

# 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 the most comprehensive molecular phylogenetic analyses of Stenopodidea using sequence data from two mitochondrial (16S and 12S rRNA) and two nuclear (histone H3 and sodium–potassium ATPase  $\alpha$ -subunit (NaK)) genes. We included all 12 nominal 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. The genera *Stenopus*, *Richardina*, *Spongiocaris*, *Odontozona*, *Spongicola* and *Spongicoloides* are showed to be poly- or paraphyletic, with monophyly of only the latter three genera strongly rejected in the analysis. The present results only strongly support the monophyly of *Microprosthema* and suggest that *Paraspongiola* should be synonymised with *Spongicola*. The three remaining genera, *Engystenopus*, *Juxtastenopus* and *Globospongicola*, may need to be expanded to include species from other genera if their statuses are maintained. All findings suggest that the morphological characters currently adopted to define genera are mostly invalid and substantial taxonomic revisions are required. As the intergeneric relationships were largely unresolved in the present attempt, 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 support that stenopodidean shrimps colonised the deep sea in multiple circumstances.

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

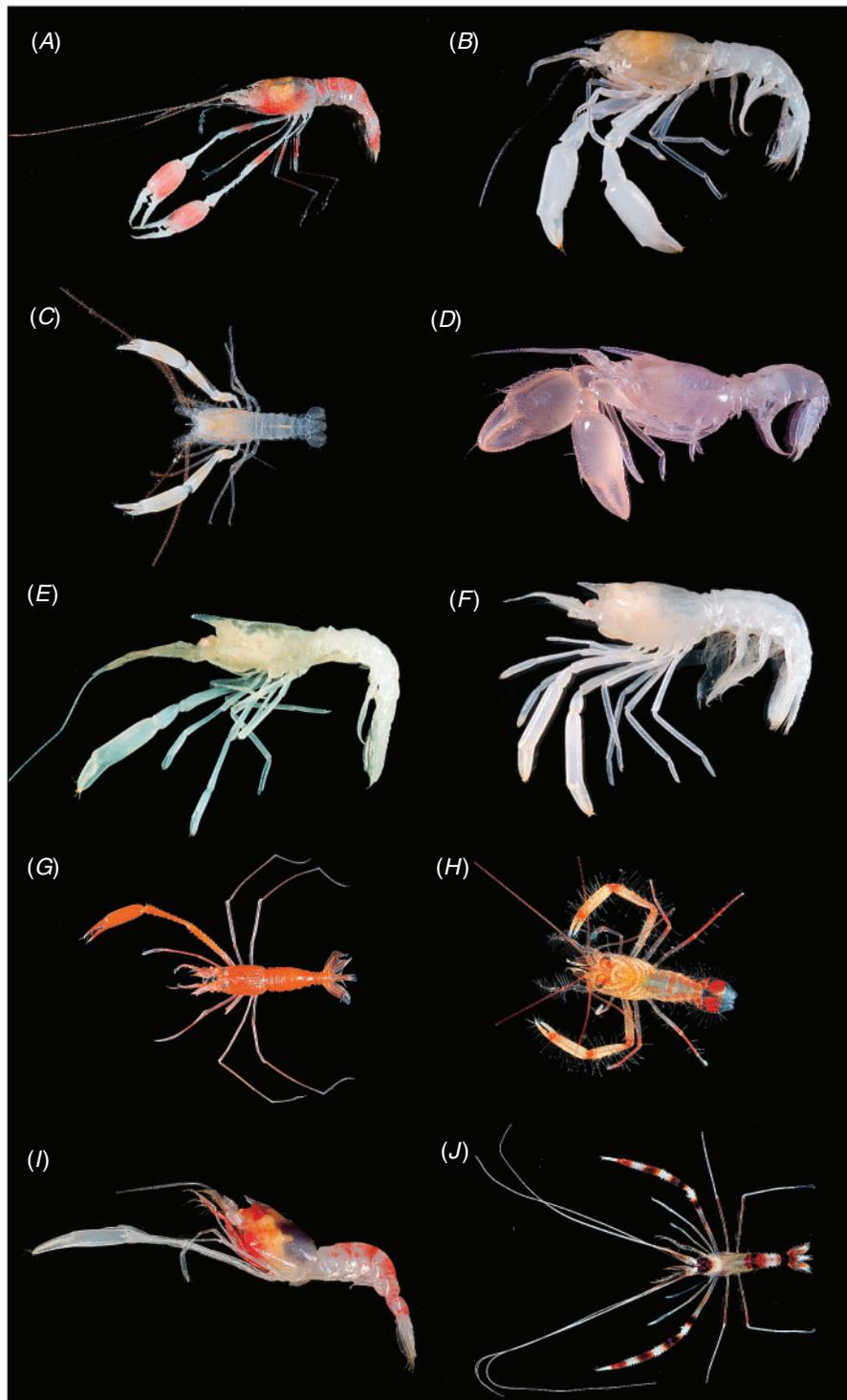
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## Introduction

