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Article type : Original Manuscript

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Phylogeny and morphological evolution of the so-called bougainvilliids (Hydrozoa, Hydroidolina)

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Running title: Evolution in bougainvilliids and allies

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Abstract

We present phylogenetic analyses (parsimony, maximum likelihood and Bayesian inference) for 69 lineages of anthoathecate hydroids based on 18 morphological characters (12 proposed for the first time) plus mitochondrial (16S and COI) and nuclear (18S and 28S) molecular markers. This study aims to test the monophyly of the present concept of the family Bougainvilliidae, assessing its phylogenetic position within Hydroidolina. Our working hypothesis is used as a context for inferring the evolution of certain morphological characters, focusing on the exoskeleton. Our results shed light on some phylogenetic uncertainties within Hydroidolina, delimiting eight well-supported linages, viz. Hydroidolina, Siphonophorae, Leptothecata, Aplanulata, Filifera II, Filifera III, Capitata, and Pseudothecata *taxon novum*, the latter supported by four morphological synapomorphies. The monophyly of several families was not supported, viz. Bougainvilliidae, Cordylophoridae, Oceaniidae, Rathkeidae, and Pandeidae. Some of the genera typically considered in Bougainvilliidae, including *Bougainvillia*, fell into the clade Pseudothecata, which is consistently reconstructed as the sister group of Leptothecata. We formally suggest that *Dicoryne* be removed from Bougainvilliidae and placed in the resurrected family Dicorynidae. The exoskeleton was a key feature in the diversification of Hydroidolina, especially with the transition from the bare hydranth to one completely enveloped within the exoskeleton. In this context, bougainvilliids exhibit several intermediate states in development of the exosarc. Although the concatenated analysis unravels some interesting hypotheses, taxon sampling is still deficient and therefore more data are necessary for achieving a more complete understanding of the evolution and ecology of bougainvilliids and their allies.

Introduction

A complex and important, but often overlooked, aspect to the natural history of Medusozoa is the development and evolution of the exoskeleton (e.g., Mendoza-Becerril *et al* 2016, 2017). In this context, some taxa have special relevance, but have yet to be explored in depth. This is the case for the hydrozoan family Bougainvilliidae Lütken, 1850, classified in the non-monophyletic order “Anthoathecata” within the subclass Hydroidolina (cf. Cartwright *et al* 2008; Schuchert 2015a). As a reference for the present concept of the family Bougainvilliidae, we follow the diagnosis by Calder (1988) and the classification

proposed by Schuchert (2007, 2012). Under this concept, bougainvilliids are widely distributed latitudinally (Mendoza-Becerril & Marques 2013), and include one genus from freshwater (*Velkovrhia* Matjašiĉ & Sket 1971 – cf. Schuchert 2007). Like many other members of Hydroidolina, bougainvilliids have two phases in their life cycles, an asexual polyp fixed to a substrate and sexual phase varying from free-swimming medusa to a gonophore that remains attached to the polyp (Russell 1953). Insufficient understanding among early naturalists of linkages between these phases resulted in a dual classification, in which the two different expressions of a single species often received separate names. For example, medusa-based species described in the genus *Bougainvillia* Lesson, 1830 were independently named as polyp-based species in the genus *Perigonimus* M. Sars, 1846. As a consequence, *Bougainvillia* is the senior synonym for six other genera (see Calder 1988; Schuchert 2015b). These taxonomic obstacles associated with morphology and different forms during the life cycle might be one of the causes for the scarcity of phylogenetic analyses of Hydrozoa based on morphology (Petersen 1990; Peña Cantero & Marques 1999; Marques & Migotto 2001; Marques *et al* 2006).

Morphology is relatively simple in most species of Bougainvilliidae, and useful characters for classification are hard to define (cf. Lütken 1850; Mayer 1910: 131; Fraser 1944: 47; Russell 1953: 143-144; Kramp 1961:74; Vannucci & Rees 1961: 57-58; Millard 1975: 88-91; Calder 1988: 12-13; Schuchert 1996: 27, 2007: 196-197). Even basic information, like defining the limits between Bougainvilliidae and other presumably related families (e.g., Cordylophoridae, Cytaeididae, Oceaniidae, Pandeidae, Russelliidae) is not an easy task (Millard 1975; Calder 1988; Schuchert 2007, 2012).

Taxonomic studies of Bougainvilliidae have tended to be limited to certain taxonomic levels and/or restricted to geographic areas. The most complete classification to date of Bougainvilliidae separates it into four subfamilies (Bimeriinae, Bougainvilliinae, Pachycordylinae, Rhizorhagiinae) based on polyp morphology, including characters like the coverage extent of the pseudohydrotheca, shape of the hydranth and hypostome, tentacle arrangement and position, and the type of gonophore (Calder 1988). Nevertheless, the current classification does not divide the family into subgroups (Schuchert 2007, 2012).

Studies using molecular data for bougainvilliids, for instance, are rare (e.g., Schuchert 2007) or dispersed in broader analyses for hydrozoans (e.g., Collins *et al* 2006; Cartwright *et al* 2008; Maronna *et al* 2016). Availability of molecular data for the family yielded the hypothesis that Bougainvilliidae falls within Gonoproxima, a clade defined by the position of gonophores, i.e., arising from hydrocauli, pedicels, or stolons rather than the

hydranth body (Cartwright *et al* 2008). Branch support for this result was low and thus monophyly and the position of the family within "Anthoathecata" remain ambiguous (Cartwright and Nawrocki 2010; Schuchert 2012; Maronna *et al* 2016).

The pseudohydrotheca, an external covering with or without detritus that is wrapped around the hydranths (Allman 1871; Calder 1988), classically has been used in the taxonomy of Bougainvilliidae and other anthoathecates. However, studies of the histology and development of the exoskeletal system of Hydroidolina, including the hydrotheca, are recent. These studies described the pseudohydrotheca as part of an extensive outer layer (the exosarc) in a bilayered exoskeleton (Mendoza-Becerril *et al* 2016, 2017). This external layer (the exosarc) and the chitin-proteic layer (the perisarc) have important implications for both evolution and ecology (Mendoza-Becerril *et al* 2016, 2017).

