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## SYSTEMATIC POSITION OF *PSEUDOCORYNOSOMA* AND *ANDRACANTHA* (ACANTHOCEPHALA, POLYMORPHIDAE) BASED ON NUCLEAR AND MITOCHONDRIAL GENE SEQUENCES

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**ABSTRACT:** Species of *Pseudocorynosoma* are North and South American acanthocephalans that use waterfowl as definitive hosts and amphipods as intermediate hosts, whereas species of *Andracantha* occur in fish-eating birds with a worldwide distribution. *Pseudocorynosoma* and *Andracantha* were originally described as *Corynosoma* (now restricted to endoparasites of marine mammals). Morphologically, *Andracantha* is distinct from other genera of Polymorphidae in possessing 2 fields of spines on the trunk, whereas *Corynosoma* and *Pseudocorynosoma* have a single field. A recent phylogenetic hypothesis based on morphological characters suggested that *Andracantha* is closely related to *Corynosoma*, whereas *Pseudocorynosoma* was of uncertain phylogenetic position within the Polymorphidae. To test the systematic affinities of these 3 genera, we sequenced 2 nuclear genes (SSU and LSU ribosomal DNA) and 1 mitochondrial gene (cytochrome *c* oxidase subunit 1; *cox 1*) of species representing *Corynosoma*, *Andracantha*, and *Pseudocorynosoma* and analyzed the data, including available sequences of other polymorphids. Maximum parsimony (MP), maximum likelihood (ML), and Bayesian analyses of the combined (SSU + LSU) sequences and the concatenated data of 3 genes (SSU + LSU + *cox 1*) placed *Andracantha* as the sister taxon to *Corynosoma* with robust support values. All analyses also showed that *Pseudocorynosoma* is an independent lineage that does not share a common ancestry with *Andracantha* and *Corynosoma*. These phylogenetic hypotheses suggest that birds were the ancestral hosts of polymorphids and that the association of *Corynosoma* with marine mammals represents a subsequent episode of colonization.

Adult polymorphid acanthocephalans are intestinal parasites of marine mammals, fish-eating birds, and waterfowl. Their life cycles typically include a crustacean (amphipod, copepod, or decapod) as intermediate host and may include fish, snakes, frogs, or toads as paratenic hosts (Schmidt, 1985; Hoberg, 1986; Pichelin et al., 1998; Nickol et al., 1999, 2002). The systematics of this family at the generic level have been unstable for decades. Schmidt (1973) considered 8 valid genera, including *Southwellina* Witenberg, 1932, which this author resurrected after a long period in which it had been considered as synonymous with *Arhythmorhynchus* Lühe, 1911. Later, Schmidt (1975) erected *Andracantha*, including 3 species classified previously as members of *Corynosoma* Lühe, 1904. More recently, Nickol et al. (1999) reintroduced *Proflicollis* Meyer, 1931, as a valid genus; it had previously been considered as a subgenus of *Polymorphus* Lühe, 1911. Finally, Aznar et al. (2006) erected *Pseudocorynosoma* Aznar, Pérez-Ponce de León, and Raga, 2006, to reallocate several species previously included in *Corynosoma*.

*Pseudocorynosoma* currently comprises 5 species that use waterfowl as definitive hosts and amphipods as intermediate hosts, with distributions ranging from North America to South America (Van Cleave, 1945; Podesta and Holmes, 1970; Farias and Canaris, 1986). The 5 species of *Pseudocorynosoma* were originally assigned to *Corynosoma* Lühe, 1904; however, Aznar et al. (2006) clearly demonstrated morphological and ecological differences among species of *Corynosoma* and *Pseudocorynosoma*. As currently defined, *Corynosoma* constitutes a monophyletic assemblage of species with a worldwide distribution that occur as adults in hosts from the marine environment (mainly pinnipeds); they use marine amphipods as intermediate

hosts and teleosts as paratenic hosts (Belopolskaja, 1958; Golvan, 1959; Zdzitowiecki, 1985; Aznar et al., 1999, 2006). Another genus proposed to have systematic affinities to *Corynosoma* is *Andracantha* Schmidt, 1975, which currently includes 7 species that occur in piscivorous birds, mainly shags and cormorants. Six of the 7 known species of *Andracantha* were formerly included in *Corynosoma* (Aznar et al., 2006; Monteiro, et al., 2006). The main diagnostic trait of *Andracantha* is the possession of 2 fields of spines on the trunk; both *Corynosoma* and *Pseudocorynosoma* have 1 field. Aznar et al. (2006) analyzed 15 morphological characters and ecological traits among *Andracantha*, *Corynosoma*, *Pseudocorynosoma*, and other polymorphids to infer the phylogenetic relationships among these taxa. Their analysis suggested a close (but weakly supported) relationship between *Andracantha* and *Corynosoma*. Similarly, taxa now recognized as a distinct genus (*Pseudocorynosoma*) were monophyletic, but with unreliable (56%) bootstrap support. Nevertheless, this result was used to support the establishment of *Pseudocorynosoma* as a separate genus, since these authors (Aznar et al., 2006) provided additional information on host-parasite relationships and a detailed comparison of foretrunk musculature.

