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Molecular Evidence for Inclusion of the Phylum Pentastomida in the Crustacea[1]

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The phylogenetic status of the phylum Pentastomida (tongue worms) was considered on the basis of comparison of nucleotide sequences of 18S ribosomal RNA from the pentastome *Porocephalus crotali,* the branchiuran crustacean fish louse *Argulus nobilis,* other crustaceans, and representatives of the Annelida, Chelicerata, Myriapoda, and Insecta. Maximum parsimony and invariants (at $P < 0.04$) analyses support an *Argulus/Porocephalus* clade, providing strong support for the proposal that tongue worms are highly modified crustaceans closely related to fish lice.

Introduction

Despite more than a century of investigation and speculation, the phylogenetic relationships of many invertebrate phyla remain unknown. This is a particular problem for parasitic groups, which often lack morphological features that suggest relationships. For these groups, molecular techniques, such as nucleotide sequencing, offer great promise. Here we use 18S ribosomal RNA nucleotide sequences to examine relationships of the phylum Pentastomida, commonly known as tongue worms.

Pentastomids are vermiform, obligate parasites that, except for a mouth flanked by two paired hooks (fig. 1), are largely devoid of morphological characters. All species attain sexual maturity in the respiratory tract of vertebrates; ~70% of the definitive hosts are snakes, followed by crocodiles, lizards, amphibians, and turtles. Two species occur in marine birds, and one occurs in the nasopharynx of canines. Intermediate hosts, when known, include fish, amphibians, lizards, snakes, insects, and mammals, although a few species may have a direct life cycle (Riley 1983). Various authors have allied pentastomes with tardigrades, mites, onychophorans, annelids, and myriapods (see Haugerud 1989) or have treated them, as do most zoology texts (Hickman 1973; Hickman et al. 1979; Engemann and Hegner 1981), as an independent phylum with arthropod relationships (Self 1969). Parasitology texts treat the group as an independent phylum (Noble and Noble 1982; Cheng 1986), a class of the Mandibulata (Beaver et al. 1984), or an order of the Arachnida (Brown and Neva 1983). Wingstrand (1972), in an elegant study of the development and structure of the spermatozoa of pentastomes and branchiuran crustaceans, concluded that these two groups share so many detailed features of the spermatozoa that they must be considered closely related, a conclusion supported by Riley et al. (1978) on the basis of their interpretation of previously published data.

1. Key words: pentastome, crustacean, ribosomal RNA, tongue worms.

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Although noted (Pearse et al. 1986), the notion that pentastomes might be crustaceans has not been integrated into recent carcinological (Schram 1986) or invertebrate textbooks (Barnes 1986). There are probably several reasons for this omission. Although crustaceans are extremely diverse morphologically and ecologically, virtually all of them, at some (usually the larval) stage in their life cycle resemble other crustaceans. No such clear evidence is found in pentastomes, as the vast majority have no free-living stage. It also seems that, for most of those who investigate crustaceans, it is difficult to embrace a taxon whose life cycle has a canine as a definitive host, a sheep as an intermediate host, and no hint of their beloved nauplius larva or some derivative of it. Finally, the relationship has been difficult to accept because it has been based on only spermatozoa, and the possibility of convergence cannot be rejected.

Material and Methods

To test the various phylogenetic hypotheses, we obtained specimens of the pentastome *Porocephalus crotali* (Humboldt) from the lungs of a water moccasin (*Agkistrodon piscivorus*) and specimens of the branchiuran crustacean *Argulus nobilis* Thiele from the body surface of a garfish (*Lepisosteus osseus*) and obtained from each species 18S rRNA nucleotide sequences of ~1,600 bases by using the methods of Lane et al. (1985). The data are available from LGA and will be deposited in GenBank. These sequences were aligned [with FASTA of Pearson and Lipman (1988)] with sequences [provided by Dr. Rudolf A. Raff from the paper by Field et al. (1988)] from representative species from the Annelida (a polychaete, *Chaetopterus* sp.), Chelicerata (the horseshoe crab, *Limulus polyphemus*), Myriapoda (a millipede, *Spirobolus marginatus*), and Insecta (a fruit fly, *Drosophila melanogaster*). These taxa provide for at least a partial test of published phylogenetic hypotheses suggesting that pentastomes are allied with annelids, chelicerates, myriapods and insects, or branchiuran crustaceans. The aligned sequences were analyzed according to two methods: invariants/operator metrics (Lake 1987a, 1987b) and maximum parsimony using PAUP (Swofford 1985).

