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Phoronid phylogenetics (Brachiopoda; Phoronata): evidence from morphological cladistics, small and large subunit rDNA sequences, and mitochondrial *cox1*

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A matrix of 24 morphodevelopmental characters and an alignment of small subunit (*SSU*) and large subunit (*LSU*) rDNA nuclear and *cox1* mitochondrial gene sequences (~4500 sites) were compiled from up to 12 phoronids including most named taxa, but probably constituting only a portion of worldwide diversity. Morphological data were analysed by weighted parsimony; sequence data by maximum and Bayesian likelihood, both with *Phoronis ovalis* as the local outgroup. Morphological and sequence-based phylogenies were similar, but not fully congruent. Phoronid rDNAs were almost free from mutational saturation, but *cox1* showed strong saturation unless distant outgroups and *P. ovalis* were omitted, suggesting that many phoronid divergences are old (≥100 Myr). rDNA divergence between named phoronid taxa is generally substantial, but *Phoronopsis harmeri* (from Vladivostock) and *Phoronopsis viridis* (from California) are genetically close enough to be conspecific. In another alignment, of 24 taxa, phoronid rDNAs were combined with data from brachiopods and distant (molluscan) outgroups. The relative ages of divergence between phoronids and their brachiopod sister-groups, of the split between the *P. ovalis* and non-*ovalis* lineages, and of other phoronid splits, were estimated from this alignment with a Bayesian lognormal uncorrelated molecular clock model. Although confidence limits (95% highest probability density) are wide, the results are compatible with an Early Cambrian split between phoronids and brachiopods and with the Upper Devonian latest age suggested for the *P. ovalis*/non-*ovalis* split by the putative phoronid ichnofossil, *Talpina*. Most other ingroup splits appear to be ~50–200 Myr old. Inclusion of phoronids with brachiopods in the crown clade pan-Brachiopoda suggests that a distinctive metamorphosis and absence of mineralization are ancestral phoronid apomorphies. Worldwide diversity and possible associations between character-states and life-history attributes deserve comprehensive further study.

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INTRODUCTION

Metazoan phylogenetics based on gene sequences is in transition from a foundation phase fuelled by sequences from small numbers of genes such as those coding for the small and large subunits of nuclear-encoded ribosomal RNA (*18S* or *SSU* and *28S* or *LSU*, respectively), to a phase using multigene and/or genomic sequences or, in some cases, miRNAs. One advantage of the foundation phase for nonmodel organisms such as phoronids (and their closest relatives, brachiopods) is that it is possible to analyse a taxonomically representative (or even near-comprehensive) sample of extant diversity, whereas cost and availability limit the range of multigene,

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genomic, or RNA-based analyses. The results reported here are the first in which both *SSU* and *LSU* rRNA nuclear genes have been sampled from the majority of named phoronid taxa, and they are supplemented by data from a mitochondrial protein-coding sequence. The molecular results broadly agree with cladistic analyses of morphological and developmental characters, suggesting that together, they reveal the main lines of phoronid evolution. Because phoronids have no certain fossil record, the relative ages of nodes in the rDNA gene tree have been estimated by a Bayesian relaxed clock method, cross-calibrated with palaeontologically dated brachiopod divergences. The relatively old node ages suggested by this analysis are supported by high saturation of the mitochondrial sequence. We also note some ways in which the results bear on aspects of phoronid life-history and ecology.

Phoronids were formerly considered a deuterostome phylum, and placed with brachiopods and bryozoans in a supraphyletic 'Lophophorata' or 'Tentaculata'. But Hyman (1959) and some others proposed instead that phoronids and brachiopods are somehow intermediate between protostomes and deuterostomes, or even belong fully amongst protostomes. This was prescient: in all phylogenetic reconstructions based on both nuclear (especially ribosomal, rDNA) and mitochondrial genes, phoronids cluster unambiguously and strongly with brachiopods and other phyla now accepted as protostomes (Cohen & Gawthrop, 1996, 1997; Cohen, Gawthrop & Cavalier-Smith, 1998; Cohen, 2000, 2007, and references therein; Halanych *et al.*, 1995; Helfenbein, 2000; Peterson & Eernisse, 2001; Helfenbein & Boore, 2004).

The nature of the association with brachiopods remained unclear until rDNA sequences from representative phoronids and the main extant brachiopod lineages (Cohen & Weydmann, 2005) confirmed the (originally controversial) suggestion (Cohen, 2000) that phoronids belong deep within the brachiopod clade as the sister-group of the three inarticulate lineages (craniids, discinids, and lingulids), a finding that led to reclassification of phoronids as a class within the Brachiopoda (Cohen & Weydmann, 2005). Despite some differences in morphogenetic movements during gastrulation and metamorphosis, phoronids and brachiopods appear to share basic body plan features (Freeman, 1991, 2003; Nielsen, 1991; Freeman & Martindale, 2002; Cohen, Holmer & Lüter, 2003; Santagata, 2004a). More recently, the first analyses based on multigene sequences from a limited selection of phoronids and brachiopods have appeared, with mixed results. One analysis, based on fragments of seven housekeeping genes (Helmkampf, Bruchhaus & Hausdorf, 2007), found a phoronid to be weakly associated with an ectoproct, but also divided ectoprocts unrealistically between two clades. This analysis also shows considerable internal conflict and little phylogenetic signal (B. L. C., unpubl. splits analyses, 2007). By contrast, two analyses based on expressed sequence tags of minimal taxon samples confirmed the close association of phoronid and brachiopod (Dunn *et al.*, 2008; Helmkampf, Bruchhaus & Hausdorf, 2008). In this early phase of multigene analyses it is well to remember that *SSU* gene trees did not reveal that phoronids nest within brachiopods (e.g. Cavalier-Smith, 1998; Zrzavy *et al.*, 1998; Peterson & Eernisse, 2001) until data from all extant brachiopod lineages were available and suitable outgroups had been identified (Cohen & Weydmann, 2005). Moreover, the newer approaches rely on protein-coding sequences, which have intrinsic limitations (e.g. Simmons, 2000; Rokas & Carroll, 2008). The new analyses should therefore be treated with some reserve until they are based on wider, more representative taxon samples, and have been more thoroughly tested. For a perspective on the new analyses as they apply to lophotrochozoans see Giribet (2008).

The analyses presented here are based on a new matrix of 24 cladistically coded morphological characters and a new sequence alignment of ~3900 nucleotides (nt) of nuclear-encoded rDNA sequence (quasi-complete *SSU* and partial *LSU*) from both phoronid genera and most of the well-established species, together with > 600 nt of mitochondrial *cox1* from all but two taxa.

Phoronids are tubiculous, epibenthic, or infaunal lophophorates found in the shallow benthos of all oceans. Extant phoronid diversity appears low (see Table 1), only 11 or 12 undisputed species in two closely similar genera, *Phoronis* (*P.*) and *Phoronopsis* (*Ph.*) (Marsden, 1959; Emig, 1979, 1982, and references therein). Most phoronid embryos form long-lived, planktotrophic (actinotroch) larvae that settle and undergo a distinctive metamorphosis (Herrmann, 1979; Santagata, 2002). The sessile, benthic adults have a lophophoral filter-feeding organ and a recurved gut, and range in length from a few mm to a few cm. The tube is chitinous, and in some species (*Phoronis architecta*, *Phoronis psammophila*) the solitary tube is decorated or strengthened with mineral grains. In others, e.g. *Phoronis vancouverensis*, the tubes form a dense mat. Tubes may be attached to or embedded in sandy or other substrates, and those of three taxa occur in distinctive associations: *Phoronis ovalis* in autogenous borings within the shells of bivalve molluscs, *Phoronis australis* on the tube sheath of cerianthid sea-anemones, and *Phoronis pallida* as a commensal in thalassinid shrimp burrows. *Phoronis hippocrepia* may also burrow into hard substrates. Phoronids are hermaphroditic or have separate sexes.

