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Evidence for cospeciation events in the host–symbiont system involving crinoids (Echinodermata) and their obligate associates, the myzostomids (Myzostomida, Annelida)

Déborah Lanterbecq [a,*], Grey W. Rouse [b], Igor Eeckhaut [a]

[a] Marine Biology Laboratory, University of Mons, 6 Av. du Champ de Mars, Bât. Sciences de la vie, B-7000 Mons, Belgium
[b] Scripps Institution of Oceanography, University of California, San Diego, La Jolla, CA 92093-0202, USA

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ABSTRACT

Although molecular-based phylogenetic studies of hosts and their associates are increasingly common in the literature, no study to date has examined the hypothesis of coevolutionary process between hosts and commensals in the marine environment. The present work investigates the phylogenetic relationships among 16 species of obligate symbiont marine worms (Myzostomida) and their echinoderm hosts (Crinoidea) in order to estimate the phylogenetic congruence existing between the two lineages. The combination of a high species diversity in myzostomids, their host specificity, their wide variety of lifestyles and body shapes, and millions years of association, raises many questions about the underlying mechanisms triggering their diversification. The phylogenetic relationships, inferred using a three-genes dataset (18S rDNA, 16S rDNA, and COI) and two-genes dataset (18S rDNA, and COI) for the myzostomids and crinoids, respectively, were congruent with the literature. The overall congruence between the two phylogenies was statistically significant according to topology-based, distance-based, and data-based approaches: a significant pattern of cophylogeny was found, though not perfect probably resulting from occasional host switches, duplications or extinction events. A minimum of 8 cospeciation events was estimated, which is significantly higher than it would have been expected due to chance alone.

1. Introduction

One of the most captivating aspects of symbioses is that distantly related organisms can be so intimately associated across millions of years of evolution, despite worldwide drastic extinction events. Surviving partners develop (or alter) adaptive traits, which can change an opportunistic, facultative relationship into a species-specific obligate association for one or both partners, a process commonly and universally accepted as “coevolution”. However, coevolution is a general term that encompasses sequential evolution and strict coevolution (see Ridley, 1996). Strict coevolution occurs in host–parasite and host–mutualist systems and implies that two separate taxa mutually influence the evolution of the other, the two taxa tending to (i) change together (coadaptation), and (ii) speciate together (cospeciation). Sequential evolution is a particular case of coevolution where the changes (morphological, physiological or behavioural) and the phylogeny of the symbionts are influenced by the host evolution, but it is not reciprocal (Ridley, 1996). At the extreme, both strict coevolution and sequential evolution might lead to completely parallel phylogenies between host and symbiont lineages (e.g., Hafner and Page, 1995; Page and Holmes, 1998). By comparing the phylogenies of hosts and their associates, it is possible to detect if a statistically significant cophylogenetic signal is present and estimate the role played by the different historical events, such as host switching, sorting or duplication events (see Paterson and Gray, 1997).

At present, cophylogenetic analyses have mainly concentrated on host–parasite and host–mutualist systems to assess whether the biological dependence in these systems was strong enough to induce the speciation of the symbionts after the host speciation (Page and Charleston, 1998). For 20 years, cospeciation events have been studied in systems as diverse as vertebrates and insects (Hafner and Nadler, 1988; Paterson et al., 1993, 1999, 2000; Page et al., 1998, 2004; Johnson et al., 2002, 2003; Banks and Palma, 2003; Weckstein, 2004), plants and insects (Weiblen, 2001; Percy et al., 2004; Kawakita et al., 2004; Quek et al., 2004), nematodes and plants (Subbotin et al., 2004), nematodes and vertebrates (Hugot, 1999), platyhelminthes and vertebrates (Hoberg et al., 2000; Skerikova et al., 2001; Desdevises et al., 2002), and fungi and algae (Piercey-Normore and DePriest, 2001). To date, no deep

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* Corresponding author. Fax: +32 65373441.
E-mail addresses: deborah.lanterbecq@umh.ac.be (D. Lanterbecq), grouse@ucsd.edu (G.W. Rouse), Igor.eeckhaut@umh.ac.be (I. Eeckhaut).

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cophylogenetic analysis has been performed on marine commensal systems, probably because commensalism is often considered as a “light” symbiosis where host-specificity is often weaker than in mutualism or parasitism (Fabricius and Dale, 1993; Deheyn et al., 2006). The present work investigates the cophylogeny of a marine host–symbiont system, involving two invertebrate taxa in which the symbionts are mainly commensals: the system involving crinoids and their myzostomid-symbionts.

Myzostomida is one of the most enigmatic protostome taxa. They are only associated with echinoderms, mainly with crinoids (>90%) (see Grygier, 2000; Eeckhaut and Lanterbecq, 2005, for reviews). The symbiosis is obligate for myzostomids, and would seem to have existed for 300 million years (Warn, 1974; Brett, 1978; Meyer and Ausich, 1983; Radwanska and Radwanski, 2005). Host specificity is high, with many myzostomid species associated with a single crinoid species (Deheyn et al., 2006; Grygier 1990; Eeckhaut et al., 1998). Myzostomids have adopted various symbiotic lifestyles (Lanterbecq et al., 2006), but 90% of them are commensals, and their external structure is as much diversified as the number of their symbiotic lifestyles (Lanterbecq et al., 2009), suggesting that the hosts’ evolution has influenced myzostomid changes. Their anatomy is such that they cannot leave their host to move on the surrounding substrata (Lanterbecq et al., 2008). They do not survive long or even die soon after removal from their host (Eeckhaut and Jangoux, 1993) and the life time of their larvae is about 10 days (Eeckhaut and Jangoux, 1993), resulting in limited dispersal capabilities. Parasitism has emerged multiple times from the ectocommensal forms and these parasitic myzostomes infest the integument, gonads, coelom, or digestive system of various echinoderms (Grygier, 2000; Lanterbecq et al., 2006). The heterogeneity of body shapes observed in myzostomids has obscured their phylogenetic position within Metazoa (see Eeckhaut and Lanterbecq, 2005, for review), but they are currently considered as part of the annelid radiation (Bleidorn et al., 2007, 2009).

Recent molecular analyses have confirmed the paleontological evidences indicating that crinoids are the sister group to the remaining extant echinoderms (see Cohen et al., 2004; Littlewood et al., 1997; Janies, 2001). An exception is Perseke et al. (2008) who found Ophiuroidea as the sister group to crinoids, asteroids, echinoids and holothuroids, though they suspected this maybe due to long branch attraction. The current crinoid classification divides the living species into Bourgueticrinida, Millericrinida, Cyrtocrinida, Isocrinida, these four groups being the “sea lilies” (about 30 genera and 95 species; Roux et al., 2002), and the “feather stars” Comatulidia (with 140 genera, 500 species; Messing, 1997). Comatulidia and to a lesser extent Isocrinida are those best know to be infested by myzostomids. Paleontological data suggest that extant crinoids compose a monophyletic grouping (Subclass Articulata; see Rasmussen, 1978) descendent from a single group following the Permian mass extinction (Hess et al., 1999). Two works exist in literature that aims at estimating the molecular phylogeny of some crinoid taxa. The most recent study (Cohen et al., 2004) focuses on the relationships between high rank crinoid taxa, of which most of them are not infested by myzostomids. The second study (White et al., 2001) concentrated on the phylogeny of Comasteridae, a comatulid family heavily infested by various myzostomid species.

The present study is the first to explore from a phylogenetic perspective the presence of a coevolutionary pattern between crinoids and myzostomids. This study first investigates relationships among a set of crinoids infested by myzostomids, and then incorporates tree-based, distance-based, and data-based methods of cophylogenetic analysis to investigate the historical association between these hosts and symbionts.

2. Material and methods

2.1. Sampling

Sixteen crinoids (belonging to 6 different families) and their 16 associated myzostomids (belonging to 15 species) were collected from Hansa Bay (Bismarck Sea, Papua New Guinea), Lizard Island Reef (Coral Sea, Australia), New Caledonia, and from Antarctic and Japanese waters (Table 1). Crinoids were examined under a binocular microscope, and the myzostomids were isolated, then preserved in 100% ethanol at 4 °C for DNA extraction. Crinoid arms were preserved in 100% ethanol and used for DNA extraction. Before DNA extraction procedures, each crinoid and associate were identified with the aid of a dissecting microscope and taxonomic keys. Amongst the 16 myzostomid specimens (Table 1), 14 were unambiguously identified as belonging to 13 species, 2 are new to science and currently described. Vouchers of the 2 new myzostomid species were deposited at the South Australian Museum (SAM).

2.2. DNA extraction and sequencing

Genomic DNA was isolated from each crinoid using organic solvents (Sambrook et al., 1989) or the DNeasy Tissue kit (QIAGEN Inc., Valencia, California). DNA fragments from the 18S rDNA and the COI were amplified by PCR using Ready-To-Go PCR Beads (Pharmacia). Each PCR was performed in a volume of 25 μl containing 1.5 U of Taq DNA polymerase, 1× PCR buffer (10 mM Tris/HCl, pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.20 mM of each dNTP, 0.6 pmol/μl of each primer, and 1 μl (approximately 10–500 ng) of genomic DNA. PCR profiles were as follows: 5 min at 95 °C, 35–40 cycles of 30 s at 95 °C, 30 s at 40 °C (COI) or 55 °C (18S), 60–90 s at 72 °C. The 18S rDNA was amplified in 3 overlapping fragments of about 600 nucleotides each using primers from Eeckhaut et al. (2000). The primers LCO1490 and HCO2198 (Folmer et al., 1994) were used to amplify the first part of COI (~500 bp), which was compared with longer fragments (1104–1125 bp) provided by co-author Greg Rouse (see Table 2). Prior to sequencing, amplification products were purified either with the Qiaquick PCR kit (QIAGEN) or from 1% agarose gels (Quantum Prep Freeze ‘N Squeeze DNA Gel Extraction Spin Columns, Biorad). Both strands of each PCR product were directly sequenced using the BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems) and products were separated electrophoretically using an Applied Biosystems 3700 automated sequencer. Sequences were edited with SEQPUP (Gilbert, 1996). Partial DNA sequences from the nuclear small ribosomal unit (18S rDNA, 1101–1659 bp) and the mitochondrial cytochrome oxidase I (COI, 1104–1125 bp) were successfully obtained for 16 myzostomid-infested crinoid species. New sequences were deposited in Genbank under accession numbers GQ913312 to GQ913344 (Table 2). The present paper is part of a long-term project investigating crinoid phylogeny (Rouse et al., in preparation) and co-author Greg Rouse provided the longer COI fragments (see Table 2), resulting in concatenated sequences for some species (e.g., *Metacrinus* spp.; see Table 2). The 16 myzostomid specimens were sequenced in a previous study (Lanterbecq et al., 2006) and their sequences are available in Genbank (see Table 2).