Exoskeletal origin, morphological and chemistry variation also have received little attention in a phylogenetic context for Hydrozoa. Few studies with evolutionary hypotheses have used exoskeletal features (e.g., Petersen 1990, their characters 15, 17 and 22; Marques & Migotto 2001, characters 5, 14, 32, 35, 38 and 40; Puce *et al* 2016, characters 1, 2, 4, 5). Despite attempts to reconstruct morphological characters using phylogenetic hypotheses, these characters have not been used to test phylogenetic inferences (cf. Cartwright & Nawrocki 2010; Miglietta & Cunningham 2012), and exoskeletal characters have seldomly been listed (Miglietta *et al* 2010).

In this study we use morphological and molecular data to discuss the monophyly and phylogenetic position(s) of Bougainvilliidae in the context of several lineages of anthoathecates – we do not intend to provide a broad hypothesis for the whole anthoathecates or hydrozoans. Our analyses allowed us to examine scenarios for exoskeletal evolution using this type of data for the first time.

Material and Methods

Terminal groups and characters

Our analysis includes 70 species, comprising 10 Bougainvilliidae (coverage of 10% of the species, 47% of the genera), 5 Capitata, 3 Aplanulata, 3 Siphonophorae, 28 other "Filifera", and 16 Leptothecata, all taxa included to provide a framework to infer the phylogenetic placement of the bougainvilliids. The species included in the analysis were

defined based on DNA availability as well as detailed morphological descriptions available in the literature, specially concerning their exoskeletons. Five species of Trachylina (monophyletic sister group to Hydroidolina) were used to root the trees (Collins *et al* 2006; Cartwright & Nawrocki 2010).

The morphological matrix followed Cartwright & Nawrocki (2010), but recoding their characters 2 and 3 as our characters 2-3 and 5-6, respectively. Character 4 was also recoded. We added 12 new morphological characters (7-18) based on well-defined species descriptions (Calder 1988; Cornelius 1995a, b; Schuchert 2010, 2012; Mendoza-Becerril *et al* 2017). Therefore, the final matrix includes 11 binary morphological characters and 7 multistate, non-additive, characters (SMA).

For the molecular analysis we used two mitochondrial (16S and COI) and two nuclear (18S and 28S) genes from sequences available in GenBank (SMB11). Of these 37 were recently published by members of Marine Evolution Laboratory.

Phylogenetic Analysis

DNA sequences were aligned using the E-INS-i strategy of software MAFFT v6 (Katoh & Toh, 2008). Ambiguously aligned regions at the 5' and 3' ends were removed using Gblocks v0.91b (Castresana 2000; Talavera & Castresana 2007) based on parameters that permit smaller final blocks and less rigor in the position of the gap with the final, end blocks. The alignments were published in FigShare with DOI: 10.6084/m9.figshare.5841981

We carried out two phylogenetic analyses. The first using only the molecular matrix with the 28S, 18S, 16S, and COI markers. The second combined the molecular with the morphological matrices. Sequences were concatenated using SequenceMatrix (Vaidya *et al* 2010). Most taxa have sequences for all markers, but some are chimeras, and only taxa with a minimum of 50% of the markers and 1,133 bp were used (Tables SMB1, SMB2). All characters were assumed to be non-additive and unweighted. Analysis criteria were as follows:

Parsimony (P). Matrices were processed using TNT (Goloboff *et al* 2008) and analyzed using the New Technology algorithm with Max. Trees = 10000, Random addition sequence = 1000, Ratchet = 100 interactions with 20 trees taken from each, with upweighting and downweighting probabilities at 4%, Drifting = 100 cycles, Tree fusing = 100 runs. Gaps were considered as a fifth state. Branch support was estimated in TNT with

bootstrap based on 100 replicates and by Bremer support (Bremer 1988, 1994) by the retention of suboptimal trees with at least 25 extra steps, obtained by random addition sequence (1000 replicates, 10 trees retained per replicate) and Tree-Bisection-Reconnection (TBR). Parsimony analyses resulting in more than one most parsimonious tree were summarized using strict consensus tree implemented by TNT.

Statistical analysis – Phylogenetic patterns were compared among the results of the different methods, detecting the insensitivity of the taxa and differences in the support index. Models of nucleotide evolution for each gene (Table SMB2) were estimated using jModelTest 2.1.1 (Darriba *et al* 2012).

Maximum likelihood (ML) analysis was carried out on the molecular character matrix only in RaxML 7.0.4 (Stamatakis 2006). The substitution model applied for three markers (16S, 18S and 28S) was GTR+GAMMA+I and the model GTR+GAMMA for COI maker). Separate nucleotide substitution models were applied to different genes in a partitioned dataset, thereby avoiding artifacts due to differential gene evolution rates (Keiner & Ianfer 2015). Support was inferred through bootstrap (500 runs).

Bayesian inference (BI) analysis was also carried out on the molecular matrix only, in Mr. Bayes v3.2.4 (Huelsenbeck & Ronquist 2001) using the same partition strategy. We ran an analysis for 5 million generations, and two independent runs were generated, each consisting of four chains of Metropolis-Coupled Markov (MCMC) beginning with a random tree. Chains were sampled every 1000 generations. Convergence of the parallel runs was determined by examining average standard deviation of split frequencies, which fell below 0.01. Posterior parameters and output from the Bayesian analysis were examined in Tracer 1.5 (Rambaut & Drummond 2007), in which the first 25% of the sampled trees were excluded as burn-in, and the remaining 75% trees were used to construct a 50% majority rule consensus tree, representing clade's posterior probability.

Phylogenetic information potential (informative sites for parsimony) of the sequences for each marker was calculated in MEGA 6.0 (Kumar *et al* 2001), and in TNT for morphological characters. Topologies were compared using the distance between trees (SPR) in TNT, which calculates the minimum number of SPR changes to transform one tree into the reference tree (Goloboff 2008).

Reconstructing ancestral characters. Ancestral states of 18 characters were reconstructed using maximum likelihood and parsimony criteria using Mesquite 2.75 (Maddison & Maddison 2011), based on the molecular topologies obtained by maximum likelihood and parsimony, respectively. Although for parsimony most characters of inner branches were

clear, ambiguities were optimized using ACCTRAN with WINCLADA 1.00.08 (Nixon 1999-2004; see Agnarsson & Miller 2008; Gainett *et al* 2014). We did not consider reconstructions based on hypotheses with spurious inner branches and inapplicable data (cf. Agnarsson & Miller 2008). Consistency index (CI), rescaled consistency index (RC), homoplasy index (IH) (Kluge & Farris 1969; Farris 1989), and retention index (RI, Farris 1989) were calculated for morphological characters and molecular markers.