The infraorder Stenopodidea Claus, 1872 (Crustacea: Decapoda) is a relatively small group of marine decapod crustaceans, with 83 species recognised to date and assigned to 12 genera (Fig. 1) in three families (Schram 1986; Goy 2010b, 2015; De Grave and Fransen 2011; Komai 2011a, 2015; Saito and Anker 2012, 2014; Anker and Tavares 2013; Goy and Martin 2013; Goy and Cardoso 2014; Komai *et al.* 2016; Wang *et al.* 2016). These small shrimps are generally uncommon and therefore not commercially important, 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, and *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 recognised as an infraorder comparable to carideans, lobsters, crabs and



**Fig. 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*, *Microprosthemata 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 *et al.* 2016 (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.

anomurans (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 Schram (1986) separated them into two families with the family Stenopodidae Claus, 1872 containing mainly free-living species, and the new family Spongicolidae, consisting 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 & Villabobos, 2006. With more genera and species being discovered, currently there are four genera in Stenopodidae and seven genera in Spongicolidae (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 octocorals (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 Stenopodidae, the most renowned genus, *Stenopus*, consists of shallow-water free-living species with many of them known to exhibit fish-cleaning behaviour (Bruce 1976; Lewinsohn and Holthuis 1978; Goy and Devaney 1980; Goy and Randall 1986; Emmerson *et al.* 1990; Goy 1992, 2010b; Calado 2008). 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).

The current classification scheme of Stenopodidea is based largely 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 diagnosing genera become questionable. For instance, the number of ungues on the ambulatory dactyli was thought to be a constant and diagnostic character for all stenopodidean taxa. 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 pereopods 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). 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), which seems to be morphologically intermediate between *Spongicola* and *Spongicoloides* Hansen, 1908 (Bruce and Baba 1973). Furthermore, the exopod of the second maxilliped appears to be 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 Cléva (1981) proposed to synonymise *Spongiocaris* under *Spongicoloides*, Komai *et al.* (2016) 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 being separated into two distinct genera and with *Engystenopus* (originally included in Stenopodidae) transferred to Spongicolidae (Goy 2010a).

Only recently, Saito and 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 that study revealed many genera to be paraphyletic and suggested that many characters used to define genera may be invalid. They further proposed that there was a reduction in several morphological features (e.g. gills, armature of carapace, pereopods and abdomen, exopods at second and third maxillipeds, setiferous organs of first pereopod) during the evolution of deep-water sponge-associated taxa from more early-derived shallow-water free-living lineages (Saito and Takeda 2003; also see Saito 2008).

On the other hand, molecular systematics of Stenopodidea is poorly documented, possibly because of many lineages being rare in nature and difficult to collect (Goy 2010b; De Grave and Fransen 2011). Very few 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 multilocus phylogeny (based on four molecular markers) that included all described families and genera of Stenopodidea. On the basis of the inferred phylogeny, we here evaluate the validity of the morphological characters that are currently applied in stenopodidean systematics and test Saito and Takeda's (2003) hypothesis that deep-sea sponge-associated species evolved from shallow-water free-living ancestors.

## Materials and methods

### Taxon sampling

We included 66 samples from 31 species (including a new species of *Spongicola*, to be described by Goy, unpubl. data) 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 to be the 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 comparisons. The samples were obtained from various expeditions, cruises and field collections as well as

**Table 1. Stenopodidean material, locality, voucher number used in this study**  
 Numbers in parentheses indicate previous catalogue numbers used in MNHN

