electron density omit maps encompassing the NADH binding site were used during refinement of the coordinates. The final model contained all 268 residues and 48 solvent molecules, with rms values of 0.021 Å and 2.3° in bond lengths and bond angles, respectively.
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Evidence from 18*S* Ribosomal DNA That the Lophophorates Are Protostome Animals

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The suspension-feeding metazoan subkingdom Lophophorata exhibits characteristics of both deuterostomes and protostomes. Because the morphology and embryology of lophophorates are phylogenetically ambiguous, their origin is a major unsolved problem of metazoan phylogenetics. The complete 18*S* ribosomal DNA sequences of all three lophophorate phyla were obtained and analyzed to clarify the phylogenetic relationships of this subkingdom. Sequence analyses show that lophophorates are protostomes closely related to mollusks and annelids. This conclusion deviates from the commonly held view of deuterostome affinity.

Understanding the phylogenetic affinities of the lophophorates (brachiopods, bryozoans, and phoronid worms) is a major unresolved issue of metazoan phylogenetics. The lophophorate taxa are thought to constitute a monophyletic subkingdom, superphylum, or phylum (*1*, *2*) because they possess a similar suspension-feeding apparatus, the lophophore [a ciliated ring of tentacles, invaded by the mesocoelomic cavity, that surrounds the mouth but not the anus (*1*, *3*)]. Because of their unusual morphologies, the phylogenetic relationships of lophophorates may provide important clues about the evolution of morphology and development in protostome and deuterostome animals.

Many phylogenetic hypotheses have been proposed for the lophophorates. They have been classified as protostomes, as deuterostomes, as members of both groups, or as an independent metazoan lineage. Earlier researchers often allied lophophorates with protostome taxa on the basis of the presence in both lophophorates and deuterostomes of chitin, the lack of sialic acids, and several embryological features (*1*, *4*). Analyses of incomplete 18*S* ribosomal DNA (rDNA) data from an inarticulate brachiopod are also consistent with protostome affinities (*5*). However, the most recent reanalyses of embryology (including blastopore fate, coelom formation, and cleavage patterns) (*6*, *7*) and morphology have led most researchers to believe that lophophorates are basal deuterostomes (*1*, *2*, *4*). The deuterostome hypothesis has been supported by recent phylogenetic analyses of morphology and embryology (*8*, *9*) on the basis of the presence in both lophophorates and deuterostomes of a tripartite coelomic arrangement, a modified radial cleavage, a ciliary food-collecting system that captures particles upstream relative to the ciliary beat, a lophophore-like apparatus (also present in echinoderms and pterobranch hemichordates), and a U-shaped adult digestive tract.

We used molecular sequence analyses to circumvent several problems associated with the interpretation of morphological and embryological characters. To reconstruct the evolution of the lophophorates, we sequenced complete 18*S* rDNAs from representative lophophorate taxa, aligned them with existing data from other metazoan taxa (*10*), and analyzed them with standard phylogenetic techniques. We chose rDNA to examine metazoan origins because it has evolved at an appropriate rate (*5*, *11*). *Anemonia sulcata* (an anthozoan) and *Tripedalia cystophora* (a scyphozoan) were used as the outgroups to triploblastic animals, which are thought to be monophyletic on the basis of morphological and molecular data (*12*).

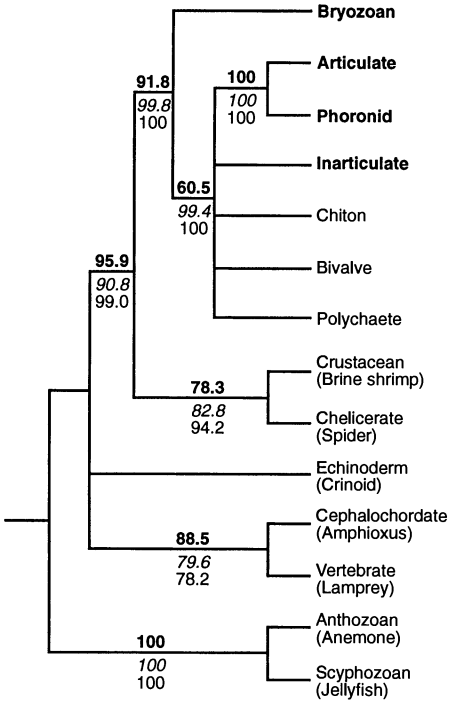

Fig. 1. Phylogenetic analyses of 18*S* rDNA sequence data to determine the position of the lophophorate metazoans (*10*). Lophophorate taxa are indicated in boldface. The consensus topology shown here is a consensus of the best trees given by four different methods: maximum likelihood analysis, bootstrap analysis of parsimony, bootstrap analysis of neighbor-joining with maximum likelihood estimates of Kimura two-parameter distances, and bootstrap analysis of paralinear distances. The numbers next to the nodes represent the bootstrap proportions (out of 500 iterations) for the parsimony analysis (top, bold numbers), the neighbor-joining analysis (middle, italic numbers), and the paralinear distance analysis (bottom, roman numbers).

