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Phylogeny of hydrothermal vent limpets ("Archaeogastropoda") based on morphological and 18S rDNA data - preliminary results

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Introduction

Despite more than 15 years of research into the evolution of hydrothermal vent fauna, the phylogeny of the endemic gastropods still remains a matter of debate. With the discovery of new taxa from hydrothermal vents and seeps, the range of "Archaeogastropoda" was markedly extended. In addition to a set of plesiomorphic characters common to all "Archaeogastropoda", the newly discovered species possess some remarkably advanced traits resulting in a confounding combination of both plesiomorphic and apomorphic characteristics. Until now, a portion of these newly discovered species has been assigned to a group labeled as "hot vent taxa" in a cladistic analysis by Ponder & Lindberg (1997). McArthur & Koop's (1999) attempt to split the heterogeneous "Archaeogastropoda" into monophyletic groups by investigating the 28S rDNA gene was only partially successful. Recently Warén & Bouchet (2001) described a high number of new "Archaeogastropod" species from hydrothermal vent and seep ecosystems. These authors divide the "Archaeogastropoda" into five subclasses and present a new classification of some taxa. Their changes, however, are not based on phylogenetic analyses. Ponder & Lindberg (1996, 1997) included Vetigastropoda in the "Archaeogastropoda", but the question of the common origin of Vetigastropoda and further "Archaeogastropoda" of hydrothermal vents and seeps remains unsolved. Moreover, the monophyly of some of the established groups of the "Archaeogastropoda" like the Neomphalina needs to be reexamined and, if necessary, revised. Hence, the "Archaeogastropoda" of hydrothermal vents remain as yet paraphyletic.

In this article we attempt to clarify their phylogeny using both morphological and molecular data for a preliminary taxa set of nine hot-vent "Archaeogastropoda" (Neolepetopsidae, Phenacolepadidae, Pyropeltidae, Neomphalidae, Peltospiridae, Lepetodrilidae). Analyses were performed with PAUP*4.0b8 based on 17 morphological characters and a 1225 base-pair long fragment of the 18S rDNA gene.

Material and methods

Nine species of hydrothermal vent "Archaeogastropoda" from several deep-sea expeditions to the East and West Pacific (Table 1) and polyplacophora as an outgroup were used for morphological analysis and DNA extraction for 18S rDNA sequences. The material had been fixed either in 95-70% ethanol or 10% formalin (Table 1) and was subsequently stored in 70% ethanol. Morphological investigations of shell and soft parts were conducted by binocular microscope observations and those of the radula by SEM. The following 17 character states where used for the morphological data matrix.

Character coding

1. Shell shape: (0) coiled, with umbilicus, (1) coiled, without umbilicus, (2) cap-shaped, (3) limpet-like.
2. Number of teleoconch whorls: (0) > 1 whorl, (1) < 1 whorl.
3. Position of apex: (0) anterior part of shell, on midline, (1) central, (2) posterior part of shell, on midline, (3) overhanging posterior margin to the right.
4. Periostracum: (0) not overhanging, (1) overhanging, (2) enveloping shell edge, (3) absent.
5. Shell septum: (0) present, (1) absent.
6. Protoconch diameter: (0) <180 µm, (1) >180 µm.
7. Operculum: (0) multispiral, (1) paucispiral, (2) anterior half corneous, posterior half calcic, intern, (3) adult rudimental, (4) only larvaly present, (5) absent.

Table 1. List of analysed "Archaeogastropoda", their current systematic position, fixation, collection site and sequence accession number.