Taxon	Species	Locality	Voucher no.	12S	16S	H3	NaK
Family Macromaxillocarididae							
Genus <i>Macromaxillocaris</i>							
	<i>Macromaxillocaris bahamaensis</i>	Eleuthera, Bahamas	UNAM/CNCR 23167	n/a	KX086378	n/a	n/a
Family Spongicolidae							
Genus <i>Engystenopus</i>							
	<i>Engystenopus palmipus</i>	The Philippines	NTOU M01900	KX086309	KX086379	KX086447	KX086508
Genus <i>Globospongicola</i>							
	<i>Globospongicola spinulatus</i>	Taiwan	NTOU M01877	KX086310	KX086380	KX086448	KX086509
Genus <i>Microprosthema</i>							
	<i>Microprosthema scabricaudatum</i>	Ryukyu Islands	NTOU M01901	KX086311	KX086381	n/a	n/a
	<i>Microprosthema semilaeve</i>	Guadeloupe	MNHN-IU-2013-4307	KX086312	KX086382	KX086449	KX086510
	<i>Microprosthema takedai</i> (1)	Vanuatu	MNHN-IU-2014-6689	KX086330	KX086400	KX086467	KX086524
	<i>Microprosthema takedai</i> (2)	Vanuatu	MNHN-IU-2014-6690	KX086331	KX086401	KX086468	KX086525
	<i>Microprosthema takedai</i> (3)	Vanuatu	MNHN-IU-2014-6693	KX086334	KX086404	KX086471	n/a
	<i>Microprosthema aff. takedai</i>	Papua New Guinea	MNHN-IU-2013-10256	KX086341	KX086410	KX086477	n/a
Genus <i>Paraspongicola</i>							
	<i>Paraspongicola inflatus</i> (1)	New Caledonia	MNHN-IU-2014-12066 (MNHN-Na 16355)	KX086322	KX086392	KX086459	n/a
	<i>Paraspongicola inflatus</i> (2)	New Caledonia	MNHN-IU-2014-12066 (MNHN-Na 16355)	KX086323	KX086393	KX086460	n/a
Genus <i>Spongicola</i>							
	<i>Spongicola andamanicus</i> (1)	Vanuatu	MNHN-IU-2014-6691	KX086332	KX086402	KX086469	n/a
	<i>Spongicola andamanicus</i> (2)	South China Sea	NTOU M01902	KX086343	KX086412	n/a	KX086530
	<i>Spongicola andamanicus</i> (3)	South China Sea	NTOU M01903	KX086346	KX086415	KX086482	KX086533
	<i>Spongicola levigatus</i> (1)	South China Sea	NTOU M01920	KX086348	KX086417	KX086483	n/a
	<i>Spongicola levigatus</i> (2)	South China Sea	NTOU M01920	KX086349	n/a	KX086484	n/a
	<i>Spongicola goyi</i> (1)	New Caledonia	MNHN IU-2014-6778	KX086352	KX086421	KX086487	n/a
	<i>Spongicola goyi</i> (2)	Papua New Guinea	MNHN-IU-2011-2036	KX086329	KX086399	KX086466	n/a
	<i>Spongicola goyi</i> (3)	South China Sea	NTOU M01905	KX086357	KX086426	KX086488	KX086538
	<i>Spongicola goyi</i> (4)	South China Sea	NTOU M01905	KX086358	KX086427	KX086489	KX086539
	<i>Spongicola robustus</i> (1)	Madagascar	MNHN-IU-2010-85	KX086325	KX086395	KX086462	KX086520
	<i>Spongicola robustus</i> (2)	Madagascar	MNHN-IU-2010-1744	KX086326	KX086396	KX086463	KX086521
	<i>Spongicola robustus</i> (3)	Madagascar	MNHN-IU-2010-1744	KX086327	KX086397	KX086464	KX086522
	<i>Spongicola robustus</i> (4)	Madagascar	MNHN-IU-2010-1744	KX086328	KX086398	KX086465	KX086523
	<i>Spongicola robustus</i> (5)	South China Sea	NTOU M01914	KX086369	KX086438	KX086500	KX086549
	<i>Spongicola</i> sp. nov.	South China Sea	NTOU M01907	KX086324	KX086394	KX086461	KX086519
Genus <i>Spongicoloides</i>							
	<i>Spongicoloides iheyaensis</i>	Taiwan	NTOU M01908	KX086355	KX086424	n/a	n/a
	<i>Spongicoloides novaezelandiae</i>	Solomon Islands	MNHN-IU-2014-6347	KX086359	KX086428	KX086490	n/a
Genus <i>Spongiocaris</i>							
	<i>Spongiocaris japonicus</i>	Japan	NTOU M01906	KX086347	KX086416	n/a	n/a
	<i>Spongiocaris koehlerii</i>	Seamount near Bermuda	MNHN-IU-2014-12841	KX086356	KX086425	n/a	n/a
	<i>Spongiocaris panglao</i>	The Philippines	NTOU M01909	KX086360	KX086429	KX086491	KX086540
	<i>Spongiocaris yaldwyni</i>	Tonga	MNHN-IU-2014-12842	KX086370	KX086439	n/a	n/a
Family Stenopodidae							
Genus <i>Juxtastenopus</i>							
	<i>Juxtastenopus spinulatus</i>	Vanuatu	MNHN-IU-2014-6692	KX086333	KX086403	KX086470	n/a
Genus <i>Odontozona</i>							
	<i>Odontozona crinoidicola</i> (1)	Papua New Guinea	MNHN-IU-2013-10251	KX086340	KX086409	KX086476	KX086528
	<i>Odontozona crinoidicola</i> (2)	Papua New Guinea	MNHN-IU-2013-10240	KX086313	KX086383	KX086450	KX086511
	<i>Odontozona crinoidicola</i> (3)	Papua New Guinea	MNHN-IU-2013-10252	KX086314	KX086384	KX086451	n/a
	<i>Odontozona crinoidicola</i> (4)	Papua New Guinea	MNHN-IU-2013-10253	KX086315	KX086385	KX086452	KX086513
	<i>Odontozona crinoidicola</i> (5)	Papua New Guinea	MNHN-IU-2013-10244	KX086337	KX086406	KX086473	n/a
	<i>Odontozona</i> aff. <i>crinoidicola</i> (1)	Papua New Guinea	MNHN-IU-2013-10238	KX086318	KX086388	KX086455	KX086516
	<i>Odontozona</i> aff. <i>crinoidicola</i> (2)	Papua New Guinea	MNHN-IU-2013-10238	KX086319	KX086389	KX086456	KX086517
	<i>Odontozona</i> aff. <i>ensifera</i> (1)	Papua New Guinea	MNHN-IU-2013-10247	KX086338	KX086407	KX086474	KX086526
	<i>Odontozona</i> aff. <i>ensifera</i> (2)	Papua New Guinea	MNHN-IU-2013-10250	KX086339	KX086408	KX086475	KX086527