The main objective of the present research was to obtain molecular sequence data to test relationships among these genera. This was accomplished by using data from 2 nuclear genes (SSU and LSU ribosomal DNA) and 1 mitochondrial gene (*cox 1*) for representative species of *Corynosoma*, *Andracantha*, and *Pseudocorynosoma* in the broader context of other polymorphid genera (including *Polymorphus*, *Proflicollis*, *Southwellina*, and *Hexaglandula* Petrochenko, 1950).

### MATERIALS AND METHODS

#### Specimens and DNA isolation

Acanthocephalans were collected from vertebrate and invertebrate hosts. Worms were washed 3 times in 0.9% (w/v) saline, preserved in absolute ethanol, and stored at 4 C. For taxonomic identification, some specimens of most species were stained with Meyer's paracarmine, cleared with methyl salicylate, and mounted on permanent slides using

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TABLE I. Specimen information and GenBank accession numbers for specimens studied in this work.

Species	Host	Locality	Vouchers (CNHE)	GenBank Access. Cox 1	GenBank Access. SSU	GenBank Access. LSU
<i>Andracantha gravida</i>	<i>Phalacrocorax auritus</i>	Yucatán, México	5997	EU267822*	EU267802*	EU267814*
<i>Corynosoma enhydri</i>	<i>Enhydra lutris</i>	Monterey Bay, California	3429	DQ089719	AF001837	AY829107
<i>Corynosoma magdalenii</i>	<i>Phoca hispida saimensis</i>	Lake Saimaa, Finland		EF467872	EU267803*	EU267815*
<i>Corynosoma strumosum</i>	<i>Phoca vitulina</i>	Monterey Bay, California		EF467870	EU267804*	EU267816*
<i>Hexaglandula corynosoma</i>	<i>Nyctanassa violacea</i>	Veracruz, México	5765	EF467869	EU267808*	EU267817*
<i>Polymorphus brevis</i>	<i>Nycticorax nycticorax</i>	Michoacán, México	5778	DQ089717	AF064812	AY829105
<i>Polymorphus minutus</i>	<i>Gammarus pulex</i>	Dijon, France		EF467865	EU267806*	EU267819*
<i>Profilicollis altmani</i>	<i>Enhydra lutris</i>	Monterey Bay, California	5777	DQ089720	AF001838	AY829108
<i>Profilicollis botulus</i> <sup>1</sup>	<i>Somateria mollissima</i>	Denmark	5768	EF467862	EU267805*	EU267818*
<i>Profilicollis botulus</i> <sup>2</sup>	<i>Anas platyrhynchos</i>	United States		DQ089721	AF064815	AY829109
<i>Pseudocorynosoma constrictum</i>	<i>Anas clypeata</i>	Estado de México, México	5720	EU267820*	EU267800*	EU267812*
<i>Pseudocorynosoma anatarium</i>	<i>Bucephala albeola</i>	Durango, México	5721	EU267821*	EU267801*	EU267813*
<i>Southwellina hispida</i> <sup>1</sup>	ND†	Baltic Sea, Finland		EF467866	EU267809*	EU267810*
<i>Southwellina hispida</i> <sup>2</sup>	<i>Tigrisoma mexicanum</i>	Veracruz, México	5769	EF467867	EU267807*	EU267811*
<i>Centrorhynchus</i> sp.	<i>Falco peregrinus</i>	California		DQ089716	AY830155	AY829104
<i>Gorgorhynchoides bullocki</i>	<i>Eugerres plumieri</i>	Quintana Roo, México		DQ089715	AY830154	AY829103
<i>Plagiorhynchus cylindraceus</i>	<i>Porcilio saber</i>	Dijon, France		DQ089724	AF001839	AY829102

\* Sequences marked with an asterisk were obtained in this study.

† ND, not determined.

Canada balsam. The acanthocephalans were identified by conventional morphological criteria following the keys of Yamaguti (1963), Petrochenko (1958), and Schmidt (1973). In addition, original and revised descriptions of the species (e.g., Van Cleave, 1945; Schmidt, 1975) were consulted as needed. Avian definitive hosts were identified using field guides by Howell and Webb (1995) and the American Ornithologists' Union (1998). Voucher specimens were deposited at the Colección Nacional de Helminths, Instituto de Biología, UNAM, México City, México (accession numbers shown in Table I).

#### Amplification and sequencing of DNA

A single specimen of each species was digested overnight at 56 C in a solution containing 10 mM Tris-HCl (pH 7.6), 20 mM NaCl, 100 mM Na<sub>2</sub>EDTA (pH 8.0), 1% Sarkosyl, and 0.1 mg/ml proteinase K. Following the digestion, DNA was extracted from the supernatant using the DNaZol reagent (Molecular Research Center, Cincinnati, Ohio) according to the manufacturer's instructions.

Two regions of nuclear ribosomal DNA (rDNA) were amplified, using the polymerase chain reaction (PCR). The near-complete SSU rDNA (~1,800 bp) was amplified in 1 fragment, using the forward primer 5'-AGATTAAGCCATGCGT and reverse primer 5'-GCA GGTTCACCTACGGAAA (Garey et al., 1996). The near-complete LSU rDNA (~2,900 bp) was amplified using 2 overlapping PCR fragments of 1,400–1,500 bp. Primers for LSU amplicon 1 were forward 5'-CAAGTACCGTGAGGGAAAGTTGC and reverse 5'-CTTCTCCA AC(T/G)TCAGTCTTCAA; amplicon 2, forward 5'-CTAAGGAGTGT GTAACAACCTACC and reverse 5'-CTTCGCAATGATAGGAAGAG CC (García-Varela and Nadler, 2005). A partial (661 bp) sequence of mitochondrial cytochrome cox 1 was amplified using the forward primer 5'-AGTTCTAATCATAA(R)GATAT(Y)GG and reverse 5'-TAAAC TTCAGGGTGACCAAAAATCA (Folmer et al., 1994).