The consensus tree derived from our reconstructions is shown in Fig. 1. In all of the reconstructions (including likelihood, parsimony, and distance methods), the lophophorate taxa clustered within the protostomate clade along with the annelid and molluscan taxa (*13*). The clade containing the lophophorates, the annelids, and the mollusks is present in 91.8, 99.8, and 100% of the trees derived from bootstrap reconstructions of data obtained through maxi-

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mum parsimony, neighbor-joining with maximum likelihood estimates of Kimura two-parameter distances, and paralinear distance reconstruction algorithms, respectively (*14*). Within this clade, the bryozoan is the deepest branching member (supported by 60.5, 99.4, and 100% of the bootstrap replicates, respectively). A topology-dependent cladistic permutation tail probability test (*15*) confirmed the significance of the lophophorate-mollusk-annelid clade ($P \leq 0.01$) in the parsimony tree. Hence, these data provide statistically significant support for this clade within the protostomes. Our interpretation of these results is shown schematically (Fig. 2); although the deuterostomes appear as a monophyletic group, this branch is not consistently supported in our analyses (Fig. 1).

Because these 18*S* rDNA analyses support the hypothesis that lophophorates, mollusks, and annelids share a common ancestor to the exclusion of arthropods and deuterostomes, we propose the node-based name (*16*) Lophotrochozoa, which is defined as the last common ancestor of the three traditional lophophorate taxa, the mollusks, and the annelids, and all of the descendants of that common ancestor. Although the similarity of the feeding apparatus among lophophorates has long suggested monophyly (*1*), the 18*S* rDNA analyses show that the Lophophorata are paraphyletic because the bryozoans are outside of the remaining Lophotrochozoa taxa. This finding raises the possibility that the lophophore has been derived multiple times or that it was present in the ancestral lophotrochozoan and has been lost in the mollusks and annelids. Clarification of this issue will require better resolution of trees and careful evaluation of other lines of evidence. Also, the position of the phoronid as the sister clade of the articulate brachiopod suggests that the Inarticulata and Articulata (that is, the Brachiopoda), contrary to traditional hypotheses (*17*), do not form a monophyletic group. The paraphyly of the Brachiopoda was strongly supported by bootstrap analyses indicating that phoronids are derived lophophorates, but additional complete 18*S* rDNA sequences will be needed before this issue can be definitively resolved. It has been proposed that phoronids evolved from a brachiopod-like ancestor (*7*, *18*) on the basis of plausible evolutionary intermediates and the evolution of the lophophore apparatus.

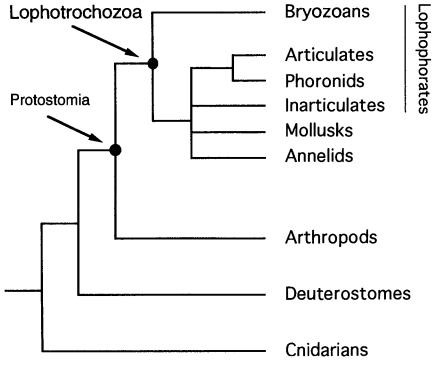

Fig. 2. The lophophorates are contained within the Protostomia. The lophophorates cluster with the mollusks and annelids to form the Lophotrochozoa (which include these taxa, their last common ancestor, and all of the common ancestor's descendants).

Some molecular and structural findings support our conclusions. For example, earlier analyses of incomplete 18*S* ribosomal RNA sequences that included an inarticulate brachiopod had suggested that it was affiliated with the mollusks (*5*), but that finding was based on only a single incomplete sequence. Also, the structure of the setae of brachiopods is similar to those of polychaetes, oligochaetes, and pogonophorans, but is unlike those of arthropods (*19*). With this removal of the chitin-synthesizing lophophorates from the deuterostomes and the recent removal of the chaetognaths as well (*20*), new attention may be focused on prior proposals (*21*) that loss of the ability to synthesize chitin may be a synapomorphy of the deuterostomes.