(continued next page)

Table 1. (continued)

Taxon	Species	Locality	Voucher no.	12S	16S	H3	NaK
	<i>Odontozona</i> aff. <i>ensifera</i> (3)	Papua New Guinea	MNHN-IU-2013-10233	KX086335	KX086405	n/a	n/a
	<i>Odontozona meloi</i> (1)	French Guinea	MNHN IU-2013-2647	KX086316	KX086386	KX086453	KX086514
	<i>Odontozona meloi</i> (2)	French Guinea	MNHN IU-2013-2887	KX086317	KX086387	KX086454	KX086515
	<i>Odontozona sculpticaudata</i>	New Caledonia	MNHN-IU-2014-6337	KX086321	KX086391	KX086458	n/a
	<i>Odontozona</i> aff. <i>sculpticaudata</i>	Papua New Guinea	MNHN-IU-2013-10239	KX086320	KX086390	KX086457	KX086518
	<i>Odontozona spongicola</i>	South China Sea	NTOU M01910	KX086344	KX086413	KX086480	KX086531
	Genus <i>Richardina</i>						
	<i>Richardina</i> aff. <i>parvioculata</i>	South China Sea	NTOU M01911	KX086345	KX086414	KX086481	KX086532
	<i>Richardina spinicineta</i>	Guadeloupe	MNHN-IU-2013-19177	KX086373	KX086442	KX086503	KX086553
	Genus <i>Stenopus</i>						
	<i>Stenopus earlei</i>	Papua New Guinea	MNHN-IU-2013-10234	KX086336	n/a	KX086472	n/a
	<i>Stenopus goyi</i>	The Philippines	NTOU M01912	KX086353	KX086422	n/a	KX086536
	<i>Stenopus hispidus</i> (1)	Ryukyu Islands	NTOU M01913	KX086342	KX086411	KX086478	n/a
	<i>Stenopus hispidus</i> (2)	Aquarium shop, Hong Kong	MSLKHC-Sthis	KX086354	KX086423	JF346323	KX086537
	<i>Stenopus hispidus</i> (3)	Papua New Guinea	MNHN-IU-2013-233	KX086351	KX086420	KX086486	KX086535
	<i>Stenopus pyrsonotus</i> (1)	Aquarium shop, Hong Kong	MSLKHC-Stpyr	KX086361	KX086430	KX086492	KX086541
	<i>Stenopus pyrsonotus</i> (2)	French Polynesia	MNHN IU-2011-8952	KX086362	KX086431	KX086493	KX086542
	<i>Stenopus scutellatus</i> (1)	Guadeloupe	MNHN IU-2013-4378	KX086363	KX086432	KX086494	KX086543
	<i>Stenopus scutellatus</i> (2)	French Guinea	MNHN IU-2013-2463	KX086350	KX086419	KX086485	KX086534
	<i>Stenopus tenuirostris</i> (1)	Marquesas Islands	MNHN-IU-2014-12843	KX086364	KX086433	KX086495	n/a
	<i>Stenopus tenuirostris</i> (2)	Aquarium shop, Hong Kong	MSLKHC-Stten	KX086368	KX086437	KX086499	KX086548
	<i>Stenopus tenuirostris</i> (3)	Papua New Guinea	MNHN-IU-2013-10246	KX086365	KX086434	KX086496	KX086545
	<i>Stenopus tenuirostris</i> (4)	Papua New Guinea	MNHN-IU-2013-10246	KX086366	KX086435	KX086497	KX086546
	<i>Stenopus tenuirostris</i> (5)	Papua New Guinea	MNHN-IU-2013-10248	KX086367	KX086436	KX086498	KX086547
	<i>Stenopus zanzibaricus</i> (1)	Papua New Guinea	MNHN-IU-2013-10241	KX086371	KX086440	KX086501	KX086551
	<i>Stenopus zanzibaricus</i> (2)	Papua New Guinea	MNHN-IU-2013-10243	KX086372	KX086441	KX086502	KX086552
	OUTGROUP						
	<i>Alvinocaris chelys</i>	Taiwan	NTOU M01671	KX086374	KX086443	KX086504	KX086554
	<i>Gnathophyllum americanum</i>	Aquarium shop, Hong Kong	MSLKHC-CA02-Game	KX086375	KX086444	KX086505	KX086555
	<i>Palaemon serrata</i>	Hawaii	MSLKHC-CA98-Paser	KX086376	KX086445	KX086506	KX086556
	<i>Procaris hawaiiiana</i>	Hawaii	OUMNH.ZC.2010-13-007	KX086377	KX086446	KX086507	KX086557

