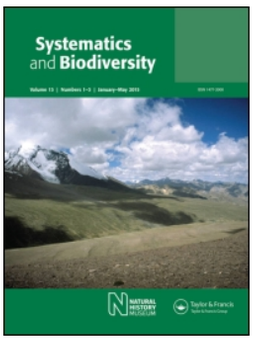

Systematics and Biodiversity

ISSN: 1477-2000 (Print) 1478-0933 (Online) Journal homepage: http://www.tandfonline.com/loi/tsab20

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To cite this article: Raúl Castro-romero, Martín M. Montes, Sergio R. Martorelli, Diego Sepulveda, Silvia Tapia & Andrés Martínez-aquino (2016): Integrative taxonomy of Peniculus, Metapeniculus, and Trifur (Siphonostomatoida: Pennellidae), copepod parasites of marine fishes from Chile: species delimitation analyses using DNA barcoding and morphological evidence, Systematics and Biodiversity

To link to this article: http://dx.doi.org/10.1080/14772000.2016.1158213

Systematics and Biodiversity (2016), 1–18

Taylor & Francis
Taylor & Francis Group

Research Article

Integrative taxonomy of *Peniculus*, *Metapeniculus*, and *Trifur* (Siphonostomatoida: Pennellidae), copepod parasites of marine fishes from Chile: species delimitation analyses using DNA barcoding and morphological evidence

RAÚL CASTRO-ROMERO[1,†], MARTÍN M. MONTES[2,†], SERGIO R. MARTORELLI[2], DIEGO SEPULVEDA[3], SILVIA TAPIA[3,1] & ANDRÉS MARTÍNEZ-AQUINO[4]

[1]Universidad de Antofagasta, Facultad Ciencias del Mar, Depto. Ciencias Acuáticas y Ambientales, Antofagasta, Chile
[2]CEPAVE, Universidad Nacional de La Plata, Buenos Aires, Argentina
[3]Universidad de Antofagasta, Depto. Biomédico, Antofagasta, Chile
[4]División Zoología Invertebrados, Museo de La Plata, FCNyM, UNLP, Paseo del Bosque s/n, 1900 La Plata, Argentina

(Received 27 October 2015; accepted 17 February 2016)

Pennellidae is a family of copepod parasites of widely distributed marine fishes. The pennellid species are usually morphologically differentiated by cephalothorax, neck, trunk, and abdomen shape. These characters, however, show high polymorphism and therefore using only this type of data, delimitation at species level of this genus is difficult. In this study, we explored the genetic distances calculated from sequences of a DNA barcoding marker (COI mt) (678 base pairs). We also explored the genetic distances of 25 *Peniculus* specimens associated within nine marine fish species, four *Metapeniculus* specimens associated within one marine fish species, and four *Trifur* specimens associated within one marine fish species. All specimens were collected in Antofagasta Bay, Chile and were calculated from sequences of a DNA barcoding marker (COI mt) (678 base pairs). The genetic distance among the *Peniculus* specimens was 0.95% from the different host species, the *Metapeniculus* specimens distance was 0.44% and the *Trifur* specimens was 2.25%. Genetic difference between *Peniculus* and *Metapeniculus* was 17.86% and *Peniculus* differ from *T. tortuosus* by 18.16%. We analysed the barcoding gene fragment using Bayesian Inference (BI) for phylogenetic reconstruction using three outgroups. Based on the phylogenetic analysis an ultrametric tree was built and a general mixed Yule-coalescent (bGMYC) model was conducted for species delimitation. Morphometrics analyses were made with Bayesian statistics. Mean and credibility limit (95%) for each parameter was calculated. Results show that based on morphology the individuals collected can be assigned to *P.* cf. *fistula* von Nordmann, 1832, *Metapeniculus antofagastensis* Castro-Romero & Baeza-Kuroki, 1985, and *Trifur* cf. *tortuosus* Wilson, 1917. High morphological polymorphism was observed for the lineage of *Peniculus* associated to several host species of marine fishes. Similar results were obtained for *Trifur* cf. *tortuosus* parasites on Chilean marine fishes.