PCR reactions (25 µl) consisted of 10 µM of each primer, 2.5 µl of 10× buffer, 2 mM of MgCl<sub>2</sub>, and 1 U of Taq DNA polymerase (Platinum Taq, Invitrogen Corporation, São Paulo, Brazil). PCR cycling parameters for rDNA amplifications included denaturation at 94 C for 3 min, followed by 35 cycles of 94 C for 1 min, annealing at 50–58 C (optimized for each rDNA amplification) for 1 min, and extension at 72 C for 1 min, followed by a postamplification incubation at 72 C for 7 min. PCR cycling conditions for the cox 1 amplifications included denaturation at 94 C for 5 min, followed by 35 cycles of 94 C for 1

min, annealing at 40 C for 1 min, and extension at 72 C for 1 min, followed by a postamplification incubation at 72 C for 5 min.

Each PCR product was purified using Millipore columns (Amicon, Billerica, Massachusetts). Purified products were cloned by ligation into pGEM-T vector (Promega, Madison, Wisconsin) and used to transform competent *Escherichia coli* (JM109). Positive clones were identified by blue/white selection, and clone (insert) size was confirmed by PCR of DNA extracts prepared from bacterial (clone) colonies. Liquid cultures for minipreps were grown in Luria broth containing 50 µg/ml of ampicillin. Plasmids for DNA sequencing were prepared using commercial miniprep kits (Qiaprep, Qiagen, Valencia, California). Plasmids were sequenced for both DNA strands using universal (vector) and internal primers. Sequencing reactions were performed using ABI Big Dye (Applied Biosystems, Boston, Massachusetts) terminator sequencing chemistry, and reaction products were separated and detected using an ABI 3730 capillary DNA sequencer. Contigs were assembled and base-calling differences were resolved using Codoncode Aligner version 1.4.5 (Codoncode Corporation, Dedham, Massachusetts). All sequences have been deposited in the GenBank database (accession numbers shown in Table I).

#### Alignments and phylogenetic analyses

The SSU and LSU data sets were aligned separately using ProAlign version 0.5 (Loytynoja and Milinkovitch, 2003). For each alignment, a ProAlign guide tree was constructed using corrected (for multiple hits) pair-wise distances; this guide tree was used to estimate the hidden Markov model parameters ( $\delta$  and  $\epsilon$ ) for progressive multiple alignment. Program (Java) memory and band-width were increased as required to complete the alignment. The minimum posterior probability of sites was used as the criterion for detecting and removing unreliably aligned sequences. To reduce the likelihood of excluding correctly aligned sites, the filter threshold was set to 60% minimum posterior probability. For the SSU sequences, using ProAlign to detect and remove unreliably aligned sequences by their posterior probabilities excluded 211 of 1,746 sites. For the LSU dataset, 666 of 2,981 sites were excluded based on posterior probability filtering. Thus, following removal of unreliably aligned sites, these combined rDNA datasets included 3,850 characters. Sequences from the mitochondrial protein coding gene cox 1 were 655 bp in all taxa. These nucleotide sequences were readily aligned based on their inferred (translated) cox 1 protein sequences. The concatenated

3-gene dataset included 4,505 characters (filtered rDNA alignments plus *cox 1* sequences).

### Phylogenetic analyses

The SSU and LSU rDNA filtered alignments were combined and analyzed as 1 rDNA dataset because these genes represent a single nuclear locus. A concatenated dataset including all 3 genes (SSU, LSU, and *cox 1*) was analyzed separately. MP and ML trees were inferred using PAUP\* 4.0b10 (Swofford, 2002). For ML analyses, the Akaike Information Criterion (AIC) was used to assess the fit of GTR (General Time Reversible) nucleotide substitution models for these data (Rodríguez et al., 1990) as implemented using Modeltest version 3.0 (Posada and Crandall, 1998). The best-fit substitution model for SSU and LSU (both as evaluated separately and as a combined dataset) was GTR + I + G, the best-fit model for *cox 1* was K81uf + I + G, and for the concatenated 3-gene dataset, the best-fit model was GTR + I + G. For phylogenetic analysis, this GTR model with invariable sites (+ I) and rate heterogeneity (+ G) (Yang, 1994) was used for rDNA and concatenated datasets, but with different estimated parameters for each as determined by Modeltest. Tree searches were performed using 100 (ML) and 1,000 (MP) random taxon addition heuristic searches with tree-bisection-reconnection branch swapping. Clade support was assessed by bootstrap resampling with 10,000 (MP) or 1,000 (ML) bootstrap pseudoreplicates. MrBayes v3.0b4 (Huelsenbeck and Ronquist, 2001) was used to analyze the concatenated data set, sampling every 5,000 trees over 5,000,000 generations (burn-in determined empirically). For this analysis, a character partition corresponding to the 3 loci (nuclear SSU, LSU, and mitochondrial *cox 1*) was invoked, and the parameters of the likelihood model were set to the GTR (SSU, LSU partitions) and K81 (*cox 1* partition) substitution models with the *invgamma* option (a proportion of sites are invariable, with the remaining sites modeled by a gamma distribution of rate variability). The priors for the proportion of invariable sites (*pinvarpr*) were fixed separately for each partition, with the values estimated by Modeltest (*pinvarpr* = 0.4156 for SSU, *pinvarpr* = 0.3945 for LSU, and *pinvarpr* = 0.3443 for *cox 1*).