from aquarium shops, and stored in ethanol ( $\geq 75\%$ ) before laboratory analysis.

#### 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  $\alpha$ -subunit (NaK). These markers have been widely applied in decapod phylogenetic analyses, including various groups of shrimps (Ma *et al.* 2009, 2011; Bracken *et al.* 2010; Li *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 stenopodidean-specific PCR primer sets: NaK-37F (5'-CAGTCWGGCTGTCA ATATGAYAA-3') and NaK-622R (5'-ACGGCGTCKGGYAC RGCRGC-3') for amplifying the NaK were designed on the basis of available sequences of different shrimp taxa in GenBank to maximise 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 analysed 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 were 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 optimised 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 3.2.1 (Ronquist *et al.* 2012) with two independent runs performed using four differentially heated Metropolis-coupled Markov Chain Monte Carlo computations for 5 000 000 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 1.5 (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 dataset 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 the 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 clade (ML + BP = 100; Bayesian posterior probability (BI PP) = 1.00) (Fig. 2). *Macromaxillocaris bahamaensis* is nested within representatives belonging to the Spongiolidae and Stenopodidae. *M. bahamaensis* is most closely related to *Microprosthemina*, yet this relationship was supported only 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 a large amount of missing data. The final four genes concatenated dataset consisted of 1523 bp with 69 taxa.

The nodal supports obtained from the ML and BI analyses of the four-gene concatenated dataset are shown together on the best ML topology (Fig. 3). The inferred molecular phylogeny did not support the reciprocal monophyly of Spongiolidae and Stenopodidae. The three stenopodid genera, *Juxtastenopus* Goy, 2010, *Odontozona* Holthuis, 1946 (except *Odontozona spongiicola* (Alcock & Anderson, 1899)) and *Stenopus* formed a strongly supported clade (ML BP = 99; BI PP = 0.99). Another clade that unites *Richardina* A. Milne-Edwards, 1881, *Odontozona spongiicola* and *Globospongiicola spinulatus* Komai & Saito, 2006 was recovered with strong statistical

support (ML BP = 100; BI PP = 1.00). Spongiolidae 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 reciprocal monophyly for both Spongiolidae and Stenopodidae ( $P < 0.001$ ).