Table 1. Phoronid phylogeny. Specimens and sequences

Genus, author, & date	Species, author, & date (local accession no.)	Locality (collector*)	*cox1* local & GenBank accession no.	*SSU* local & GenBank accession no.	*LSU* local & GenBank accession no.	Reference and other information
Ingroup						
Phoronis Wright, 1856	*architecta* Andrewes, 1890/*psammophila* Cori, 1899	Panacea, Florida (Gulf Specimen Supply)	**AY368231.1** (mitochondrial genome)	D1205 **AF025946**	D1205/1659 **EU334109**	*cox1*: (Helfenbein & Boore, 2004). *SSU*: (Cohen *et al.*, 1998; Cohen & Weydmann, 2005). *LSU*: this paper
Phoronis	*australis* Haswell, 1883 (97-2)	10 m., in *Cerianthus*, Canala, eastern New Caledonia, (BrdeF)	D1269 **EU484457**	D1269 **AF202111** **EU334121**	D1269 **EU334110**	*cox1* and *LSU*: this paper. *SSU*: (Cohen, 2000)
Phoronis	*australis* (98-5-1)	in *Cerianthus,* Shimoda, Japan (RU, *via* KE†)	D1270 **EU484458**	D1270 **EU334122**	D1270 **EU334111**	this paper
Phoronis	*australis* (99-8-1)	3 m., in *Cerianthus,* Port Jackson, Sydney Harbour, Australia (VM‡)	D1738 (heteroplasmic?)	D1738 **EU334123**	D1738 **EU334112**	this paper
Phoronis	*australis*	Girona, Spain, Mediterranean Sea	—	**AF119079**	**AF119079**	gift sequences, G. Giribet, from voucher MCZ DNA100052.
Phoronis	*hippocrepia* Wright, 1856 (F4)	Marseilles harbour, Mediterranean Sea (CE/PB§)	D1257 **EU484459**	D1257 **AF202112** **EU334124**	D1257 **AY839251**	(Cohen, 2000; Cohen & Weydmann, 2005) and this paper
Phoronis	*muelleri* Selys-Longchamps, 1903 (2005-8-1)	16 m., Kristineberg, Sweden (CN¶)	**EU484460**	D1599 **EU334125**	D1599 **EU334114**	this paper
Phoronis	*ovalis* Wright, 1856	Irish Sea, south of Isle of Man, in *Glycmeris* shells (LV††)	D1409 **EU484461**	D1409 **EU334126**	D1409 **EU334115**	this paper

Phoronis	*pallida* Silén, 1952	San Juan Is, WA (SS‡‡)	D1600 (heteroplasmic?)	D1600 **EU334127**	D1600 **EU334116**	this paper
Phoronis	*vancouverensis* Pixell, 1912/ijimai Oka, 1897	Pier, Monterey, CA. (Sea Life Supply)	D1328 **EU484462**	D1328 **AF202113** **EU334128**	D1328 **AF342797**	(Cohen, 2000) and this paper
Phoronopsis Gilchrist, 1907	*californica* Hilton, 1930 07-07-1	9 m., Black Cavern, San Clemente Is., (SS‡‡)	D1737 **EU484463**	D1737 **EU334129**	D1737 **EU334118**	this paper
Phoronopsis	*harmeri* Pixell, 1912 06-11-01	SCUBA, Vostok Bay, Sea of Japan (VM§§)	D1731 **EU484464**	D1731 **EU334130**	D1731 **EU334119**	this paper
Phoronopsis	*viridis* Hilton, 1930	California, USA	D1405 **EU484465**	**AF123308**	D1405 **EU334120**	*cox1* & *LSU* from DNA gift of G. Giribet;
Outgroups Select taxa						(Cohen & Weydmann, 2005)

LSU, large subunit; *SSU*, small subunit.

The *cox1* sequence AY428841, which was determined independently from the same *Phoronopsis viridis* DNA sample, agreed perfectly but was slightly shorter at each end (not shown).

A few other phoronid *SSU* sequences are available in GenBank. Those identified to species were found to agree well with our new data and added no useful information.

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Soft-bodied phoronids have no certain fossil record, but tunnels of the trace fossil *Talpina* v. Hagenow, 1840 in bivalve shells appear to represent a form similar to extant *P. ovalis*. *Talpina* is not known before the Upper Devonian, 385–359 Mya (Bromley, 2004, and references therein; Voigt, 1975; Gradstein & Ogg, 2004; Wilson & Palmer, 2007). *Iotuba* Chen & Zhou, 1997, a large, worm-like fossil from the Lower Cambrian Chengjiang fauna has been referred to the Phoronida (e.g. Chen & Zhou, 1997), but this is disputed (Z. Zhang, pers. comm., 2006) and it is now thought more likely to be a priapulid (Huang, 2006, and pers. comm., 2007).

Until recently, relationships amongst phoronids had been inferred only from morphology (Marsden, 1959; Emig, 1974, 1979, 1982, 1985) and by analyses of *SSU* rDNA sequences from a limited taxon sample (Cohen *et al.*, 1998; Cohen, 2000), but a cladistic analysis of morphological characters and another phylogenetic reconstruction based on *SSU* rDNAs have recently appeared (Grobe, 2008). However, *SSU* rDNA alone cannot adequately resolve phoronid interrelationships; even the larger number of variable sites in the *SSU* + *LSU* analyses reported here do not robustly resolve all nodes. Overall, the molecular and morphological analyses presented here agree in important particulars. The sequence analyses confirm the association with brachiopods and suggest an outline of phoronid evolution in which many divergence events are unexpectedly old.

MATERIAL AND METHODS

Specimens and imported sequences

Details of specimens and new sequences are given in Table 1. Additional rDNA sequences were from the alignment used by Cohen & Weydmann (2005), and other *cox1* sequences from Saito, Kojima & Endo (2000). Other phoronid sequences (mainly *SSU* rDNA) were obtained from GenBank (Benson *et al.*, 1998) or were received as gifts from their authors, and all those from specimens determined to species were included in preliminary analyses. Except for the first published phoronid *SSU* sequence, these sequences agreed closely with one another and with our data, and are not further employed. The first phoronid *SSU* sequence to be reported (Halanych *et al.*, 1995) is highly discordant with all others and has been identified as an artefactual brachiopod–phoronid chimaera (e.g. Cohen & Gawthrop, 1996; Cohen, 2000). Further evidence of chimaerism appears in Supporting Information Appendix S1.

Diversity, taxonomy, taxonomic vouchers, and sample identification

Phoronid diversity has not generally been investigated by the comparison of large samples of animals collected from multiple locations, and their taxonomy has been largely based on a small number of characters, some ascertained by microscopy of stained thin sections (Marsden, 1959). Other potentially useful features, e.g. of reproductive biology or ultrastructure, have not been defined for all species (see Zimmer, 1991; Herrmann, 1997; Santagata & Zimmer, 2002). Thus, the current list of named species may be an uncertain guide to diversity, a fact highlighted by the existence of actinotroch larvae of doubtful taxonomic affinity (e.g. see discussion and references in Temereva, Malakhov & Chernyshev (2006). The long-lived planktotrophy of most phoronid larvae may imply that apparently cosmopolitan species (e.g. *P. australis*) constitute a shared gene pool, but this is unlikely to be entirely true (Medlin, 2007). Thus our samples, which are mostly drawn at single times from single localities, may also be an uncertain guide to diversity.

Specimen identity is outlined in Table 1 and is supported by the following evidence. For *P. ovalis* and *P. australis* the source, macroscopic morphology and associations were diagnostic. In addition, *P. ovalis* was positively identified by C. Lüter, who had arranged its collection and by L. Veale, the collector. *Phoronis muelleri* from Sweden was identified by the collector, C. Neilsen, and by its *SSU* rDNA sequence, which was almost identical to one from a Helgoland collection kindly provided by P. Grobe some years earlier. *Phoronis hippocrepia* was collected in his home locality and identified by C. C. Emig. *Phoronis pallida* and *Phoronopsis californica* were identified by S. Santagata. *Phoronopsis harmeri* was identified by the collector, V. Malakhov, and DNA of a specimen from California named as *Phoronopsis viridis* was received from G. Giribet. However, the status of *Ph. viridis* as a junior synonym of *Ph. harmeri* seems well established (Marsden, 1959), and is confirmed by the molecular results reported here. Issues surrounding the identification of *P. architecta* and *P. vancouverensis* are detailed below. Where available, voucher specimens have been deposited in the Hunterian Museum, Glasgow University under registration numbers GLHM 126497–126500, 126512, 126513.

Two taxa presented particular taxonomic problems. The difficult differentiation of *P. architecta* (from the west coast of North America) and *P. psammophila* (from Europe) has been reported (Marsden, 1959), and the former has been described as a junior synonym of *P. psammophila* (Emig, 1982). This assessment is currently reflected in the GenBank taxonomy records for accessions derived from specimens collected in Florida as '*Phoronis architecta*' by the Gulf Specimen Supply Company. However, the longitudinal muscles of a separate batch of such specimens received from this source by S. Santagata

are consistent with descriptions of *P. architecta*. Furthermore, these specimens had separate sexes, the females lacked nidamental glands, and freely spawned fertilized primary oocytes (60 μm in diameter) that developed into larvae (S. Santagata, pers. observ.). These details are consistent with descriptions of *P. architecta* (Andrews, 1890; Brooks & Cowles, 1905) and with comments by Marsden (1959: 100–101), but inconsistent with *P. psammophila*, which has nidamental glands and broods embryos. We therefore believe that the differentiation of these species is valid and have re-adopted the original name, *P. architecta*, for sequences derived from specimens from Florida. Because no sequence has yet been obtained from a specimen definitely referable to, or from a locality typical of, *P. psammophila* (e.g. Messina, Italy), all sequences currently identified in GenBank as *P. psammophila* (e.g. accession AF025946, Cohen *et al.*, 1998), should be redesignated as *P. architecta*.

A similar problem exists for *P. vancouverensis*, interpreted as a junior synonym of *Phoronis ijimai* by Emig (1974). Current information on the morphology and reproductive characteristics of these forms (Wu, Chen & Sun, 1980; Zimmer, 1991) suggests that they could be a single species but, lacking sequences from Japan, we have re-adopted the name *P. vancouverensis* for our sample of adults collected at Monterey, CA (see Cohen, 2000; Santagata & Zimmer, 2002).