Results

Phylogenetic hypotheses

We found four equally parsimonious topologies (L=19,933 steps) for molecular markers (Table SMB2). The consensus of these topologies had 10 lineages with bootstrap values > 70 and Bremer > 8 (Fig. 1; Table SMB3). Eudendriidae had lower support values even though its monophyly is supported by several autapomorphies (cf. Marques *et al* 2000; Marques 1996, 2001). Both ML and BI analyses found the same clades with similar and strong support (bootstrap) (Figs. 2, SMC1; Table SMB3).

Some phylogenetic topologies differ between them depending on which method (P, ML, BI) was used, but P and ML were more congruent with previous hypotheses (e.g., Collins *et al* 2006; Cartwright *et al* 2008; Maronna *et al* 2016), with an SPR distance of 8 between them (Table SMB4). Topologies due to P and ML include a well-supported clade of filiferans as sister group to Leptothecata, which we call Pseudothecata *taxon novum* (Figs. SMA1, 2). However, Pseudothecata + Leptothecata has weak support with P and ML.

The parsimony analysis of the combined matrix (molecular and morphological) resulted in eight equally parsimonious trees (20,356 steps). The strict consensus included the same 10 lineages as in the molecular analysis, but with one large basal polytomy, in which only one group was defined, Pseudothecata + Leptothecata (Fig. SMC2). Upon using first-order jackknife, we found that the exclusion of the character exosarc covering the hydranth (character 18) did not change the topology in comparison with that obtained by molecular markers (Fig. 1). Optimization of character 18 resulted in a homoplastic pattern among Hydroidolina, but it proved to be useful to resolve some less inclusive taxa. We adopted the molecular most parsimonious hypotheses as the working model because it is consistent with the optimization method used.

Monophyly was supported for Hydroidolina, Siphonophorae, Leptothecata, Aplanulata, Filifera III (*sensu* Cartwright *et al* 2008), and Capitata (Fig. 1). With the exception of Eudendriidae (=Filifera I in Cartwright *et al* 2008), less inclusive clades of filiferans, such as the current concepts of Bougainvilliidae, Cordylophoridae, Oceaniidae, Rathkeidae, and Pandeidae, were non-monophyletic (cf. Cartwright *et al* 2008; Schuchert 2012). Pseudothecata *taxon novum* is divided into two well-supported clades that reject traditional hypotheses: clade A includes only Oceaniidae and clade B includes Bougainvilliidae (except *Dicoryne conybearei* (Allman, 1864), whose position remains ambiguous), Cordylophoridae and Rathkeidae. Clade B comprises two lineages: C [*Koellikerina fasciculata* (Péron & Lesueur, 1810), *Podocorynoides minima* (Trinci, 1903), *Bougainvillia carolinensis* (McCady, 1859), and *Bougainvillia fulva* Agassiz & Mayer, 1899] and D [*Nemopsis bachei* L. Agassiz, 1849, *Garveia grisea* (Motz-Kossowska, 1905), *Cordylophora caspia* (Pallas, 1771), *Bimeria vestita* Wright, 1859, *Bougainvillia muscus* (Allman, 1863), *Pachycordyle michaeli* (Berrill, 1948), and *Pachycordyle pusilla* (Motz-Kossowska, 1905)]. Genera in Bougainvilliidae with more than one species were monophyletic with the exception of *Bougainvillia* (Fig. 1).

Reconstruction of ancestral morphological character states

Sixteen out of the 18 morphological characters were informative for parsimony, although including some homoplasy (Tables SMA1, SMB5, Table 1). Uninformative characters for parsimony analysis were also used for character reconstruction due to their relevance in defining less-inclusive groups. Each of the statements on character evolution below are hypothetical, dependent upon our working hypothesis of phylogeny, character coding, and taxon sampling.

For completeness, we reconstructed ancestral states with ML and P criteria (Table 1; SMD, E). P results have fewer ambiguities, but differ in 33.83% from ML results, considering the 11 clades and a total of 198 ancestral states. Additionally, both methodologies have the same result for 17 out of the 18 characters for Pseudothecata *taxon novum*. Therefore, we opt to adopt the parsimonious results to discuss of reconstruction of ancestral morphological characters states.

Discussion

Phylogenetic relationships

Although progress is being made, the phylogeny of Hydroidolina remains uncertain in many respects. Some uncertainty stems from incongruences related to the variety of data sets and algorithms employed (Lemmon *et al* 2009; Simmons & Goloboff 2013). To explore different possibilities is interesting to raise new perspectives, especially under a limited number of characters. For example, when gaps are considered as a fifth character state, internal nodes and some novel hypotheses of relationships within Hydroidolina are revealed, such as Capitata plus the "Filifera" group of *Rhizogeton nudus* Broch, 1910, *Lizzia blondina* Forbes, 1848 and *Rathkea octopunctata* (M. Sars, 1835), contrasting with other inferences not using gaps as a source of information (cf. Cartwright *et al* 2008; Kayal *et al* 2015; Maronna *et al* 2016). Ultimately, more character-rich analyses will be needed to assess the phylogenetic hypotheses raised here and in earlier studies of hydroidolinan phylogeny.

Our results support monophyly in Hydroidolina, Siphonophorae, Leptothecata, Aplanulata, Filifera III, and Capitata (Cartwright *et al* 2008; Cartwright & Nawrocki 2010; Nawrocki *et al* 2010, 2013; Kayal *et al* 2015; Maronna *et al* 2016). However, some discrepancies remain with respect to the position of some "Filifera", such as *Hydrichthella epigorgia* Stechow, 1909, which here is (perhaps spuriously) close to Eudendriidae. Also somewhat surprising, many species that previously were ascribed to Gonoproxima (Cartwright *et al* 2008) are herein included in Pseudothecata (and supported by characters such as oral tentacles in two or more close-set whorls and exosarc on the hydranth). Thus, monophyly of Gonoproxima is not supported.