Results

Both methods yielded the same result (fig. 2); the *Argulus*-Pentastomida clade occurs in the single most parsimonious tree and is supported at the $P < 0.04$ level. On the basis of nucleotide sequences of 18S rRNA, we are currently reconsidering the classification of the Crustacea; but for the present we include the Pentastomida and Branchiura in the subclass Maxillopoda along with the Copepoda, Cirripedia, Mystacocarida, and Tantulocarida. Preliminary results from a maximum-parsimony analysis of the Maxillopoda focusing on 18S rRNA sequences from copepods (*Cyclops* sp.) and cirripeds (*Balanus eburneus*) also suggest an argulid-pentastome clade. An invariants/operator-metrics analysis is equivocal, offering some support for both an argulid-pentastome clade [$P(9;12,0.5) = 0.146$] and a copepod-pentastome clade [$P(6;8,0.5) = 0.289$]. However, a close examination of the sequences of argulids, pentastomes, and copepods reveals that, of 156 informative sites, 97 (48 transversions, pattern 113; 49 transitions, pattern 112) support an argulid-pentastome clade, whereas only 30 sites (16 transversions, pattern 133; 14 transitions, pattern 122) support a copepod-pentastome clade. The results thus provide strong support for Wingstrand's proposal that pentastomes are highly modified crustaceans most closely related to branchiurans.

FIG. 1.—Anterior, ventral surface of *Argulus nobilis* (*top*) and anterior portion of *Porocephalus crotali* (*bottom*). Both photomicrographs are by SEM.
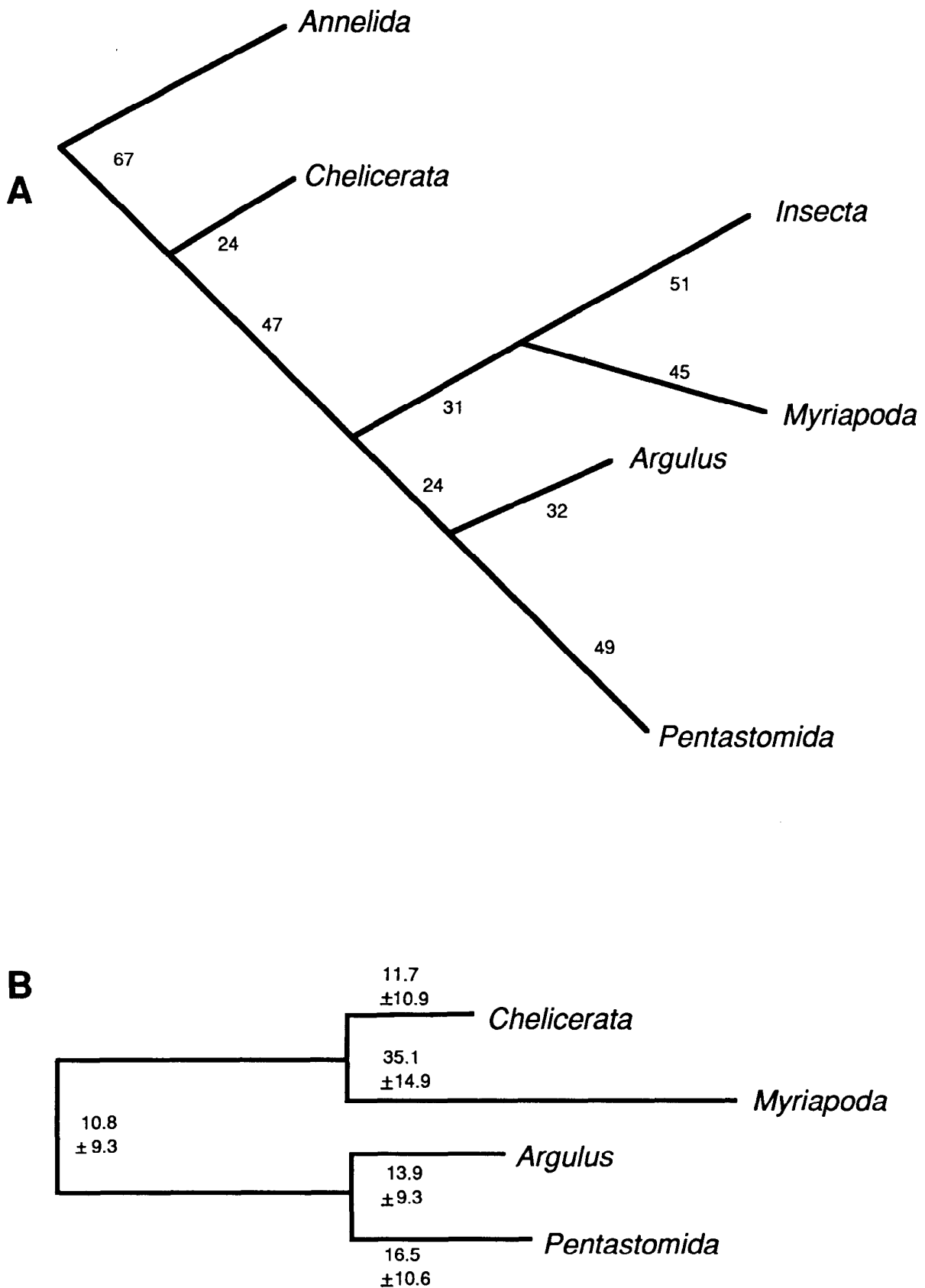
FIG. 2.—A, Relationships of the groups as suggested by the ALLTREES option of PAUP; branch lengths are indicated; total length = 370, CI = 0.681, on the basis of 164 informative sites. The next shortest tree is 374 steps and has *Argulus* and the Pentastomida coming off the main branch adjacent to each other. In the next five shortest trees (two at 375 steps and three at 376 steps), *Argulus* and the Pentastomida either come off the main branch adjacent to each other or share a common node. B, Relationships as suggested by the method of invariants/operator metrics. For this invariant ($X = E + u - H - J = 8 + 2 - 0 - 2$), the hypothesis that $E + u = H + J$ is a two-tailed binomial, where $P(E+u;E+u+H+J,0.5) = P(10;12,0.5)$ = 0.038 (see Holmquist et al. 1988). Branch lengths indicated are number of transversions/1,000 nucleotides and are to scale.

