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Phylogeny of Stenopodidea (Crustacea: Decapoda) shrimps inferred from nuclear and mitochondrial genes reveals non-monophyly of the families Spongicolidae and Stenopididae, and most of their composite genera

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ABSTRACT

The infraorder Stenopodidea is a relatively small group of marine decapod crustaceans including the well-known cleaner shrimps, but their higher taxonomy has been rather controversial. This study provides~~d~~ the most comprehensive molecular phylogenetic analyses of ~~the infraorder~~ Stenopodidea using sequence data from two mitochondrial (16S and 12S rRNA) and two nuclear (H3 and NaK) genes. We include~~d~~ all twelve nominated genera from the three stenopodidean families in order to test the proposed evolutionary hypothesis and taxonomic scheme of the group. The inferred phylogeny did not support the familial ranking of Macromaxillocarididae and rejected the reciprocal monophyly of Spongicolidae and Stenopididae. ~~Six out of seven genera with multiple exemplars analyzed were poly- or paraphyletic in our molecular phylogeny.~~ The~~se~~ genera ~~are~~ *Stenopus, Richardina*, *Spongiocaris*, *Odontozona*, ~~*Spongiocaris*,~~ *Spongicola* and *Spongicolodes* are showed to be poly- or paraphyletic, with the monophyly of the latter three~~four~~ genera strongly rejected in the analysis. The present results only strongly support the monophyly of *Microprosthema* and suggest that *Paraspongiola* should be synonymized with *Spongicola*. The three remaining genera, *Engystenopus*, *Juxtastenopus* and *Globospongicola*, may need to be expanded to include species from other genera if their status are maintained. All findings suggest that the morphological characters

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currently adopted to define genera are mostly invalid and substantial taxonomic revisions are required. ~~Although a number of well-supported clades were revealed in the molecular phylogeny,~~ As the intergeneric relationships were largely unresolved in the present attempt~~. Thus~~, the hypothesis of evolution of deep-sea sponge associated taxa from shallow water free-living species could not be verified here. The present molecular phylogeny, nevertheless, provides some supports that stenopoidid shrimps colonized the deep sea in multiple circumstances.

Running title: Molecular phylogeny of stenopodidean shrimps.

Additional keywords*:* shrimps, molecular phylogeny, Stenopodidea, classification.

INTRODUCTION

The infraorder Stenopodidea Claus, 1872 (Crustacea: Decapoda) is a relatively small group of marine decapod crustaceans, with 83~~2~~ species recognized to date and assigned to 12 genera (Figure 1), 3 families (Schram 1986; Goy 2010b; De Grave and Fransen 2011; Komai 2011a; Saito and Anker 2012; Anker and Tavares 2013; Goy and Martin 2013; Goy and Cardoso 2014; Saito and Anker 2014; Goy 2015; Komai 2015; Wang *et al.* 2016; Komai *et al.* in press). These small shrimps are generally uncommon and therefore not ~~a~~ commercially important ~~species~~, but several species of *Stenopus* Latreille, 1819 are popular ornamental shrimps in the aquarium trade (Calado 2008; Goy 2010b); e.g. *Stenopus hispidus* (Olivier, 1811) is well known as the barber pole or coral banded boxing shrimp. Furthermore, deep water genera of the family Spongicolidae Schram, 1986 (e.g. *Globospongicola* Komai & Saito, 2006, *Spongicola* De Haan, 1844) are famous for living as a monogamous pair entrapped in the internal cavity of deep water hexactinellid sponges (Saito and Takeda 2003; Komai and Saito 2006; Saito and Komai 2008; Goy 2010b, 2015).

Stenopodidean shrimps, though low in diversity and numbers, possess many unique characters and have long been recognized as an infraorder comparable to carideans, lobsters, crabs and anomurans~~hermit crabs~~ (see De Grave *et al.* 2009; Goy 2010b). Although of high taxonomic rank, these shrimps were generally treated under

a single family (see Holthuis 1946, 1955) until Schaarm (1986) separated them into two families with the family Stenopodidae Claus, 1872 containing mainly free living species, and the new family Spongicolidae, consistinged of mostly sponge associated species. Recently, an additional monotypic family, Macromaxillocarididae Alvarez, Iliffe & Villabobos, 2006, was created for a single anchialine cave dwelling species *Macromaxillocaris bahamaensis* Alvarez, Iliffe & Villalobos, 2006. With more genera and species discovered, currently there are four genera in Stenopodidae and seven genera in Spongicolodae (Goy 2010b; De Grave and Fransen 2011).

With the exception of *Microprosthema* Stimpson, 1860, which is a free-living shallow water inhabitant, all of the remaining spongicolid genera are symbionts with deep-sea hexactinellid sponges or octocoral (Kubo 1942; Bruce and Baba 1973; Berggren 1993; Komai and Saito 2006; Ortiz *et al.* 2007; Saito 2008; Saito and Komai 2008; Goy 2010b, 2015). In the family Stenopodidae, the most renown genus, *Stenopus,* consists of shallow water free-living species with many of them known to have fish cleaning behavior (Bruce 1976; Lewinsohn and Holthuis 1978; Goy and Devaney 1980; Goy and Randall 1986; Emmerson *et al.* 1990; Goy 1992; Calado 2008; Goy 2010b). The other three genera exhibit diverse ecological niches from shallow to deep waters and from free living, cave dwelling to association with sponges, crinoids or corals (Pretus 1990; Hendrickx 2002; Okuno 2003; Saito and

Fujita 2009; Saito and Komatsu 2009; Goy 2010b; De Grave and Fransen 2011; Komai 2011a; Goy and Cardoso 2014).

Current classification scheme of Stenopodidea is largely based on the key characters proposed by Holthuis (1993) and Goy (2010b). However, with more stenopodidean materials discovered in recent years, many key characteristics previously used for ~~generic~~ diagnosing genera~~s~~ become questionable. For instance, the number of ungues on~~at~~ the ambulatory dactyli was thought to be a constant and diagnostic character for all stenopodidean taxa~~shrimps~~. However, variations in the number of ungues were observed in the recently described species *Stenopus goyi* Saito *et al*. 2009 which has the ambulatory dactyli varied from simple to triunguiculate, and even among the pereiopods of the same specimen (Saito *et al.* 2009). Furthermore, it has been argued that *Spongicola japonicus* Kubo, 1942 and *S. cubanicus* Ortiz, Gómezx & Lalana, 1994 should not belong to *Spongicola* because they lack an exopod on the third maxilliped (Saito and Komai 2008; Goy 2015~~also see Komai *et al.* in press~~). It has been suggested that these two species, as well as *Spongicoloides koehleri* (Caullery, 1896), be transferred to *Spongiocaris* Bruce & Baba, 1973 (Saito 2008; Goy 2010b, 2015~~; also see Komai *et al.* in press~~) which seems to be morphologically intermediate between *Spongicola* and *Spongicoloides* Hansen, 1908 (Bruce and Baba 1973). Furthermore, the exopod at the second maxilliped appears to

be actually well developed and not absent in *Spongiocaris koehleri* (García Raso 1996), and therefore, closer to the definition of *Spongiocaris* than *Spongicoloides*. While de Saint Laurent and Cleva (1981) proposed to synonymize *Spongiocaris* under *Spongicoloides*, Komai et al. (in press) followed Saito (2008) in assigning *Spongicola japonicus*, *S. cubanicus* and *Spongicoloides koehleri* to *Spongiocaris*. On the other hand, the availability of more specimens for examination in the rare genus *Engystenopus* Alcock & Anderson, 1894 has resulted in the two species contained in this genus separated into two distinct genera and with *Engystenopus* (originally included in Stenopodidae) transferred to Spongicolidae (Goy 2010a).