2.3. DNA sequence alignments

Myzostomid alignments were obtained from Lanterbecq et al. (2006). Multiple alignments using a combination of SOAP (Löytynoja and Milinkovitch, 2001) and ProAlign (Löytynoja and Milinkovitch, 2003) were performed for the 18S rDNA and 16S rDNA sequences (sites with minimum posterior probability <90% were

Table 1
List of myzostomids examined in this study, along with their lifestyle, collection locality, and crinoid host species.

Symbiont			Host		
Myzostomid species	Myzostomid lifestyle	Collection locality	Crinoid species	Taxonomic status (family)	
Myzostoma mortenseni Jägersten, 1940	Ectocommensal on calyx	Hansa Bay (Bismarck Sea, Papua New Guinea)	*Clarkcomanthus albinotus* Rowe, Hogget, Birtles and Vail, 1986	Comasteridae	CC
Myzostoma cuniculus Eeckhaut, Grygier and Deheyn, 1998	Ectocommensal on pinnules	Lizard Island Reef (Coral Sea, Australia)	*Comanthus mirabilis* Rowe, Hogget, Birtles and Vail, 1986	Comasteridae	CC
Myzostoma ambiguum Graff, 1887	Ectocommensal on calyx	Hansa Bay (Bismarck Sea, Papua New Guinea)	*Oxycomanthus bennetti* (Müller, 1841)	Comasteridae	CC
Myzostoma capitocutis Eeckhaut, VandenSpiegel and Grygier, 1994	Ectocommensal on calyx	Hansa Bay (Bismarck Sea, Papua New Guinea)	*Phanogenia gracilis* (Hartlaub, 1890)	Comasteridae	CC
Myzostoma furcatum Graff, 1887	Ectocommensal on pinnules	Lizard Island Reef (Coral Sea, Australia)	*Himerometra robustipinna* (Carpenter, 1881)	Himerometridae	CC
Myzostoma coriaceum Graff, 1884	Ectocommensal on calyx	Lizard Island Reef (Coral Sea, Australia)	*Comanthina schlegelii* (Carpenter, 1881)	Comasteridae	CC
Notopharyngoides aruensis (Remscheid, 1918)	Endoparasitic in digestive tube	Lizard Island Reef (Coral Sea, Australia)	*Oxycomanthus comanthipinna* (Gislén, 1922)	Comasteridae	CC
Hypomyzostoma sp. aff. *crosslandi* a (Boulenger, 1913)	Ectocommensal on arms	Lizard Island Reef (Coral Sea, Australia)	*Liparometra articulata* (Müller, 1849)	Mariametridae	CC
Hypomyzostoma sp. aff. *crosslandi* b (Boulenger, 1913)	Ectocommensal on arms	Lizard Island Reef (Coral Sea, Australia)	*Stephanometra indica.*	Mariametridae	CC
Hypomyzostoma n. sp. 1 (SAM)	Ectocommensal on arms	Lizard Island Reef (Coral Sea, Australia)	*Colobometra perspinosa* (Carpenter, 1881)	Colobometridae	CC
Endomyzostoma clarki (McClendon, 1906)	Endoparasitic in galls	Japanese waters (Japan)	*Metacrinus* spp.	Isocrinidae	SC
Endomyzostoma tenuispinum (Graff, 1884)	Endoparasitic in galls	New Caledonia	*Saracrinus* spp.	Isocrinidae	SC
Endomyzostoma deformator (Graff, 1884)	Endoparasitic in galls	Japanese waters (Japan)	*Endoxocrinus* spp.	Isocrinidae	SC
Endomyzostoma cysticolum (Graff, 1883)	Endoparasitic in cysts	Antarctic waters	*Promachocrinus kerguelensis* (Carpenter, 1888)	Antedonidae	CC
Mesomyzostoma reichenspergi Remscheid, 1918	Endoparasitic in gonads	Lizard Island Reef (Coral Sea, Australia)	*Himerometra magnipinna* A.H. Clark, 1908	Himerometridae	CC
Mesomyzostoma n. sp. 1a (SAM)	Endoparasitic in gonads	Lizard Island Reef (Coral Sea, Australia)	*Clarkcomanthus littoralis* (Carpenter, 1888)	Comasteridae	CC

Vouchers of the new myzostomid species are deposited at the South Australian Museum (SAM). Lifestyle comments: ectocommensals move around on the external surface of crinoids; some stay on the crinoid calyx and divert food particles from the host's ambulacral grooves; other stay preferably on the pinnules or the arms. Endoparasites can be located in the anterior part of the digestive system, in the gonads, or in the integument (where they form galls or cysts). Cysticolous parasites live in a soft and uncalcified cyst located on the crinoid host's arm or mouth. Gallicolous parasites induce galls on arms by deformation of the original crinoid's ossicles. Host abbreviations: CC = comatulid crinoid, SC = stalked crinoid.

excluded). COI sequences were aligned according to the corresponding amino acid alignment (see Lanterbecq et al., 2006, for details) using ClustalX (Thompson et al., 1997). The myzostomid combined dataset included 16 ingroup (plus 1 outgroup; *Pulvinomyzostomum pulvinar)* and 2417 bp, of which 540 were variable sites and 414 parsimony-informative sites (580 and 425 when including the outgroup, respectively).

The crinoid dataset was obtained in aligning 18S rDNA sequences using ClustalX (Thompson et al., 1997) and the alignment was corrected by eye. Of the 1659 bp of 18S rDNA examined (including outgroup), 92 were potentially parsimony informative, whereas only 17 were informative when considering the ingroup only (and despite the inclusion/exclusion of the 18S sequence of *Saracrinus* spp. that has to be taken with caution). Crinoid COI sequences were aligned using ClustalX (Thompson et al., 1997) according to the corresponding amino acid alignment. Of the 1125 bp of COI examined (including outgroup), 367 were potentially parsimony informative. The crinoid combined dataset (18S + COI) included 16 crinoid sequences (Table 2) and 2784 bp, of which 435 were variable sites and 277 parsimony-informative sites (635 and 459 when including the seastar sequence, respectively). The crinoid 18S dataset is clearly less informative than the COI dataset and could certainly not be used alone to resolve phylogenetic relationships in a "whole crinoid phylogeny" context (see Rouse et al., in preparation), but was considered as useful in the present cophylogenetic framework (e.g., Parafit analyses).

2.4. Host and symbiont phylogenetic analyses

Base composition bias was evaluated and departure from average base composition was determined for each taxon using a chi-square (v2) test implemented in PAUP*4.0b4a (Swofford, 1998). To test for incongruence between genes in the two datasets, we conducted an incongruence length difference test (ILD; Farris et al., 1994) in PAUP*4.0b4a (Swofford, 1998) (partition homogeneity method with 100 replicates). Invariable characters were removed before starting the analysis (Cunningham, 1997). The two ILD tests showed that the gene fragments in both datasets were not significantly incongruent (ILD $p > 0.05$) and could consequently be combined in two multiple-genes datasets (see Lanterbecq et al., 2006 for details on the myzostomid dataset).

Rooting of the present myzostomid tree was based on Lanterbecq et al. (2006)'s analyses: the outgroup chosen was the myzostomid *Pulvinomyzostomum pulvinar* (Accession Nos.: DQ238114, DQ238150, and DQ238180). The seastar *Asterina gibbosa* was selected as outgroup to root the crinoid trees according to Cohen et al. (2004), and its 18S rDNA (AF088801) and COI (U50058) sequences were taken from GenBank.

Maximum parsimony (MP) and maximum likelihood (ML) analyses were performed on the two datasets. MP analyses were performed with PAUP*4.0b4a (Swofford, 1998) using a heuristic search (SeqAdd and TBR branch-swapping). Clade supports were estimated by bootstrapping (Felsenstein, 1985) (Simple SeqAdd

Table 2
GenBank accession numbers of the sequences used in the present study.

Species	18S rDNA	16S rDNA	COI
Symbiont			
Myzostoma mortenseni	DQ238143	–	DQ238207
Myzostoma cuniculus	DQ238138	DQ238174	DQ238203
Myzostoma ambiguum	DQ238142	–	DQ238206
Myzostoma capitocutis	DQ238144	DQ238177	DQ238209
Myzostoma furcatum	DQ238145	DQ238178	DQ238211
Myzostoma coriaceum	DQ238146	DQ238179	DQ238212
Notopharyngoides aruensis	AF260587	–	DQ238210
Hypomyzostoma sp. aff. *crosslandi* a	DQ238133	DQ238168	DQ238197
Hypomyzostoma sp. aff. *crosslandi* b	DQ238134	DQ238169	DQ238198
Hypomyzostoma n. sp. 1	DQ238132	DQ238167	DQ238196
Endomyzostoma clarki	DQ238124	DQ238159	DQ238188
Endomyzostoma tenuispinum	DQ238128	DQ238163	DQ238192
Endomyzostoma deformator	DQ238126	DQ238161	DQ238190
Endomyzostoma cysticolum	DQ238130	DQ238165	DQ238194
Mesomyzostoma reichenspergi	DQ238116	DQ238152	DQ238182
Mesomyzostoma n. sp. 1a	DQ238122	DQ238157	–
Host			
Clarkcomanthus albinotus	xxxxxx	–	xxxxxx
Comanthus mirabilis	xxxxxx	–	xxxxxx
Oxycomanthus bennetti	xxxxxx	–	xxxxxx
Phanogenia gracilis	xxxxxx	–	xxxxxx
Himerometra robustipinna	xxxxxx	–	xxxxxx
Comanthina schlegelii	xxxxxx	–	xxxxxx
Oxycomanthus comanthipinna	xxxxxx	–	xxxxxx
Liparometra articulata	xxxxxx	–	xxxxxx
Stephanometra indica	xxxxxx	–	xxxxxx
Colobometra perspinosa	xxxxxx	–	xxxxxx
Metacrinus spp.	xxxxxx	–	xxxxxx
Saracrinus spp.[a]	xxxxxx	–	xxxxxx
Endoxocrinus spp.	xxxxxx	–	xxxxxx
Promachocrinus kerguelensis	xxxxxx	–	xxxxxx
Himerometra magnipinna	xxxxxx	–	xxxxxx
Clarkcomanthus littoralis	xxxxxx	–	xxxxxx

All our crinoid sequences were deposited in GenBank under accession numbers GQ913312 to GQ913344.

[a] Has to be taken with caution (the sequence contains ambiguous parts).

and TBR branch-swapping; 1000 replicates) and Bremer support (BS; Bremer, 1994).

Heuristic likelihood analyses (SeqAdd and branch-swapping) were performed using PAUP*4.0b4a (Swofford, 1998) with the likelihood model selected by Modeltest v3.6 (Posada and Crandall, 1998). The general time reversible (GTR) model, with rate heterogeneity and estimated proportion of invariable sites (GTR + I + G model), was identified as best fitting the observed combined data for both myzostomid and crinoid datasets). Clade supports were estimated by bootstrapping (Felsenstein, 1985) (100 replicates).