	Current Systematic Position (after Warén & Bouchet, 2001)		Species	Fixation	Collection Site and Accession No.
Rhipidoglossa (Sasaki, 1998, Beck, in press) Neomphalina (McLean, 1990)	Patellogastropoda	Neolepetopsidae	*Eulepetopsis vitrea* McLean, 1990	formalin	East Pacific Rise 9°N, AF 534976
	Neritimorpha	Phenacolepadidae	*Olgasolaris tollmanni* Beck, 1992	formalin	Manus Back-Arc Basin, AF 534978
	Cocculiniformia	Pyropeltidae	*Pyropelta musaica* McLean & Haszprunar, 1987	formalin	Juan de Fuca Ridge, Axial Seamount, AF 534981
	Subclass "uncertain"	Neomphalidae	*Cyathermia naticoides* Warén & Bouchet, 1989	70% ethanol	East Pacific Rise 9°N, AF 534982
	Subclass "uncertain"	Neomphalidae	*Symmetromphalus hageni* Beck, 1992	formalin	Manus Back-Arc Basin, AF 534984
	Subclass "uncertain"	Peltospiridae	*Depressigyra globulus* Warén & Bouchet, 1989	95% ethanol	Juan de Fuca Ridge, Endeavour Segment, AF 534986
	Subclass "uncertain"	Peltospiridae	*Rhynchopelta concentrica* McLean, 1989	formalin	East Pacific Rise 21°N, AF 534988
	Vetigastropoda	Lepetodrilidae	*Lepetodrilus fucensis* McLean, 1988	formalin	Juan de Fuca Ridge, Endeavour Segment, AF 534993
	Vetigastropoda	Lepetodrilidae	*Clypeosectus curvus* McLean, 1989	95% ethanol	Juan de Fuca Ridge, Endeavour Segment, AF 534990

8. Snout: (0) with oral lappets, (1) tapered, (2) oral disc, (3) furrow with lateral lappets.
9. Cephalic tentacles: (0) uniform in both sexes, (1) males' left tentacle transformed into a penis, (2) males' right tentacle transformed into a penis, (3) absent.
10. Jaw: (0) absent, (1) single, (2) paired.
11. Ctenidia size: (0) medium, (1) large.
12. Heart configuration: (0) diotocard, (1) monotocard.
13. Renal organ: (0) right and left present, (1) only left present, (2) left very small, right normal size.
14. Shell muscle characters: (0) horseshoe-shaped, arms evenly broad, (1) horseshoe-shaped, area of contact between arms narrow, (2) one pair, (3) horseshoe-shaped, with inwardly directed hook-shaped anterior processes, (4) 8 pairs.
15. Epipodial tentacles: (0) present, (1) absent.
16. Rectum: (0) penetrating pericard, (1) not penetrating pericard.
17. Type of radula: (0) stereoglossate, (1) flexoglossate.

DNA extraction, PCR amplification and sequencing

DNA was extracted from foot or gonad tissue, or in the case of minute specimens from the complete animal. The extraction of ethanol-fixed material followed the tissue protocol of the QIAamp-DNA-Mini-Kit (Qiagen). That of formalin-fixed material followed modifications after Shedlock et al. (1997) and Chase et al. (1998) adapted to the QIAamp-DNA-Mini-Kit: formalin-fixed material was washed in GTE three times 24 hours before extraction, and 10 µl 1M DTT was added to ATL-buffer of QIAamp-Kit. PCR amplification was performed in two parts with two primer-pairs after Winnepenninckx et al. (1994) and Wollscheid & Wägele (1999). Primer pair I: forward 5'-CTGGTTGAT(CT)CTGCCAGT-3' and reverse 5'-TCTCAGGCTCC(CT)TCTCCGG-3' to amplify the first section (~450bp) of the 18S rDNA gene. Primer pair II: forward 5'-CGGAGA(CT)GGAGCCTGATAAACGG-3' and reverse 5'-CCGTCAATTCCTTTAAGTTTCAG-3' for the second section (~ 800 bp) of the 18S rDNA gene. Both sections were amplified without internal primers. PCR amplifications were conducted using 20 µl reaction mix with thermal cycling conditions of 94°C for 3 min, followed by 35 cycles of 1 min at 94°C, 1 min at 50°C, 1 min at 72°C, and a final extension for 5 min at 72°C. The double-stranded PCR products were ligated into the pCR2.1-/TOPO-Vector with the TOPO TA Cloning Kit (Invitrogen) under the conditions recommended by the manufacturer, and finally transformed in *E. coli* cells. Plasmid DNA sequencing was performed using "Thermo sequenase fluorescent labelled primer cycle sequencing Kit with 7-deaza-dGTP" (Amersham Pharmacia) and a LI-COR 4200.