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Only recently Saito & Takeda (2003) published the first phylogenetic hypothesis of stenopodidean shrimps. Their cladistic analysis was based on 38 morphological characters of 30 spongicolid species, with two outgroups from Stenopodidae. Results from this study revealed many genera to be paraphyletic and suggested many characters used to define genera may be invalid. They further proposed that there was a reduction in a number of morphological features (e.g. gills, armature of carapace, ~~and third~~ pereiopods and abdomen, exopods at second and third maxillipeds, setiferous organs of first pereiopod) during the evolution of deep water sponge associated taxa from more early-derived shallow water free-living lineages (Saito and Takeda 2003, also see Satio 2008).

On the other hand, molecular systematics of Stenopodidea is poorly documented, possibly attributed to many lineages being rare in nature and difficult to collect (Goy 2010b; De Grave and Fransen 2011). Only very limited species (i.e. *Stenopus hispidus* and *Microprosthema inornatum* Manning & Chace, 1990) have been studied and included in research examining the higher classification of Decapoda (Kim and Abele 1990; Ahyong and O'Meally 2004; Tsang *et al.* 2008; Bracken *et al.* 2009). Jiang *et al*. (2015) provided the first and only molecular phylogenetic attempt to elucidate the phylogenetic relationships among genera in Stenopodidae and Spongicolidae based on only one genetic marker, the mitochondrial~~,~~ 16S rRNA gene. Their topology supports the monophyly of Stenopodidae, but not Spongicolidae. However, only eight species from six genera were included in the study, limiting the scope and robustness of the results.

Due to the many unanswered questions that still remain concerning the higher classification of these shrimp, we reconstructed a comprehensive molecular phylogeny of the infraorder Stenopodidea. We generated a multi-locus phylogeny (based on four molecular markers) which included all described families and genera of the Stenopodidea. Based on the inferred phylogeny, we would like to evaluate the validity of the morphological characters that are currently applied in stenopodidean systematics and test Saito and Takeda's (2003) ~~the~~ hypothesis of deep-sea sponge

associated species were evolved from the shallow water free-living ancestors.

Materials and Methods

Taxon sampling

We included 66 samples from 31 species (including a new species of *Spongicola* going to be described in Goy in preparation) that cover all of the 12 genera from the three families, Spongicolidae, Stenopodidae and Macromaxillocarididae of Stenopodidea (Table 1). Exemplars from the other two infraorders, Caridea and Procarididea, which are considered as sister group of Stenopodidea (Tsang *et al.* 2008; Fransen and De Grave 2009; Bracken *et al.* 2010; Shi *et al.* 2012) were included as outgroup comparison. The samples were obtained from various expeditions, cruises, field collections as well as aquarium shops, and stored in ethanol (≥75%) before laboratory analysis.

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Laboratory protocol and phylogenetic analyses

Total genomic DNA was extracted from the pleopod or abdominal muscle tissue by using the commercial QIAamp Tissue Kit (QIAGEN) or QIAamp DNA Micro Kit (QIAGEN). We attempted to sequence four molecular markers, namely, the mitochondrial 12S and 16S rRNA genes, nuclear histone 3 (H3) and

sodium-potassium ATPase α-subunit (NaK). These markers have been widely applied in decapod phylogenetic analyses, including various groups of shrimps (Ma *et al.* 2009; Bracken *et al.* 2010; Li *et al.* 2011; Ma *et al.* 2011). Polymerase chain reaction (PCR) profiles and primers for the 12S, 16S and H3 loci followed those described previously (Colgan *et al.* 1998; Tsang *et al.* 2014). Novel stenopodideans specific PCR primer sets: NaK-37F (5'- CAGTCWGGCTGTCAATATGAYAA-3') and NaK-622R (5'- ACGGCGTCKGGYACRGCRGC-3') for amplifying the NaK were designed based on available sequences of different shrimp taxa in the GenBank to maximize the success rate of amplification. Successful amplicons were then purified using the QIAquick gel purification kit (QIAGEN) or QIAquick PCR purification kit (QIAGEN) according to the manufacturer's instructions. Sequencing reactions were performed using the same sets of primers and the ABI Big-dye Ready-Reaction mix kit according to the standard cycle sequencing protocol on an ABI3700 automated sequencer.

Sequences were aligned with MUSCLE (Edgar 2004), in which the default parameter settings were applied, and the results were checked manually. The sequences from the four molecular markers were first individually analyzed using maximum likelihood (ML) analyses to determine any conflict amongst the gene trees. The sequences were subsequently concatenated and partitioned by genes if the

supports for the conflicting topologies from different markers are not significant. The best-fit models of nucleotide substitution for each partition were determined using jModelTest 2.1 (Darriba *et al.* 2012). The ML analysis was implemented using RAxML 8.0.2 (Stamatakis 2014). The GTRGAMMAI model was used for all the six partitions. The gamma distribution with individual shape parameters, GTR rates, and base frequencies were estimated and optimized for each partition during the analyses. We performed 1000 bootstrap (BP) runs and searched for the ML tree with the highest score. Bayesian inference (BI) was conducted using MrBayes v.3.2.1 (Ronquist *et al.* 2012) with two independent runs performed using four differentially heated Metropolis-coupled Markov chain Monte Carlo computations for five million generations that started from a random tree. Model parameters were estimated during the analysis, and chains were sampled every 500 generations. Convergence of the analyses was validated by the standard deviation of split frequencies reaching <0.01 and by graphically monitoring the likelihood values over time by using Tracer v1.5 (Rambaut and Drummond 2009). The trees were created before stable log likelihood values (5000 trees) were discarded as burn-in. A 50% majority-rule consensus tree was constructed from the remaining trees to estimate posterior probabilities (PP).

Alternative *a priori* phylogenetic hypotheses from current taxonomic groupings (e.g., family and genus assignments) were statistically tested using the

likelihood-based approximately unbiased (AU) test (Shimodaira 2002). The null hypothesis for all topology testing was that no difference existed between trees in the AU test. Alternative tree topologies were constructed using RAxML by setting constraints on taxa monophyly according to the *a priori* hypotheses. The per-site log likelihood values of individual sites for the trees were estimated using the same program and subsequently the confidence values of the tree topology were calculated using CONSEL (Shimodaira and Hasegawa 2001) with 1000 BP replicates to access the p values of the testing topology.