Keywords: Antofagasta Bay, Bayesian morphometric, crustacean, genetic divergence, GMYC, molecular phylogenetic, Pennellidae, SEM, systematic

Introduction

The family Pennellidae Burmeister, 1835 (Copepoda: Siphonostomatoida), from the coast of Chile, have been represented by the genera *Peniculus* von Nordman, 1832, *Metapeniculus* Castro-Romero and Baeza-Kuroki, 1985, and *Trifur* Wilson, 1917 (Atria, 1977; Castro-Romero & Baeza-Kuroki, 1985; Muñoz & Olmos, 2007; Sepúlveda, Marin, & Carvajal, 2004; Wilson, 1917). For instance, *Peniculus fistula* von Nordmann, 1832, a cosmopolitan species and considerate generalist parasite (Appendix S1, see online supplemental material, which is available from the article's Taylor & Francis Online page at http://dx.doi.org/10.1080/14772000.2016.1158213), was recorded from *Prolatilus jugularis* Valenciennes, 1833 (Pinguipidae) and *Cheilodactylus variegatus* Valenciennes, 1833 (Cheilodactylidae) from Chile's coast (Atria, 1977; Sepúlveda et al., 2004; Wilson, 1917). On the other hand, *Metapeniculus antofagastensis* Castro-Romero & Baeza-Kuroki, 1985, an endemic parasitic from the South Pacific

Correspondence to: Andrés Martínez-Aquino. E-mail: maandres_@hotmail.com
†These authors contributed equally to this work

ISSN 1477-2000 print / 1478-0933 online

http://dx.doi.org/10.1080/14772000.2016.1158213

coast of Chile, was recorded as associated only with *Anisotremus scapularis*Tschudi, 1846 (Haemulidae) (Castro-Romero & Baeza-Kuroki, 1985). Finally, *Trifur tortuosus* Wilson, 1917 was registered from Chile parasitizing *Salilota australis* Günther, 1878 (Moridae), *Merluccius gayi* Guichenot, 1848, and *Merluccius australis* Hutton, 1872 (Merlucciidae) (Atria, 1977; Fernández, 1985; González & Carvajal, 1994; Muñoz & Olmos, 2007; Talice, 1936; Wilson, 1917).

Pennellids have been presented with great homogeneity of the appendages, especially those like legs (Alexander, 1983; Kabata, 1979). Furthermore, pennellids show a morphological variability in some characters, especially those used for the attachment on the hosts, which has probably been misinterpreted by some authors when describing some species (e.g., Castro-Romero & Baeza-Kuroki, 1988; Hogans, 1987; Castro-Romero & Joyeux, pers. obs.). For the delimitation of species with high morphological polymorphism molecular information such DNA barcoding can be applied (Jones, Ghoorah, & Blaxter, 2011). In the last decade an increasing interest in the use of DNA barcoding for species identification and taxonomy was observed (Bucklin, Steinke, & Blanco-Bercial, 2011; Hebert, Cywinska, Ball, & de Waard, 2003; Kress, García-Robledo, Uriarte, & Erickson, 2015). DNA barcoding is a powerful tool to detect species with highly conserved morphological features (morphological stasis), which make it difficult to distinguish between related species, and also distinct populations of the same species (Fontaneto, Giordani, Melone, & Serra, 2007; Hansen, Bakke, & Bachmann, 2007; Weigand, Jochum, Pfenninger, Steinke, & Klussmann-Kolb, 2010). In the case of parasite organisms of marine fishes, barcoding may be useful for delimiting populations/species. For example, barcoding was used in several studies in siphonostomatoid copepods to find morphological and molecular differentiation at the intra- and interspecific level, to find species boundaries, to detect complex cryptic species, and to support their phylogenetic relationship (Boulding, de Waard, Ang, & Hebert, 2009; Bucklin et al., 2010; Diamant et al., 2014; Dippenaar, 2009; Dippenaar, Mathibela, & Bloomer, 2010; Easton, Darrow, Spears, & Thistle, 2014; Gollner, Fontaneto, & Martínez-Arbizu, 2011; Mangena, Jordaan, & Dippenaar, 2014; McBeath et al., 2006; Morales-Serna, Pinacho-Pinacho, Gómez, & Pérez-Ponce de León, 2014; Nowak, Hayward, González, Bott, & Lester, 2011; Øines & Heuch, 2005; Øiness & Schram, 2008; Skern-Mauritzen, Torrissen, & Glover, 2014; Tjensvoll, Glover, & Nylund, 2006; Yazawa et al., 2008). However, molecular data from siphonostomatoid copepods of the family Pennellidae are scarce (Huys, Llewellyn-Hughes, Conroy-Dalton, Spinks, & Johnston, 2007; Muñoz, Landaeta, Palacios-Fuentes, López, & González, 2015; Yasuike et al., 2012).

In order to attend the description of the taxonomic diversity of siphonostomatoid pennellids we collected several parasitic specimens of *Peniculus*, *Metapeniculus*, and *Trifur* genus from 10 host species of marine fishes from Antofagasta Bay (South Pacific coast of Chile). Therefore, the main aim in this study was to unravel their taxonomic status based on an integrative taxonomic approach using scanning electron micrography (SEM) and morphometric analysis, and applied state-of-the-art phylogenetic tools on sequences of the barcoding to estimating species boundaries using general mixed Yule-coalescent (GMYC) models. Finally, we established an overview on the systematic position of *Peniculus*, *Metapeniculus*, and *Trifur* within the phylogeny of the Penellidae.