ML analyses were also performed using the Metapopulation Genetic Algorithm (MetaGA; Lemmon and Milinkovitch, 2002) using the software Metapiga 1.0.2b with the following settings: 4 populations of 4 individuals each, probability consensus pruning, random starting trees, HKY nucleotide substitution model (i.e., the most parameter-rich model implemented in MetaGA), with estimated proportion of invariable sites and rate heterogeneity (4 categories). MetaGA branch support values (PBS, which approximate posterior probabilities of branches) were computed from 1000 MetaGA samples (250 replicates with four populations) Bayesian analyses were performed with MrBayesv3.0b4 (Ronquist and Huelsenbeck, 2003). The model selected by MrModelTest 1.0b (Nylander, 2002) was applied for each combined dataset. Four Markov chains were run simultaneously for 5×10^5 generations, and trees were sampled every 100 cycles for a total of 5000 trees.

The first 1000 trees with preasymptotic likelihood scores, i.e., the 100,000 first generations, were discarded as "burn-in". The remaining trees were used to compute. Bayesian posterior probabilities (BPP) for each clade of the consensus tree. The run was repeated twice to ascertain convergence towards the same posterior parameter distribution (see Huelsenbeck et al., 2002).

Shimodaira–Hasegawa (SH) tests were used to compare alternative MP and ML topologies obtained during the analyses (Shimodaira and Hasegawa, 1999), as implemented in PAUP* 4.0b4a (Swofford, 1998).

2.5. Cophylogenetic analyses

Several methods for testing cospeciation are available (see Paterson and Banks, 2001, for review). Three of these methods were used to analyze the host–symbiont interactions in the present system: two topology-based methods (TreeMap 1.0; Page, 1995; 2.0; Charleston and Page, 2002), one distance-based method (Parafit; Legendre et al., 2002), and one data-based method (KH and SH tests; Kishino and Hasegawa, 1989; Shimodaira and Hasegawa, 1999). Tree-based methods, such as reconciliation analysis, compare the branching structure of host and symbiont trees to determine if more cospeciation/codivergence events are present than would be expected by chance. Distance-based methods determine if the hosts and their parasites are associated randomly by comparing genetic distances from homologous gene regions for the associated taxa. Data-based methods are used to determine the possible origin of any topological incongruence between host and associate trees.

MP and ML crinoid trees were respectively compared to MP and ML myzostomid trees using the software TreeMap 1.0 (Page, 1995) in a preliminary analysis (exact searches) in the aim to estimate the number of events (cospeciation, duplication, host-switch, and sorting event) that could explain the congruence/incongruence between the host and symbiont phylogenies. The maximum number of cospeciation events is the optimality criterion used in TreeMap 1.0 to find the "best" scenarios. However, TreeMap 1.0 does not take into account some types of host-switching events (see Charleston, 1998; Charleston and Page, 2002) that are correctly considered in TreeMap 2.02. Consequently, the results obtained in TreeMap 1.0 serve principally to fix the parameters requested to run the program TreeMap 2.02 (i.e., the number of the four events) to save computational time and memory. Also, randomisation tests with the proportional-to-distinguishable search algorithm were conducted with TreeMap 1.0 using 5000 random trees (for host plus symbiont simultaneously) in order to estimate the number of cospeciation events that could be expected by chance alone.

Reconciliation analyses were then performed on a MP host–symbiont tree pair using TreeMap 2.02 (Charleston and Page, 2002) via the Jungle algorithm (Charleston, 1998) to find the least costly reconstruction of host–parasite relationships while maximizing the number of cospeciation events. Given a tree for the hosts, a tree for the symbionts, and a mapping of extant symbionts onto extant hosts, this analysis exhaustively searches to find all possible solutions for the relationship between the two trees within a bound "jungle" of potential solutions (Charleston, 1998). The jungle for a given host tree and associated tree is a graph which contains all the potentially optimal solutions (see Charleston, 1998, for more details). TreeMap 2.02 then presents the solution(s) that fall within those bounds, displays congruence, and attempts to explain incongruence by postulating evolutionary events such as duplication of parasite lineages, sorting events and host-switching. The total number of non-cospeciation events postulated by the reconciled tree is the 'cost' of the tree (Page and Holmes, 1998). The jungle was bound with following parameters: 0 minimum codivergence (cospeciation events), and at most 50 non-cospeciation events, 30 lineage duplications (independent parasite speciation), 6 host-switches and 50 lineage losses (parasite extinction). Default settings were taken for weighing the evolutionary events (a weight of 0 was assigned to cospeciation, and 1 for duplication, sorting event and host-switch). With these parameters, two analyses were performed in which host trees were allowed a maximum symbiont load of 1 in the first analysis, and 2 in the second, the symbiont load being the number of symbionts accepted at any node of the host tree. These numbers are based on information given by Eeckhaut et al. (1998), who observed most of the time only one or two myzostomid species on a single crinoid species.

Although reconciliation analysis works with various numbers of symbionts per host (and hosts per symbiont), it ideally requires 1-to-1 host–parasite associations; otherwise the complexity of the problem could become too great to find an optimal solution without requiring too much time and computational memory. However, reconciliation analysis compares the topologies of the host and symbiont phylogenies and proposes precise evolutionary scenarios for the history of the association, implying that the trees are well known. Since a topology is sensitive to the presence or absence of taxa, the method assumes a thorough knowledge and good sampling of the clades under study. But even when all species are known and sampled, consideration of an extinct species could alter the topology of the actual tree and could change the proposed coevolutionary scenario (see Brooks and McLennan, 1991). We therefore used another method in addition to reconciliation analyses, implemented in Parafit (Legendre et al., 2002), especially designed to assess a cophylogenetic pattern of the data. Parafit implements a general test of cophylogeny between host and symbiont datasets that accommodates with multiple associations per host and uncertainty in tree topologies. This method consists in testing the null hypothesis (H_0) that the host phylogeny and the symbiont phylogeny are independent. First, two uncorrected distance matrices, one for crinoids and one for myzostomids, were built from sequences datasets that were then converted in principal coordinates matrices with the DistPCoA program (Legendre and Anderson, 1998). Information on host–symbiont associations were summarised in a third matrix (i.e., the presence/absence of a symbiont on a host). ParaFit combined these three matrices into a single one, called "the fourth-corner", and apply a global statistic test that compares the observed distribution with a null distribution generated by randomising host–symbiont associations (a total of 999 permutations were performed). The significance of individual associations between host–symbiont pairs was also tested by sequentially deleting each host–symbiont pair from the analysis. We completed this analysis on the 18S rDNA alone, the COI alone, and on combined datasets (18S + 16S + COI for myzostomids, and 18S + COI for crinoids).

Tree-based and distance-based methods are used to test significance of cophylogeny between associated taxa, whereas data-based methods can be used to estimate the origin of any topological incongruence between host and associate trees. Data-based methods test the hypothesis that host and associate datasets are consistent with an identical topology, thus indicating if sampling error occurred and lead to differences between topologies rather than biological processes such as host switching or extinction events (Clark et al., 2000; Page, 2003; Jackson, 2004a, 2004b; Kawakita et al., 2004). If the null hypothesis is not rejected, topological incongruence is assumed to be due to sampling error (e.g., incomplete taxa sampling, or deficiency in informative sites). However, if the null hypothesis is rejected, it is assumed that biological processes (i.e., host switch, extinction, etc.) resulted in the observed topological incongruence. KH and SH tests (Kishino and Hasegawa, 1989; Shimodaira and Hasegawa, 1999; Goldman et al., 2000) were used to compare trees inferred from alternative datasets in both a parsimony and likelihood basis (Peek et al., 1998; Clark et al., 2000). The likelihood and parsimony scores obtained for the best associate trees (i.e., myzostomid) given the associate data were compared to the score of the alternative host trees, also given the associate data (i.e., given the myzostomid dataset, the best myzostomid trees are compared to the best crinoid trees in which crinoid taxa have been replaced by their respective associate taxa). Similarly, the best host trees are compared to the score of the alternative associate trees given the host dataset. The differences between tree scores were determined using RELL optimization and 1000 nonparametric bootstrap replicates as implemented in PAUP*b4.10 (Swofford, 1998).

3. Results

3.1. Phylogeny of the crinoid-hosts

Sequence lengths were of 1101–1659 bp for 18S rDNA and 1104–1125 bp for COI in crinoids (total matrix of 2784 bp; including outgroup). The base composition was homogenous and the uncorrected pairwise sequence divergences were higher for COI (1–17%) than for 18S rDNA (0.06–15%). Saturation plots (data not shown) for each gene, as well as the third positions of the COI codons, indicated no obvious Ti or Tv saturation for the whole range of pairwise distances. The ILD test showed that the two gene fragments were not significantly incongruent and could consequently be combined in a two-gene dataset.

Of the 1659 bp of 18S rDNA examined (including outgroup), 92 were potentially parsimony informative (17 were informative when including the ingroup only). Parsimony analysis of the 18S rDNA resulted in 8 most parsimonious trees (not shown; length 215, CI = 0.9535) in which only a few clades were resolved (bootstrap support = 64 for clade B, 62 for clade E, 96 for clade G, 73 for clade J, referring to Fig. 1A which is based on the combined dataset). Of the 1125 bp of COI examined (including outgroup), 367 were potentially parsimony informative. Parsimony analysis of the COI alone resulted in one most parsimonious tree (not shown; length 1256, CI = 0.5533) in which most of the clades were resolved (bootstrap support = 71 for clade A, 58 for clade B, 100 for clade C, 66 for clade D, 53 for clade E, 57 for clade H, 100 for clade J, 58 for clade K, 64 for clade L, 100 for clade N, referring to Fig. 1A which is based on the combined dataset).

The combined dataset (18S rDNA + COI) resulted in 2784 bp, of which 459 were potentially parsimony informative (considering outgroup). Parsimony analysis of the combined dataset resulted in one most parsimonious tree (length 1483, CI = 0.6069) in which most of the clades were resolved (Fig. 1A).