Morphological characters, cladistic coding, and phylogenetic inference

Morphological characters and character-states were compiled from publications (Emig, 1982; Zimmer, 1991; Santagata, 2002; Santagata & Zimmer, 2002; Grobe, 2008) and personal observations of *P. architecta*. A data-matrix of 24 characters was constructed in MacClade 4.0 (Maddison & Maddison, 2001) using both binary and multistate coding as appropriate, and analysed by parsimony in PAUP*4 (Swofford, 2000) as unordered characters, with branch-and-bound (B&B) search. When a search with equally weighted characters produced multiple equally most parsimonious trees (MPT) a single least-homoplasious tree was found by successive approximation reweighting (Farris, 1969) using the maximum value of the rescaled consistency index (RCI) with B&B search, repeated until tree length stabilized (WP, weighted parsimony). Retention index reweighting gave the same tree. Bootstrap 50% consensus trees were found by B&B search (500 pseudoreplicates) both before and after reweighting (but see Lidén, 1999), with uninformative characters excluded. Gaps were treated as 'missing' and zero-length branches collapsed. See Supporting Information Appendix S2 for the character list and data matrix. MacClade's 'Trace all changes' function was used to locate character-state changes on trees.

DNA methods, including sequence choice, alignment, and phylogenetic reconstruction

Methods for the PCR amplification, purification, and sequencing of gene fragments were as previously described (Cohen & Weydmann, 2005). Because *SSU* rDNA contains too few variable sites to resolve phoronid relationships robustly, we added ~2 kb from the 5′ end of *LSU* rDNA (details in Cohen & Weydmann, 2005). In addition, because both rDNAs give little resolution of relatively recent divergences some mitochondrial sequence was sought. Mitochondrial small or large subunit rDNAs would have been preferred because they generally show little saturation, but neither could reliably be amplified with available universal primers. Instead, universal primers (Folmer *et al.*, 1994) were used to amplify and sequence 621 nt of mitochondrial *cox1* from all but two taxa. The rDNA sequences were aligned using ClustalX 1.83 (Thompson *et al.*, 1997; Jeanmougin *et al.*, 1998; Chenna *et al.*, 2003) with default open and extend gap penalties (10/6.66), and a few (generally invariant) terminal nucleotides were trimmed away to remove ragged ends. The *cox1* sequences were aligned by eye without indels and used as nucleotides for phylogenetic reconstruction.

Two alignments were prepared by concatenating the available sequences: alignment '13tx' comprised all 12 phoronid ingroup *SSU* + *LSU* rDNAs, the ten ingroup *cox1* nucleotide sequences, and the corresponding chiton and/or pectinid outgroup sequences. Alignment '24tx' contained the ingroup rDNAs plus the brachiopod rDNAs used previously, together with pectinid and chiton outgroup rDNAs (Cohen & Weydmann, 2005). The brachiopod sequences came from two representatives of each of the three extant inarticulate lineages, and two from each of the three main articulate lineages. *cox1* sequences were not included in the 24tx alignment.

Regions of potential rDNA alignment ambiguity were excluded separately for each alignment with GBlocks 0.91 (Castresana, 2000) using default parameter settings (e.g. minimum number of sequences for a conserved block, 13; minimum number for a flanking position, 20; minimum number of noncontiguous conserved positions, 8; minimum block length, 5; maximum number of included sequences with a gap, half). Phylogenetic analyses using parsimony and maximum likelihood (ML) were made with PAUP*4b10 (Swofford, 2000), with ML models selected separately for each alignment under the Aikake information criterion (AIC) in MODELTEST

3.06 (Posada & Crandall, 1998; Posada & Buckley, 2004). Iterative selection (Sullivan, 2005) led to the same model. Parsimony trees and bootstrap trees were found by B&B search with tree bisection-reconnection (TBR) branch exchange, which is guaranteed to find the shortest tree(s). ML and ML bootstrap trees were found by heuristic search with TBR branch exchange using initial trees obtained by neighbor joining. The number of branch exchanges permitted in the ML bootstrap searches was restricted to 10^4.

The molecular clock hypothesis was assessed by a likelihood ratio test on ML trees ($2 \times$ difference in –lnL with and without the clock assumed to be distributed as χ^2 with df = number of taxa – 2) and relative rate tests were applied to taxonomically grouped data using RRTree (Robinson *et al.*, 1998; Robinson-Rechavi & Huchon, 2000). Base composition heterogeneity was tested using the χ^2 method in PAUP*4. Bayesian maximum likelihood (BML) analyses were made with MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). The MrBayes log file showing all parameters was inspected in TRACER 1.4.7 (Drummond *et al.*, 2006) to check that the number of sampling generations and effective sample sizes were large enough for reliable parameter estimates. Dating with a relaxed molecular clock was performed with BEAST 1.4.7 and associated programs (Drummond *et al.*, 2006) on the 24tx alignment and the ML tree derived from it (see legend to Fig. 4 for details).

Nucleotide sequence alignments were tested for saturation by plotting pairwise transition and transversion '*p*' (uncorrected) distances against pairwise ML total distances. Conflicting phylogenetic signals in aligned sequence data (nontree-like signal) were visualized as equal-angle networks in SPLITSTREE 4 using HKY + gamma + I distances (Hasegawa, Kishino & Yano, 1985) with split decomposition and neighbor-net (Huson, 1998; Huson & Bryant, 2005). The influence of alignment polarity ('Heads or Tails', Landan & Graur, 2007) was tested by reversing the 24tx data before CLUSTAL realignment (with all gaps removed), followed by parsimony and distance (LogDet) bootstrap analyses in PAUP*4.

Outgroup selection

Criteria for optimal outgroup selection include low substitution rate and ingroup-like base composition (Rota-Stabelli & Telford, 2008). Using such criteria it was previously found in exploratory analyses with many potential outgroups including basal deuterostomes and other lophotrochozoans (polychaetes, bryozoans, nemertines, other molluscs, etc) that chiton and pectinid sequences were optimal for the analysis of brachiopod and phoronid rDNAs in part because these outgroups, but not others, resulted in the recovery of brachiopod benchmark clades (Cohen *et al.*, 1998; Cohen & Weydmann, 2005). However, saturation analyses show that when the focus is on phoronid inter-relations a more local outgroup is required. In a wide variety of outgroup-rooted preliminary analyses, and with midpoint rooting (Hess & de Moraes Russo, 2007), *P. ovalis* behaved as the sister-group of the remaining ingroup (in agreement with Emig, 1974; Grobe, 2008), the topology of which was constant. *Phoronis ovalis* therefore was used as the local outgroup.

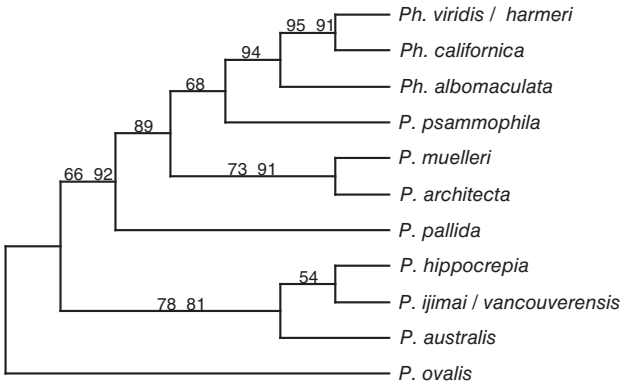

Figure 1. Phoronid phylogeny. Reweighted parsimony cladogram (length = 36.75, consistency index = 0.907, retention index = 0.899) based on a 24-character morphological data matrix with bootstrap support (%; 500 pseudoreplicates; first figure unweighted, second figure reweighted).

RESULTS

Cladistic analysis of morphology

The states of 24 characters (21 informative) that describe aspects of larval and adult anatomy and reproductive traits of 11 terminal taxa and the resulting data-matrix (PAUP or MacClade inFile) are given in Supporting Information Appendix S2. With equal weighting, B&B search gave eight MPT [length (L) = 61, consistency index (CI) = 0.738, retention index (RI) = 0.719]. After two successive rounds of reweighting and search with the rescaled consistency index (or the retention index) these reduced to one MPT (L = 36.75, CI = 0.907, RI = 0.899), shown in Figure 1. On this tree 11 informative characters had weight = 1.0, nine had weight < 1, and one weight = zero (i.e. uninformative). Bootstrap values are shown both before and after reweighting (the latter are controversial, Lidén, 1999), and are therefore shown with reservations). They suggest that in this analysis only the clade joining *P. hippocrepia* with *P. vancouverensis*/*ijimai* is weakly supported. Overall, the result is highly congruent with previous

analyses (Emig, 1974; Grobe, 2008). The basal split between the *P. australis* clade and the rest is associated with one nonhomoplasious change: character 17 (longitudinal muscle type) from bushy to feathery. However (see below), this split does not appear in such a simple state in the molecular analyses.

Molecular analyses: missing data, saturation, base composition, relative rates, and data conflict

Two *cox1* amplicons, from *P. australis* D1738 and *P. pallida* D1600 could not be sequenced. Repeated PCR syntheses under different high stringency annealing conditions gave products that appeared monodisperse in long, high-concentration, agarose gels, but interspersed long stretches of the sequence traces were unreadable. *SSU* and *LSU* from the same DNAs were readily sequenced, suggesting that the problem was caused by mitochondrial sequence (not length) heteroplasmy. Completed *cox1* sequences showed no internal stop codons when conceptually translated with the protostome mitochondrial code (Helfenbein, 2000; Helfenbein & Boore, 2004) and were alignable without indels, and preliminary analyses showed that essentially the same topology with similar bootstrap support resulted when analysed as nucleotides or after translation.