Results

Sequence characteristics and phylogenetic analyses

We have generated 69, 68, 62 and 46 new sequences for the 12S, 16S, H3 and NaK genes, respectively. The aligned data set contained 353 base pairs (bp)(12S), 408 bp (16S), 294 bp (H3) and 468 bp (NaK) for the four gene fragments and the individual gene tree inferred from maximum likelihood (ML) analyses revealed no significantly conflicting nodes (here defined as ML bootstrap (BP) > 70). Therefore, we concatenated the data from the four genes. However, only 16S gene could be obtained from the monotypic family Macromaxillocarididae. Hence, we also performed the phylogenetic analyses based on a mitochondrial genes only dataset

(12S and 16S; 761 bp). The mitochondrial phylogeny indicated that the three stenopodidean families formed a strongly supported ~~monophyletic~~ clade (ML BP = 100; Bayesian posterior probability (BI PP = 1.00, Figure 2). *Macromaxillocaris bahamaensis* is nested within representatives belonging to the Spongicolidae and Stenopodidae. *Macromaxillocaris bahamaensis* is most closely related to *Microprosthema,* yet this relationship was only supported in the ML topology (ML BP = 73) but not in the BI analyses. We subsequently excluded *M. bahamaensis* in the final concatenated analyses to avoid the negative effect of large amount of missing data. The final four genes concatenated dataset consisted of 1,523 bp with 69 taxa.

The nodal supports obtained from the ML and BI analyses of the four gene concatenated dataset a~~we~~re shown together on the best ML topology (Figure 3). The inferred molecular phylogeny did not support the reciprocal monophyly of Spongicolidae and Stenopodidae. The three stenopodid genera, *Juxtastenopus* Goy, 2010, *Odontozona* Holthuis, 1946 (except *Odontozona spongicola* (Alcock & Anderson, 1899)) and *Stenopus* formed a strongly supported ~~monophyletic~~ clade (ML BP = 99; BI PP = 0.99). Another clade that unities *Richardina* A. Milne-Edwards, 1881, *Odontozona spongicola* and *Globospongicola spinulatus* Komai & Saito, 2006 was recovered with strong statistical support (ML BP = 100; BI PP = 1.00). Spongicolidae was paraphyletic with respect to Stenopodidae in the inferred

phylogeny yet the statistical supports for these arrangements were low at several deep nodes. Nevertheless, AU tests clearly rejected the reciprocal monophyly for both Spongicolidae and Stenopodidae ($p < 0.001$).

Six out of seven genera with multiple exemplars analyzed (*Odontozona*, *Richardina*, *Spongiocaris*, *Spongicola*, *Spongicolodes* and *Stenopus*) were poly- or paraphyletic in our molecular phylogeny and only *Microprosthema* was supported to be monophyletic. *Juxtastenopus* was placed within the genus *Stenopus* and being sister to *Stenopus goyi* and *Stenopus earlei* Goy, 1984, making the genus *Stenopus* a paraphyletic assemblage. *Odontozona* was polyphyletic and split into three major lineages. *Odontozona spongicola* clustered with *Richardina* and *Globospongicola* (ML BP = 100; BI PP = 1.00) and this clade was more closely related to the genera in Spongicolidae than the taxa of Stenopodidae. The remaining species of *Odontozona* were paraphyletic with *Juxtastenopus* + *Stenopus* clade nested within this group. In all instances, the AU tests rejected a priori hypothesis of a monophyletic *Odontozona*, regardless if *Odontozona spongicola* was included ($p < 0.001$) or excluded ($p = 0.002$). Furthermore, several species of *Odontozona* (e.g. *Odontozona crinoidicola*) were represented by more than one –lineage in the phylogeny, indicating the possible presence of cryptic species (which may also be present in the specimens of *Microprosthema takedai* Saito & Anker, 2012 analyzed). Although two species of

Stenopus (i.e. *Stenopus goyi* and *Stenoopus earlei*) formed a clade with *Juxtastenopus*, AU test cannot reject the monophyly of *Stenopus* (p = 0.01). Similarly, the AU test cannot reject the monophyly of *Richardina* (p = 0.01) despite of the two species of *Richardina* show a non-sister relationship in the molecular trees

The twothree species of *Spongicoloides* did not form a clade in the phylogeny, with *Spongicoloides iheyaensis* Saito, Tsuchida & Yamamoto, 2006 grouping with *Engystenopus palmipes* Alcock & Anderson, 1894 (ML BP = 100; BI PP = 1.00) and *Spongicoloides novaezelandiae* Baba, 1979 and *Spongicoloides koehleri* clustered with different species of *Spongiocaris* and *Spongicola japonicus* (ML BP = 98; BI PP = 1.00). *Spongicola* sp. nov. aligned with *Microprosthema* in the four genes combined dataset (Figure 3; ML BP =59; BI PP = 0.97), but clustered with other species of *Spongicola* and *Paraspongicola* in the mitochondrial gene tree (Figure 2). The remaining species of *Spongicola* formed a strongly supported clade but with *Paraspongicola* nested within this group (ML BP = 100; BI PP = 1.00). AU tests clearly rejected a priori hypotheses of reciprocal monophyly of *Spongiocaris*, *Spongicola* and *Spongicolodes* ($p < 0.001$ in all both cases), but not *Spongiocaris* ($p = 0.164$). Although a number of well-supported clades were revealed in the molecular phylogeny, the intergeneric relationships were largely unresolved in the present attempt.

Discussion

Familial level relationship and life style evolution

The inferred phylogeny did not support the familial-level ranking of Macromaxillocarididae and rejected the reciprocal monophyly of Spongicolidae and Stenopodi~~d~~idae. Macromaxillocarididae is represented by a single cave species and considered to be unique for its habitat and a combination of extremely peculiar morphological characters, including the presence of a massive third maxilliped, pereiopods that increase in length posteriorly, and a reduced branchial formula (Alvarez *et al.* 2006). Moreover, *M. bahamaensis* possesses a bifid palp of the first maxilla and an unsegmented palp of the first maxilliped, which are absent in the Spongicolidae and Stenopodidae (Alvarez *et al.* 2006). However, *Macromaxillocaris* was nested deep inside spongicolids and stenopodids in the mitochondrial gene tree. Furthermore, the genetic divergence among *Macromaxillocaris* and other stenopodideans was not pronounced. *Macromaxillocaris* is revealed to be most closely related to *Microprosthema* in our gene tree, though~~however~~ the statistical support is only high in the maximum likelihood analysis. *Microprosthema* comprises ~~of~~ shallow water inhabitants found in tropical and subtropical water worldwide. Therefore, it is possible that they shared a common shallow water ancestor with

Macromaxillocaris, with the latter subsequently colonized shallow water anchialine cave. In any case, the unusual morphology of *Macromaxillocaris* is likely derived adaptations instead of representing pleisomorphic characters. Thus, the familial status of *Macromaxillocaris* may be unwarranted; a situation similar to the specialized chemosynthetic squat lobster *Shinkaia crosineri* Baba & Williams, 1998, which was formerly treated as a distinct subfamily (Ahyong *et al.* 2010).