Six of seven genera with multiple exemplars analysed (*Odontozona*, *Richardina*, *Spongiocaris*, *Spongiicola*, *Spongioloides* and *Stenopus*) were poly- or paraphyletic in our molecular phylogeny and only *Microprosthemina* was supported to be monophyletic. *Juxtastenopus* was placed within *Stenopus* as a sister to *Stenopus goyi* and *Stenopus earlei* Goy, 1984, making *Stenopus* a paraphyletic assemblage. *Odontozona* was polyphyletic and split into three major lineages. *O. spongiicola* clustered with *Richardina* and *Globospongiicola* (ML BP = 100; BI PP = 1.00) and this clade was more closely related to the genera in Spongiolidae than the taxa of Stenopodidae. The remaining species of *Odontozona* were paraphyletic with the *Juxtastenopus* + *Stenopus* clade nested within this group. In all instances, the AU tests rejected an *a priori* hypothesis of a monophyletic *Odontozona*, regardless of whether *O. spongiicola* 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 *Microprosthemina takedai* Saito & Anker, 2012 analysed). Although two species of *Stenopus* (i.e. *S. goyi* and *S. earlei*) formed a clade with *Juxtastenopus*, the 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 the two species of *Richardina* showing a non-sister relationship in the molecular trees.

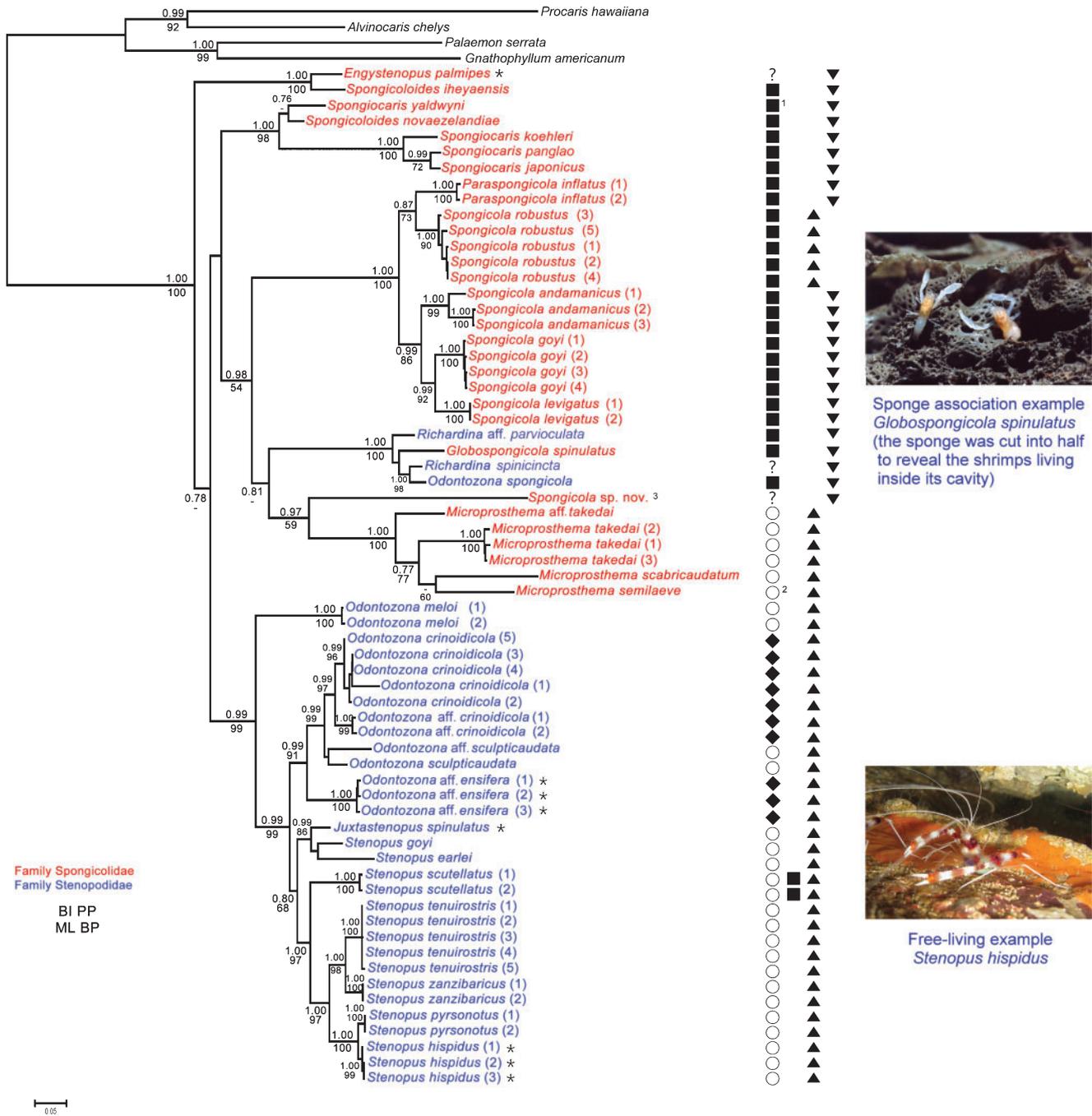
The two species of *Spongioloides* did not form a clade in the phylogeny, with *Spongioloides iheyaensis* Saito, Tsuchida & Yamamoto, 2006 grouping with *Engystenopus palmipes* Alcock & Anderson, 1894 (ML BP = 100; BI PP = 1.00) and *Spongioloides novaezelandiae* Baba, 1979 clustered with different species of *Spongiocaris* (ML BP = 98; BI PP = 1.00). *Spongiicola* sp. nov. aligned with *Microprosthemina* in the four-genes combined dataset (Fig. 3) (ML BP = 59; BI PP = 0.97), but clustered with other species of *Spongiicola* and *Paraspongiicola* in the mitochondrial gene tree (Fig. 2). The remaining species of *Spongiicola* formed a strongly supported clade but with *Paraspongiicola* nested within this group (ML BP = 100; BI PP = 1.00). The AU tests clearly rejected *a priori* hypotheses of reciprocal monophyly of *Spongiicola* and *Spongioloides* ( $P < 0.001$  in both cases), but not *Spongiocaris* ( $P = 0.164$ ). Although several 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 Spongiolidae and Stenopodidae. Macromaxillocarididae is represented by a single cave species and is considered to be unique