The only substantial missing rDNA sequence was ~800 nt at the 5′ end of the *LSU* of a Mediterranean *P. australis*, received as a gift from G. Giribet and used only in preliminary analyses. Of the 3885 sites in the rDNA alignment '24tx', 1786 were from *SSU* and 2099 from *LSU* (one was a spacer); 2563 were invariant, 607 variable but parsimony-uninformative, and 715 parsimony-informative. The proportions of variable + parsimony-informative sites (*SSU*, 10.1%; *LSU*, 22.3%) demonstrate the greater informativeness of the *LSU* partition. The concatenated rDNAs showed remarkable base composition homogeneity ($P = 1.00$) both amongst all taxa and amongst selected small groups. Previous analyses of comparable rDNA sequences showed saturation to be negligible or absent (e.g. Cohen & Weydmann, 2005) and this was confirmed: ingroup rDNAs showed only slight saturation, with scatter-plots of '*p*' transition and transversion pairwise distances against ML total pairwise distance best fitted by slight power curves, $r^2 = 0.94$–0.95; details not shown).

Relative rate tests on the 24tx alignment (pectinid excluded, chiton as outgroup) compared all 21 pairs of seven taxon groups: 1. *Phoronis ovalis*; 2. other *Phoronis* spp; 3. *Phoronopsis* spp; 4. Craniids; 5. Discinids; 6. Lingulids; 7. Articulate brachiopods. All relative rates amongst or between phoronids and inarticulate brachiopods (i.e. taxon groups 1 to 6) gave nonsignificant differences ($P > 0.05$), whereas all comparisons between articulate brachiopods and other groups showed the former to be significantly faster ($P < 0.001$). Thus, any departure from clock-like sequence evolution in this alignment is mainly attributable to articulate brachiopods, which show about 1.3 times as much change as phoronids and inarticulate brachiopods. The 24tx rDNA data were re-aligned and re-analysed by parsimony after sequence reversal (Landan & Graur, 2007) but the resulting trees were not materially different from those obtained before reversal. Thus, the phylogenetic results do not reflect misalignment arising from sequence orientation.

Of the 4515 nucleotides in the 13tx alignment 621 were from *cox1* and 3894 from rDNA. 3082 characters were constant, 964 variable but parsimony-uninformative, and 469 informative. Base composition was not heterogeneous (all taxa, all data, $P = 0.93$). In saturation analysis of *cox1* from this alignment, with the chiton included, even third-position transversions were strongly saturated. With the chiton and *P. ovalis* omitted, transitions at all codon positions were saturated, but transversions at codon positions 1 + 2 were not (linear best-fit $r^2 = 0.46$), and transversions at position 3 showed mild saturation (power curve best-fit, $r^2 = 0.91$; details not shown). Thus *cox1* of phoronids alone (*P. ovalis* excluded) is not so saturated as to be unusable. In relative rate tests (chiton outgroup) with each phoronid as a separate lineage only one test showed a significant rate difference ($P < 0.05$). Thus, use of this alignment for phylogenetic reconstructions of phoronid relationships is unlikely to be misled by differences in base-composition or lineage-specific evolutionary rate. All SPLITSTREE analysis modes showed that neither outgroup: ingroup nor ingroup inter-relationships were affected by more than slight nontree-like signal (details not shown). Thus, tree-reconstruction methods are appropriate for these alignments.

Relationship of phoronids to brachiopods and other taxa: phylogenetic analysis of the 24tx alignment

Previous analyses of *SSU* rDNAs found that phoronids are more closely related to brachiopods than to other lophotrochozoan protostome phyla (e.g. Cohen, 2000) and, with the addition of *LSU* rDNAs, that they behave as the sister-group of inarticulate brachiopods (Cohen & Weydmann, 2005). This sister-group relationship was confirmed by analysis of the 24tx rDNA alignment, as was the sister-group relationship between *Phoronis ovalis* and all other phoronids (Fig. 2). Thus, inclusion of sequence data from the majority of extant phoronid named taxa confirms the

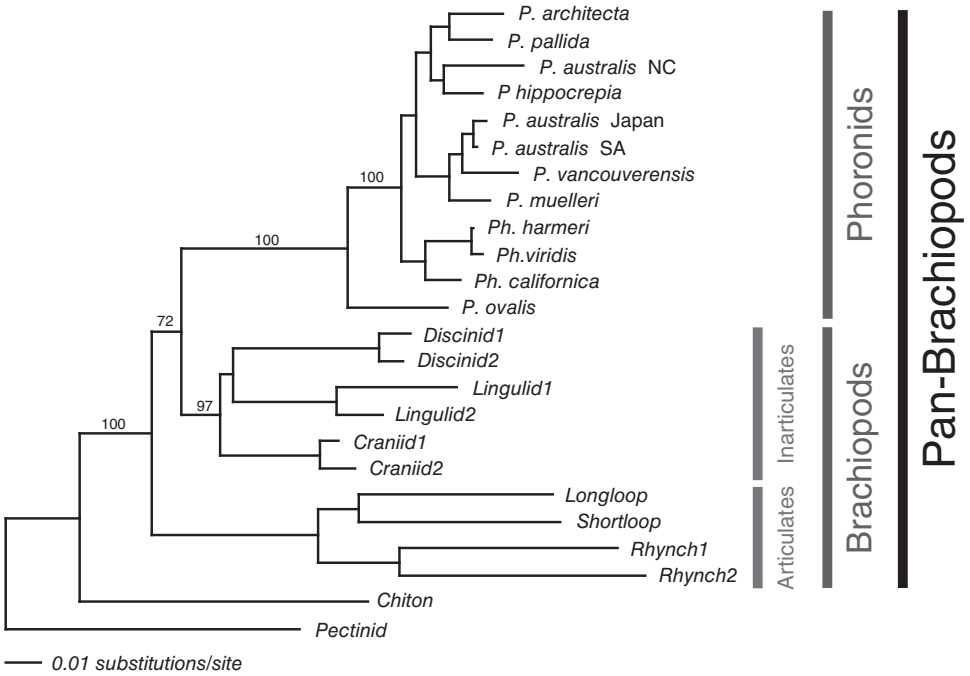

Figure 2. Phoronid phylogeny. Maximum likelihood phylogram based on 24tx alignment of concatenated rDNA sequences, with selected bootstrap values (%, 100 pseudoreplicates).

identification of the previously observed phoronid + brachiopod clade, which may be designated 'pan-Brachiopoda', and does not interfere with recovery of brachiopod benchmark clades.

Relationships amongst phoronids

Preliminary analyses of the 13tx *cox1* + rDNA alignment showed that bootstrap support in parsimony and ML analyses was substantially reduced with chiton or pectinid as outgroup. Therefore a distant outgroup was excluded from some analyses and *P. ovalis* was used as the local outgroup in 12-taxon analyses. Separate MODELTEST analyses of *cox1* and the concatenated rDNAs (13tx) identified general time reversible (GTR) + I + G models with similar gamma distribution shape coefficients (0.43, 0.57) and invariant site estimates (0.61, 0.63). Base composition and substitution rate matrices were also reasonably similar, and therefore the complete *cox1* + *SSU* + *LSU* alignment (4515 nt) was analysed with a single model. (The relative homogeneity of the rDNA sequences and unpublished data indicate that the use of separate models for stem and loop secondary structure regions would have a small effect on branch lengths but little or no effect on topology.) Heuristic search with random addition and TBR branch exchange quickly found one ML tree (–lnL = 15420.53). Bootstrap support was estimated with heuristic search of 100 pseudoreplicates.

Partitioned Bayesian likelihood analyses of the 13tx alignment with the chiton and/or the local outgroup were performed in multiple runs of MrBayes 3.1.2 with GTR + I + G equivalent models, whose parameters had been separately estimated from five partitions comprising the first, second, and third codon sites of *cox1* and the two rDNAs. Topology and all partitions were unlinked. After 10^6 generations effective sample sizes were > 200 and parameter estimates had stabilized. From one of these runs 20 002 trees were obtained, of which the first 2002 were discarded. Amongst the remaining 18 000, one tree had a high probability (0.68) and provides the best Bayesian topology estimate (Fig. 3); all other trees had low probabilities. All 18 000 trees were used to compile a consensus and the Bayesian clade credibility values were transferred to the most probable tree (Fig. 3).

In PAUP*4 the log-likelihood of the most probable Bayesian ML tree was -lnL 15432.81 compared with –lnL = 15420.53 for the ML tree. Apart from small differences in relative branch length the only disagreement affects the position of *P. pallida* relative to *P. hippocrepia* and *P. architecta*, and involves the clade with the lowest ML and Bayesian support. Thus, the inter-relationships of these three taxa remain uncertainly resolved. Although the morphological and molecular trees agree on a basal split amongst the non-*ovalis* taxa, they disagree substantially on its composition.