Our molecular phylogeny also clearly rejected the monophyly of the other two Stenopodidea families, Spongicolidae and Stenopodidae. The stenopodid *Richardina* and *Odontozona spongicola* are more closely related to members of Spongicolidae than other stenopodids. Furthermore, Spongicolidae is paraphyletic with respect to Stenopodidae even when *Richardina* and *Odontozona spongicola* are not considered. Saito & Takeda (2003) hypothesized that deep-water sponge associated taxa evolved from more basal shallow water free-living lineages (also see Saito 2008)~~(Saito and Takeda 2003)~~. This hypothesis cannot be verified confidently given the low nodal support at higher relationships and the lacking of life history information in some species. However, the present molecular phylogeny reveals an early branching lineage comprises ~~of~~ *Engystenopus* and *Spongicoloides iheyaensis* (Figures 2, 3). Although whether *Engystenopus* forming an association with other animals remains unclear, both *Engystenopus* and *Spongicoloides iheyaensis* (sponge associated) are deep-sea

inhabitants. Thus, the current molecular data provide some evidence of the earliest branching lineages in the Stenopo~~i~~didea are deep-sea inhabitants and the shrimps colonized the deep sea in multiple circumstances. Moreover, it appears that habitat depth and sponge association may be more informative than morphological characters currently adopted in stenopodidean systematics. For example, all of the shallow water free-living stenopodidean species analyzed forms a strongly supported ~~monophyletic~~ clade. On the contrary, *Odontozona spongicola* and a number of *Richardina* species are confirmed in association with hexactinellid sponge in deeper water (Saito and Komatsu 2009). These similarities in ecology are congruent with the close affinity between *Richardina*/*Odontozona spongicola* and the family Spongicolidae in the phylogeny, and transfer of the two taxa into Spongicolidae (or other family if Spongicol~~o~~idae will be split) appears to be more appropriate.

Validity of the genera

The present molecular phylogeny trees show that all except one stenopodidean genera with multiple exemplars are para- or polyphyletic. The only monophyletic genus is the shallow water free-living *Microprosthema*. The monotypic genus *Juxtastenopus* was erected by Goy (2010a) for *J. spinulatus,* which is formerly placed under *Stenopus*. *Juxtastenopus* is considered to be morphologically close to but yet

different from *Stenopus* in the dactyli of the ambulatory pereiopods being long, slender and uniunguiculate whereas most those of *Stenopus* are biunguiculate (Goy 2010a). The shape and armature of the dactyli of the ambulatory pereiopods have been considered to be important characters in stenopodideans at generic level (see Holthuis 1993, Goy, 2010b). However, variations in the number of ungues are found in the recently described species *Stenopus goyi*, sometimes even among the pereiopods of the same specimen (Saito *et al.* 2009). Interestingly, the present molecular analyses suggested that *Juxtastenopus* forms a clade with *Stenopus goyi* and *Stenopus earleri*, and this clade is sister to the remaining *Stenopus* species. Thus, if the genus *Juxtastenopus* is to be retained, it may be necessary to expanded by including some species of *Stenopus* and redefining its generic characters. Further analyses including more species of *Stenopus* may provide more insights on the status as well as coverage of *Juxtastenopus*.

The genus *Odontozona* is revealed to be polyphyletic in the present analysis. *Odontozona. spongicola* is distantly separated from the other species of the genus, and the Atlantic species *O. meloi* Anker & Tavares, 2013 does not form a ~~monophyletic~~ clade with the other *Odontozona* species from the Indo-West Pacific. With the recent discoveries of a number of new species, *Odontozona* becomes one of the two most species rich genera in stenopodideans (with 16 species, as in *Microprosthema*).

Odontozona species exhibit a wide range of lifestyle, from shallow to deep waters, and free living to association with sponges or other invertebrates (Figures 2 and 3). The present results strongly suggest that this genus needs to be redefined with the transfer of some species currently included under *Odontozona* to other genera (e.g. *Odontozona spongicola*) or new genera (e.g. *O. meloi*). *Odontozona spongicola* shows a close relationship with *Richardina* and *Globospongicola* in our analyses. *Globospongicola* is believed to be unique within Stenopodidea in having simple gills whereas all other stenopodidean taxa share trichobranchiate gills (Komai and Saito 2006). Nevertheless, *Richardina* somewhat resembles *Globospongicola* in the reduced armament on the body and third pereiopod, the well-developed exopod of the second and third maxillipeds, as well as the integument of carapace and pleon being glabrous. It has been suggested that the simple gills were derived from the typical trichobranchiate gills with complete loss of gill filaments and thickening of the rachis (Alvarez *et al.* 2006; Komai and Saito 2006; Goy 2010b). *Odontozona spongicola*, originally described under *Richardina*, was transferred to *Odontozona* on the basis of the biunguiculate dactyli of the fourth and fifth pereiopods (Holthuis 1946). However, Saito and Komatsu (2009) pointed out that *O. edwardsi* (Bouvier, 1908), *O. foresti* Hendrickx, 2002, and *O. spongicola* appear closer to *Richardina* rather than *Odontozona*. The three species are very similar to *Richardina* in almost all of its

diagnostic features except having biunguiculate dactyli in the fourth and fifth pereiopods. Goy and Cardoso (2014) also suggested that *O. spongicola* lacks the spinous propodal margins of the third perieopods observed in the deep water members of *Odontozona* (e.g. *O. edwardsi*, *O. lopheliae* Goy & Cardoso, 2014 and *O. foresti*). Furthermore, *O. spongicola* is the only *Odontozona* species reported to be associated with hexactinellid sponge in the deep sea, similar to *Globospongicola* (Holthuis 1946; Saito and Fujita 2009). Some of the recently described species of *Richardina* (e.g. *R. ohtsukai* Saito & Komatsu, 2009 and *R. parvioculata* Saito & Komatsu, 2009) are commensals of hexactinellid sponges like most of the members of the family Spongicolidae, so it is possible that some more ~~other~~ ~~or all~~ species of *Richardina* are sponge commensals (though at least *R. rupicola* Komai, 2011a seems to be free-living). Therefore, the present results suggest to transfer *O. spongicola* back to *Richardina* or re-assign it to *Globospongicola*, which may later prove to be merged with *Richardina*. The formal taxonomic placement for *Richardina*, *Globospongicloa* and *Odontozona spongicola* should be decided in future attempts given only two of the six~~even~~ species of *Richardina* and only a single species of *Globospongicola* are included in this analysis, and the type species of these two genera are not included.