Discussion

There are many parasitic crustaceans whose adult morphology is as devoid of crustacean features as is that of any pentastome. These groups, as noted previously, have a life stage, usually the nauplius or some derivative of it, that clearly indicates their crustacean affinities. Not all crustaceans, however, reveal affinities so clearly, and viewing the branchiurans as the pentastomes' closest relatives closes the putative gap between pentastomes and crustaceans considerably. The newly hatched larva of the branchiuran *Chonopeltis brevis* is morphologically unique within the Crustacea (Fryer 1961). This species is entirely parasitic; the larva uses one species of fish as an intermediate host, and adults parasitize a different fish species as a definitive host. It is not difficult to see a similarity between the life cycle and larval morphology of *Chonopeltis* and those of a pentastomid, especially one utilizing fish as an intermediate host and a fish-eating crocodile as a definitive host. In addition, the branchiuran genus *Dolops* has a mouth flanked by hooks not dissimilar in appearance to pentastomid hooks. However, the chitin of pentastome cuticle is of the β type, whereas most arthropods have chitin of the α type (Karuppaswamy 1976). Unfortunately, nothing is known about the chitin composition of the hooks of either group. Inclusion of the pentastomes in the subclass Maxillopoda will extend only the ecological, not the morphological, range of the Maxillopoda, as this group already is partially characterized by morphological simplification and by many independent excursions into parasitism, exemplified by many groups of copepods, all branchiurans, the Tantulocarida, and several groups of barnacles. Pentastomes are, however, the only crustaceans to parasitize terrestrial vertebrates. (A species of branchiuran, *D. ranarum,* has been found on tadpoles, but the more typical association of this species is with fish; Avenant et al. 1989).

It is possible to estimate indirectly the times of origin and divergence of branchiurans and pentastomes, which lack a fossil record. A lower limit for the origin of the branchiurans would be the Devonian [400 Myr ago (Mya)], during the diversification of their fish hosts (Romer 1966). The distribution of the branchiuran genus *Dolops* (South America, Africa, and Tasmania) suggests that the branchiurans had diversified by the early Cretaceous (135 Mya), when these freshwater species could have attained their present distribution (Fryer 1969). There are two orders of pentastomes, the Cephalobaenida and the Porocephalida. The former is considered more primitive and contains two species known to parasitize the amphibian genus *Bufo* as the definitive host (Ali et al. 1982), but very little is known about the phylogeny of pentastomes. Within the porocephalids are genera that exclusively parasitize crocodiles and that use fish as an intermediate host (Riley 1983). These groups were probably extant before the Triassic (225 Mya), when their crocodilian hosts first appeared. The wide geographic distribution of pentastome genera (e.g., *Raillietiella;* Ali et al. 1985) suggests that these groups were in existence during the late Triassic ($\sim$180 Mya). If pentastomes first parasitized amphibians, then the origin might be as early as the Carboniferous ($\sim$350 Mya), when amphibian hosts would have been available. Haugerud (1989) suggests that pentastomes gained access to the lungs of amphibians through their earlier association with the gill chambers of early fishes. The divergence of branchiurans and pentastomes probably occurred, then, sometime between 350 Mya and 225 Mya. *Argulus* and *Porocephalus* differ at 174 sites (93 transitions and 81 transversions) out of 1,603 nucleotides (excluding gaps). Thus, over a period of time very roughly estimated to be 287 $\pm$ 62 Myr, the 18S rRNA of these two groups has diverged $\sim$10.8%, or about 1.9%/50 Myr, a higher rate than the 1%/50 Myr

previously reported for eucaryote 18S rRNA (Ochman and Wilson 1987). Given the potential errors in making such estimates, the significance of this difference remains unknown.

Acknowledgments

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