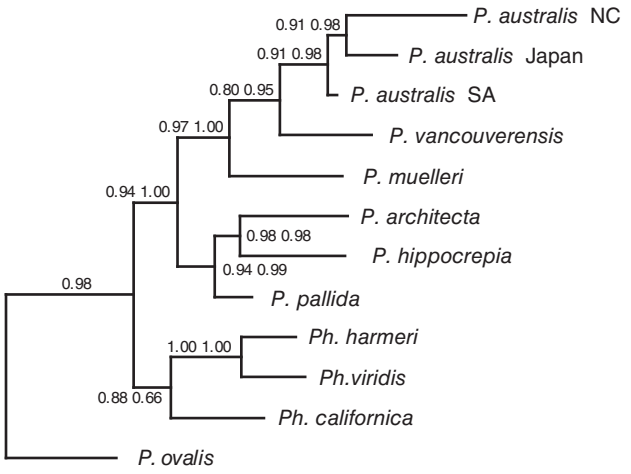

Figure 3. Phoronid phylogeny. Most probable Bayesian likelihood tree ($P = 0.68$) from analysis of the 13tx alignment with clade credibility values from the majority rule consensus tree, which had the same topology. Where two clade credibility values are shown the first was from a run with the chiton as outgroup (not shown), and the second from a run with *Phoronis ovalis* as outgroup. MrBayes run commands were: charset coding_1st = 1-621\3; charset coding_2nd = 2-621\3; charset coding_3rd = 3-621\3; charset non-coding = 622-4515; partition all_4 = 4: coding_1st, coding_2nd, coding_3rd, non-coding; set partition = all_4; lset applyto=(all) nst = 6 rates = invgamma; databreaks 621 2386; unlink shape = all, pinvar = all, statefreq = all, revmat = all; prset ratepr = variable; mcmc ngen = 10^6 samplefreq = 100 printfreq = 5000 savebrlens = yes; plot match = all; sumt burnin = 2002. The tree shown was drawn in PAUP*4. The .con file was imported and displayed as a phylogram. Clade credibility values were added in a graphics editor.

Divergence within named forms (species)

rDNA divergence was estimated between geographically well-separated members of two phoronid species: (1) *Phoronis australis* from Japan, New Caledonia and South Australia, plus partial data from a Mediterranean specimen, and (2) *Ph. harmeri* from Vladivostock and material received as *Ph. viridis* from California. For *P. australis*, pairwise '*p*' distances were calculated after excluding the ~870 nucleotides missing from the Mediterranean specimen's sequence. These distances were quite uniform ($N = 6$, range 0.0080–0.0150, mean = 0.0113 ± SD 0.0028). When the Mediterranean specimen was excluded all 3919 aligned rDNA nucleotides were used and distances were less uniform ($N = 3$, range 0.0084–0.0318, mean = 0.0229 ± SD 0.0126). These data do not strongly suggest cryptic speciation in *P. australis* but all analyses show a relatively distant relationship between the New Caledonia and other specimens, suggesting that with more extensive sampling the *P. australis* complex might break up into distinct clusters.

The rDNA '*p*' distance between *Ph. harmeri* and *Ph. viridis* samples was 0.0043 ($n = 1$). For comparison, mean between-species and between-genus phoronid '*p*' distances ranged somewhat higher ($N = 41$, range 0.0087–0.0391, mean 0.0252 ± SD 0.0069). The close relationship between these *Phoronopsis* species, and the relatively large distance between them and *Ph. californica* is clear, and comparable with the divergences within *P. australis*. This result is compatible with the suggested synonymy of *Ph. harmeri* and *Ph. viridis* (Marsden, 1959; Emig, 1979, 1982). Again, more extensive sampling would be required to test whether distinct species-like clusters of genotypes exist.

Except for the case of artefactual chimaerism (Supporting Information Appendix S1), replicate or conspecific *SSU* and *LSU* sequences determined in different laboratories or by different sequencing methods from samples obtained from the same or closely similar localities showed negligible differences.

Timing of phoronid clade origins

Only one palaeontological calibration point is potentially available to date directly a node in the phoronid molecular tree: if the trace fossil *Talpina* is correctly identified as the borings of a phoronid similar (and presumably ancestral) to *P. ovalis*, divergence between this and other extant phoronid lineages must have occurred not long before the Late Devonian, *c*. 385–359 Mya; not long before because the dramatic Ordovician diversification of macroboring taxa (Wilson & Palmer, 2007) makes it unlikely that earlier *Talpina*-like borings have been overlooked. In addition, one divergence (*P. hippocrepia* vs. *P. architecta*/*P. vancouverensis*) has been tentatively dated to around 120 Mya (Cohen & Weydmann, 2005: fig. 2 and table 2, node I). Given this scanty evidence we have made a Bayesian relaxed clock analysis (Fig. 4) by adding all the phoronid *SSU* + *LSU* sequences to those of the taxonomically representative brachiopods (and mollusc outgroups) that were shown (Cohen & Weydmann, 2005) to allow the recovery of palaeontologically validated benchmark clades. To avoid dependence on potentially controversial calibrations, age priors were not used and node ages are reported relative to a root node age = 1.00. For this analysis topology and starting branch lengths were constrained by the 24tx ML tree from PAUP*4 (shown in Fig. 2), so that the BEAST package was used only to find the posterior means and distributions of relative node ages. It should be noted, however, that when topology was unconstrained, the BEAST and ML

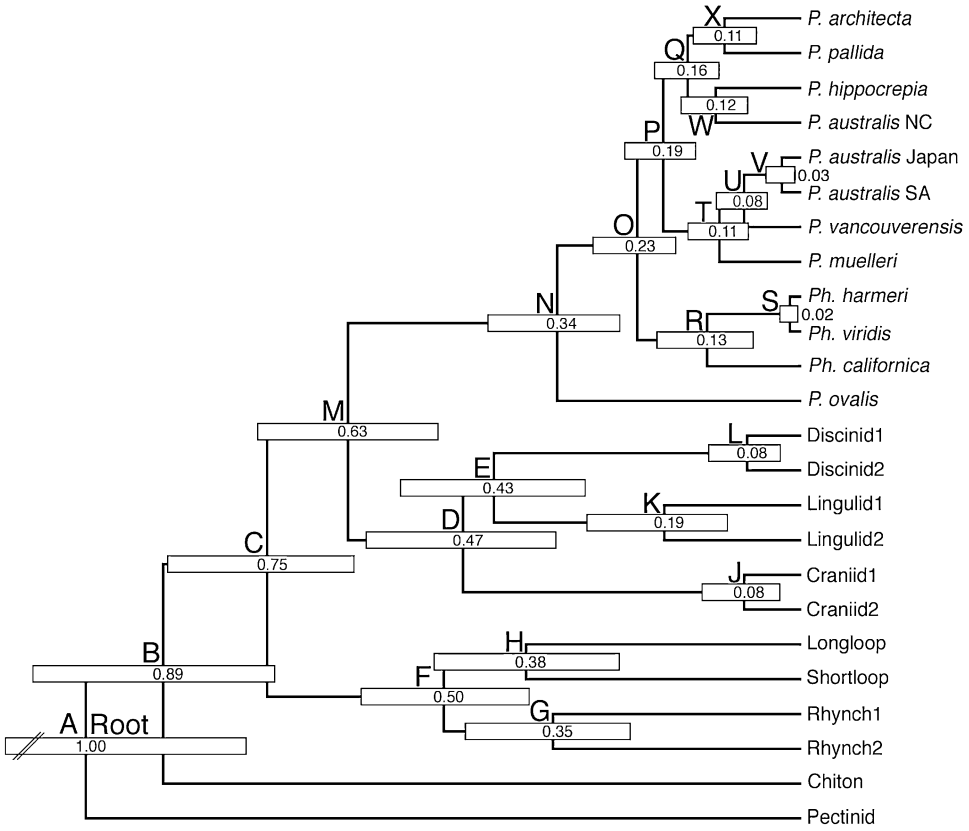

Figure 4. Phoronid phylogeny. Bayesian relaxed clock analysis of rDNA sequences. At each node is shown the relative node height, the 95% highest posterior density (HPD) limits, and the node label. Labels at brachiopod nodes are as used previously (Cohen & Weydmann, 2005). The 24tx alignment file was loaded into the utility BEAUTI to prepare an xml input file for BEAST and taxon groups corresponding to nodes B, D, F, M, N, and R were defined. The general time reversible + gamma + invariant sites model was selected, with four gamma categories and the mean substitution rate was fixed = 1.0 (the default). The uncorrelated lognormal clock rate variation model was selected, with all priors set to 'Uniform'. Operators that would change tree topology were switched off, and auto-optimize selected. The xml file was edited to include the maximum likelihood starting tree, which had been exported from PAUP*4 in Newick format with branch lengths. Multiple analyses were run with closely comparable results; the particular analysis shown was stopped after 1.3×10^7 generations with default data-logging, when inspection of the log file in TRACER revealed that all parameter distributions had smooth, quasi-normal curves and the lowest effective sample size was > 450. The best resulting tree was selected in TreeAnnotator, with 10% discarded as burnin, displayed in FigTree as relative heights (root = 1.0) with 95% HPD node bars, and the graphics file exported for editing. Diagonal lines mark a truncated node bar.

trees were identical except for alternative resolutions of a weakly-supported three-member terminal node and minor differences in branch length (not shown).