**Fig. 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 colour of the taxon names indicates the familial classification, with an asterisk referring to type species of the genera. 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; ◆, crinoid associated; ?, association unknown; <sup>1</sup>, reports of association with gorgonian octocoral; <sup>2</sup>, reports of association with sea anemone and flame scallops; <sup>3</sup>, new species to be described by Goy (unpubl. data).

Spongicolidae and Stenopodidae (Alvarez *et al.* 2006). However, *Macromaxillocaris* was nested deep within spongicolids and stenopodids in the mitochondrial gene tree. Furthermore, the genetic divergence among *Macromaxillocaris*

and other stenopodideans was not great. *Macromaxillocaris* is revealed to be most closely related to *Microprosthem* in our gene tree, though the statistical support is only high in the maximum-likelihood analysis. *Microprosthem* comprises

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 colonising shallow-water anchialine caves. 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 specialised 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 stenopodids *Richardina* and *Odontozona spongicola* are more closely related to members of Spongicolidae than to other stenopodids. Furthermore, Spongicolidae is paraphyletic with respect to Stenopodidae even when *Richardina* and *Odontozona spongicola* are not considered. Saito and Takeda (2003) hypothesised that deep-water sponge-associated taxa evolved from more basal shallow-water free-living lineages (also see Saito 2008). 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 comprising *Engystenopus* and *Spongicoloides iheyaensis* (Figs 2, 3). Although whether *Engystenopus* forms associations with other animals remains unclear, both *Engystenopus* and *Spongicoloides iheyaensis* (sponge-associated) are deep-sea inhabitants. Thus, the current molecular data provide some evidence that the earliest branching lineages in Stenopodidea are deep-sea inhabitants and the shrimps colonised 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 analysed form a strongly supported clade. On the contrary, *Odontozona spongicola* and several *Richardina* species are confirmed associates of hexactinellid sponges 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 another family if Spongicolidae were to be split) appears to be more appropriate.

#### Validity of the genera

The present molecular phylogeny trees show that all except one stenopodidean genus 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 was formerly placed under *Stenopus*. *Juxtastenopus* is considered to be morphologically close to, but yet different from, *Stenopus* in that the dactyli of the ambulatory pereopods are long, slender and uniunguiculate whereas most of those of *Stenopus* are biunguiculate (Goy 2010a). The shape and armature of the dactyli of the ambulatory pereopods have

been considered to be important characters in stenopodideans at the generic level (see Holthuis 1993; Goy 2010b). However, variations in the number of ungues have been found in the recently described species *Stenopus goyi*, sometimes even among the pereopods 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 expand it 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*.