All analyses (MP, ML, MetaGA and Bayesian analyses) support the rooting of the crinoid tree on a lineage including the Isocrinida (stalked crinoids) in one hand (clade A) and the other crinoids (all comatulids) in the other hand (clade B; see Figs. 1A and 2A; only MP and ML trees are shown). The topology is congruent with the results of Cohen et al. (2004). Comatulids split in two main clades, Comasteridae (clade D) and the other families (Himerometridae, Mariametridae and Colobometridae) (clade E). Among the non-comasterids, *Promachocrinus kerguelensis* (Antedonidae) is sister to the grouping comprised of Himerometridae (clade J) and Mariametridae (clade I) each of which are monophyletic (Fig. 1). Within Comasteridae (clade D), *Phanogenia gracilis* is the sister to the remaining comasterids, *Clarkcomanthus* (clade N) is monophyletic, whereas *Oxycomanthus* is not. The differences between MP and ML trees are (i) the position of *Colobometra perspinosa* and (ii) the relationship between the four species within clade L. ML (Fig. 2A) and Bayesian analyses yielded almost identical trees (differences are observed within clades K and G: clade N was the sister group of the clade *Comaster schegelii*/*Oxycomanthus comanthipinna*; *Colobometra perspinosa* was found at the base of clade G, whereas only clade J was retrieved) that were only slightly different from the topology generated by parsimony analysis (Fig. 1A). These

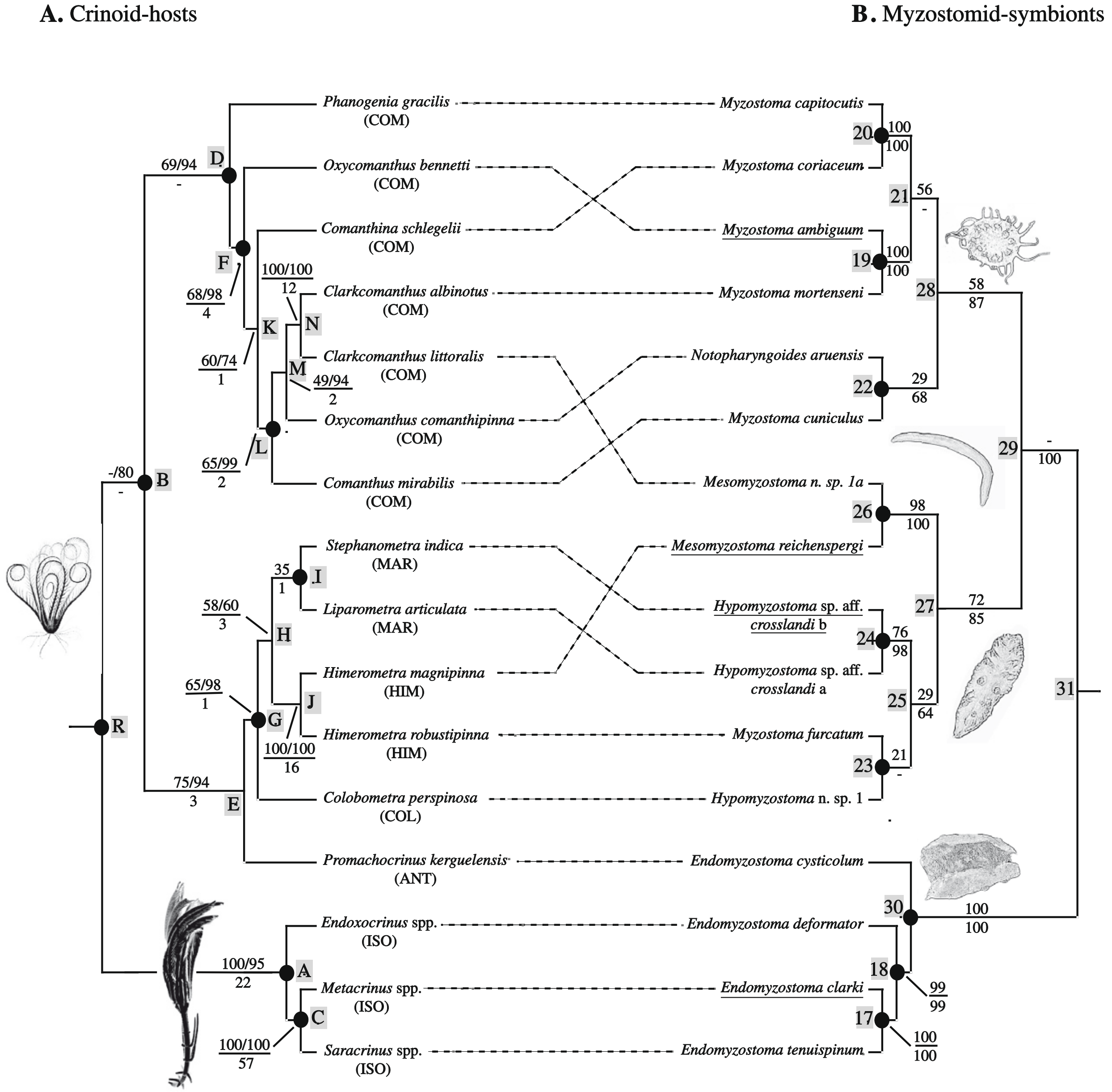

Fig. 1. Tanglegram of MP topology for 16 species of crinoid-hosts and MP topology of their obligate associates (16 myzostomids). (A) Crinoid-host MP tree (bootstrap 50% majority-rule consensus tree, length = 1492, CI = 0.6032, RI = 0.59) inferred on a two genes-dataset (18S rDNA + COI, 2784 bp): numbers above branches indicate bootstrap values (1000 replicates) of MP analyses (left side) and posterior branch support value of ML analyses with Metapiga (right side); numbers below branches indicate Bremer support. (B) Myzostomid-symbiont MP tree (bootstrap 50% majority-rule consensus tree, length = 1384, CI = 0.5542, RI = 0.5994) inferred among 16 specimens (15 species) on a three genes-dataset (18S + 16S + COI, 2417 bp, pp >90%, see Lanterbecq et al., 2006, for details): numbers above branches indicate bootstrap values (1000 replicates) of MP analyses and numbers below branches indicate posterior branch support value of ML analyses with Metapiga. Lines connecting taxa indicate host–symbiont associations. Solid black circles on nodes are cospeciation events inferred from reconciliation analysis (TreeMap 1.0). Letterings and numbers represent each node of the host and symbiont topology, respectively. The number of cospeciation events (eight) was significantly higher than expected by chance in 5000 randomizations (see text and Fig. 3A). Drawings on the left illustrate stalked (bottom) and comatulid (top) crinoid-hosts, and a few representative myzostomids are illustrated on the right (species names corresponding to drawings are underlined in the tree). Host families: Antedonidae (ANT), Colobometridae (COL), Comasteridae (COM), Himerometridae (HIM), Isocrinidae (ISO), Mariametridae (MAR).

topological differences were not significant by SH tests (MP versus Bayesian topology $p = 0.485$; MP versus ML topology $p = 0.245$; and ML versus Bayesian topology $p = 0.250$).

3.2. Phylogeny of the myzostomid-symbionts

A total of 1513 bp for 18S rDNA, 271 bp for 16S and 633 bp for the COI were used to infer the myzostomid-symbiont phylogeny (total matrix of 2417 bp; see Lanterbecq et al., 2006, for details). The base composition was homogenous and uncorrected pairwise sequence divergences are higher for 16S rDNA (4–20%) and COI (1–25%) than for 18S rDNA (0.3–9%). Saturation plots (data not shown) for each of the three genes, as well as the third positions of the COI codons, indicate no obvious Ti or Tv saturation for the whole range of pairwise distances. In the present paper, we concentrated our analyses on the combined dataset, as exhaustive analyses have already been completed on the different genes combinations and on the whole dataset (see Lanterbecq et al., 2006, for details), and the inferred topologies are congruent with their results. All analyses (MP, ML, and MetaGA analyses) found the four

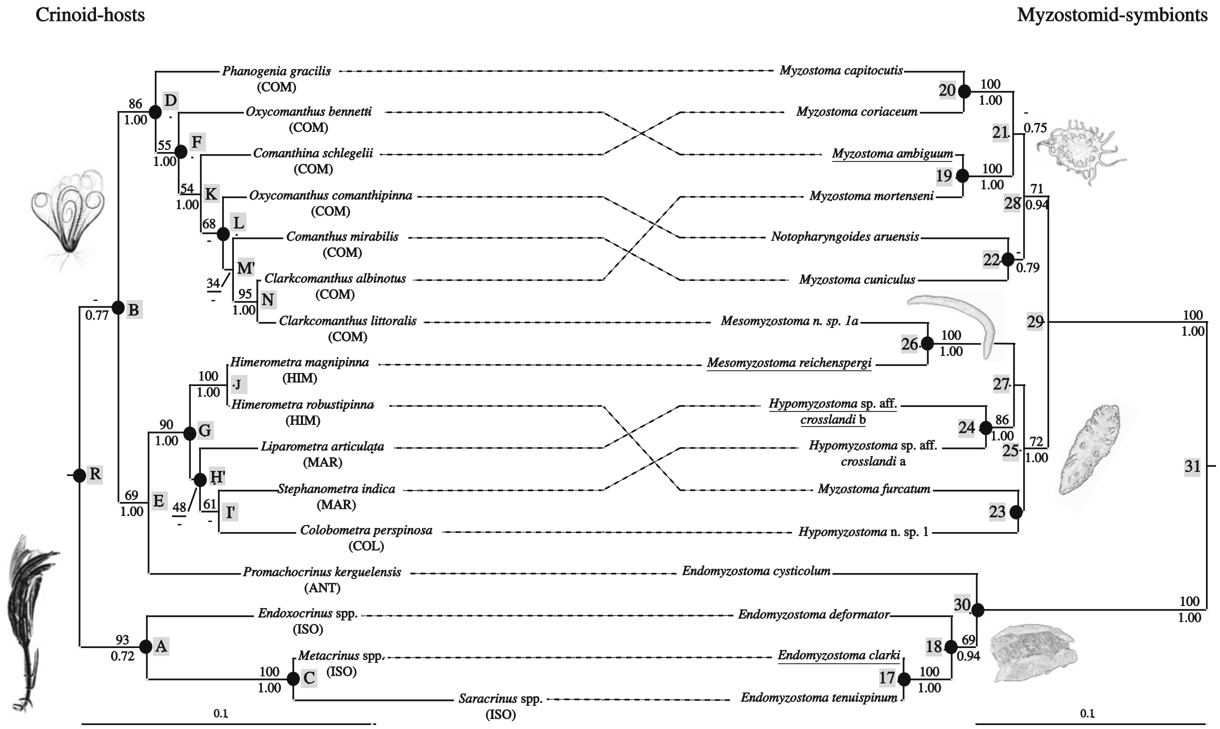

Fig. 2. Tanglegram of ML topology for 16 crinoids and ML topology of their obligate associates (16 myzostomids). (A) Crinoid-host ML tree inferred on a two genes-dataset (18S rDNA + COI, 2784 bp) based on the specific model selected by Modeltest v3.6 (Posada and Crandall, 1998) (i.e., GTR + I + G). (B) Myzostomid-symbiont ML tree inferred among 16 specimens (15 species) on a three genes-dataset (18S + 16S + COI, 2417 bp, pp > 90%, see Lanterbecq et al., 2006 for details) based on the specific model selected by Modeltest v3.6 (Posada and Crandall, 1998) (i.e., GTR + I + G). Numbers above and below branches indicate bootstrap values (100 replicates) and bayesian posterior probabilities, respectively. Lines connecting taxa indicate host–symbiont associations. Solid black circles on nodes are cospeciation events inferred from reconciliation analysis (TreeMap 1.0). The branch lengths are proportional to the number of substitutions per site (see scale). Lettering and numbers represent each node of the host and symbiont topology, respectively, allowing the comparison with the MP trees (see Fig. 1), except for H′, I′ and M′. The number of cospeciation events (nine) was significantly higher than expected by chance in 5000 randomizations (see text and Fig. 5B). Drawings on the left illustrate stalked (bottom) and comatulid (top) crinoid-hosts, and a few representative myzostomids are illustrated on the right (species names corresponding to drawings are underlined in the tree). Host families: Antedonidae (ANT), Colobometridae (COL), Comasteridae (COM), Himerometridae (HIM), Isocrinidae (ISO), Mariametridae (MAR).