Another interesting hypothesis is the sister group relationship of Pseudothecata and Leptothecata. Members of these groups have skeletal characters that, if related, may imply an evolutionary series from an uncovered (no exoskeleton) hydranth (e.g., Capitata) to a rigid and laminar exoskeleton (Leptothecata). The gap between these states could be the intermediate stage with exosarc (e.g., Bougainvilliidae). If so, Bougainvilliidae may be related to Haleciidae (Stechow 1909), or more directly to Leptothecata (Maronna *et al* 2016). However, because Bougainvilliidae is non-monophyletic, we refrain from making strong inferences about its phylogenetic relationship with other groups – we understand that a better taxon sampling of bougainvilliid terminals is still needed before redefining Bougainvilliidae.

Exosarc on the hydranth (= pseudohydrotheca) is possessed by species presently classified as "Bougainvilliidae" and other families, sometimes considered to be related

(Rees 1956; Calder 1988; Cartwright *et al* 2008). Our results suggest that the exosarc is widespread within "Filifera" and that the trait, when covering the hydranth, would not be diagnostic for family-level taxa (Table 2). In fact, "Bougainvilliidae" needs to be redefined, with the inclusion of more taxa and a variety of types of data, such as medusa morphology, considered. This is even more important given that the distinctive features in filiferan polyps, such as dimensions and number of tentacles, depend on developmental stage and ecophysiological conditions in which the colony was sampled (Prudkovsky 2012; Prudkovsky & Neretina 2016). Other characters associated with a general definition of Bougainvilliidae, such as the arrangement of the oral tentacles and the position and development of the gonophores, are also inconsistent with recent molecular analyses that place some species of Cytaeididae as close relatives of species of "Bougainvilliidae" (Prudkovsky *et al* 2016).

Some species and genera have also to be redefined, such as the non-monophyletic *Bougainvillia*, originally proposed for medusae (Lesson 1830), which are easily recognized when mature (Vannucci & Rees 1961). Relationships based on the hydroid stage are even more complicated due to strong similarity with other genera (Millard 1975; Calder 1988). Hydroids without gonophores or sufficiently developed medusa buds are difficult to identify. Also, the diagnostic exosarc covering the hydranth is influenced by contraction of the organisms (Schuchert 2007; Mendoza-Becerril *et al* 2017) and environmental conditions (Vannucci & Rees 1961; Mendoza-Becerril *et al* 2017).

Dicoryne is another genus presently classified as a member of Bougainvilliidae due to the presence of exosarc on the hydranth, the gonophore (plesiomorphic), trophosome similar to *Bougainvillia*, and similarity of gonadal development with *Bougainvillia superciliaris* (L. Agassiz, 1849) (Ashworth & Ritchie 1915). However, both our topology and previous works (e.g., Cartwright *et al* 2008; Prudkovsky *et al* 2016) contradict a hypothetical bougainvilliid nature of *Dicoryne*. In fact, *Dicoryne* has unique characters, not shared with Bougainvilliidae and Pseudothecata (e.g., gonophores on specialized blastostyles and swimming ciliated sporosacs; Schuchert 2007). We therefore suggest that the genus be removed from Bougainvilliidae and instead be placed within a resurrected family Dicorynidae Allman, 1864.

Reconstruction of ancestral characters

Character evolution is likely tied quite closely to the ecology of different hydrozoan lineages. The character medusa, for instance, varies widely across the group and directly influences biotic interactions with planktonic and benthic communities, as well as its obvious impact on dispersal. The medusa stage is considered to be ancestral in Medusozoa (cf. Marques & Collins 2004; Van Iten *et al* 2006; Cartwright & Nawrocki 2010 – for an alternative view see Salvini-Plawen (1987) with many losses or transformations in non-swimming stages (Leclère *et al* 2007; Maronna *et al* 2016). Reconstruction of the character on the working hypothesis we adopt surprisingly suggests a transformation for the expression of sporosac in Hydroidolina, and later reverted back to the medusa stage (Fig. SMD1, character 1) one or more times. In Pseudothecata, there could be three reductions, or one with re-evolution of medusa, or two reductions and one re-evolution. Cunningham & Buss (1993) and Miglietta & Cunningham (2012) argued that reversals to medusae would be less likely than multiple independent reductions (Miglietta & Cunningham). This hypothesis deserves further study, but may shed light on alternatives to the classical acceptance of an universal ancestral medusa.

Coloniality, another fundamental character for understanding the evolutionary history of hydrozoan lineages, is also influenced by the presence of a medusa stage (Fig. SMD1, character 2). Modular (colonial) species are found in a variety of cnidarian groups, especially in Anthozoa, and seems to be ancestral for the phylum, and one might think it likely that it is also ancestral for Hydrozoa. However, our analysis supports the hypothesis that a solitary polyp stage was ancestral for hydroids (Rees 1957). Indeed, the loss of coloniality may be underestimated in Hydroidolina (cf. Maronna *et al* 2016; Cunha *et al* 2017) because there are several solitary "Anthoathecata" that were not included in out analysis (e.g., Halimedusidae, *Brinckmannia hexactinellidophila* Schuchert & Reiswig, 2006; Cartwright & Nawrocki 2010). Our analysis supports the hypothesis that a solitary polyp stage would be the ancestral for hydroids (Rees 1957).

Tentacle type has long been used as a character to separate anthoathecates into "Filifera" and Capitata, even though the "filiform" state has long been thought to be plesiomorphic (Petersen 1990). The results of our phylogenetic analysis support the hypothesis that the capitate state is apormorphic, but homoplastic between two groups (Fig. SMD4 character 7). In contrast to past hypotheses, our results do not support scattered tentacles along the body as ancestral for Capitata and "Filifera" (Fig. SMD5, character 9; Rees 1957; Millard 1975; Petersen 1979, 1990).

The relationship between Pseudothecata and Leptothecata is supported by the colonial organization of the polyp stage (character 3), gonophore position (character 4), chitinous skeleton type (character 14), and exosarc covering hydranth (character 18) (4 of 18 morphological characters), indicating a general hypothetical morphology for the ancestor of the most diversified clade in Medusozoa. Gonophores on the hydrocaulus, two or more close-set whorls of tentacles, connections between the skeletal layers (perisarc and exosarc), and the complete covering of the hydranth at least by exosarc would be ancestral for Pseudothecata. The gonophore position had used to place bougainvilliid lineages in Gonoproxima (Cartwright *et al* 2008), but this clade did not have significant support when it was introduced, and so it is perhaps not too surprising that it is contradicted here.