*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 clade with the other *Odontozona* species from the Indo-West Pacific. With the recent discoveries of several new species, *Odontozona* becomes one of the two most species-rich genera in the Stenopodidea (with 16 species, as in *Microprosthema*). Species of *Odontozona* exhibit a wide range of life styles, from shallow to deep waters, and from free-living to being associated with sponges or other invertebrates (Figs 2, 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. *O. spongicola*) or new genera (e.g. *O. meloi*). *O. 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 have trichobranchiate gills (Komai and Saito 2006). Nevertheless, *Richardina* somewhat resembles *Globospongicola* in the reduced armament on the body and third pereopod, the well developed exopod of the second and third maxillipeds, as well as the integument of the 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 pereopods (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* than to *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 pereopods. Goy and Cardoso (2014) also suggested that *O. spongicola* lacks the spinous propodal margins of the third pereopods 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 sponges 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

Spongicolidae, so it is possible that some more species of *Richardina* are sponge commensals (though at least *R. rupicola* Komai, 2011a seems to be free living). Therefore, the present results suggest the transfer of *O. spongicola* back to *Richardina* or reassign it to *Globospongicola*, which may later be merged with *Richardina*. The formal taxonomic placement for *Richardina*, *Globospongicola* and *O. spongicola* should be decided in the future, given that only two of the six 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 japonicus* from *Spongicola* and *Spongiocaris koehleri* from *Spongicoloides*, form a strongly supported clade with only one of the two other species of *Spongiocaris* in the 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 *Spongiocaris* (including *S. 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 that the characters adopted to define the genera may be variations within a continuum. The present molecular phylogeny indicates that *Spongiocaris* may need to be synonymised with *Spongicoloides*, even though our results somewhat support the recent generic reassignment of *Spongiocaris japonicus* and *Spongiocaris koehleri* by Saito (2008) and Komai *et al.* (2016). However, the type species of *Spongiocaris*, *Spongicoloides* and *Spongiocicola* are not included in the present analysis, so particular caution will be necessary in future works in redefining these three genera.

With respect to the monotypic genus *Engystenopus*, which was first assigned to Stenopodidae (Holthuis 1946, 1955, 1993) though de Saint Laurent and Cléva (1981) suggested that *Engystenopus palmipes* is closer to *Spongicola* than to *Stenopus*. Goy (2010a) rediagnosed and transferred *E. palmipes* to Spongicolidae. The presence of a well developed exopod at the third maxilliped and the unguiculate dactyli of the fourth and fifth pereopods in *E. palmipes* is unique within Spongicolidae (Goy 2010a, 2010b). Our phylogeny corroborated the view of de Saint Laurent and Cléva (1981), Goy (2010a, 2010b) 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 a hepatic groove, and bearing small but numerous eggs (versus the carapace lacking postorbital spines and hepatic groove, and bearing large and few eggs). Whether *S. iheyaensis* should be transferred to *Engystenopus* awaits more extensive studies on *Spongicoloides*, as only two of the eight species known

in this genus are included in this analysis and the two 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 & Cléva, 1981 to *Spongicola* on the basis of the similarity in the armature of the carapace in spite of the fact that the species has a well developed exopod on the third maxilliped that resembles that of *Paraspongicola*. Saito and Takeda (2003) revealed a sister relationship for *Spongicola inflatus* and *Paraspongicola pusillus* de Saint Laurent & Cléva, 1981 in their cladogram inferred from adult morphology. Thus, Saito and Komai (2008) transferred *Spongicola inflatus* to *Paraspongicola*. The other known species of *Paraspongicola*, namely *P. acantholepis* Komai, 2011b, is also superficially rather more similar to species of *Spongicola* than to the type species, *P. pusillus* (Komai, 2011b). The presence or absence of an exopod on the third maxillipeds has been considered to be of great importance in the generic classification within Spongicolidae (de Saint Laurent and Cléva 1981; Holthuis 1993; Saito and Takeda 2003; Saito and Komai 2008; 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 imply that its species should be transferred back to *Spongicola*. In so doing, the present results support the view of Saito and Anker (2014) in considering the development of an exopod at the third maxilliped as not being an informative character in spongicolid 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 recognised 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 strongly supports the validity of the genus *Microprosthema* whilst the genus *Paraspongicola* is invalid and should be synonymised under *Spongicola*. The genera *Odontozona*, *Spongicola*, *Spongicoloides* and *Spongiocaris* need to be redefined and revised. Further studies with more extensive taxon coverage will need to determine whether the two recently established genera *Juxtastenopus* and *Globospongicola* are valid and whether *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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