To compare trees representing specific alternative phylogenetic hypotheses, topological constraints were defined on trees obtained from MP and ML analyses of the concatenated (SSU + LSU + *cox 1*) dataset. Differences between unconstrained (best) and constrained trees representing alternative hypotheses were evaluated using the Shimodaira and Hasegawa likelihood test (Shimodaira and Hasegawa, 1999) and Templeton's modified parsimony test (Templeton, 1983) as executed in PAUP\*. Trees were drawn using RETREE and DRAWGRAM from PHYLIP (Felsenstein, 1999). The observed (uncorrected) genetic differentiation between taxa was represented using the uncorrected (p-distance) method on the filtered concatenated dataset. Alignments and tree files have been deposited in TreeBASE (Sanderson et al., 1994).

## RESULTS

### SSU + LSU dataset

The combined (SSU + LSU) rDNA dataset included 17 taxa, with 3,850 sites and 495 parsimony informative characters. MP analysis of this dataset yielded 3 trees, with a C.I. = 0.73 and length of 1,877 steps. The MP consensus tree (Fig. 1) supported the monophyly of Polymorphidae. This tree was composed of 4 main clades. The first clade contained 2 species of *Profilicollis* + *Polymorphus minutus* Goeze, 1782 and was strongly supported (100%) by bootstrap resampling. The second clade included the only sampled representative of *Hexaglandula*, plus 2 isolates of *S. hispida* Van Cleave, 1925 and *Polymorphus brevis*, Van Cleave, 1916. However, only the sister-group relationship between the *S. hispida* isolates and the clade consisting of *P. brevis* plus *Southwellina* received reliable bootstrap support. The third clade was composed of 2 species representing *Pseudocorynosoma* and had a bootstrap value of 100%. The fourth clade was composed of species representing *Andracantha* and *Corynosoma* and had 100% bootstrap support; however,

a polytomy was recovered that included *Pseudocorynosoma* and *Andracantha* + *Corynosoma*, so this analysis did not resolve relationships among all 3 genera (Fig. 1). ML analysis of the combined nuclear rDNA yielded 1 tree (Fig. 2). This ML tree was fully resolved and yielded 4 clades with the same clade composition as the MP trees, except for the position of *Pseudocorynosoma*. Each of these 4 clades also received strong ML bootstrap support (Fig. 2). Relationships among these 4 clades differed between MP and ML methods; however, these among-clade differences were not reliably supported, as assessed by bootstrap resampling for ML or MP inference.

### Combined SSU + LSU + *cox 1* dataset

The concatenated dataset of 3 genes consisted of 4,505 sites, with 806 parsimony informative characters. MP analysis of these SSU + LSU + *cox 1* data yielded a single tree with a C.I. = 0.60 and length of 3,290 steps. This tree (Fig. 3) had the same general topology as the combined SSU + LSU MP analysis (Fig. 1), but had more resolved nodes and higher bootstrap values for clades (Fig. 3). The ML analysis of the concatenated dataset yielded a single tree (Fig. 4) showing the same topology as the ML tree for the combined SSU + LSU analysis (Fig. 2). Bootstrap support was higher for certain clades in the concatenated ML tree. Bayesian analysis of the concatenated dataset also depicted these same relationships among genera as ML trees for the combined and concatenated datasets. However, Bayesian posterior probabilities (BPP) of clades were much higher than bootstrap values, suggesting that relationships among all genera were well resolved in the sampled posterior probability distribution (Fig. 4).

## DISCUSSION

The MP, ML, and Bayesian analyses of the combined rDNA (SSU + LSU) and concatenated dataset of 3 genes showed that Polymorphidae is a monophyletic assemblage, and this result is strongly supported by bootstrap resampling and Bayesian posterior probabilities. These phylogenetic analyses included 3 of the 30 species of *Corynosoma* considered valid by Aznar et al. (2006), including the type species *Corynosoma strumosum* Rudolphi, 1802. *Corynosoma* was recovered as monophyletic with high bootstrap values and BPP. These results are congruent with those of previous studies based on morphological and molecular data, which support the monophyly of *Corynosoma* spp. (García-Varela et al., 2005; Aznar et al., 2006).