Exoskeletal development in Hydroidolina came about through a series of systems as demonstrated in studies of chitin, GAGs and other components of the skeletal system (Wagner 1994; Miglietta *et al* 2010; Mendoza-Becerril *et al* 2016; Puce *et al* 2016). The first is the molecular synthesis system (MSS), which includes the biosynthetic mechanism to produce the molecules. The second system is the molecular matrix (MM), which organizes components of the first system at or above the epidermis. Finally, the third system is the morphological expression (ME), which refers to the structure of the exoskeleton itself, resulting from interactions between the environment and the first two systems.

Patterns of exoskeleton character reconstruction corroborate previous phylogenetic hypotheses (Mendoza-Becerril *et al* 2016) for the skeletal system of Hydroidolina (the most complex among Medusozoa) (Fig. 3). There would have occurred multiple and independent origins of skeletal types, with some possible transitions among them. The ancestral state of the MM system in Hydroidolina comprises aminopolysacharides (AP), glycoproteins (GP), and glucosaminoglycans (GAGs), while ME is a single structure covering polyp base and hydrocaulus (Fig. 3).

GAGs and APs in basal groups of Hydroidolina (e.g., Aplanulata) may indicate a transition towards the development of a rigid exoskeleton (e.g., chitin or calcium carbonate). Exoskeletons with greater AP and lower GP concentrations (typical of solitary species) are soft and may easily be lost. A greater GP may be the base for the formation of anchoring filaments, representing a possible step in the process of chitinization of the exoskeleton and favoring coloniality (Vervoort 1966). In calcareous exoskeletons, increasing AP functions as an organic matrix for calcium deposition (Vervoort 1966), a pattern expressed at least twice in Hydroidolina. This feature may have clear phylogenetic

basis, such as the calcareous skeleton acquired by the ancestor of Stylasteridae that is maintained throughout its descendants (Puce *et al* 2016).

The chitin exoskeleton was apparently lost in some species of "Filifera" (e.g., *Corymorpha nutans* M. Sars, 1835, as well as in species with calcareous exoskeletons of Filifera III and Capitata). This loss of the ME does not strictly imply the lack of an MSS for production of chitin or other sources of exoskeletal development. Hydractiniidae (e.g., *Hydrissa sodalis* (Stimpson, 1859), *Hydractinia symbiolongicarpus* Buss & Yund, 1989, *Schuchertinia conchicola* (Yamada, 1947), *Podocoryna hayamaensis* Hirohito, 1988) have grains of calcium carbonate even without production of a calcareous exoskeleton (Miglietta *et al* 2010). Similarly, the loss of chitin does not imply the loss of the MSS for chitin, because chitin is expressed in a variety of ways in different Metazoa and Medusozoa (cf. Wagner 1994; Mendoza-Becerril *et al* 2016). Thus, these lines of evidence suggest that the ancestral MSS is conserved, with independent modifications in Hydroidolina. In light of our most robust phylogenetic hypothesis. Calcium carbonate and chitinous exoskeletons (internal or external) are found in terminal Capitata and Filifera III. Character optimization suggest that the internal ME in the form of a disc arose independently in the Siphonophorae (as internal chitinous layer, pneumatophore) and Capitata, and the internal anastomosed ME in some Capitata may have originated subsequently (Fig. 3).

The clade Pseudothecata + Leptothecata has an exoskeletal structure restricted to chitin with a GAG base, which is not externally evident in some Oceaniidae and Leptothecata. However, they may be present in the MSS and MM, as suggested due to the GAGs in *Clytia gracilis* (Sars, 1850) and early developmental phases in *Turritopsis* sp. (cf. Mendoza-Becerril *et al* 2017). AP and GP production increases and GAG production in the MM decreases in Leptothecata, resulting in the development of a rigid ME (albeit with varying levels of cover; Fig. 3). The different expressions of the exoskeleton in Pseudothecata and Leptothecata may have the same origin, although accumulating peculiarities during the evolution of each clade.

Our inferences here are the first approximation of broadly distributed and obscure patterns, and we offer alternatives for further avenues of study. Among these, we highlight studies on (1) the origin of Hydroidolina; (2) the developmental mechanisms at the transitions of the skeleton from granules of calcium carbonate, GAGs or other kinds of skeletons; (3) to test whether there is an interruption in the expression of the ancestral MSS or the genetic capacity for the production of some skeletal components was lost; (4)

evolutionary and ecological implications of the extent of coverage of the polyp; (5) type of exoskeletal structure. We are aware about the caveats of taxon sampling in our phylogenetic inference, but an integrated analysis of morphology and molecular data is lacking among cnidarians, therefore making this tentative also important for future developments towards understanding the evolution of the group.

Acknowledgments

We thank Amanda F. Cunha and Maximiliano M. Maronna for their help and provided sequences for this study, our colleagues at the Marine Evolution Laboratory Molecular Evolution Laboratory at the University of São Paulo (USP). Peter Schuchert, Thaís P. Miranda, Adriana Morales and Lucília S. Miranda read previous versions of the manuscript and contributed with constructive criticisms and suggestions. James J. Roper helped with the English version of the manuscript. This study was funded by a fellowship from CAPES/CNPq - IEL Nacional - Brasil (Proc. 6101100-2011), PROCAD, CNPq (Proc. 490348/2006-8, 562143/2010-6, 477156/2011-8, 305805/2013-4, 309995/2017-5, 445444/2014-2) and the *Fundação de Amparo à Pesquisa do Estado de São Paulo* (FAPESP, Proc. 2011/50242-5, 2013/50484-4). This is a contribution of the NP-BioMar (USP). The authors have declared that no conflicts of interests exist.