The two species recently transferred to *Spongiocaris*, namely *Spongiocaris* ~~*cola*~~ *japonicus* from *Spongicola* and *Spongicaris* ~~*oloides*~~ *koehleri* from *Spongicoloides*,

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form a strongly supported clade with only one of the two other species of *Spongicaris* in the~~our~~ present analyses. Furthermore, *Spongicoloides iheyaensis* was separated into another lineage distantly related to all other spongicolids except the monotypic *Engystenopus*. Nevertheless, these results are largely consistent with the cladogram by Saito and Takeda (2003), which also indicated a close relationship between ~~among~~ *Spongiocaris* (including~~,~~ *Spongiocaris~~cola~~ japonicus*) and *Spongicoloides*. The development of the exopod on the second maxilliped is the major characteristic used to distinguish between *Spongiocaris* and *Spongicoloides*. However, Bruce and Baba (1973) proposed that *Spongiocaris* appears to be intermediate between *Spongicola* and *Spongicoloides*, suggesting the characters adopted to define the genera may be variations within a continuum. ~~They further argued that *Spongicola japonicus* is morphologically more similar to *Spongiocaris* and this view is supported by Saito and Komai (2006). On the other hand, the exopod at the second maxilliped appears to be actually well developed and not absent in *Spongicoloides koehleri* (García Raso 1996), and therefore, closer to the definition of *Spongiocaris* than *Spongicoloides*.~~ The present molecular phylogeny indicates that *Spongiocaris* may need to be synonymized with *Spongicoloides*, even though our results somewhat support the recent genus re-assignment of ~~with~~ *Spongiocaris~~ola~~ japonicus* and *Spongicaris koehleri* by Satio (2008) and Komai *et al.* (in press). ~~transferred to the latter genus. On the other hand,~~

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~~the exopod at the second maxilliped appears to be actually well developed and not absent in *Spongicoloides koehleri* (García Raso 1996), and therefore, closer to the definition of *Spongiocaris* than *Spongicoloides*.~~ However, the type species of *Spongiocaris*, *Spongicoloides* and *Spongiocola* are not included in the present analysis, particular caution will be necessary in future works in redefining these three genera.

With respect to the monotypic genus *Engystenopus,* which was firstly assigned to Stenopodidae (Holthuis 1946, 1955, 1993) though de Saint Laurent and Cleva (1981) suggested that this species is closer to *Spongicola* than to *Stenopus*. Goy (2010a) re-diagnosed and transferred *Engystenopus palmipes* to the family Spongicolidae. The presence of a well-developed exopod at the third maxilliped and the unguiculate dactyli of the fourth and fifth pereiopods in *E. palmipes* is unique within Spongicolidae (Goy 2010a, b). Our phylogeny corroborated the view of de Saint Laurent and Cleva (1981), Goy (2010a, b) and Jiang et al. (2015) that *Engystenopus* has higher affinity with the genera of Spongicolidae. However, *Engystenopus* formed a robust clade with *Spongicoloides iheyaensis*, and this clade is inferred to be an early-branching lineage of all stenopodideans. *Spongicoloides iheyaensis* is indeed similar to *Engystenopus* and different from other species of *Spongicoloides* in the carapace having postorbital spines and hepatic groove, and bearing small but

numerous eggs (vs. carapace lacking postor~~i~~bital spines and hepatic groove, bearing large and few eggs). Whether *Spongicoloides iheyaensis* should be transferred to *Engystenopus* awaits for more extensive studies on *Spongicoloides*, as only two~~hree~~ out of the eight~~nine~~ species known in this genus are included in the analysis and the two ~~hree~~ studied species are separated on the gene tree.

Paraspongicola is morphologically very similar to *Spongicola* except for the possession of a well-developed, flagellum-like exopod on the third maxilliped (de Saint Laurent and Cléva 1981; Holthuis 1993). De Saint Laurent and Cléva (1981) originally assigned *Spongicola inflatus* de Saint Laurent and Cléva, 1981 to *Spongicola* on the basis of the similarity in the armature of the carapace in spite of the species has a well-developed exopod on the third maxilliped that resembles *Paraspongicola*. Saito and Takeda (2003) revealed a sister relationship for *Spongicola inflatus* and *Paraspongicola pusillus* de Saint Laurent & Cleva, 1981 in their cladogram inferred from adult morphology. Thus~~is leads to~~ Saito and Komai (2006) transferred *Spongicola inflatus* to *Paraspongicola*. The other known species of *Paraspongicola*, namely *P. acantholepis* Komai, 2011a, is also superficially rather similar to species of *Spongicola* than to the type species *P. pusillus* (Komai, 2011a). The presence or absence of exopod on the third maxillipeds has been considered to be of great importance in the generic classification within Spongicolidae (de Saint

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Laurent and Cléva 1981; Holthuis 1993; Saito and Takeda 2003; Saito and Komai 2006; Goy 2010b). However, Saito and Anker (2014) argued that the variation in the development of the exopod of the third maxilliped may compromise or at least introduce ambiguities to some key characters presently used to define spongicolid genera. Our analyses are strongly against the validity of *Paraspongicola* and suggest to transfer its species back to *Spongicola*. In so doing, the present results support the view of Saito and Anker (2014) in considering the development of exopod at the third maxilliped being not an informative character in Spongicolidae systematics.

Suggested classification of Stenopodidea

The present molecular phylogeny strongly refutes most of the higher classification schemes in the infraorder Stenopodidea. All the three families currently recognized are shown to be poly- or paraphyletic. Thus, it may be more appropriate to unify all the stenopodideans back to a single family Stenopodidae before a detailed redefinition of the families and reassignment of species is made. The current result only strongly supports the validity of the genus *Microprosthema* whilst the genus *Paraspongicola* is ~~appears to be~~ invalid and should be synonymized under *Spongicola*. The genera *Odontozona, Spongicola*, *Spongicoloides* and *Spongiocaris* need to be redefined and revised. Further studies with more extensive taxon coverage will need

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to determine if the two recently established genera *Juxtastenopus* and *Globospongicola* are valid and if *Stenopus* and *Richardina* need to be split. Once a robust molecular phylogeny on stenopodideans is reached, higher taxa in this infraorder can then be fully redefined and with their diagnostic characters elucidated.

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Captions.

Table 1. Stenopodidean material, locality, voucher number used in this study.