*Andracantha* is composed of 7 species parasitizing shags, cormorants, and other piscivorous birds (Monteiro et al., 2006). The only diagnostic morphological difference between *Andracantha* and *Corynosoma* is the possession of spines in 2 fields of the trunk in the former. Based on this morphological feature, Schmidt (1975) transferred 3 species of *Corynosoma* (*C. gravida* Alegret, 1941, *C. mergi* Lundström, 1941, and *C. phalacrocoracis* Yamaguti, 1939) to *Andracantha*. In the present study, we used sequences of the type species, *A. gravida*. The MP, ML, and Bayesian trees derived from the rDNA and concatenated datasets placed *Andracantha* as the sister taxon of *Corynosoma*, with strong support as assessed by bootstrap resampling and Bayesian posteriors. This topological result is not inconsistent with generic status for *Andracantha*, but neither does it provide topological evidence for recognition of *Andra-*

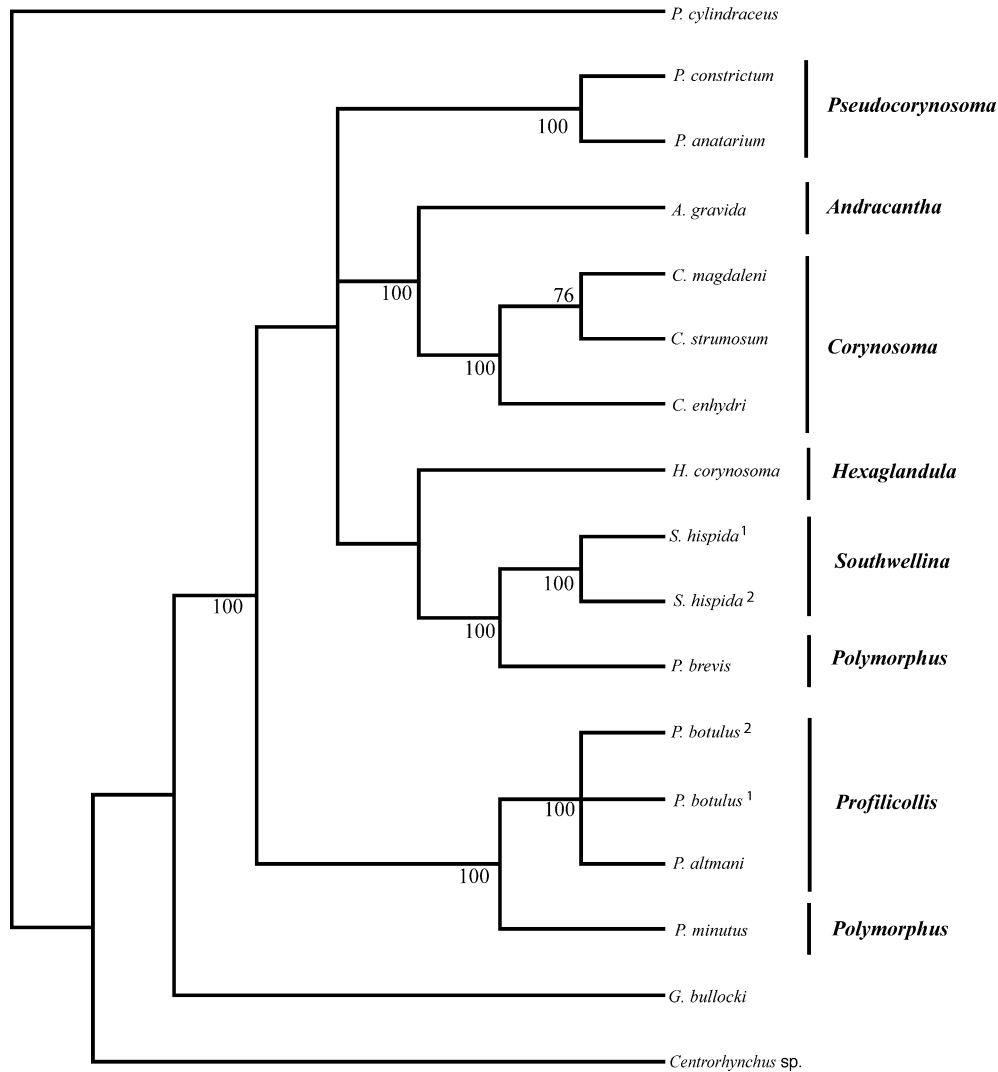


FIGURE 1. Strict consensus of 3 equally parsimonious trees (1,877 steps) inferred for the combined SSU + LSU rDNA dataset. Numbers below internal nodes show MP bootstrap clade frequencies.

*cantha* as separate from *Corynosoma*. However, the genetic divergence (estimated from the filtered concatenated dataset) between *Andracantha* and *Corynosoma* is 5%; this divergence value is similar to those representing inter-generic comparisons in Polymorphidae, i.e., *P. minutus*, + *Proflicollis* and *P. brevis* + *Southwellina* is 5%, lending support to the hypothesis of *Andracantha* as a distinct genus. The inclusion of more congeners in the molecular analysis is necessary to more fully test this notion. The phylogenetic pattern obtained in this study is also congruent with previous analyses based on morphological and ecological data, where *Andracantha* was closely related to *Corynosoma*. The known life cycles of both genera involve marine fish as paratenic hosts (see Aznar et al., 2006). Since both pinnipeds (definitive hosts of *Corynosoma*) and cormorants (definitive hosts of *Andracantha*) feed on fish, opportunities for host-switching of polymorphids between definitive hosts might have been frequent (Hoberg and Adams, 2000; Aznar et al., 2006).