Conservative inferences from the relaxed clock analysis include:

1. the broad overlap of the 95% highest posterior density (HPD) ranges of nodes A–F and M indicate that the data provide no effective time-resolution at these basal nodes. This is consistent with palaeontological evidence that each of the organismal lineages descending from nodes A–F first appeared not later than the Early Cambrian (N.B.: divergence of rDNA lineages antedates organismal divergence by unknown, and perhaps very substantial, time-spans).
2. the origin of the phoronid lineage at node M suggests that this took place not later than the Early Cambrian.
3. the origin of the *P. ovalis* lineage at node N is dated on trace fossil evidence to 385–359 Mya at the latest. This puts node M (by arithmetic) at 713–480 Mya, a broad range that encompasses both the earliest record of the somewhat chiton-like, unmineralized mollusc *Kimberella*, *c.* 550 Mya (in the Ediacaran period, Knoll *et al.*,

2006; Fedonkin, Simonetta & Ivantsov, 2007) and the first appearance of brachiopods (Early Cambrian, Atdabanian, 530–524 Mya). Node M's broad range does not overlap the 95% HPD range of node N.

4. The 95% HPD limits around node N overlap the HPD limits of brachiopod nodes D–F, G, H, and K, which have palaeontologically established originations not later than Cambrian (D), Ordovician (E), Ordovician–Silurian (F), Triassic (G), Devonian–Triassic (H), and ?Cretaceous (K) (Selden, 2007, table 41, Ranges of Taxa, pp. 2966–3081). Thus, although of low resolution, brachiopod cross-calibration does not exclude (and is broadly consistent with) the trace fossil-derived date for node N.
5. if an Upper Devonian age for node N is accepted, the split between *Phoronis* and *Phoronopsis* (node O) would have occurred around 260–243 Mya and node P would have been at *c.* 215–200 Mya, whereas later splits (other than the most recent, nodes S and V) would have occurred around 200–100 Mya. These ages are broadly consistent with the mitochondrial *cox1* saturation results, which can be calibrated independently by comparison with data from the relatively fast-evolving laqueoid articulate brachiopods, whose *cox1* shows strong saturation after divergences of *c.* 130 Myr (Saito *et al.*, 2000). Thus, the *cox1* data supply independent, but indirect, support for the considerable ages of the phoronid lineage splits inferred from the relaxed clock rDNA analysis.

Overall, independent lines of evidence suggest that, despite the complete absence of phoronid body fossils, it is fair to conclude that the rDNA gene lineage represented by these organisms originated in the same radiation that gave rise to the articulate and inarticulate brachiopods, and that the deepest split amongst extant phoronids had developed by the Late Devonian, roughly 400 Mya.

DISCUSSION

Analyses of phoronid relationships based on morphological characters are limited in resolution by the relatively small number of characters available. Nevertheless, all analyses including ours agree that extant phoronids are monophyletic, that *P. ovalis* is the sister-group of the other extant forms and that *Phoronopsis* forms a distinct sub-clade (Marsden, 1959; Emig, 1974; Grobe, 2008). Differences of detail affect the composition of the basal split between non-*ovalis* taxa, the position of *P. ovalis* relative to other species that brood larvae (see below), and (because of disagreement over character-states) the positions of *P. architecta* and *P. psammophila*.

Topological discrepancies between morphological and molecular phylogenetic trees are common (Pisani, Benton & Wilkinson, 2007). Our morphology-based tree tends to separate brooding species from free-spawners, but this is not supported by our molecular data. This suggests that some reproductive characters such as brooding and sex of the adult (hermaphroditic or separate sexes) may be evolutionarily plastic amongst phoronid species. One possible source of homoplasy amongst reproductive characters may be linked to the distribution preferences of adult phoronids. For example, simultaneous hermaphroditism occurs in taxa whose individuals form isolated conspecific aggregates (20–50 individuals; *P. vancouverensis* and *P. hippocrepia*), and in taxa with commensal relationships that favour small clusters of adults (10–20 individuals; *P. australis* and *P. pallida*), but not in species with less specific settlement sites or adult distributions. Another association appears to exist between spermatophoral gland type (which controls spermatophore morphology) and conspecific aggregation. Simple spermatophoral glands generally produce bean- or club-like spermatophores thought to have lower dispersal capacity, whereas composite spermatophoral glands produce more elaborate, sail-like, spermatophores, thought to disperse better (Zimmer, 1964, 1991). Although details are not known for all species, taxa such as *Ph. harmeri* (= *Ph. viridis*) that produce sail-like spermatophores are generally found spread out across relatively uniform substrates such as sandy flats, whereas taxa such as *P. vancouverensis* (Zimmer, 1991) and *P. pallida* (S. Santagata, pers. observ.) that produce simple spermatophores occur in mat-like aggregates or as relatively isolated small groups. Whether such suggestive associations reflect shared descent or functional convergence cannot be assessed reliably without a high-resolution molecular analysis.

A key question in phoronid evolution is whether the absence of a mantle (with its correlated potential to form a mineralized shell or tube) is a plesiomorphic or apomorphic state. Because the relaxed clock analysis shows that confidence limits are wide, the order in which the articulate brachiopod, inarticulate brachiopod, and phoronid lineages separated from one another may not be reliably shown by the ML tree and, to be conservative, a polytomy may be assumed. Articulates are characterized by calcite (calcium carbonate) mineralization, whereas inarticulates have calcite in craniids or apatite (calcium phosphate) in linguliforms. Moreover, the shell of the putative stem-group articulate *Eoobolus* contained both calcite and apatite (Balthasar, 2007), whereas molluscs (some of which deposit calcite in or on the mantle) appear to be

a near sister-group of pan-brachiopods. Thus, the potential to form a mantle and the capacity to mineralize it with either apatite, calcite, or both was present (by inference) in the common ancestor of brachiopods and phoronids, and probably in a more remote common ancestor too. Thus, the absence of a mineralized (or potentially mineralized) mantle in phoronids must be derived. In fact, no embryonic or larval structures equivalent to the mantle-forming lobes of brachiopod larvae are present in larvae of extant phoronids, whereas, compared with brachiopods, metamorphosis follows a radically different programme. Thus, whereas the early stages of phoronid and brachiopod ontogeny appear to be homologous (Freeman, 1991, 2000), later stages of phoronid ontogeny are apomorphic. Moreover, the phoronid tube is secreted mainly by the trunk (extraverted metasomal sac) epithelium (Pourreau, 1979), which is also apomorphic, bearing no close correspondence to the shell-secreting tissues of any brachiopod. It is therefore unlikely that Early Cambrian linguliform brachiopods with an elongated, unmineralized, ventral pseudointerarea (e.g. *Wangyuia*, Zhang *et al.*, 2007) have any relevance for the origin of phoronids. Further, the epidermal fold that characterizes *Phoronopsis* must be a relatively late-evolved apomorphy, not a mantle relic.

Despite any limitations, this study provides evidence for a broad outline of phoronid evolutionary history, together with some evidence of the nature and distribution of present-day genetic diversity. It seems probable that there was an early split into two surviving lineages, one leading (probably via *Talpina*) to extant *P. ovalis*, the other leading to the remaining extant taxa. The *Talpina–P. ovalis* lineage is distinguished from the others by its habit of tunnelling into bivalve mollusc shells, by budding (possibly), and by possession of a morphologically simplified, nonplanktotrophic larva, all of which appear to be derived character-states. The non-*ovalis* lineages seem to represent three main clades, amongst which *Phoronopsis* is clearly separate and (from molecular evidence) sister to the *Phoronis* residue. The latter divides (on molecular evidence) into two sub-clades (*architecta*, *hippocrepia*, *pallida*) and (*muelleri*, *vancouverensis*, *australis*). Relationships within these subclades are not all strongly supported, are partially contradicted by the morphology-based analysis, and may be affected by missing molecular data (*cox1* of two taxa and all sequences of *P. psammophila*). They should therefore be considered provisional until further tested.

If *Talpina* is correctly identified as an ancestral member of the lineage represented by *P. ovalis*, the basal divergence leading to extant phoronids must have occurred some time (probably not very long) before the Upper Devonian, 385–359 Mya, and the relaxed clock analysis reported here makes this date appear not unreasonable. The suggestion that other splits within the phoronid clade such as the origin of *Phoronopsis* occurred in the region of 200–100 Mya, prompted by the high saturation in the mitochondrial sequence, also fits with the evidence that the mitochondrial genomes of a phoronid and a brachiopod share (with a chiton) a highly conserved, ancestral gene order, indicative of having experienced generally similar, low, rates of sequence evolution (Stechmann & Schlegel, 1999; Helfenbein, 2000; Helfenbein & Boore, 2004). A comparison with taxonomic evolution in (terebratellidine) articulate brachiopods confirms the idea of slow phoronid evolution. An endemic terebratellidine fauna of several clear-cut genera evolved around New Zealand during ~ 90 Myr of geographical isolation [Bitner *et al.*, 2008 (for 2007)]. Yet, phoronid populations of similar apparent age (e.g. *P. australis*) in the same general area, remain apparently conspecific. This difference in taxonomic diversity may truly reflect slow taxic evolution in phoronids, but it may also result in part from the low gene flow characteristic of most articulate brachiopods contrasted with high gene flow caused by dispersal of long-lived phoronid larvae, or simply reflect a lack of intensive study of phoronids.