Figure 1. Representatives of some genera within Stenopodidea: A. *Engystenopus palmipes* Alcock & Anderson, 1894 (Spongicolidae), Papua New Guinea; B. *Globospongicola spinulatus* Komai & Saito, 2006 (Spongicolidae), Taiwan; C. *Microprosthema takedai* Saito & Anker, 2012 (Spongicolidae), Vanuatu; D. *Spongicola venustus* De Haan, 1844 (Spongicolidae), the Philippines; E. *Spongicoloides iheyaensis* Saito, Tsuchida & Yamamoto, 2006 (Spongicolidae), Taiwan. F. *Spongiocaris panglao* Komai, De Grave & Saito, in press (Spongicolidae), the Philippines; G. *Juxtastenopus spinulatus* (Holthuis, 1946) (Stenopodidae), the Philippines; H. *Odontozona crinodicola* Saito & Fujita, 2009 (Stenopodidae), Papua New Guinea; I. *Richardina spinicincta* A. Milne-Edwards, 1881 (Stenopodidae), Guadeloupe; J. *Stenopus hispidus* (Olivier, 1811) (Stenopodidae), Papua New Guinea.

Figure 2. Maximum likelihood topology for the combined mitochondrial 12S and 16S gene sequences. Nodal supports are denoted on the corresponding branches for a bootstrap value >50% for ML or posterior probability >0.70 for Bayesian analysis. The color of the taxon names indicates that the familial classification with * referring

to type species of the genus. Symbols next to the taxon names show the lifestyle of the species reported from literature: ▲Shallow water; ▼Deep sea; ○Free living; ※ Cave dwelling; ■ Sponge associated; ◆ Crinoid associated; ? Association unknown; [1]Reports of association with gorgonian octocoral; [2]Reports of association with sea anemone and flame scallops; [3]New species going to be described in Goy in preparation.

Figure 3. Maximum likelihood topology for the combined mitochondrial 12S and 16S, and nuclear H3 and NaK gene sequences. Nodal supports are denoted on the corresponding branches for a bootstrap value >50% for ML or posterior probability >0.70 for Bayesian analysis. The color of the taxon names indicates that the familial classification with * referring to type species of the genus. Symbols next to the taxon names show the lifestyle of the species reported from literature: ▲Shallow water; ▼ Deep sea; ○ Free living; ※ Cave dwelling; ■ Sponge associated; ◆ Crinoid associated; ? Association unknown; [1]Reports of association with gorgonian octocoral; [2] Reports of association with sea anemone and flame scallops; [3]New species going to be described in Goy in preparation.