Phylogenetic Analyses

To avoid contamination, the BLAST programme (http://www.ncbi.nlm.gov/BLAST) was employed to verify the identity of sequences. Subsequently, both sequence-sections were combined using BioEdit 5.0.9 (Hall, 1999) and submitted to GenBank (for Accession No. see Table 1).

The alignment was performed with Clustal X (Thompson et al., 1997), followed by manual editing by eye. The alignment of 10 taxa with 1225 bp is available from the authors on request. The phylogenetic tree reconstruction was conducted using PAUP*, version 4.0b8 (Swofford, 2001). Maximum-parsimony analysis were performed using the exhaustive search option, ACCTRAN, and gaps were treated as missing data. 181 characters were parsimony-informative. Maximum likelihood analysis was conducted using the HKY85 model, the branch-and-bound search option and addition sequence "as-is". The GenBank sequence of *Acanthochitona crinita* (Polyplacophora: AF120503) was used as outgroup. Bootstrap analyses (100 replicates) were performed (heuristic search option).

For analysis of 17 multistate coded morphological characters the following settings were used: Maximum parsimony, exhaustive search option, ACCTRAN, all characters treated as unordered and weighted equally, non coded as irreversible. The outgroup characters of "*Chiton*" are generalized characters of polyplacophora after Sasaki (1998).

Table 2. The character states for the taxa included in the morphological analysis. See Material and methods for character coding. Abbreviations: *Cc* = *Clypeosectus curvus*, *Cn* = *Cyathermia naticoides*, *Dg* = *Depressigyra globulus*, *Ev* = *Eulepetopsis vitrea*, *Lf* = *Lepetodrilus fucensis*, *Ot* = *Olgasolaris tollmanni*, *Pm* = *Pyropelta musaica*, *Rc* = *Rhynchopelta concentrica*, *Sh* = *Symmetromphalus hageni*, *Chi* = *Chiton*.

	Character state	*Cc*	*Cn*	*Dg*	*Ev*	*Lf*	*Ot*	*Pm*	*Rc*	*Sh*	*Chi*
1	Shell shape	2	1	0	3	2	3	3	3	3	?
2	Number of teleconch whorls	0	0	0	1	1	1	1	1	1	1
3	Position of apex	2	3	3	0	3	1	1	2	1	?
4	Periostracum	2	?	?	0	2	1	3	2	1	?
5	Shell septum	1	1	1	1	1	0	1	0	1	1
6	Protoconch diameter	?	1	?	1	0	1	?	1	0	?
7	Operculum	4	1	0	5	5	2	5	3	0	5
8	Snout	0	3	2	2	2	0	2	1	3	2
9	Cephalic tentacles	0	1	0	0	2	0	2	0	1	3
10	Jaw	1	0	0	1	2	0	2	2	2	0
11	Ctenidia size	0	0	1	0	0	0	0	0	1	0
12	Heart configuration	0	1	1	1	0	0	1	1	0	1
13	Renal organ	2	?	1	0	0	1	2	1	1	0
14	Shell muscle characters	0	?	?	0	1	2	3	1	1	4
15	Epipodial tentacles	0	0	0	1	0	0	0	1	0	1
16	Rectum	0	1	1	1	?	1	0	1	1	1
17	Type of radula	1	1	1	0	1	1	1	1	1	0