Little can be said about extant within-species diversity and geographical distribution because both require detailed molecular study. The predominant finding reported here, of large genetic distances between phoronid specimens and taxa has one clear exception: the shortest distance observed (transition '*p*' distance = 0.0192; transversion '*p*' distance = 0.0073) was between a *Ph. harmeri* specimen from Vladivostock and a specimen of *Ph. viridis* from California. Thus, the molecular data agree with the suggestion (Marsden, 1959) that these forms may be conspecific. Comparable findings are known from Pacific specimens of craniid brachiopods which, unlike phoronids, have short-lived larvae and low dispersal capacity and, as it happens, are a close sister-group of phoronids (Cohen & Weydmann, 2005). In *Novocrania*, using mitochondrial *LSU* rDNA and other fast-evolving sequences, specimens of *Novocrania californica* from Vancouver Island were found to be most closely allied to (potentially conspecific with) specimens from near Taiwan, whereas some specimens from near Japan were close to those from a South-east Pacific seamount and from the Weddell Sea (Cohen, Long & Saito, 2008). Such close relationships between geographically separate specimens serve to emphasize how little we know and understand about modes and rates of evolution, dispersal, and vicariance in the oceans.

Our attempt to combine cladistic and molecular phylogenetic analyses of phoronids is limited by

sparse worldwide sampling and the possible underestimation of species-level diversity (Santagata & Zimmer, 2002). Nevertheless, it has revealed some unexpected features and allows far-reaching inferences. Clearly, however, a complete taxonomic revision based on much wider and deeper sampling is needed, along with the collection of more detailed information on the reproductive biology of adults and their larval forms. Even within 'well-studied' species such as *P. pallida* where reliable information exists on reproductive biology, there are differences in adult habitat and ecological associations amongst geographically distant populations (Santagata, 2004b). The large genetic distances separating most phoronid 'species' were unexpected, and they made largely fruitless the work carried out to collect *cox1* sequences. However, the evident saturation of this gene provided useful, if unexpected, evidence of deep and ancient divergence in the cosmopolitan *P. australis* and highlighted, in stark contrast, the small distance between specimens of *Phoronopsis* spp. from opposite sides of the Pacific Ocean. Possible associations between character-states and life-history attributes should repay future detailed study using sequencing targets better adapted to the levels of divergence and rates of evolution involved.

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REFERENCES

Andrews EA. 1890. On a new American species of the remarkable animal Phoronis. *Annals and Magazine of Natural History* **5:** 445–449.

Balthasar U. 2007. An Early Cambrian organophosphatic brachiopod with calcitic granules. *Palaeontology* **50:** 1319–1325.

Benson DA, Boguski M, Lipman DL, Ostell J, Ouellette BFF. 1998. GenBank. *Nucleic Acids Research* **26:** 1–7.

Bitner MA, Cohen BL, Long SL, Richer de Forges B, Saito M. 2008 (for 2007). *Gyrothyris williamsi* sp. nov. and inter-relationships of some taxa from waters around New Zealand and the southern oceans (Rhynchonelliformea: Terebratelloidea). *Earth and Environmental Sciences Transactions of the Royal Society of Edinburgh* **98:** 425–435.

Bromley RG. 2004. A stratigraphy of marine bioerosion. In: McIlroy D, ed. *The application of ichnology to palaeoenvironmental and stratigraphic analysis*. London: The Geological Society, 455–479.

Brooks WK, Cowles RP. 1905. *Phoronis architecta*: its life history, anatomy and breeding habits. *Memoirs of the National Academy of Sciences, Washington* **10:** 72–113.

Castresana J. 2000. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Molecular Biology and Evolution* **17:** 540–552.

Cavalier-Smith T. 1998. A revised six-kingdom system of life. *Biological Reviews* **73:** 203–266.

Chen J, Zhou G. 1997. Biology of the Chengjiang fauna. *Bulletin of the National Museum of Science* **10:** 11–105.

Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG, Thompson JD. 2003. Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Research* **31:** 3497–3500.

Cohen BL. 2000. Monophyly of brachiopods and phoronids: reconciliation of molecular evidence with Linnaean classification (the subphylum Phoroniformea nov.). *Proceedings of the Royal Society of London. Series B. Biological Sciences* **267:** 225–231.

Cohen BL. 2007. The brachiopod genome. In: Kaesler RL, ed. *Treatise on invertebrate paleontology*. Lawrence, KS: Geological Society of America and University of Kansas Press, 2356–2372.

Cohen BL, Gawthrop AB. 1996. Brachiopod molecular phylogeny. In: Copper P, Jin J, eds. *Brachiopods: proceedings of the third international brachiopod congress, Sudbury, Ontario, 1995*. Rotterdam: Balkema. 73–80.

Cohen BL, Gawthrop AB. 1997. The brachiopod genome. In: Kaesler RL, ed. *Treatise on invertebrate paleontology*. Lawrence, KS: Geological Society of America and University of Kansas Press, 189–211.

Cohen BL, Gawthrop AB, Cavalier-Smith T. 1998. Molecular phylogeny of brachiopods and phoronids based on nuclear-encoded small subunit ribosomal RNA gene sequences. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* **353:** 2039–2061.

Cohen BL, Holmer LE, Lüter C. 2003. The brachiopod fold: a neglected body plan hypothesis. *Palaeontology* **46:** 59–65.

Cohen BL, Long SL, Saito M. 2008. Living craniids: preliminary molecular evidence of their inter-relationships. *Fossils and Strata* **54:** 283–287.

Cohen BL, Weydmann A. 2005. Molecular evidence that phoronids are a subtaxon of brachiopods (Brachiopoda: Phoronata) and that genetic divergence of metazoan phyla began long before the Early Cambrian. *Organisms, Diversity and Evolution* **5:** 253–273.

Drummond AJ, Ho SYW, Phillips MJ, Rambaut A. 2006. Relaxed phylogenetics and dating with confidence. *PLoS Biology* **4:** e88.

Dunn CW, Hejnol A, Matus DQ, Pang K, Browne WE,

Smith SA, Seaver E, Rouse GW, Obst M, Edgecombe GD, Sorensen MV, Haddock SHD, Schmidt-Rhaesa A, Okusu A, Kristensen RM, Wheeler WC, Martindale MQ, Giribet G. 2008. Broad phylogenomic sampling improves resolution of the animal tree of life. *Nature* **452:** 745–750.

Emig CC. 1974. The systematics and evolution of the phylum Phoronida. *Zeitschrift für zoologische Systematik und Evolutions-forschung* **12:** 128–151.

Emig CC. 1979. British and other Phoronids. In: Kermack DM, Barnes RSK, eds. *Synopses of the British Fauna (new series)*. London: Academic Press, 57.

Emig CC. 1982. The biology of Phoronida. *Advances in Marine Biology* **19:** 1–89.

Emig CC. 1985. Phylogenetic systematics in Phoronida (Lophophorata). *Zeitschrift für Zoologische Systematik und Evolutions-forschung* **23:** 184–193.

Farris JS. 1969. A successive approximations approach to character weighting. *Systematic Zoology* **18:** 374–385.

Fedonkin MA, Simonetta A, Ivantsov AY. 2007. New data on *Kimberella*, the Vendian mollusc-like organism (White Sea region, Russia): palaeoecological and evolutionary implications. In: Vickers-Rich P, Komarower P, eds. *The rise and fall of the Ediacaran biota*. London: Geological Society, 157–179.

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* **3:** 294–299.

Freeman G. 1991. The bases for and timing of regional specification during larval development in *Phoronis*. *Developmental Biology* **147:** 157–173.

Freeman G. 2000. Regional specification during embryogenesis in the Craniiform brachiopod Crania anomala. *Developmental Biology* **22:** 219–238.

Freeman G. 2003. Regional specification during embryogenesis in Rhynchonelliform brachiopods. *Developmental Biology* **261:** 268–287.

Freeman G, Martindale MQ. 2002. The origin of mesoderm in *Phoronids*. *Developmental Biology* **252:** 301–311.

Giribet G. 2008. Assembling the lophotrochozoan (= spiralian) tree of life. *Philosophical Transactions of the Royal Society. Series B, Biological Sciences* **363:** 1513–1522.

Gradstein F, Ogg JG. 2004. Geologic Time Scale 2004 – why, how and where next! *Lethāia* **37:** 175–181.

Grobe P. 2008. *Larval development, the origin of the coelom and the phylogenetic relationships of the Phoronida*. Dissertation, Freie Universitët Berlin. Available at: http://www.diss.fu-berlin.de/diss/receive/FUDISS_thesis_000000003523.

Halanych KM, Bacheller JD, Aguinaldo AMA, Liva SM, Hillis DM, Lake JA. 1995. Evidence from 18S ribosomal DNA that the lophophorates are protostome animals. *Science* **267:** 1641–1643.