Species	Locality	Voucher no.	12S	16S	H3	NaK
Family MACROMAXILLOCARIDIDAE						
Genus *Macromaxillocaris*						
Macromaxillocaris bahamaensis	Eleuthera, Bahamas	ULLZ 11769	N/A	KX086378	N/A	N/A
Family SPONGICOLIDAE						
Genus *Engystenopus*						
Engystenopus palmipes	the Philippines	NTOU M01900	KX086309	KX086379	KX086447	KX086508
Genus *Globospongicola*						
Globospongicola spinulatus	Taiwan	NTOU M01877	KX086310	KX086380	KX086448	KX086509
Genus *Microprosthema*						
Microprosthema scabricaudatum	Ryukyu Islands	NTOU M01901	KX086311	KX086381	N/A	N/A
Microprosthema semilaeve	Guadeloupe	MNHN-IU-2013-4307	KX086312	KX086382	KX086449	KX086510
Microprosthema takedai (1)	Vanuatu	MNHN-IU-2014-6689	KX086330	KX086400	KX086467	KX086524
Microprosthema takedai (2)	Vanuatu	MNHN-IU-2014-6690	KX086331	KX086401	KX086468	KX086525
Microprosthema takedai (3)	Vanuatu	MNHN-IU-2014-6693	KX086334	KX086404	KX086471	N/A
Microprosthema aff. *takedai*	Papua New Guinea	MNHN-IU-2013-10256	KX086341	KX086410	KX086477	N/A
Genus *Paraspongicola*						
Paraspongicola inflatus (1)	New Caledonia	MNHN-IU-2014-12066 (MNHN-Na 16355)	KX086322	KX086392	KX086459	N/A
Paraspongicola inflatus (2)	New Caledonia	MNHN-IU-2014-12066 (MNHN-Na 16355)	KX086323	KX086393	KX086460	N/A
Genus *Spongicola*						
Spongicola andamanicus (1)	Vanuatu	MNHN-IU-2014-6691	KX086332	KX086402	KX086469	N/A
Spongicola andamanicus (2)	South China Sea	NTOU M01902	KX086343	KX086412	N/A	KX086530
Spongicola andamanicus (3)	South China Sea	NTOU M01903	KX086346	KX086415	KX086482	KX086533
Spongicola levigatus (1)	South China Sea	NTOU M01920	KX086348	KX086417	KX086483	N/A
Spongicola levigatus (2)	South China Sea	NTOU M01920	KX086349	N/A	KX086484	N/A
Spongicola goyi (1)	New Caledonia	MNHN IU-2014-6778	KX086352	KX086421	KX086487	N/A
Spongicola goyi (2)	Papua New Guinea	MNHN-IU-2011-2036	KX086329	KX086399	KX086466	N/A
Spongicola goyi (3)	South China Sea	NTOU M01905	KX086357	KX086426	KX086488	KX086538
Spongicola goyi (4)	South China Sea	NTOU M01905	KX086358	KX086427	KX086489	KX086539
Spongicola robustus (1)	Madagascar	MNHN-IU-2010-85	KX086325	KX086395	KX086462	KX086520
Spongicola robustus (2)	Madagascar	MNHN-IU-2010-1744	KX086326	KX086396	KX086463	KX086521
Spongicola robustus (3)	Madagascar	MNHN-IU-2010-1744	KX086327	KX086397	KX086464	KX086522
Spongicola robustus (4)	Madagascar	MNHN-IU-2010-1744	KX086328	KX086398	KX086465	KX086523
Spongicola robustus (5)	South China Sea	NTOU M01914	KX086369	KX086438	KX086500	KX086549
Spongicola sp. nov.	South China Sea	NTOU M01907	KX086324	KX086394	KX086461	KX086519
Genus *Spongicoloides*						
Spongicoloides iheyaensis	Taiwan	NTOU M01908	KX086355	KX086424	N/A	N/A
Spongicoloides novaezelandiae	Solomon Islands	MNHN-IU-2014-6347	KX086359	KX086428	KX086490	N/A
Genus *Spongiocaris*						
Spongiocaris japonicus	Japan	NTOU M01906	KX086347	KX086416	N/A	N/A
Spongiocaris koehleri	Seamount near Bermuda	MNHN-IU-2014-12841	KX086356	KX086425	N/A	N/A
Spongiocaris panglao	the Philippines	NTOU M01909	KX086360	KX086429	KX086491	KX086540
Spongiocaris yaldwyni	Tonga	MNHN-IU-2014-12842	KX086370	KX086439	N/A	N/A
Family Stenopodidae						
Genus *Juxtastenopus*						
Juxtastenopus spinulatus	Vanuatu	MNHN-IU-2014-6692	KX086333	KX086403	KX086470	N/A
Genus *Odontozona*						
Odontozona crinoidicola (1)	Papua New Guinea	MNHN-IU-2013-10251	KX086340	KX086409	KX086476	KX086528
Odontozona crinoidicola (2)	Papua New Guinea	MNHN-IU-2013-10240	KX086313	KX086383	KX086450	KX086511
Odontozona crinoidicola (3)	Papua New Guinea	MNHN-IU-2013-10252	KX086314	KX086384	KX086451	N/A
Odontozona crinoidicola (4)	Papua New Guinea	MNHN-IU-2013-10253	KX086315	KX086385	KX086452	KX086513
Odontozona crinoidicola (5)	Papua New Guinea	MNHN-IU-2013-10244	KX086337	KX086406	KX086473	N/A
Odontozona aff. *crinoidicola* (1)	Papua New Guinea	MNHN-IU-2013-10238	KX086318	KX086388	KX086455	KX086516
Odontozona aff. *crinoidicola* (2)	Papua New Guinea	MNHN-IU-2013-10238	KX086319	KX086389	KX086456	KX086517
Odontozona aff. *ensifera* (1)	Papua New Guinea	MNHN-IU-2013-10247	KX086338	KX086407	KX086474	KX086526
Odontozona aff. *ensifera* (2)	Papua New Guinea	MNHN-IU-2013-10250	KX086339	KX086408	KX086475	KX086527
Odontozona aff. *ensifera* (3)	Papua New Guinea	MNHN-IU-2013-10233	KX086335	KX086405	N/A	N/A
Odontozona meloi (1)	French Guinea	MNHN IU-2013-2647	KX086316	KX086386	KX086453	KX086514
Odontozona meloi (2)	French Guinea	MNHN IU-2013-2887	KX086317	KX086387	KX086454	KX086515
Odontozona sculpticaudata	New Caledonia	MNHN-IU-2014-6337	KX086321	KX086391	KX086458	N/A
Odontozona aff. *sculpticaudata*	Papua New Guinea	MNHN-IU-2013-10239	KX086320	KX086390	KX086457	KX086518
Odontozona spongicola	South China Sea	NTOU M01910	KX086344	KX086413	KX086480	KX086531
Genus *Richardina*						
Richardina aff. *parvioculata*	South China Sea	NTOU M01911	KX086345	KX086414	KX086481	KX086532
Richardina spinicincta	Guadeloupe	MNHN-IU-2013-19177	KX086373	KX086442	KX086503	KX086553
Genus *Stenopus*						
Stenopus earlei	Papua New Guinea	MNHN-IU-2013-10234	KX086336	N/A	KX086472	N/A
Stenopus goyi	the Philippines	NTOU M01912	KX086353	KX086422	N/A	KX086536
Stenopus hispidus (1)	Ryukyu Islands	NTOU M01913	KX086342	KX086411	KX086478	N/A
Stenopus hispidus (2)	Aquarium shop, Hong Kong	MSLKHC-Sthis	KX086354	KX086423	JF346323	KX086537
Stenopus hispidus (3)	Papua New Guinea	MNHN-IU-2013-233	KX086351	KX086420	KX086486	KX086535
Stenopus pyrsonotus (1)	Aquarium shop, Hong Kong	MSLKHC-Stpyr	KX086361	KX086430	KX086492	KX086541
Stenopus pyrsonotus (2)	French Polynesia	MNHN IU-2011-8952	KX086362	KX086431	KX086493	KX086542
Stenopus scutellatus (1)	Guadeloupe	MNHN IU-2013-4378	KX086363	KX086432	KX086494	KX086543
Stenopus scutellatus (2)	French Guinea	MNHN IU-2013-2463	KX086350	KX086419	KX086485	KX086534
Stenopus tenuirostris (1)	Marquesas Islands	MNHN-IU-2014-12843	KX086364	KX086433	KX086495	N/A
Stenopus tenuirostris (2)	Aquarium shop, Hong Kong	MSLKHC-Stten	KX086368	KX086437	KX086499	KX086548
Stenopus tenuirostris (3)	Papua New Guinea	MNHN-IU-2013-10246	KX086365	KX086434	KX086496	KX086545
Stenopus tenuirostris (4)	Papua New Guinea	MNHN-IU-2013-10246	KX086366	KX086435	KX086497	KX086546
Stenopus tenuirostris (5)	Papua New Guinea	MNHN-IU-2013-10248	KX086367	KX086436	KX086498	KX086547
Stenopus zanzibaricus (1)	Papua New Guinea	MNHN-IU-2013-10241	KX086371	KX086440	KX086501	KX086551
Stenopus zanzibaricus (2)	Papua New Guinea	MNHN-IU-2013-10243	KX086372	KX086441	KX086502	KX086552
OUTGROUP						
Alvinocaris chelys	Taiwan	NTOU M01671	KX086374	KX086443	KX086504	KX086554
Gnathophyllum americanum	Aquarium shop, Hong Kong	MSLKHC-CA02-Game	KX086375	KX086444	KX086505	KX086555
Palaemon serrata	Hawaii	MSLKHC-CA98-Paser	KX086376	KX086445	KX086506	KX086556
Procaris hawaiiana	Hawaii	OUMNH.ZC.2010-13-007	KX086377	KX086446	KX086507	KX086557

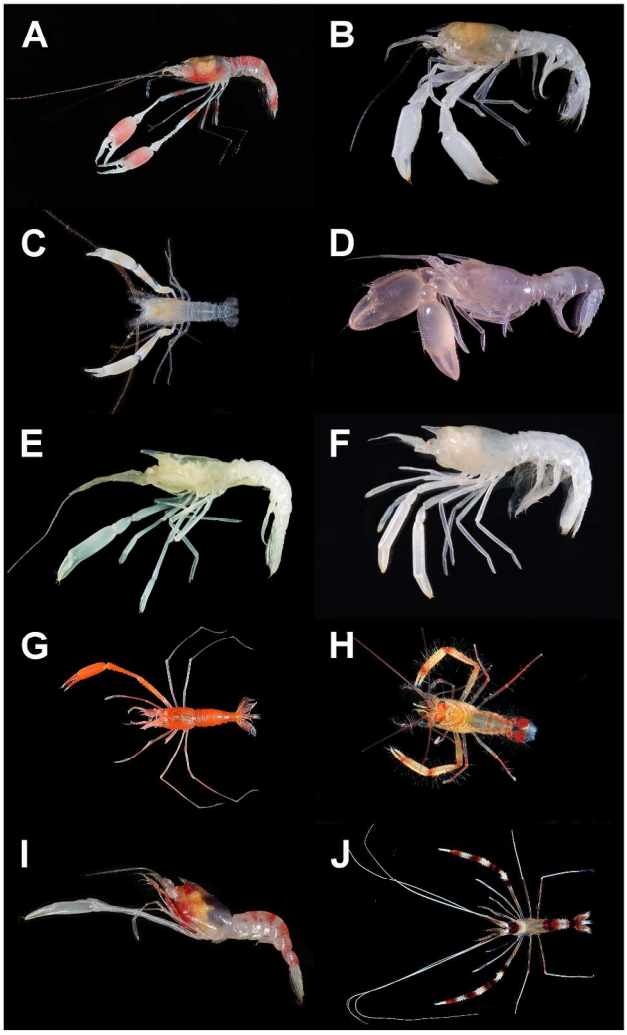

Fig. 1

874x1237mm (72 x 72 DPI)