Results

Resulting morphological data matrix (Table 2) was analysed as detailed in the Methods. Fig. 1 shows the 50% majority-rule consensus tree of the six most parsimonious trees based on the 17 parsimony-informative morphological characters. In this morphological tree, the positions of three groups are remarkable. 1) The tree assigns a relatively plesiomorphic position to *Rhynchopelta concentrica* placing the species outside the Neomphalina-group (*Cyathermia naticoides*, *Depressigyra globulus*, *Symmetromphalus hageni*). Hence, peltospirids are presented as paraphyletic, separating *Rhynchopelta concentrica* from the other peltospirid *Depressigyra globulus.* We suggest that peltospirids should be divided in two groups: one with limpet-like shells and one with coiled shells. 2) The neritomorph *Olgasolaris tollmanni* is grouped with *Symmetromphalus hageni*. This placement is based upon the anatomical synapomorphy of the diotocard heart configuration. 3) The morphological tree illustrates the close relationship between the cocculiformian species *Pyropelta musaica* and the vetigastropod clade of *Clypeosectus curvus* and *Lepetodrilus fucensis*. This group shares three synapomorphies: protoconch diameter < 180 µm, left renal organ very small, right normal size, rectum penetrating pericard.

The maximum likelihood analysis based on 18S rDNA-data produced a tree with ln L= -3864.99 (Fig. 2). Under the maximum parsimony criterion PAUP* found 31 equally parsimonious trees of 456 steps. Their strict consensus tree is given also in Fig. 2, because it is identical with the ML-analysis.

Both trees based on molecular data show a surprising topology. They illustrate discrepancies between the morphological tree mentioned above and currently accepted trees. The ML-tree and the MP-consensus tree illustrate a plesiomorphic and sister-group position of the coiled species *Cyathermia naticoides* and *Depressigyra globulus* and a near relationship of them to *Pyropelta musaica*. In contrast to morphological observations the patellogastopod species, *Eulepetopsis vitrea* shows not a plesiomorphic position, but groups with peltospirid and lepetodrilid species. Also notable is the polytomie of *Clypeosectus curvus*, *Rhynchopelta concentrica*, *Symmetromphalus hageni*, *Lepetodrilus fucensis* and *Olgasolaris tollmanni*, which combine neomphalid, vetigastopod and neritimorph species.

Discussion

After the addition of hydrothermal vent species, gastropod phylogeny is in disarray. In this study we sought to clarify the phylogeny of a preliminary taxa set of "Archaeogastropoda". In agreement with recent theories (Koufopanou et al., 1999, Harasewych & McArthur, 2000), our morphologically based results suggests the patellogastropod species *Eulepetopsis vitrea* as the earliest gastropod offshoot. It shares the plesiomorphic stereogloss radula with the outgroup. In contrast to McArthur and Koop's (1999) results, this study indicates that the Peltospiridae are paraphyletic. The limpet-like peltospirid *Rhynchopelta concentrica* is separated from the other peltospirid *Depressigyra globulus*, due to its rudimentary operculum and the shell septum, both of which are considered plesiomorphic characters.

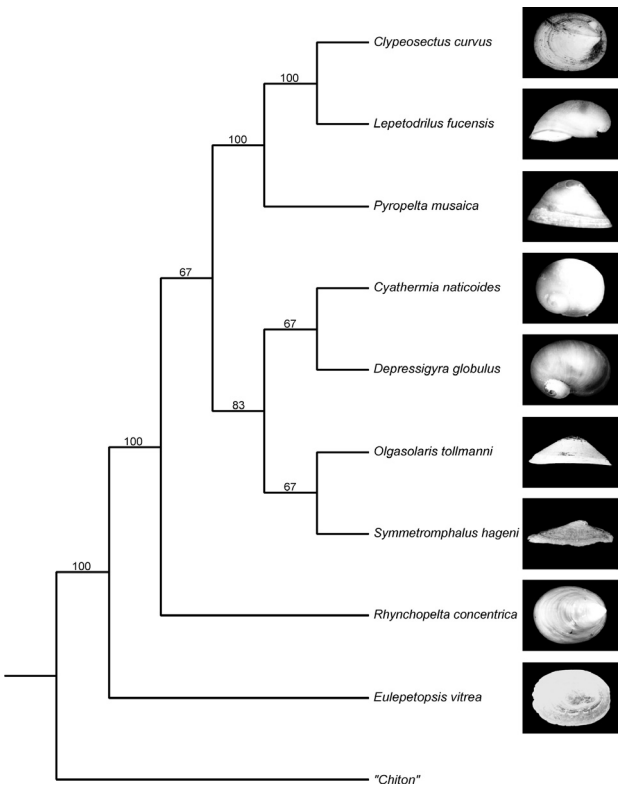

Figure 1. 50% majority-rule consensus tree (consensus % values above branches) of 6 most parsimonious trees based on morphological data produced with PAUP* 4.0b8: MP, exhaustive search, 10 taxa, 17 parsimony-informative characters, ACCTRAN, all characters weighted equal and unordered, non coded as irreversible, tree length 51, CI=0.71, RI=0.48, RC=0.34, outgroup characters of "*Chiton*" are generalized characters of Polyplacophora after Sasaki (1998).