Common perceptions of morphological, developmental, and genomic evolution are intricately tied to phylogenetic hypotheses. Traditional hypotheses posit that the lophophorates are protostomes, deuterostomes, or a separate metazoan subkingdom. Molecular data from 18*S* rDNA genes, however, support the position of lophophorates within the protostome lineage and suggest that some embryological features (such as coelom formation and cleavage patterns) are more plastic than has been traditionally recognized.

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13. The sequence alignment of the 14-terminal taxa included 1605 unambiguously aligned nucleotide positions, of which 502 are variable and 294 are phylogenetically informative (that is, parsimony sites). D. L. Swofford's program PAUP version 3.1.2d5 [*Phylogenetic Analysis Using Parsimony* (Smithsonian Institution, Washington, DC, 1993)] was used for the parsimony analyses. The program package PHYLIP version 3.5 (J. Felsenstein, Department of Genetics, University of Washington) was used for the neighbor-joining and maximum likelihood analyses. A revision of the PHYLIP maximum likelihood program, fastDNAml version 1.0 (G. J. Olsen, University of Illinois at Urbana-Champaign), was also used. For paralinear distances [J. A. Lake, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 1455 (1994); P. J. Lockhart, M. A. Steel, M. D. Hendy, D. Penny, *Mol. Biol. Evol.* **11**, 605 (1994)], the Bootstrapper's Gambit multiple taxon reconstruction algorithm [D. A. Maslov, H. A. Avila, J. A. Lake, L. Simpson, *Nature* **368**, 345 (1994); J. A. Lake, in preparation] was used. The parsimony and Kimura analyses incorporated a transversion–transition ratio of 1.4:1. We obtained this ratio by determining the number of unambiguous transition and transversion events of 10 randomly selected trees and calculating the average ratio. The maximum likelihood score is also maximized at this ratio. Analyses with transversion–transition ratios of 1:1 to 3:1 did not alter our conclusions. Bootstrap analyses [J. Felsenstein, *Annu. Rev. Genet.* **22**, 521 (1988)] were used to examine the confidence of nodes within the resultant topologies.
14. Empirical studies of bootstrap analyses [D. M. Hillis and J. J. Bull, *Syst. Biol.* **42**, 182 (1993)] indicate that bootstrap values represent highly conservative estimates of phylogenetic accuracy. Typically, bootstrap proportions of ≥70% correspond to a probability of ≥95% that the respective clade is an historical lineage.

15. The topology-dependent cladistic permutation tail probability (T-PTP) test determines whether the difference in length between the shortest tree supporting the monophyly of this clade (997 steps) and the shortest tree not supporting monophyly (1003 steps) is significantly different from the difference in length expected from randomized data. If the difference in length between the monophyly and nonmonophyly trees is outside 95% of the distribution based on randomized data, it can be concluded that the data significantly support monophyly of the clade [D. P. Faith, *Syst. Zool.* **40**, 366 (1991); J. W. O. Ballard *et al.*, *Science* **258**, 1345 (1992)]. We used 200 randomized data sets that were created with the program Shuffle (J. P. Huelsenbeck, University of Texas at Austin) and were analyzed by means of the parsimony criterion.
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Sodefrin: A Female-Attracting Peptide Pheromone in Newt Cloacal Glands

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A decapeptide called sodefrin was isolated from the abdominal gland of the cloaca of the male red-bellied newt, *Cynops pyrrhogaster*. The native peptide, as well as the synthetic one, had a female-attracting activity. Sodefrin was found in the apical portion of the epithelial cells of the abdominal gland. Sodefrin is apparently species specific because it did not attract females of *Cynops ensicauda*. This is the first amphibian pheromone to be identified and the first peptide pheromone identified in a vertebrate.

In urodeles, chemical stimuli contribute to sex recognition and courtship behavior (*1*). It has been suggested that males emit olfactory attractants or pheromones to lure females (*2*). The abdominal gland of the cloaca has been thought to be the site where these substances are produced (*3*). We have found that the water in which sexually active male newts (*Cynops pyrrhogaster*) were kept attracted conspecific females (*4*). The attractant pheromone was secreted by or through the abdominal gland of the cloaca because the water in which abdominal gland–ablated males had been kept did not attract females (*4*).