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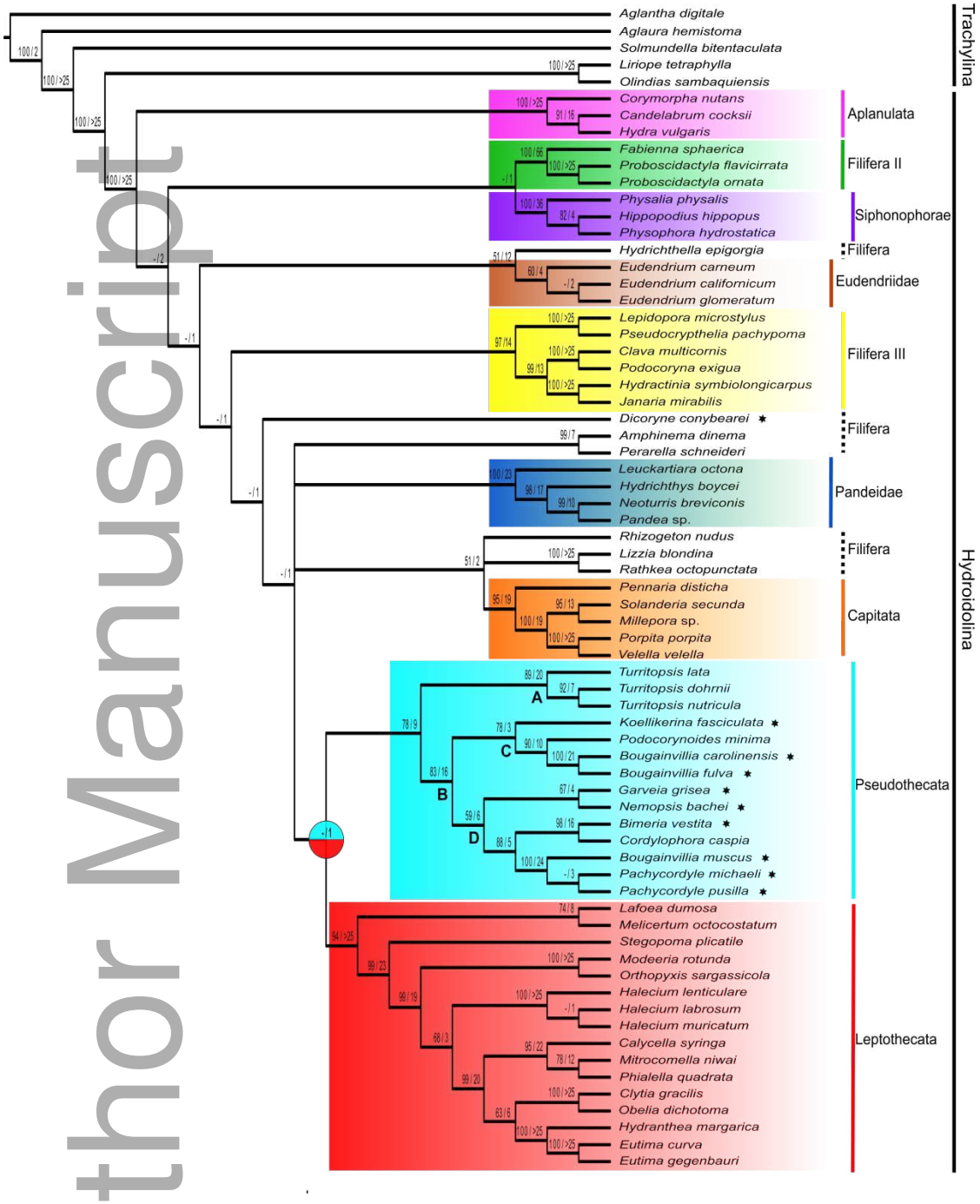

Figure 1. Phylogenetic hypothesis from parsimony analysis (P) using concatenated molecular markers (markers 28S, 18S, 16S, and COI). Numbers above the branches indicate bootstrap/Bremer values. Main monophyletic groups are separated by color. * - species classically considered to be Bougainvilliidae; - Pseudothecata + Leptothecata, sister groups; A, B, C and D, subgroups of Pseudothecata.