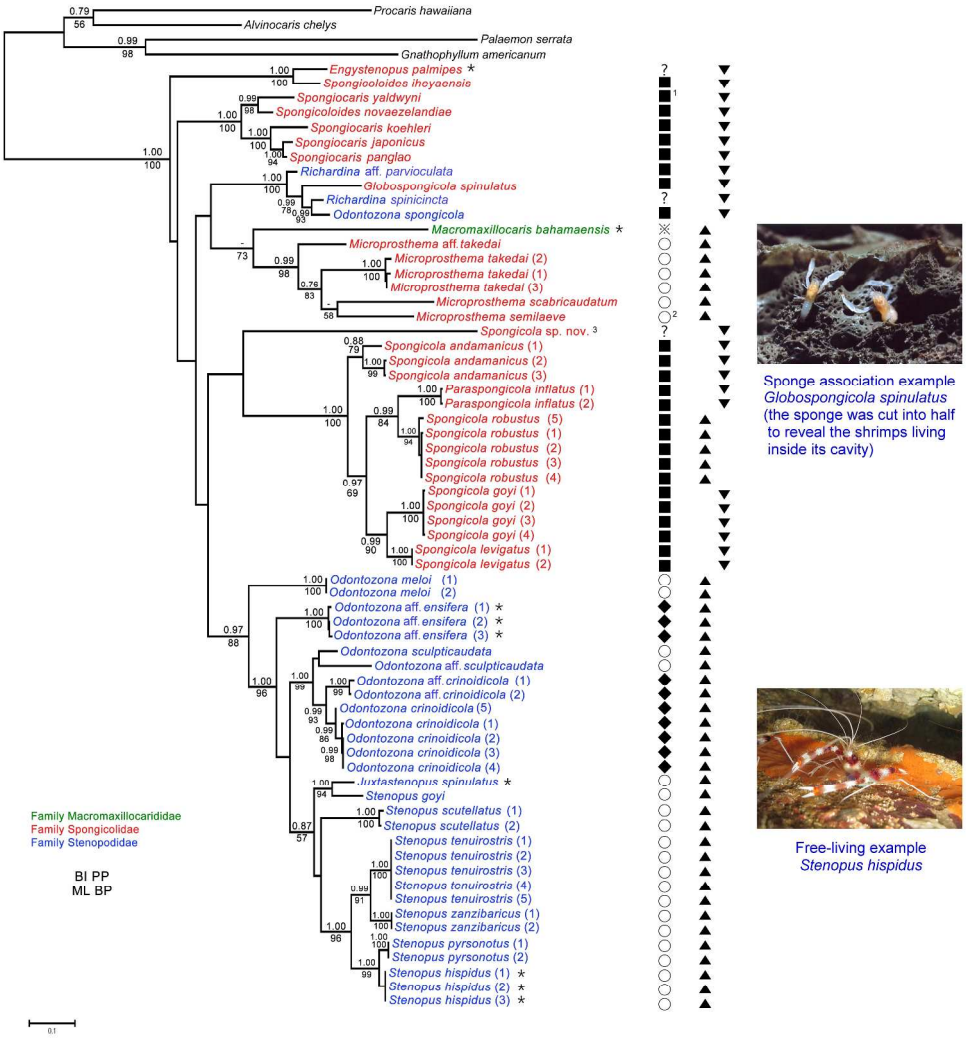

Fig. 2
1363x1634mm (72 x 72 DPI)

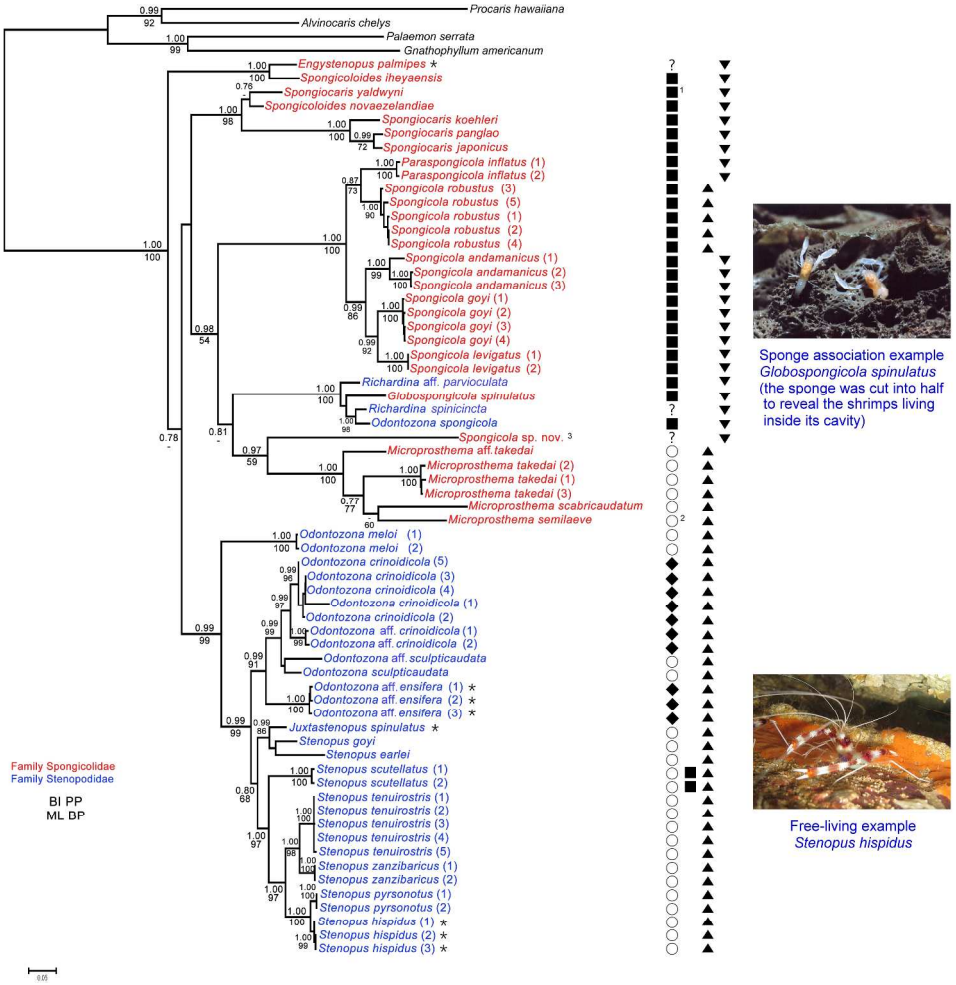

Fig. 3
1416x1634mm (72 x 72 DPI)