We report here the isolation and characterization of the female-attracting pheromone from the abdominal glands of male newts. Female-attracting pheromone activity was monitored by a preference test (*5*). An aqueous extract of the abdominal glands had a female-attracting pheromone activity. The minimum effective amount of extract in a sponge block that attracted a female placed in a container filled with 3000 ml of water was the equivalent of 0.1% of the abdominal gland content (Fig. 1). The active substance in the abdominal gland was soluble in water but not in organic solvent. When the water-soluble fraction was subjected to gel-filtration column chromatography, the female-attracting pheromone activity emerged in a fraction with a relative molecular mass below 5000. Pronase digestion eliminated the activity, indicating that the active substance is a peptide.

To isolate the active peptide from an aqueous extract of the abdominal glands, we used two purification cycles of reversed-

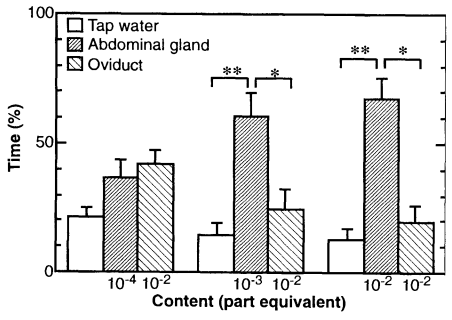

Fig. 1. Female-attracting activity in the abdominal gland of the male newt. Twenty abdominal glands were homogenized in 20 ml of distilled water. The homogenate was centrifuged at 5000*g* for 1 hour at 4°C. The supernatant was lyophilized and was used as the test substance. Oviducal extract was prepared similarly and was used as a control substance. Preference testing was done as described (*5*). Each sponge block contained tap water or extract from the indicated amount of the abdominal glands or oviduct. Results represent mean values (± SE) of eight tests. *Probability of significant difference (P) < 0.05; **P < 0.01.

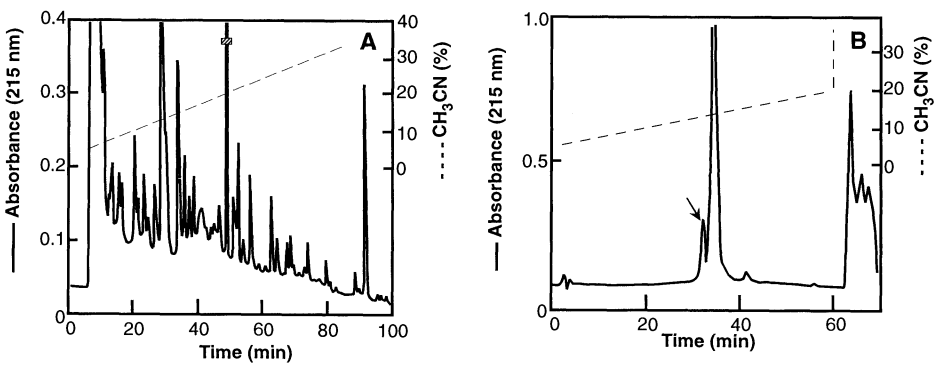

Fig. 2. Purification of female-attracting substance from the abdominal gland of the male newt. (**A**) The first reversed-phase HPLC. Fifty abdominal glands were homogenized in 100 ml of distilled water. After centrifugation at 5000*g* for 1 hour, the supernatant was lyophilized, dissolved in 0.15 M NH_4HCO_3 (pH 8.0), and applied to a Sephadex G-100 column (2.5 cm by 80 cm; Pharmacia) equilibrated with the same solution. Fractions with relative molecular mass below 5000, which had female-attracting activity, were pooled and lyophilized. The lyophilized sample was dissolved in 0.1% trifluoroacetic acid (TFA) and applied to an octadecyl silyl–silica cartridge (C_{18} Sep-Pak; Waters). The adsorbed substances were eluted with acetonitrile containing 0.1% TFA. The lyophilized C_{18}-adsorbed fraction was dissolved in 0.1% TFA and applied to a 4 mm by 125 mm column [Superspher 60 RP-8(e) column; Merck] equilibrated with 0.1% TFA. A gradient of acetonitrile was used at a flow rate of 1 ml min^{-1}. The active fraction, designated by the small hatched bar on the appropriate peak, was collected. (**B**) The second reversed-phase HPLC. The active fraction from (A) was further purified on a 4 mm by 150 mm phenyl column (Inertsil; Gas-Liquid Science, Tokyo) with a gradient of acetonitrile at a flow rate of 1 ml min^{-1} in the presence of 0.1% TFA. Female-attracting activity was detected in the peak fraction designated by an arrow. Detection of female-attracting activity was done as described (*5*). Yield of the final product was 0.6 μg per gland.