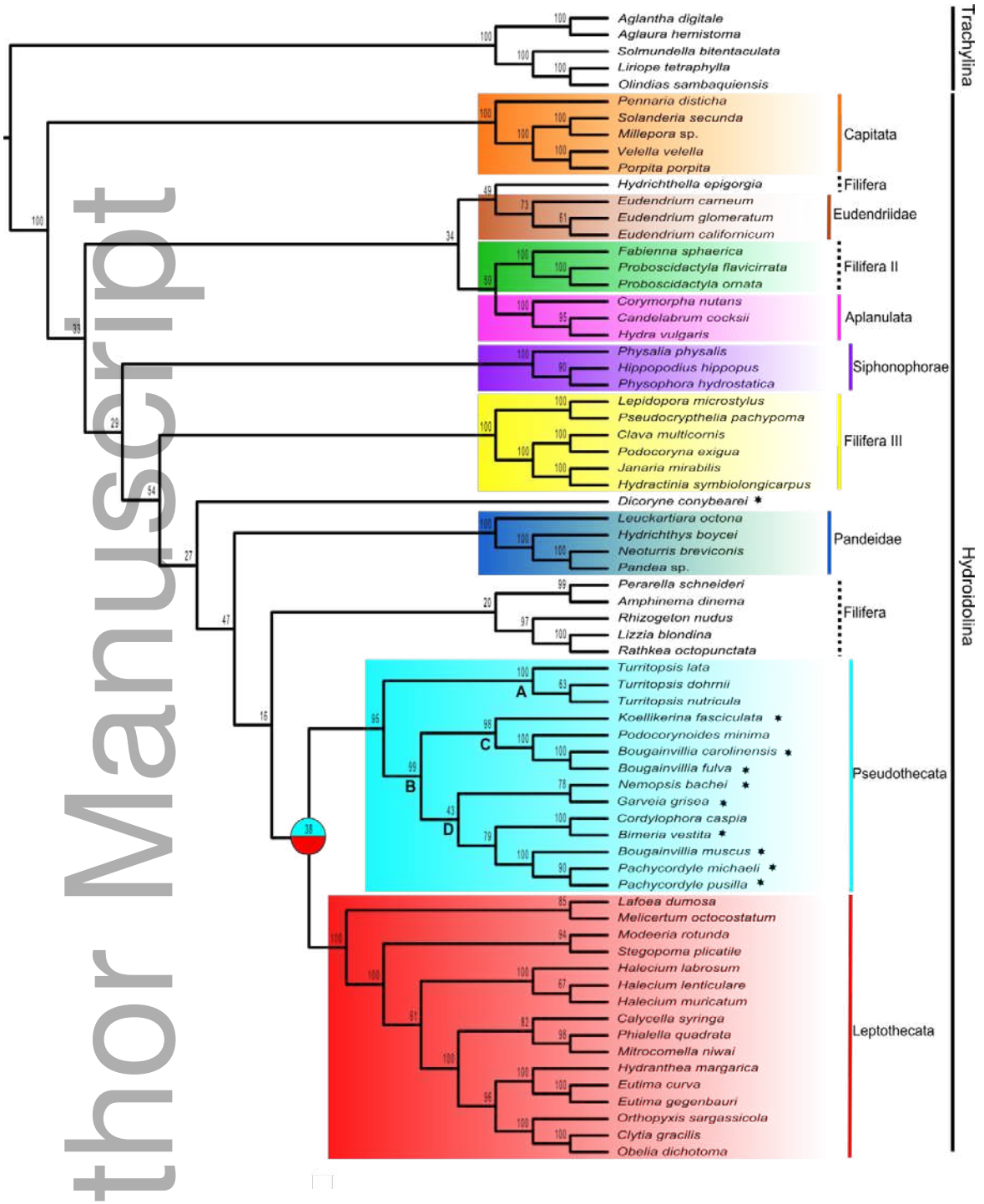

Figure 2. Phylogenetic hypothesis from maximum likelihood analysis (ML) using concatenated molecular markers (markers 28S, 18S, 16S, and COI). Numbers above branches indicated bootstrap values. Main monophyletic groups are indicated by color. * , species classically as Bougainvilliidae; , Pseudothecata + Leptothecata, sister groups; A, B, C and D subgroups of Pseudothecata..

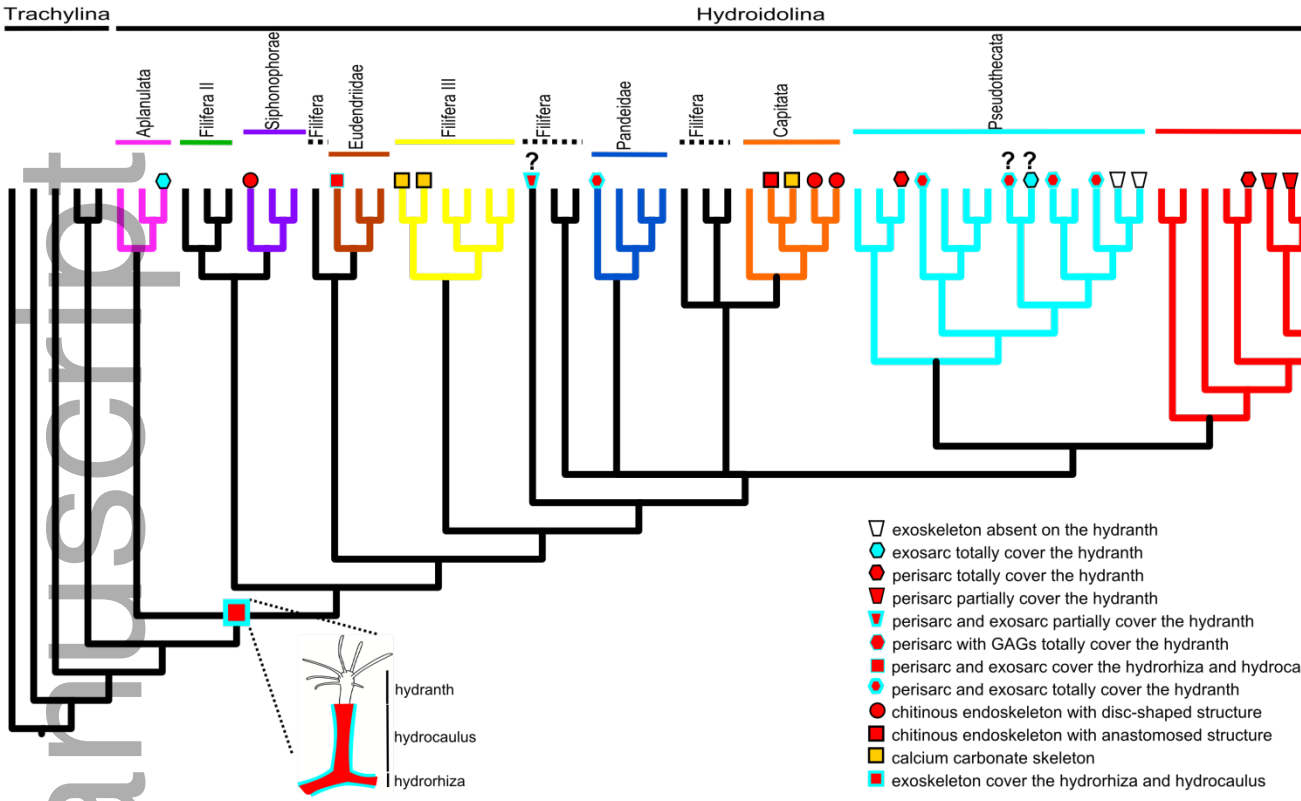

Figure 3. Evolutionary hypothesis for the skeleton within the major groups of Hydroidolina, using the molecular matrix (MM) and morphological expression (ME) of the exoskeleton. "?" indicates absence of SSE. Colors and symbols indicate some separate groups. Cyan, have some type of GAGs in the skeleton; Orange, calcium carbonate dominates in the skeleton; Red, chitin is major skeletal component.

Table 1. Reconstructions of ancestral characters using maximum likelihood (ML) and parsimony (P) criterion.

