent results. The most striking example affects the megathyrids (MacKinnon & Smirnova 1995; Williams 1965) which have generally been included among long-looped (terebratellacean) forms, although they have somewhat atypical loops. From the available molecular results two genera, *Megerlia* and *Megerlina*, certainly do belong in this group, but two other genera, *Platidia* and *Kraussina*, appear to belong in the short-looped clade, implying either that megathyrid loop ontogeny is more diverse than has been recognized or that it arose at least twice, in parallel. However, the *Platidia* and *Kraussina* results require confirmation.

One further possible discrepancy between the molecular and morphological phylogenies should be noted: among the long-looped articulates, sequences from *Laqueus* and *Fallax* cluster together so closely that they are not resolved by parsimony analysis, yet these genera are morphologically very different and their loop ontogenies differ substantially (D. I. MacKinnon, personal communication). Furthermore, the samples come from geographically very distant sites (table 1). Independent confirmation of this unexpected molecular result is required.

Among the inarticulate brachiopod and phoronid sequence results, there are four points that should be noted.

1. The close concordance between independently determined sequences from *Lingula* spp. and *Phoronis* spp.
2. The basal position of *Glottidia* and its deep divergence from *Lingula* spp. is surprising and may owe something to residual sequencing errors (Conway Morris *et al*. 1996; Halanych *et al*. 1996). However, ongoing shell fabric studies indicate that *Glottidia* may indeed be remotely related to *Lingula* (Williams 1997); clearly a second *Glottidia* sequence is needed.
3. None of the individual analyses reported here resolves the discinids and lingulids into the same clade, although they alone among Recent brachiopods share a chitino-phosphatic shell fabric and planktotrophic larvae (Holmer *et al*. 1995; Williams *et al*. 1996). This putative failure of molecular analysis may be explained if discinid–lingulid synapomorphies were originally few and have largely been erased or balanced by homoplastic similarities with other taxa since these lineages diverged. Undoubted discinids first appeared in the Lower Ordovician (L. Holmer, personal communication), and undoubted lingulids are known from the Lower Cambrian, but it would not be surprising if the underlying genomic divergence predated this. Thus, there has been ample time for the erasure of synapomorphies.
4. The molecular analyses all concur in excluding the calcareous-shelled craniids from the articulate brachiopods, thus indicating that a calcareous shell fabric arose twice. This conclusion is consistent with current classification (Holmer *et al*. 1995; Williams *et al*. 1996) and with the markedly different calcification ultrastructures of craniid and articulate brachiopods (Williams 1956) and inconsistent with an earlier and now abandoned taxonomic proposal (Gorjansky & Popov 1986).

Brachiopod systematics has been enlivened by the somewhat controversial application of shell immunotaxonomy (Cohen 1994; Curry *et al*. 1993) and it is sufficient to note that some relationships suggested by that method are inconsistent with the SSU rDNA gene phylogeny.

(c) *Evolutionary and biogeographic inferences*

The correlation between genetic divergence and palaeontologically determined age of the 16 nodes for which 'molecular' ages have been estimated (table 6 and figure 7) contains three main discrepancies, involving nodes Q, M and T. The time-position of node Q is based on palaeontological advice regarding the Cretaceous–Palaeocene first appearance of a family considered to be ancestral to extant hemithirids, and this time of first appearance conflicts with its long branch length. The interpretation of rhynchonellid relationships is recognized

to be somewhat subjectively based and this molecular result predicts that lineages descending from node Q actually diverged considerably earlier in rhynchonellid history. It is unlikely, however, that this prediction will be capable of disproof. The molecular position of node M and its tree position also predict a much earlier origin for the lineage to which its diagnostic taxon, *Gwynia*, belongs than follows from known fossils. Today, this is a morphologically unique, minute, infaunal organism (the smallest extant brachiopod) with few homologous fossils (Logan *et al.* 1997) and, if this life habit is not derived, it is possible that early fossils have escaped notice. Thus, this prediction is capable of disproof by future work. The deeply divergent position of *Gwynia* may argue against the suggestion that it originated by paedomorphosis. Node T represents an important discrepancy between the deep molecular divergence of *Lingula* spp. from *Glottidia* and the relatively recent first fossil occurrence of this genus. However, the molecular divergence may reflect residual errors in the *Glottidia* sequence (B. L. Cohen, unpublished data; Conway Morris *et al.* 1996; Halanych *et al.* 1996) and therefore needs to be confirmed. It is, however, consistent with new observations of a substantial difference in shell micro-fabric between these genera (Williams 1997).

For three nodes, W, X and Y, the molecular ages interpolated from figure 7 (see table 6) have no palaeontological counterpart. Node W arises from the (surprisingly) low divergence between morphologically distinguishable northern and southern hemisphere species of the craniid *Neocrania*. This node's date is much too recent to fit the hypothesis that divergence of these taxa resulted from the break-up of Gondwanaland and it raises a series of open questions about craniid dispersal and divergence. Node X identifies species-level divergence among phoronids, and is likely to remain untested by palaeontological evidence. Node Y, which suffers from the simplifying assumption that the rate of molecular evolution in the chiton lineage equals that in brachiopods, places the last common ancestor of these lineages deep in Precambrian time, well before the first appearance of shelly fossils. We can only hope either that future palaeontological discoveries will lead to recognition of the predicted soft-bodied ancestors (see, for example, Fedonkin & Waggoner 1997) or that, with more molecular data, it will become possible to make better inferences of evolutionary rates. Overall, and despite the qualifications that must be attached to these analyses, the rough agreement between most nodes and geologically determined time lends some, perhaps spurious, confidence to the outcome.

The results described here are based on a single gene tree. In ongoing work, DNA sequence is being collected from the more rapidly evolving mitochondrial genome, especially of short-looped articulate brachiopods where the SSU rDNA resolution is weakest. As so far analysed, this genetically independent evidence supports the pattern of relationships reported here, especially the cancellothyrid clade and the protostome affinities of brachiopods (Cohen *et al.* 1998).

Note added in proof

Further analyses have indicated that the unexpectedly low bootstrap support for the cancellothyrid subclade of short-looped brachiopods resulted from inclusion of the imperfect *Kraussina* sequence (B. L. Cohen, unpublished data).

5. CONCLUSIONS

Results of the first DNA-based, taxonomically representative analysis of brachiopod phylogeny are in broad agreement with current morphology-based views on classification and systematics; in particular the hypothesis that brachiopod shell ontogeny and morphology are a good guide to phylogeny is supported, although with minor reservations. It seems likely, though not proven, that phoronids belong within the clade of inarticulate brachiopods.

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