Endomyzostoma species at the basis of the tree, sister group of the remaining twelve species. The latter split in two clades of six species: the first includes five *Myzostoma* species and *Notopharyngoides aruensis*, the second comprises three *Hypomyzostoma* species, two *Mesomyzostoma* species and *Myzostoma furcatum* (Figs. 1B and 2B).

ML (Fig. 2B) and Bayesian analyses yielded almost identical trees (except that *M. furcatum* was found at the base of the *Hypomyzostoma* clade, sister group of the *Mesomyzostoma*) that were only slightly different from the topology generated by parsimony analysis (Fig. 1B). These topological differences were not significant by SH tests (MP versus Bayesian topology $p = 0.428$; MP versus ML topology $p = 0.279$; and ML versus Bayesian topology $p = 0.274$).

3.3. Reconciliation analysis

The tanglegrams in Figs. 1 and 2 illustrate the relationships between myzostomid and crinoid MP (Fig. 1) and ML-tree pairs (Fig. 2). The two tanglegrams do not illustrate a perfect match between the two tree pairs, meaning that a strict co-speciation between crinoids and myzostomids is not corroborated by the present data.

Exact searches (performed in TreeMap 1.0) made on the MP (Fig. 1) and ML-tree pairs (Fig. 2) retrieved 85 and 6 “best” equally probable reconstructions with 9 and 10 cospeciation events, respectively. The significance of the results was estimated by generating 1000 random symbiont and host trees (simultaneously) and recalculating the number of cospeciation events. The results demonstrated that the observed number of cospeciation events (9 and 10) in the 2 host–symbiont trees (MP- and ML-tree pairs, Figs. 1 and 2) was significantly higher than what was observed in the 1000 random associations (Fig. 3A and B). Most of the randomized host–symbiont tree pairs showed five cospeciation events (30.36% and 30.20% of randomized MP- and ML-tree pairs, respectively), whereas 9 (in MP) or 10 (in ML) cospeciation events are only retrieved in 0.34% and 0.04% of the randomized trees (Fig. 3). The occurrence of 9 (in MP) and 10 (in ML) cospeciation events is thus significantly higher than the number expected by chance alone (Fig. 3). The significance of the results was also estimated by generating either 1000 random host trees or 1000 random symbiont trees for each tree pair (MP or ML) and both tests confirmed that the observed number of cospeciation events (9 and 10) in the 2 host–symbiont tree pairs (MP and ML) was significantly higher than that observed in the 1000 random associations (not shown). Most of the randomized host trees pairs showed seven cospeciation events (56.78% and 56.04% of randomized MP- and ML-tree pairs, respectively), whereas 9 (in MP) or 10 (in ML) cospeciation events are only retrieved in 5.02% and 0.36% of the randomized trees (not shown). Most of the randomized symbiont tree pairs showed five cospeciation events (30.7% and 31.9% of randomized MP- and ML-tree pairs, respectively) whereas 9 (in MP) or 10 (in ML) cospeciation events are only retrieved in less than 0.2% of the randomized trees (not shown).

Jungle analyses using TreeMap 2.0b detected significant cophylogeny between the myzostomids and their crinoid hosts MP-tree pairs. Reconciliation analysis found 12 and 36 equally probable reconstructions (Table 3) when a symbiont load of 1 and 2 is

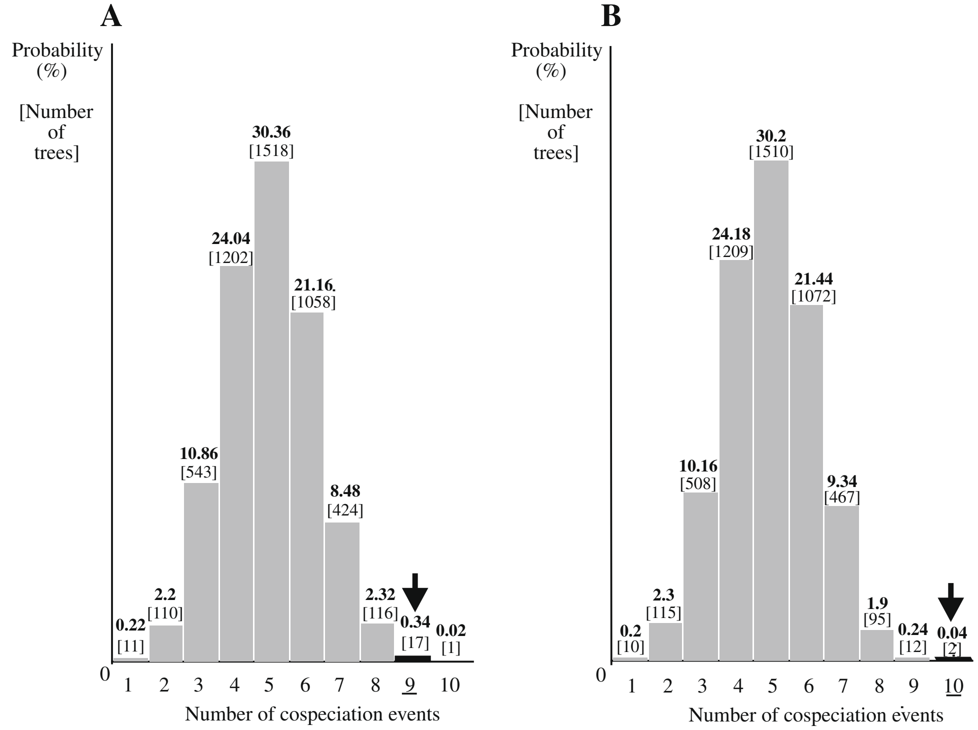

Fig. 3. Histogram showing the distribution of the number of cospeciation events produced in the randomization analysis (TreeMap 1.0) on the MP topology (A) and ML topology (B) of the host–symbiont tree pairs. The number of tree pairs (in bracket) and their percentage retrieved in 5000 simultaneously host and associate randomized trees pairs are indicated above the bars of the histograms. The arrow indicates the number of cospeciation events inferred from reconciliation analysis on the real host-associate tree pairs (9 and 10 in MP and ML topologies, respectively).

Table 3
Results of Jungle analyses (TreeMap 2.02) on MP-tree pairs and allowing a symbiont load of 1 or 2 at each node of the host tree.

Symbiont load	Total number of solutions	Number of solutions	C	D	L	S	T[a]
1	12	12	8	1	8	6	28
2	36	6	8	1	7	6	27
		2	**9**	**0**	**9**	**6**	**27**
		2	**9**	**1**	**10**	**5**	**27**
		14	9	2	13	4	29
		12	9	3	16	3	31

Abbreviations: T = total cost of non-cospeciation events; C = cospeciation, D = duplication, L = loss (i.e., all sorting events as extinction, "missing the boat" and failure to speciate); S = host-shift. Four solutions are emphasised in bold as they count a maximum number of cospeciations (9) and a minimum total cost (27). The jungle was bound with the following prior parameters: 0 minimum codivergence (cospeciation events), and at most 50 non-cospeciation events, 30 lineage duplications (independent parasite speciation), 6 host-switches and 50 lineage losses (parasite extinction). Host trees were allowed a maximum symbiont load of 1 and 2, the symbiont load being the number of symbionts accepted at any node of the host tree. These numbers are based on information given by Eeckhaut et al. (1998), who observed most of the time only one or two myzostomid species on a single crinoid species. Default settings were taken for weighing the evolutionary events (a weight of 0 was assigned to cospeciation, and a weight of 1 for duplication, sorting event and host-switch).

[a] T = L + 2D + 3S.

successively allowed (the symbiont load being the number of symbionts accepted at any node of the host tree). Reconciliation analysis found 12 equally probable reconstructions (Table 3) when a symbiont load of 1 is allowed with 8 cospeciation events, 1 duplication, 8 sorting events (or extinction), 6 host switches, and a net cost of 28 (Table 3). The 8 reconstructed cospeciation events were greater than expected by chance (Fig. 3).

Fig. 4A summarizes the number of evolutionary events observed in these 12 reconstructions when Comasteridae, non-comasterid comatulids and Isocrinidae are taken into consideration. The cophylogenetic pattern between Isocrinidae and myzostomids is fully explained by 2 cospeciations. The cophylogenetic patterns between Comasteridae and myzostomids on one hand, and non-comasterid comatulids and myzostomids on the other hand, are each explained by 2 cospeciations, 3 sorting events and 2 to 4 host-switches. Internal host-switches (i.e., occurring within the clade) are either equal (in non-comasterid comatulids) or higher (in Comasteridae) than external host-switches (i.e., shift to another clade) (Fig. 4A). Reconciliation analysis found 36 equally probable reconstructions (Table 3) when a symbiont load of 2 is allowed with 8–9 cospeciation events, 0–3 duplication, 7–16 sorting events (or extinction), 3–6 host switches, and a net cost of 27–31 (Table 3). The 8–9 reconstructed cospeciation events were greater than expected by chance (Fig. 3).