Materials and methods

Collection of hosts and copepod parasites

Between January 2012 and December 2012 a total of 575 individual hosts were caught in order to collect copepod parasites from the Pennellidae family. The hosts represent 10 wild fish species included in nine families and four orders. Samples were collected from Antofagasta Bay (Fig. 1). Specimens of *Peniculus* genus were found in nine host species:

Anisotremus scapularis, *Cheilotrema fasciatum* Tschudi, 1846, *Chromis crusma* Valenciennes, 1833, *Girella laevifrons* Tschudi, 1846, *Hemilutjanus macrophthalmos* Tschudi, 1846, *Isacia conceptionis* Cuvier, 1830, *Mugil cephalus* Linnaeus, 1758, *Odontesthes regia* Humboldt, 1821, and *P. jugularis*, specimens of *M. antofagastensis* were found as a parasite of *A. scapularis*, specimens of *Trifur* genus were found as a parasite of both *I. conceptionis* and *Sebastes oculatus* Valenciennes, 1833 (Table 1). Additionally, we collected specimens of two species of copepods, *Prokroyeria meridionalis* Ramírez, 1975 (Siphonostomatoida: Kroyeriidae) as a

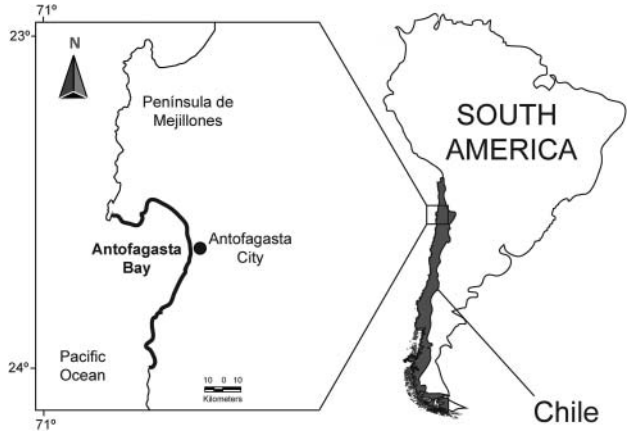

Fig. 1. Map of Antofagasta Bay, Chile.

Table 1. Details of copepod parasite species of marine fishes from Chile used in this study.

Copepod parasites species	Host (Family) [Code]	N	Prevalence (%)	Vouchers	GenBank accession
Siphonostomatoida					
Pennellidae					
Peniculus cf. *fistula* Von Nordmann, 1832	*Anisotremis scapularis* Tschudi, 1846 (Pomadasidae) [Asc]	21	4.5		KU557437
	Cheilotrema fasciatum Tschudi, 1846 (Sciaenidae) [Cfa]	5	25	15118	KU557429
	Chromis crusma Valenciennes, 1833 (Pomacentridae) [Ccr]	25	12	15113	KU557427-28
	Girella laevifrons Tschudi, 1846 (Kyphosidae) [Gla]	32	13.9	15117	KU557438-41
	Hemilutjanus macrophthalmus Tschudi, 1846 (Haemulidae) [Hma]	10	70	15116	KU557417-22
	Isacia conceptionisn Cuvier, 1830 (Haemulidae) [Ico]	100	4	15119	KU557425-26
	Mugil cephalus Linnaeus, 1758 (Mugilidae) [Mce]	115	3	15120	KU557430-32
	Odonthestes regia Humboldt, 1821 (Atherinopsidae) [Ore]	125	20.7	15114	KU557433-36
	Prolatilus jugularis Valenciennes, 1833 (Pinguipedidae) [Pju]	16	56.3	15115	KU557423-24
Metapeniculus antofagastensis Castro & Baeza, 1985	*Anisotremis scapularis* (Tschudi, 1846) (Pomadasidae) [Asc]	21	43.75		KU557413-16
Trifur cf. *tortuosus* Wilson, 1917	*Isacia conceptionisn* Cuvier, 1830 (Haemulidae) [Ico]	100	4	15122	KU557442-43
	Sebastes oculatus Valenciennes, 1833 (Sebastidae) [Soc]	5	30	15121	KU557444-45
Kroyeriidae					
Prokroyeria meridionalis Ramírez, 1975	*Callorhynchus callorhynchus* Linnaeus, 1758 (Callorhinchidae) [Cca]				KU557410
Poecilostomatoida					
Ergasilidae					
Ergasilus sp.	*Mugil liza* Valenciennes, 1836 (Mugilidae) [Mli]				KU557411-12

N = Number of examined host, Code = Code use for each individual of copepod sequenced, as shown in the terminal taxa names of figure 1 (three letters for species host and number, respectively), Voucher = Catalogue material, and GenBank = Accession number.

parasite of *Callorhynchus callorhynchus* (Linnaeus, 1758) from Antofagasta Bay, and *Ergasilus* sp. (Poecilostomatoida: Ergasilidae) as a parasite of *Mugil liza* Valenciennes, 1836 from Samborombon Bay, Argentina. These specimens were used as outgroups for the phylogenetic analysis in the study.