The type species of *Pseudocorynosoma* was originally described as *Corynosoma constrictum*, Van Cleave 1918, for spec-

imens obtained from an American scoter or possibly of a surf scoter from Yellowstone Park, Wyoming (Van Cleave, 1945). Subsequently, 4 additional species from waterfowl were added to the genus (*P. peposacae* Porta, 1914; *P. anatarium* Van Cleave 1945; *P. enrietti* Molfi and Fernandes, 1953; and *P. inheringi* Machado Filho, 1961). Moreover, another 2 species, *C. longilemniscatus* Machado Filho, 1961, and *C. molfifernandesii* Machado Filho, 1962, were described and recently were considered as synonyms of *P. peposacae* and *P. enrietti*, respectively (Aznar et al., 2006). Based on a phylogenetic analysis of 15 morphological characters, comparative analysis of trunk musculature, and data derived from host-parasite associations of 7 species of *Corynosoma* from marine mammals and 4 species from waterfowl, Aznar et al. (2006) suggested that the species from freshwater waterfowl belonged to a distinct genus, *Pseudocorynosoma*. In the present study, we analyzed 2 species of parasites representing *Pseudocorynosoma*, including the type species *P. constrictum*. The genetic distance between *P. constrictum* and *P. anatarium* is 3%; this level of divergence is similar to that of other congeneric comparisons, for example,

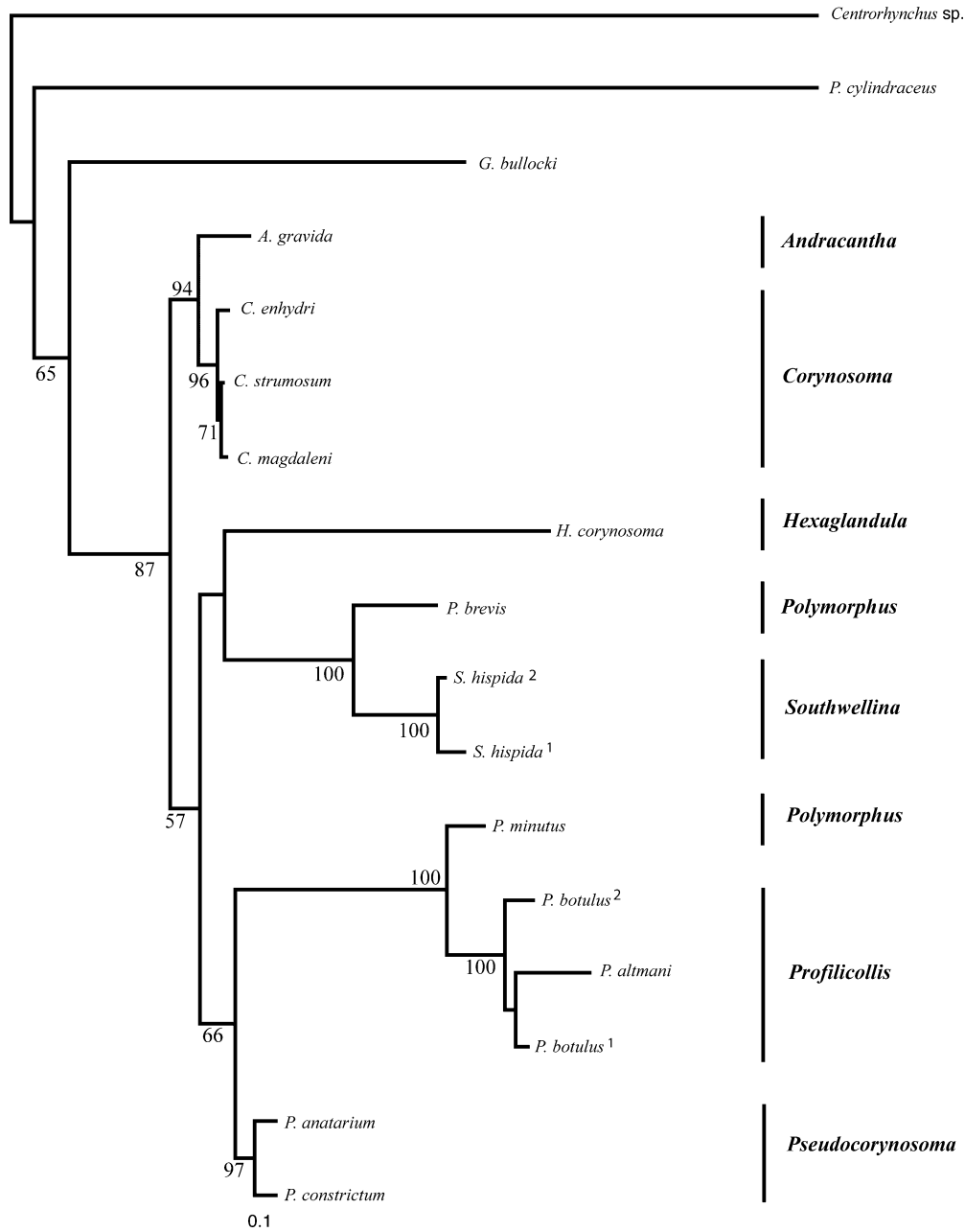


FIGURE 2. ML tree ( $-\ln$  likelihood 14,475.67) inferred from combined SSU + LSU rDNA dataset. Numbers near internal nodes show ML bootstrap clade frequencies.

among species of *Corynosoma* (0.7–2%) and species of *Profilicollis* (up to 4%). The 2 species of *Pseudocorynosoma* were recovered as a monophyletic group, consistent with its recognition as a separate genus. However, to test the taxonomic validity of *Pseudocorynosoma*, the alternative hypothesis of (*Andracantha* (*Corynosoma*, *Pseudocorynosoma*)) was evaluated with MP and ML analyses using the concatenated dataset of 3 genes. The constrained analyses of MP and ML showed a tree with a length of 3,331 steps and  $-\ln = 21,324.60$ , respectively, whereas original hypotheses of MP and ML yielded a tree with a length of 3,290 and  $-\ln = 21,129.49$ , respectively. Based on both the Shimodaira and Hasegawa (1999) likelihood test and

Templeton's (1983) parsimony test as executed in PAUP\*, the alternative hypotheses of *Pseudocorynosoma* as the sister group of *Corynosoma* is significantly worse than the best trees represented in Figures 3 and 4.