Four of these reconstructions (in bold in Table 3) show the highest number of cospeciation events (9) and the lowest total cost (27). Fig. 5 illustrates one of these four reconstructions. The cophylogenetic pattern retrieved for the 16 myzostomids and their hosts is first explained by a primary association of the ancestors. An early cospeciation (event 1, Fig. 5) gives birth to two lineages, the first leads to all the *Endomyzostoma* species and the second to the remaining myzostomids. All the *Endomyzostoma* species

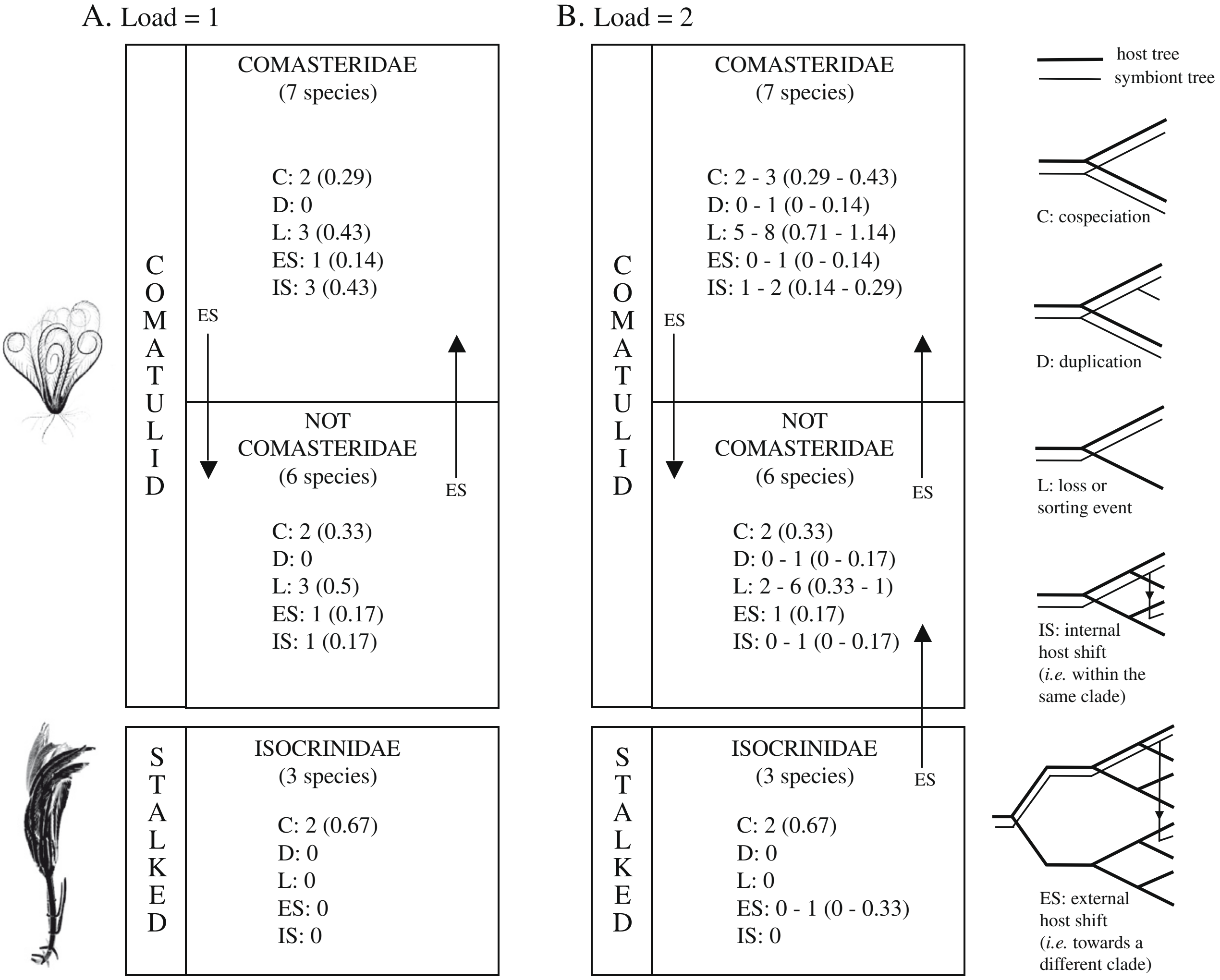

Fig. 4. Summary of the results obtained in a Jungle analysis on MP-tree topology pairs when a symbiont load of 1 (A) and 2 (B) is allowed at each node of the host tree. The number of evolutionary events observed in the 12 and 36 retrieved reconstructions, respectively, are summarised when Comasteridae, non-comasterids and Isocrinidae are taken into consideration. The different biological events are represented on the right. Values in brackets are the ratio between the number of the given event and the number of host-species in the clade considered. Abbreviations: C = cospeciation, D = duplication, L = losses (or sorting events), IS = internal host-switch, ES = external host-switch.

are associated with stalked crinoids except *Endomyzostoma cysticolum,* which infests the antedonid, *Promachocrinus kerguelensis*. The latter is explained by a host-switch (event I) that occured on the lineage leading to the three *Endomyzostoma* species (appeared by "pure" cospeciation, events 2 and 3, Fig. 5). At the base of the comatulids, a fourth cospeciation event (4; Fig. 5), gives rise to two lineages, one leading to most of the myzostomids associated with Comasteridae, with the exception of *Mesomyzostoma* n. sp. 1 that comes from an external host-switch (event II). The other lineage leads to most myzostomids associated with non-comasterids, with the exception of *E. cysticolum* and *Mesomyzostoma reichenspergi* that appear to be external host-switches (events I and III). Aside from these external host-switches, two cospeciations (events 5 and 6), 1 internal host-switch (event IV) and 2 sorting events explain the cophylogenetic pattern between non-comasterid comatulids and their associated myzostomids. Similarly, 3 cospeciations (events 7, 8 and 9), 2 internal host-switches (events V and VI), and 7 sorting events are necessary to explain the cophylogenetic pattern among Comasteridae and their associated myzostomids.

Fig. 4B summarizes the number of evolutionary events observed in these 36 reconstructions (symbiont load of 2) when Comasteridae, non-comasterid comatulids and Isocrinidae are taken into consideration. The cophylogenetic pattern between Isocrinidae and myzostomids is again fully explained by 2 cospeciations. The reconstructions consider that 2–3 cospeciations happened between Comasteridae and myzostomids and that 2 cospeciations occurred between non-comasterid comatulids and myzostomids. The number of sorting events is high: it is up to 8 between Comasteridae and myzostomids, and up to 6 between non-comasterid comatulids and myzostomids. The number of duplication is always less than 2 in each group. Internal switches of myzostomids occur in both Comasteridae and non-comasterid comatulids. External switches also occur from one of these two groups to the other group of comatulids. External switches are also present from Isocrinidae to non-comasterid comatulids.

3.4. Parafit tests

The tests performed on the COI and the combined datasets each independently indicated that a significant cophylogenetic pattern exists between crinoid and myzostomid trees (Table 4). Global tests of cophylogeny using ParaFit (Legendre et al., 2002) resulted in rejection of random association between host and parasite taxa ($p = 0.003$ and 0.013, respectively). The cophylogenetic pattern is not significant with the 18S rDNA datasets that could probably

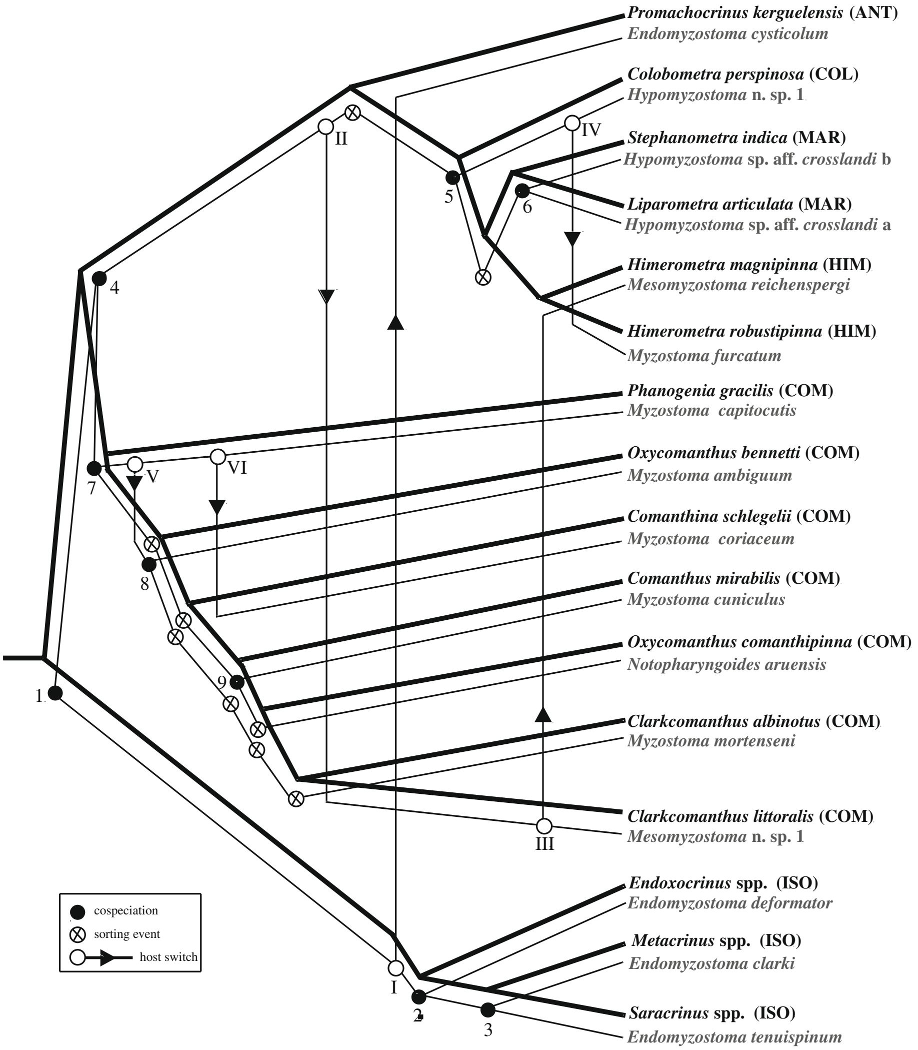

Fig. 5. Reconstruction of one of 36 potentially optimal solutions of reconciled trees recovered from a Jungle analysis (Treemap 2.0) on the MP topologies pair allowing a symbiont load of two in each node of the reconstruction. Thick and thin lines represent the host and associate trees, respectively. Black circles in the symbiont tree (near nodes on the host tree) represent cospeciation events, white circles represent duplication events, crossed circles represent losses (extinction or sorting event) from the lineage that the symbiont tree fails to track, and arrows indicate the direction of a host-switch (always coupled with a duplication in these jungles). The jungle reconstruction (out of 2) shown here has the largest number of cospeciation events, the fewest number of non-codivergent events and the lowest total cost (see Table 3). Host families: Antedonidae (ANT), Colobometridae (COL), Comasteridae (COM), Himerometridae (HIM), Isocrinidae (ISO), Mariametridae (MAR).

Table 4
Results of the Parafit Statistical Tests. The null hypothesis of the global test corresponds to a random choice of the hosts by the symbionts (i.e., the overall structure is not cophylogenetic). The null hypothesis of the individual association is that the link between host and symbiont is chosen randomly.