Hasegawa M, Kishino H, Yano T. 1985. Dating the human-ape splitting by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution* **22:** 160–174.

Helfenbein K. 2000. *Phoronids and articulate brachiopods: mitochondrial genomes, phylogeny and molecular evolution. The Millennium Brachiopod Congress*. London: Natural History Museum.

Helfenbein K, Boore JL. 2004. The mitochondrial genome of *Phoronis architecta*–comparisons demonstrate that phoronids are lophotrochozoan protostomes. *Molecular Biology and Evolution* **21:** 153–157.

Helmkampf M, Bruchhaus I, Hausdorf B. 2007. Multigene analysis of lophophorate and chaetognath phylogenetic relationships. *Molecular Phylogenetics and Evolution* **46:** 206–214.

Helmkampf M, Bruchhaus I, Hausdorf B. 2008. Phylogenetic analyses of lophophorates (brachiopods, phoronids, bryozoans) confirm the Lophotrochozoa concept. *Proceedings of the Royal Society of London. Series B, Biological Sciences* **275:** 1927–1933.

Herrmann K. 1979. Larval development and metamorphosis of *Phoronis psammophila* (Phoronida, Tentaculata). *Helgoländer Wissenschaftliche Meeresuntersuchungen* **32:** 550–581.

Herrmann K. 1997. Phoronida. In: Harrison FW, Woollacott RM, eds. *Microscopical anatomy of invertebrates*. New York: Wiley-Liss, 207–235.

Hess PN, de Moraes Russo CA. 2007. An empirical test of the midpoint rooting method. *Biological Journal of the Linnean Society* **92:** 669–674.

Huang D. 2006. The early body plan, origin and evolutionary radiation of Priapulida. In: Rong J, Fang Z, Zhou Z, Zhan R, Wang X, Yuan X, eds. *Originations, radiations and biodiversity changes-evidences from the Chinese fossil record*. Beijing: Science Press, 125–137.

Huson DH. 1998. SplitsTree: analysing and visualizing evolutionary data. *Bioinformatics* **14:** 68–73.

Huson DH, Bryant D. 2005. Application of phylogenetic networks in evolutionary studies. *Molecular Biology and Evolution* **23:** 254–267.

Hyman LH. 1959. *The invertebrates: smaller coelomate groups*. New York, Toronto, London: McGraw-Hill.

Jeanmougin F, Thompson JD, Gouy M, Higgins DG, Gibson TJ. 1998. Multiple sequence alignment with Clustal X. *Trends in Biological Sciences* **23:** 403–405.

Knoll AH, Walter MR, Narbonne GM, Christie-Black N. 2006. The Ediacaran Period: a new addition to the geologic time scale. *Lethaia* **39:** 13–30.

Landan G, Graur D. 2007. Heads or tails: a simple reliability check for multiple sequence alignments. *Molecular Biology and Evolution* **24:** 1380–1383.

Lidén M. 1999. Boots-traps. *Taxon* **48:** 349–350.

Maddison DR, Maddison WP. 2001. *MacClade 4: analysis of phylogeny and character evolution*, version 4.02. Sunderland, MA: Sinauer Associates.

Marsden JR. 1959. Phoronidea from the Pacific coast of North America. *Canadian Journal of Zoology* **37:** 87–111.

Medlin LK. 2007. If everything is everywhere, do they share a common gene pool? *Gene* **46:** 180–183.

Nielsen C. 1991. The development of the brachiopod *Crania (Neocrania) anomala* (O. F. Muller) and its phylogenetic significance. *Acta Zoologica* **72:** 7–28.

Peterson K, Eernisse DJ. 2001. Animal phylogeny and the ancestry of bilaterians: inferences from morphology and 18S rDNA sequences. *Evolution & Development* **3:** 170–205.

Pisani D, Benton MJ, Wilkinson M. 2007. Congruence of morphological and molecular phylogenies. *Acta Biotheoretica* **55:** 269–281.

Posada D, Buckley TR. 2004. Model selection and model averaging in phylogenetics: advantages of Aikake information criterion and Bayesian approaches over likelihood ratio tests. *Systematic Biology* **53:** 793–808.

Posada D, Crandall KP. 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics* **14:** 817–818.

Pourreau C. 1979. Morphology, distribution and role of the epidermal gland cells in *Phoronis psammophila* Cori. *Tethys* **9:** 133–136.

Robinson M, Gouy M, Gautier C, Mouchiroud D. 1998. Sensitivity of the relative-rate test to taxonomic sampling. *Molecular Biology and Evolution* **15:** 1091–1098.

Robinson-Rechavi M, Huchon D. 2000. RRTREE: relative-rate tests between groups of sequences on a phylogenetic tree. *Nucleic Acids Research* **16:** 296–297.

Rokas A, Carroll SB. 2008. Frequent and widespread parallel evolution of protein sequences. *Molecular Biology and Evolution* **25:** 1943–1953.

Ronquist FR, Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19:** 1572–1574.

Rota-Stabelli O, Telford MJ. 2008. A multi-criterion approach for the selection of optimal outgroups in phylogeny: recovering some support for Mandibulata over Myriochelata using mitogenomics. *Molecular Phylogenetics and Evolution* doi: 10.1016/j.ympev.2008.03.033.

Saito M, Kojima S, Endo K. 2000. Mitochondrial COI sequences of brachiopods: genetic code shared with protostomes; limits of utility for phylogenetic reconstruction. *Molecular Phylogenetics and Evolution* **15:** 331–344.

Santagata S. 2002. Structure and metamorphic remodelling of the larval nervous system and musculature of *Phoronis pallida* (Phoronida). *Evolution & Development* **4:** 28–442.

Santagata S. 2004a. Larval development of Phoronis pallida (Phoronida): implications for morphological convergence and divergence among larval body plans. *Journal of Morphology* **259:** 347–358.

Santagata S. 2004b. A waterborne behavioural cue for the actinotroch larva of *Phoronis pallida* (Phoronida) produced by *Upogebia pugettensis* (Decapoda; Thalassinidea). *Biological Bulletin* **207:** 103–115.

Santagata S, Zimmer RL. 2002. Comparison of the neuromuscular systems among actinotroch larvae: systematic and evolutionary implications. *Evolution & Development* **4:** 43–54.

Selden PA, ed. 2007. *Treatise on invertebrate paleontology. Brachiopoda, revised*. Part H6., 2321–3226. Boulder, Colorado and Lawrence, KS: Geological Society of America and University of Kansas.

Simmons MP. 2000. A fundamental problem with amino-acid sequence characters for phylogenetic analysis. *Cladistics* **16:** 274–282.

Stechmann A, Schlegel M. 1999. Analysis of the complete mitochondrial DNA sequence of the brachiopod *Terebratulina retusa* places Brachiopoda within the protostomes. *Proceedings of the Royal Society of London. Series B. Biological Sciences* **266:** 2043–2052.

Sullivan J. 2005. Maximum-likelihood methods for phylogeny estimation. In: Zimmer EA, Roalson EH, eds. *Methods in enzymology*, Amsterdam: Elsevier, 757–779.

Swofford DL. 2000. *Phylogenetic analysis using parsimony (*and other methods)*. Sunderland, MA: Sinauer Associates.

Temereva M, Malakhov VV, Chernyshev AV. 2006. Giant actinotroch, a larva of Phoronida from the South China Sea: the giant larva phenomenon. *Doklady Biological Sciences* **410:** 410–413.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25:** 4876–4882.

Voigt E. 1975. Tunneling of recent and fossil phoronids. *Paläontologische Zeitschrift* **49:** 135–167.

Wilson MA, Palmer TJ. 2007. Patterns and processes in the Ordovician bioerosion revolution. *Ichnos* **13:** 109–112.

Wu B, Chen M, Sun R. 1980. On the occurrence of *Phoronis ijimai* in the Huang Hai, with notes on its larval development. *Studies on Marine Sinica* **16:** 101–112.

Zhang Z, Han J, Zhang X, Liu J, Guo J, Shu D. 2007. Note on the gut preserved in the Lower Cambrian *Lingulellotreta* (Lingulata, Brachiopoda) from southern China. *Acta Zoologica, Stockholm* **88:** 65–70.

Zimmer R.L. 1964. Reproductive biology and development of Phoronida. PhD Dissertation. University of Washington, Seattle, pp. 1–416.

Zimmer RL. 1991. Phoronida. In: Giese AC, Pearse JS, Pearse VB, eds. *Reproduction of marine invertebrates*. Pacific Grove, CA: Boxwood Press, 2–45.

Zrzavy J, Mihulka S, Kepka P, Bezdek A, Tietz D. 1998. Phylogeny of the metazoa based on morphological and 18S ribosomal DNA evidence. *Cladistics* **14:** 249–285.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Artefactual, chimaeric nature of the first phoronid *SSU* rDNA. Variable sites from an alignment of *SSU* rDNAs of *Terebratalia transversa* (Tt) and a variety of phoronids.

Appendix S2. Phoronid phylogeny. (A) Short descriptions of 24 morphological characters and associated character-states, coded respectively a–e in order shown. (B) morphological data-matrix in Nexus format (Maddison, Swofford & Maddison, 1997).

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