Taxon		Character Number																	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Hydroidolina	P	medusa	solitary	encrusting colony	hydrocaulus	absent	absent	filiform	absent	single whorl	external	absent	absent	absent	With GAGs	laminar	absent	absent	absent
	ML	=	colonial	=	=	present	absent	filiform	absent	=	external	absent	absent	absent	=	heterogeneous	?	absent	absent
Aplanulata	P	medusa	solitary	N	hydranth	absent	absent	filiform	absent	single whorl	external	absent	absent	absent	With GAGs	laminar	absent	absent	absent
	ML	medusa	solitary	N	hydranth	absent	absent	filiform	absent	single whorl	external	absent	absent	absent	N	N	N	N	N
Filifera II	P	medusa	colonial	encrusting	hydrocaulus	present	absent	filiform	absent	single whorl	external	absent	absent	absent	With GAGs	laminar	absent	absent	absent
	ML	medusa	?	?	?	?	?	?	?	N	?	?	absent	?	N	N	N	N	N
Siphonophorae	P	medusoid	colonial	pelagic	hydrocaulus	present	absent	N	N	N	internal	absent	absent	absent	N	N	N	N	N
	ML	medusoid	colonial	pelagic	hydrocaulus	present	absent	N	absent	N	internal	absent	absent	absent	N	N	N	N	N
Eudendriidae *	P	sporosac	colonial	upright	hydranth	present	absent	filiform	absent	single whorl	external	absent	absent	absent	With GAGs	laminar	absent	absent	absent
	ML	sporosac	colonial	upright	hydranth	present	absent	filiform	absent	single whorl	external	absent	absent	absent	?	?	?	N	?
Filifera III	P	sporosac	colonial	encrusting	ambíguo•	present	present	filiform	absent	single whorl	external	absent	absent	absent	With GAGs	heterogeneous	present	total	partial

	ML	sporosac	colonial	encrusting	hydranth	present	present	filiform	absent	single whorl	external	absent	absent	absent	N	N	N	N	N
Pandeidae	P	medusa	colonial	encrusting	hydrocaulus	absent	absent	filiform	absent	single whorl	external	absent	absent	absent	With GAGs	heterogeneous	present	total	total
	ML	medusa	colonial	encrusting	hydrocaulus	absent	absent	filiform	absent	single whorl	external	absent	absent	absent	?	?	?	?	?
Capitata	P	medusoid	colonial	upright	hydrocaulus	absent	absent	capitate	absent	scattered on no clear whorls	external	absent	absent	absent	With GAGs	heterogeneous	present	absent	absent
	ML	medusa	colonial	upright	hydrocaulus	absent	present	capitate	absent	scattered or no clear whorls	internal	absent	absent	absent	?	?	N	?	?
Pseudothecata taxon novum	P	medusa	colonial	upright	hydrocaulus	absent	absent	filiform	absent	two or more close-set whorls	external	absent	absent	absent	With GAGs	heterogeneous	present	total	total
	ML	medusa	colonial	upright	hydocaulus	absent	absent	filiform	absent	two or more closet-set whorls	external	absent	absent	absent	=	?	?	total	?
Leptothecata	P	medusa	colonial	upright	hydrocaulus	present	absent	filiform	absent	single whorl	external	absent	absent	absent	Without GAGs	laminar	present	total	total
	ML	medusa	colonial	upright	hydocaulus	present	absent	filiform	absent	single whorl	external	absent	absent	absent	?	?	N	=	N
Pseudothecata+Leptothecata*	P	medusa	colonial	upright	hydrocaulus	absent	absent	filiform	absent	single whorl	external	absent	absent	absent	Without GAGs	heterogeneous	present	total	total
	ML	medusa	colonial	upright	hydrocaulus	absent	absent	filiform	absent	single whorl	external	absent	absent	absent	=	heterogeneous	?	total	?

N, not applicable;*, weak support; •, ambiguous (hydranth/hydrocaulus/hydrorhiza);?, no resolve; =, same probability. 1, Gonophore development upon sexual maturity; 2,

Organization of the polyp stage; 3, Organization of the colonial polyp stage; 4, Gonophore position; 5, Gonozooid as a type of polyp in the colony; 6, Dactylozooid as a polyp in the colony; 7, Type of oral tentacles; 8, Aboral tentacles on gastrozooids; 9, Arrangement of oral tentacles; 10, Chitin present as skeletal structure; 11, Anastomosed skeleton; 12, Disc-shaped skeletal structure; 13, Calcium carbonate skeleton; 14, Chitinous skeleton type; 15, Morphology of perisarc and exosarc; 16, Connection between the perisarc and exosarc; 17, Perisarc covering hydranth; 18, Exosarc covering hydranth. Cells in grey indicate reconstruction using ACCTRAN optimization and two cells with possible incorrect reconstruction indicated by grey with border.

Table 2. Historical classification the groups with pseudohydrotheca, included so-called bougainvilliids. *, taxa with pseudohydrotheca; ?, Lizzia, whose polyp phase is unknown..

Study	Suborder	Superfamily	Family	Subfamily	Genus
Allman, 1864			Eudendriidae*		Bimeria*
					Bougainvillia*
					Garveia*
					Rhizorhagium*
			Dicorynidae*		Dicoryne*
			Tubulariidae		Nemopsis
Allman, 1871			Bimeriidae*		Bimeria*
					Garveia*
			Bougainvilliidae*		Bougainvillia*
			Dicorynidae*		Dicoryne*
			Nemopsidae		Nemopsis
Hickson & Gravely, 1907			Bougainvilliidae*	Bougainvilliidae*	Rhizorhagium*
				Margelinae	
				Dicorynae*	
				Eudendriinae*	
				Bimeriinae*	
Kramp, 1926			Margelidae*		Bougainvillia*
					Lizzia (?)
					Rhatkea
			Tiaridae*		Leuckartiara*
Petersen, 1979	Pandeidae	Bougainvillioidea*	Cytaeidae		
			Bougainvillidae*		
			Heterotentaculidae		

	Pandeoidea*	Pandeidae*		
Calder, 1988		Bougainvilliidae*	Bimeriinae*	Bimeria*
			Pachycordylinae	Millardiana
				Pachycordyle
				Silhoueta
			Rhizorhagiinae*	Parawrightia*
				Rhizorhagium*
			Bougainvilliinae*	Bougainvillia*
				Nemopsis
				Dicoryne*
				Garveia*
		Pandeoidea*	Pandeidae*	
Schuchert, 2007		Bougainvilliidae*		Bougainvillia*
				Dicoryne*
				Koellikerina*
				Nemopsis