Symbionts	Hosts	18S dataset: probability	COI dataset: Probability	Combined[a] dataset: probability
Pairwise test				
Endomyzostoma cysticolum	*Promachocrinus kerguelensis*	0.293	0.504	0.825
Endomyzostoma tenuispinum	*Sarracrinus* spp.	**0.041**[*]	**0.001**[*]	**0.009**[*]
Endomyzostoma clarki	*Metacrinus* spp.	0.448	**0.001**[*]	**0.016**[*]
Endomyzostoma deformator	*Endoxocrinus* spp.	0.423	**0.029**[*]	0.113
Notopharyngoides aruensis	*Oxycomanthus comanthipinna*	0.690	0.113	0.110
Myzostoma cuniculus	*Comanthus mirabilis*	0.653	0.136	0.102
Myzostoma capitocutis	*Phanogenia gracilis*	0.667	0.208	0.267
Myzostoma coriaceum	*Comanthina schlegelii*	0.647	0.134	0.113
Myzostoma ambiguum	*Oxycomanthus bennetti*	0.643	**0.046**	0.087
Myzostoma mortenseni	*Clarkcomanthus albinotus*	0.671	**0.047**	0.105
Mesomyzostoma n. sp. 1	*Clarkcomanthus littoralis*	0.617	/	0.147
Mesomyzostoma reichenspergi	*Himerometra magnipinna*	0.418	0.445	0.258
Hypomyzostoma sp. aff. *crosslandi* b	*Stephanometra indica*	**0.024**[*]	0.153	**0.033**[*]
Hypomyzostoma sp. aff. *crosslandi* a	*Liparometra articulata*	0.459	0.241	0.172
Hypomyzostoma n. sp. 1	*Colobometra perspinosa*	0.496	0.253	0.158
Myzostoma furcatum	*Himerometra robustipinna*	0.447	0.607	0.306
Global test		0.166	**0.003**[*]	**0.013**[*]

[a] Combined dataset corresponds to 18S + 16S + COI for myzostomids and 18S + COI for crinoids.

[*] Significant association are in bold ($p \leqslant 0.05$).

be explained by the low number of informative sites in crinoids (92 or 17 potentially parsimony informative out of 1659 bp when including or excluding outgroup, respectively).

A random association between hosts and symbionts was also rejected in two to five association tests depending on the dataset considered (Table 4). The individual association between *Endomyzostoma tenuispinum* and *Sarracrinus* spp. was significant for the three datasets ($p = 0.041$, 0.001, and 0.009, respectively; Table 4). The association between *Endomyzostoma clarki* and *Metacrinus* spp. appeared significant for the COI and combined datasets ($p = 0.001$, and 0.016, respectively; Table 4). The COI datasets also significantly support the association between *Endomyzostoma deformator* and *Endoxocrinus* spp., *Myzostoma ambiguum* and *Oxycomanthus bennetti*, and *Myzostoma mortenseni* with *Clarkcomanthus albinotus* ($p = 0.029$, 0.046, and 0.047, respectively; Table 4). The association between *Hypomyzostoma* sp. aff. *crosslandi* b and *Stephanometra indica* was only significant for the 18S rDNA and combined datasets ($p = 0.024$, and 0.033, respectively; Table 4).

3.5. Data-based method

KH and SH analyses all revealed significant differences ($p < 0.05$) between the host and associate datasets, thereby rejecting the null hypothesis that observed topological differences were caused by sampling error. Thus, these tests indicate that differences between crinoid and myzostomid phylogenies are probably the consequence of historical events, such as host switching or extinction.

4. Discussion

4.1. Phylogeny of the crinoid-hosts

In this study, we reconstructed for the first time the phylogeny of some crinoid taxa based on a two-genes dataset (Figs. 1A and 2A). Our results show that stalked crinoids and comatulid crinoids separate early in two clades. These results are congruent with those obtained by Cohen et al. (2004), who used morphological and molecular data to estimate extant crinoid high-level interrelationships, and with the analysis of White et al. (2001), which focused on Comasteridae by comparing 16S rDNA sequences from 25 species (11 genera). In both works, *Clarkcomanthus* is monophyletic while *Oxycomanthus* is not. The present analysis is the first including non-comasterid comatulids in the ingroup. We found that Comasteridae and non-comasterid families are two sister clades. In the non-comasterid families, Himerometridae is monophyletic, Mariametridae is either monophyletic (Fig. 1A) or includes *Colobometra perspinosa* (Fig. 2A).

4.2. Phylogeny of the myzostomid-symbionts

The topologies inferred in the present analysis (Figs. 1B and 2B) are congruent with those presented by Lanterbecq et al. (2006), who analyzed the whole combined dataset and included many more species than included here (their goals were to infer the phylogeny of the Myzostomida based on 37 species). All of the current analyses placed the four *Endomyzostoma* species as sister group of the remaining 12 species (all living on comatulid crinoids), which split into two clades: one including five *Myzostoma* species (ectocommensal living on the host calyx or pinnules) and *Notopharyngoides aruensis* (an endoparasite living in the digestive tube), the second comprising three *Hypomyzostoma* species (ectocomensal living on arms), two *Mesomyzostoma* species (endoparasites living in the gonads) and *Myzostoma furcatum* (ectocommensal living on the pinnules).

4.3. Cophylogenetic analyses

The present study is the first to test for the presence of a coevolutionary pattern between crinoids and myzostomids. Tree-based, distance-based, and data-based methods were all informative regarding the historical associations among the crinoids and myzostomids examined in this study. The tree-based reconciliation analyses (Treemap) detected a significant pattern of cophylogeny between the crinoid lineages and their myzostomid symbionts. The reconciliation analyses indeed support the view that topological congruence between myzostomid and crinoid trees are not due to chance alone with the 16 studied crinoid–myzostomid pairs supporting the presence of at least 8 cospeciations (via cophylogenetic analyses). The distance-based method used in this study (ParaFit) reveals a global significant nonrandom association pattern between host and symbiont datasets and identified significant individual association pairs. It also identified host–symbiont pairs that associated randomly, most probably as a result of a process other than cospeciation such as host switching (Table 4). The data-based method used to compare the hosts and symbionts in this study (KH and SH) confirmed that at least some of the differences in the datasets did not result from sampling error, but rather historical processes such as host switching or extinction (see Clark et al., 2000; Page, 2003; Jackson, 2004a, 2004b; Kawakita et al., 2004).

4.4. Interpretation of the results

Despite the cophylogenetic pattern detected by several methods, we have to keep in mind that the taxa included in the present analysis do not represent an exhaustive list of the associations observed in the system crinoid–myzostomids: more than 170 species of myzostomids have been described in literature (Grygier, 2000; Eeckhaut and Lanterbecq, 2005). For now, assuming that the evolutionary scenarios obtained with these data might be representative of the twin history of crinoids and myzostomids, our analyses show that (i) cospeciations occur in both stalked crinoids and comatulid crinoids, (ii) the number of host-switches is much higher in comatulids than in stalked crinoids, (iii) internal host-switches occur in Comasteridae and in the non-comasterid comatulid clade, although not evident in stalked crinoids, (iv) external host-switches evidently exist from Comasteridae to non-comasterid and vice versa, and may exist from stalked crinoids to non-comasterid (observed in one scenario), (v) the number of duplications is low and the number of sorting events is high (Fig. 4).

The present analysis supports the hypothesis that the topological congruence between the myzostomid and crinoid trees is not due to chance alone. Speciation of crinoids sometimes induced speciation in myzostomids (Fig. 6A), that would presumably occur when a set of symbiont individuals of a given species became genetically isolated. Such isolation could exist during cospeciations, host switches, and/or duplications (Fig. 6). These events occur both in myzostomid parasites and commensals and are at the origin of myzostomid diversification. Host-switches, however, are more common in commensals than in parasites, which can be explained by their ability to contact other hosts. Whether parasites or commensals, myzostomid populations living on crinoids are in a similar situation to terrestrial populations living on islands: if batches of individuals stay genetically isolated for long enough, they will be liable to speciate by allopatry.

If cospeciations are currently occurring, examples of myzostomid pairs (living on taxonomically related crinoids), in which species have acquired a very close anatomy, should be present in nature. The best example is probably the pair involving *Myzostoma cuniculus* and *Myzostoma pseudocuniculus*, in which the two species are extremely similar morphologically except that the latter have