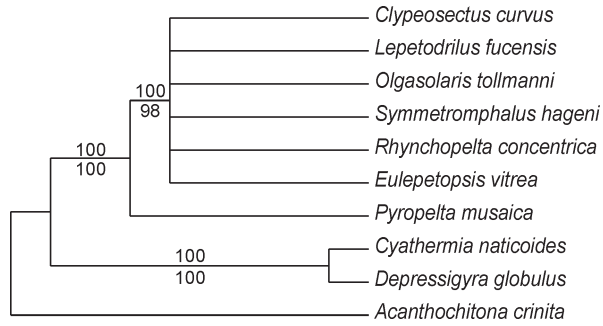

Figure 2. Strict consensus tree of 31 MP-trees, based on 1225 bp of the 18S rDNA gene of 10 taxa, produced with PAUP* 4.0b8: exhaustive search, gaps are treated as "missing", ACCTRAN, 181 parsimony-informative characters, 456 steps, CI=0.93, RI=0.91, RC=0.848; *Acanthochitona crinita* (Polyplacophora: GenBank accession No. AF120503) used as outgroup; MP-bootstrap values (100 replicates) above branches. ML-branch-and-bound search (HKY85 model) produced the same tree, -ln L=3864.99, ML-bootstrap values (100 replicates) under branches.

The position of *Pyropelta musaica* supports the results of Sasaki (1998) who found that the Cocculiniformia are more closely related to Vetigastropoda than to Neritimorpha. This is in strong opposition to the findings of Haszprunar (1988) and Ponder & Lindberg (1997).

Generally the combined analysis of species with doco- and rhipidogloss radulae causes problems. As doco- and rhipidogloss radulae teeth cannot be homologized, only the type of radula and not specific radula features are included. Lack of detailed radula features leads to the position of *Olgasolaris tollmanni* in the neomphalid clade in this presentation. The fact that *Cyathermia naticoides* and *Depressigyra globulus* were clustered in all three analyses suggests two possibilities of reclassification: a) *D. globulus* within the Neomphalidae, b) *C. naticoides* within a newly erected group of "coiled-peltospiridae". Fig.1 favours possibility b, because the limpet-like peltospirid *Rhynchopelta concentrica* shows a plesiomorphic position within the Rhipidoglossa.

The discrepancies between the morphological and the molecular trees, and the differences between the latter and recently discussed topologies, lead to consideration of possible problems created by sequencing DNA of formalin-fixed specimens. Shedlock et al. (1997) showed that formalin causes cross-links between DNA and surrounding proteins but these authors also showed that this does not influence the sequence. Sequences from formalin- and ethanol-fixed species are identical. The surprising position of the patellogastropod *Eulepetopsis vitrea* among the Rhipidoglossa in molecular data based trees cannot been explained by problems related to fixation. McArthur & Koop (1999) studied the 28S rDNA sequence of *Eulepetopsis vitrea* and some rhipidoglossate species and obtained the same results, i.e. a patellogastropod among the Rhipidoglossa. This might lead to the conclusion that the Patellogastropods cannot be considered as an early offshoot of the Rhipidoglossa, but gene trees may not correspond exactly with patterns of species relationships, particularly at low taxonomic levels (Brower et al., 1996).

Hence, we favour the morphological tree. To solve the problem of the impossibility of homologizing of doco- and rhipidogloss radula features, a larger set of only rhipidoglossate taxa is in preparation. To improve the construction of trees based on molecular data, an analysis of a larger taxa set is also underway.

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