The phylogenetic position of *Pseudocorynosoma* within Polymorphidae varies depending on the inference method. The MP trees derived from the concatenated dataset yielded the topology: (*Pseudocorynosoma* ((*Andracantha*, *Corynosoma*), (*Hexaglandula* (*Southwellina*, *P. brevis*))))), whereas the ML and Bayesian analyses (rDNA and concatenated) differed, yielding (*Pseudocorynosoma* (*P. minutus*, *Profilicollis*)). Aznar et al. (2006) indicated that species of *Pseudocorynosoma* are nearly

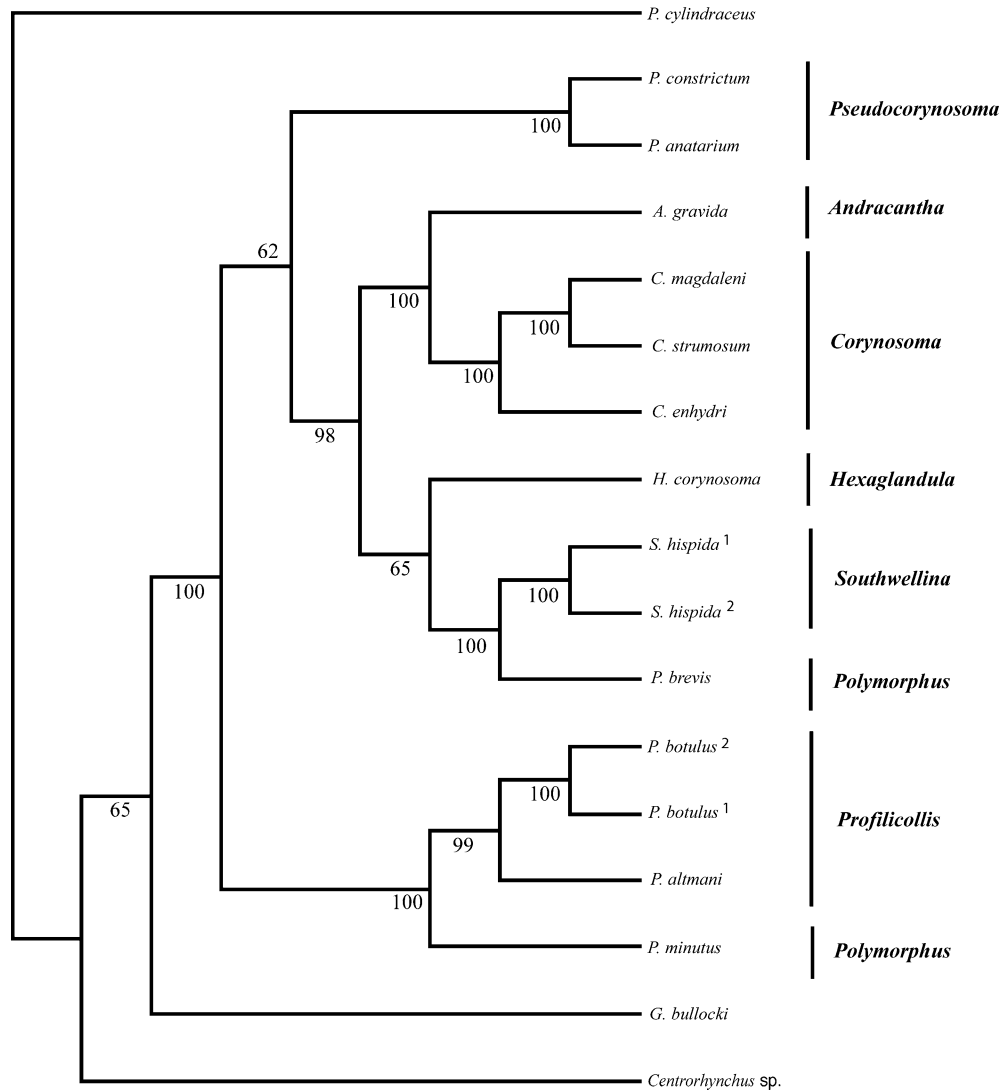


FIGURE 3. MP tree (3,290 steps) inferred from the concatenated SSU + LSU + *cox 1* dataset. Numbers below internal nodes show MP bootstrap clade frequencies.

identical to *Polymorphus* with respect to morphology and life cycle. Indeed, only the possession of genital spines as an isolated field, at least in males, and 6 cement glands in some species allows *Pseudocorynosoma* to be separated from *Polymorphus*. It is important to note that the other species of *Polymorphus* included in the analysis, *P. brevis*, and *P. minutus*, are paraphyletic in all analyses. An alternative topology test (Shimodaira-Hasegawa) showed that the monophyly of *Polymorphus* was a significantly worse interpretation (result not shown) of the concatenated (SSU + LSU + *cox 1*) data than the tree shown in Fig. 4. Therefore, the appropriate generic designation for *Polymorphus* taxa requires further consideration (see Amin, 1992).