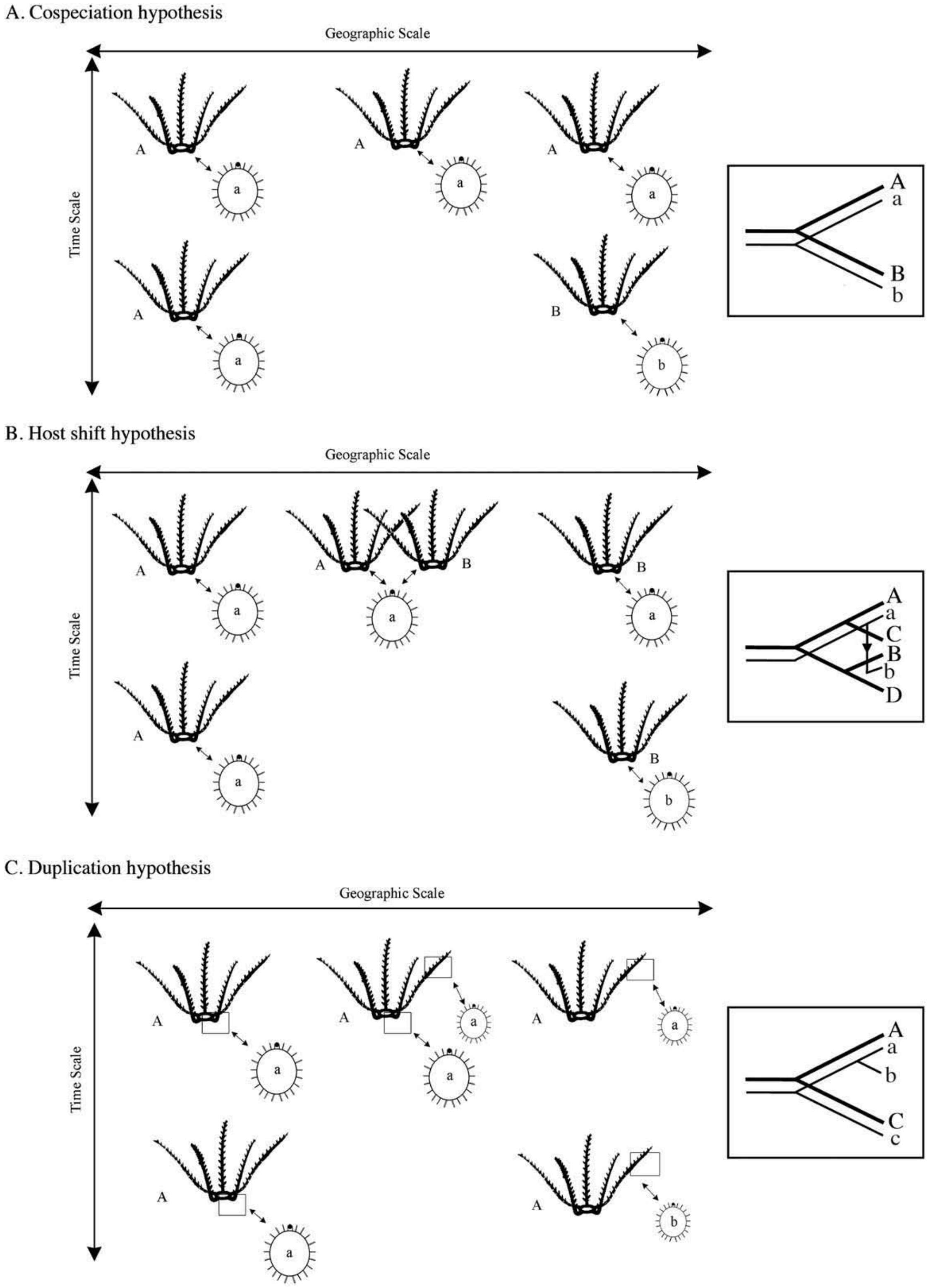

Fig. 6. Schematic representation of the hypothesis of cospeciation (A), host-shift (B), or duplication events (C) that possibly occured in the crinoid–myzostomid system, considering geographical and time scales. Different colours (black and grey) and letters refer to different species. The different biological events are represented on the right, with thick and thin lines representing the host and associate trees, respectively. (A) Cospeciation hypothesis: when a crinoid species *A* has speciated in a new species *B*, the myzostomid-associate *a* cospeciated and became a new species *b* through time. (B) Host-shift hypothesis (followed by speciation): if two crinoid species (*A* and *B)* live together in the same geographic area, it is thinkable that the myzostomid *a*, associated with the crinoid species *A*, has colonised the crinoid species *B*, then became a new species *b* by geographic isolation on the crinoid *B* (through time). (C) Duplication hypothesis: the isolation of myzostomid individuals on one crinoid species is possible, as some ectocommensal myzostomids often show a preferred location on crinoids (e.g., the pinnules or the calyx), and thus, regarding at large geographical scale, individuals batches of a given myzostomid species *a* might show such preferences that could eventually lead to its speciation in *b* if the preferred location is maintained through time.

many long marginal cirri (Lanterbecq and Eeckhaut, 2003). The first species is observed on *Comanthus mirabilis* in Australia (Rouse, *pers. obs.*) and on *Clarkcomanthus* spp. in Papua New Guinea (Eeckhaut et al., 1998), the second on *Comaster whalbergii* in Madagascar (Lanterbecq and Eeckhaut, 2003), and the two crinoid-host species are phylogenetically close (White et al., 2001). Crinoids and myzostomids however do not have strict parallel phylogenies and evolutionary events other than cospeciation are needed to explain the topological incongruence found in the crinoid–myzostomid tree pairs. Sorting events, host-switches and, to a lower degree, duplications, were present all along the twin history of these organisms.

In the present system, host-switching (Fig. 6B) requires two elements to be successful: (i) the symbiont must have the opportunity in time and in space to make the switch, and (ii) the symbiont must have adequate morphological characters, or must have the capacity to develop adaptive traits, to establish and survive on the new host. In myzostomids, host-switches could, in theory, occur at the larval or postmetamorphic stages. In the first case, old and new colonized host species must not necessarily contact each other: the eggs pawned into the water column by myzostomids living on the "old" host species develop into free-swimming larvae that may infest the "new" host species. In the second case, "old" and "new" host species must contact each other as myzostomids are not able to move on the substratum between crinoids (Eeckhaut and Jangoux, 1993) and transmission must be direct from one crinoid to another. Both types of transmission thus explain why host-switches are less frequent from stalked crinoids than from comatulid crinoids: it is common in nature to see many species of comatulids in a restricted area (Fabricius and Dale, 1993; Eeckhaut et al., 1998). Moreover, most myzostomids infesting stalked crinoids, such as *Endomyzostoma* species, are gallicolous parasites (i.e., they live in galls generally induced on crinoid arms, with dwarf males living on large females) and their displacements are probably limited to the inside of these shelters (as such parasites spent most of their life walled up, the sensory and locomotory organs became reduced or totally disappear in both the females and dwarf males; Lanterbecq et al., 2009).

Once host-switches occur, new associations persist through time (Fig. 6B) if the "new" host presents the "ideal" conditions for the settlement of myzostomids. Host-switches between crinoids of relative similar morphology would logically be easier than between dissimilar crinoids. Our results agree with this assumption as the transmission between comatulids and stalked crinoids is rare and the number of external host-switches is never higher than the number of internal host-switches. Though rare, the estimated scenarios allow for myzostomids to switch from stalked crinoids to comatulids, but not the reverse. Host-switches do not only imply that myzostomid individuals colonize a new crinoid species but they also allow these individuals to speciate (Fig. 6B). In nature, the occurrence of crinoid-switches by myzostomids could be explained by the opportunism that characterized some symbiont species. Indeed, on a small geographical scale, some myzostomids

Table 5
Some relevant references of cophylogenetic studies.

Authors	Symbiosis type	'Symbiont'	'Host'		*Program*	Cospeciation events	Switch events	Duplication events	Sorting events
Paterson et al. (2000)	Parasitism	Lice (Ischnocera) (14 species, 6 genera)	Seabird (Procellariiformes, Sphenisciformes and Charadriiformes) (11 species)		TreeMap 1.0	9	0–1	3–4	11–14
Johnson et al. (2003)	Parasitism	Lice (*Columbicola*) (19 taxa, 5 genera)	Birds (columbiformes) (25 taxa, 22 genera)		TreeMap 1.0	9	/	11	61
					TreeMap 2.02	11	0	9	53
						12	1	8	47
						12	2	8	59
						13	3	7	51
Page et al. (2004)	Parasitism	Lice (Phthiraptera: Ischnocera) (39 species)	Seabirds (Procellariiform: petrels, albatrosses, and their relatives) (39 species)	Subtree 1 (12P/12H)	TreeMap 2.02	9	2	2	1
				Subtree 2 (13P/13H)	TreeMap 2.02	7	0–2	5	11
				Subtree 3 (14P/14H)	TreeMap 2.02	7	2	6	15
				Subtree 4 (9P/9H)	TreeMap 2.02	5	3	3	1
Weckstein (2004)	Parasitism	Lice (5 subtrees of 1 species)	Birds (Toucans, 11 species)		TreeMap 1.0	1	/	3	17–22
Percy et al. (2004)	Parasitism	Insects (legume-feeding Psyllids) (46 species)	Plants (Genistae) (44 species)		TreeMap 1.0	16	0	29	220
Subbotin et al. (2004)	Parasitism	Nematodes (gall-forming; Anguinidae) (21 taxa)	Plants (Grasses) (16 taxa)	126 solutions	TreeMap 1.0	12	1–4	4–6	
Skerikova et al. (2001)	Parasitism	Cestode (*Proteocephalus*) (7 taxa)	Fishes (7 taxa)	3 solutions	TreeMap 1.0	3	/	0–1	1–4
Desdevises et al. (2002)	Parasitism	Cestode (*Lamellodiscus*: Monogenea, Diplectanidae) (20species)	Fishes (Teleostei, Sparidae) (16 species)		TreeMap 1.0	5	/	14	62
Piercey-Normore and DePriest (2001)	Mutualism	Algae	Fungi 33 taxa	9–94 solutions	TreeMap 1.0	10–11	3–5	7–9	65–81
Hugot et al. (2001)	Parasitism	Arenavirus (5 species)	Rodent (11 species)	10 solutions	TreeMap 1.0	2–4	0–2	0–2	4–11
Jackson and Charleston (2003)	Parasitism	RNA viruses (15 Avian sarcoma leucosis virus, ASLV)	Birds (Galliform) (13 species)	66 solutions	TreeMap 2.0	7	3	4	9

often appear to be species-specific, but these myzostomids often infest more than one crinoid species when viewed on a wider geographical scale (Eeckhaut et al., 1998). For instance, *Myzostoma fissum,* which is found in the whole Indo-West Pacific Ocean, has only been recorded on *Dichrometra flagellata* in Hansa Bay (Papua New Guinea), where 25 crinoid species co-occur, while it is sometimes observed on other species (mainly on Mariametridae) in other regions (Eeckhaut et al., 1998). Such opportunistic myzostomids would be prone to speciation if the host species become separated (Fig. 6B).

Speciation during duplication (Fig. 6C) is probably more difficult to imagine and to explain because genetic isolation of batches of myzostomid individuals on one crinoid species should be less feasible. However, myzostomids often show a preferred location on crinoids, e.g., the pinnules or the calyx (Lanterbecq et al., 2006). At a large geographical scale, batches of individuals of a given myzostomid species might show such preferences that could eventually lead to speciation if the preferred location is maintained through time (Fig. 6C).

4.5. Comparison with other models

Based on the present results, it appears that the crinoid–myzostomid system is a good model of marine cophylogeny involving commensals. It is difficult to compare the present results with those presented in literature, which involve exclusively parasites or mutualists, because (i) the number of hosts and symbionts used in the various existing studies is extremely variable, and (ii) the taxonomical range of symbionts and hosts is also extremely different from one study to another (Table 5). In the first instance, the number of cospeciation and host-switches is generally not extremely dissimilar in models involving parasites, mutualists or commensals (Table 5). For example, in a study of the mutualistic association involving algae and 33 fungi in lichens, Piercey-Normore and DePriest (2001) recorded 10–11 cospeciations and 3–5 host-switches. Yet, in a study involving the parasitic louse (19 species) and birds (25 species), Johnson et al. (2003) observed at most 13 cospeciations and 3 host-switches. At the extreme opposite, a notable exception is the Sparidae (Teleostei)-Diplectanidae (Monogenea) system, where no cospeciation occurs, but where host-switch of flukes from fish to fish is the main driving force leading to speciation (Desdevises et al., 2002). In most of the host–parasite systems analyzed cophylogenetically, speciation of hosts was revealed to be an important driving force of parasite speciation. The more specialized a parasite is, the more cospeciation events should occur and the more a parasite should mirror its host phylogeny. Pocket gophers (Geomyidae) and lice (Trichodectidae) association is a good example of strict cospeciation (Hafner and Nadler, 1988; Hafner and Page, 1995; Hafner et al., 1994; Page and Hafner, 1996). However, an increasing number of studies showed that mismatches between host and parasite or co-existing species are common (Charleston and Robertson, 2002; Huyse and Volckaert, 2005; Johnson et al., 2001; Weiblen and Bush, 2002). This suggests that the type of symbiosis is not sufficient to expect a cophylogenetic pattern, and that the importance of one evolutionary event on another within a host–symbiont system varies from case to case.

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