The Polymorphidae is composed of an assemblage of genera with a long history of conflicting status. The taxonomic position among some representatives has been tested using either morphological (Aznar et al., 2006) or molecular (García-Varela and Pérez-Ponce de León, 2008) characters. However, the present study represents the first phylogenetic analysis that includes a

more complete representation of the family (7 of 10 genera), thereby providing a better phylogenetic framework to infer the evolution of host-parasite associations. Parsimony mapping of definitive host associations onto MP, ML, and Bayesian trees indicates that birds were the ancestral hosts of polymorphids, and the association of *Corynosoma* with marine mammals would represent a subsequent episode of colonization. Sampling of *Bolbosoma*, *Arythmorhynchus*, and *Diplospinifer* are fundamental to providing a comprehensive phylogenetic framework for this family and to provide additional tests of the hypotheses discussed herein.

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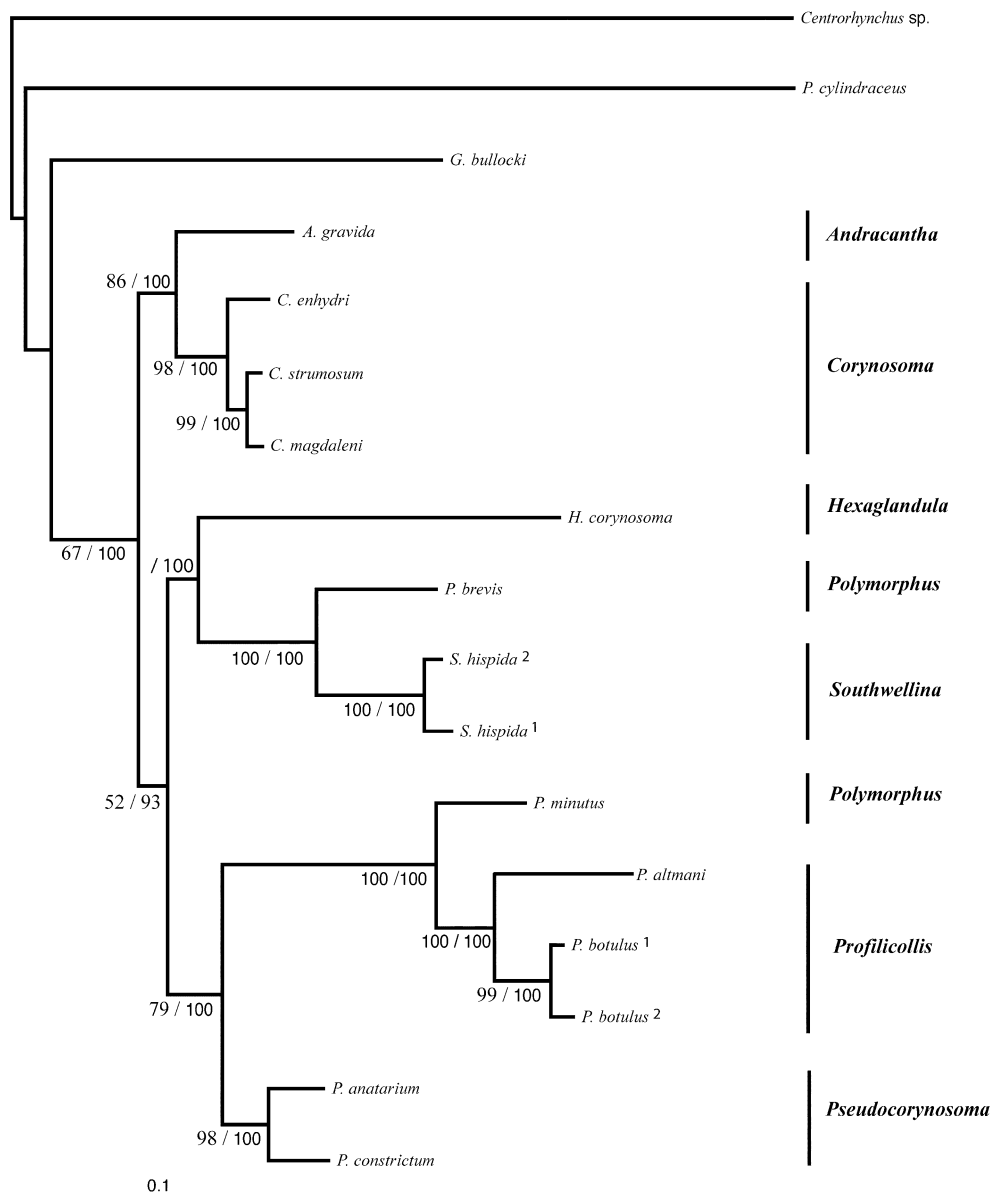


FIGURE 4. ML and Bayesian trees inferred from the concatenated SSU + LSU + *cox* 1 dataset. Branch lengths are scaled to the expected number of substitutions per site ( $-\ln$  of 21,129.49) and are based on the likelihood analysis. Numbers near internal nodes show ML bootstrap clade frequencies and Bayesian posterior probabilities, respectively (ML/Bayesian).

of Spain to MG. FJA benefits from a "Ramón y Cajal" contract from the MEC.

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