Fish were caught by hand line or were obtained from commercial artisanal capture and then carried to the laboratory for visual or stereomicroscope inspection. The specimens for morphological studies were collected and fixed in 75% ethanol, and for molecular analysis fixed with 100% ethanol. The specimens for morphological study were cleared in lactic acid, when necessary, or they were cleaned with a diluted solution of KOH to draw the body or the appendages. Drawings were made using a binocular microscope equipped with millimetre eye piece and drawing tube. Morphological aspects and terminology follow Kabata (1979) and Huys and Boxshall (1991). Voucher specimens were deposited in the Museo Nacional de Historia Natural de Santiago de Chile, Chile.

Morphological data and Bayesian morphometrics

Particularly, the specimens of *Peniculus* genus were analysed with morphological meristic data because they showed a high morphological variation within and between their hosts. In the case of *Metapeniculus* and *Trifur* samples morphometric analysis was not necessary. For morphology studies, the length and width measurement were expressed in micrometres with mean and range (minimum−maximum), unless indicated otherwise in the text. The cephalothorax term is used as defined by Venmathi Maran, Moon, Oh, Ho, and Myoung (2012). Also the ratios between the cephalothorax length/neck length, fourth segment length/fourth segment width; fourth segment length/trunk length, trunk length/width for the *P.* cf. *fistula* specimens were calculated from different hosts. Specimens of *P. fistula* were donated by Z. Kabata and A. Otkener (Turkey), and used for morphological comparison. The description of *P.* cf. *fistula* was based on Boxshall (1986), Claus (1864), Delamare-Deboutteville and

Nunes (1951), Lewis (1964), Moon and Choi (2014), Vidjack, Zorica, and Sinovic (2008), and von Nordmann (1832). Comparison with other *Peniculus* species was based on Boxshall (1986), Kabata (1979), Leigh-Sharpe (1934), Lewis (1964), Moon and Choi (2014), Okawachi, Uyeno, Ogino, and Nagasawa (2012), Shiino (1956), Venmathi Maran et al. (2012), Wilson (1917), and Yamaguti (1939). Morphometric analyses were made with EPIDAT 4.1 (Hervada et al., 2014). Mean and credibility limit (to the 95% significance level) for each parameter was obtained using Bayesian statistics (McCarthy, 2007).

DNA extraction, PCR amplification, and sequencing

Sequence fragments corresponding to the standard animal DNA barcoding marker were obtained with a range of 571 to 678 base-pairs (bp). To obtain a range of the genetic variability in copepods (while avoiding sequencing individuals from the same host), DNA was extracted from one individual of pennellid (*Peniculus* sp., *M. antofagastensis*, and *Trifur* sp.), for each host (in all analysed 25 *Peniculus* specimens; four *Metapeniculus* specimens and four *Trifur* specimens all associated with nine marine fish all from Antofagasta Bay, as suggested in another study on molecular data of parasites) (Martínez-Aquino, Ceccarelli, & Pérez-Ponce de León, 2013). In addition, specimens of *Ergasilus* sp. and *P. meridionalis* were used for DNA extraction. The DNA extraction was performed using releasing reagent GeneReleaser® DNA Full Size (BioVentures, Inc.), with some modifications (Shizas, Street, Coull, Chandler, & Quattro, 1997). Each assay was performed with replicates. *Drosophila melanogaster* Meigen, 1830 larvae were used as positive control. The mtDNA COI mitochondrial gene region was amplified by Polymerase Chain Reaction (PCR) (Saiki et al., 1988), using LCO1490 fwd (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′) and HCO2198 reverse (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) (Folmer, Black, Hoen, Lutz, & Vrijenhoek, 1994) for the barcode fragment. The reactions were prepared using Green GoTaq 5× Buffer (Promega), 2.5 mM MgCl2 (Promega), 0.2 mM of NEB Nucleotide Mix and Flexi GoTaq polymerase enzyme (Promega). This procedure was carried out using a PTC-100 thermocycler Pelter. PCR protocol follows that of Burgos et al. (2003). The PCR products were analysed by electrophoresis in 1% agarose gel using TAE 1× buffer supplemented with 2 μl of ethidium bromide in the presence of UV light. Sequencing was carried out in a specialized laboratory (Macrogen, Korea).

Molecular data, phylogenetic reconstruction, and species delimitation analyses

All sequences were edited using the platform Geneious Pro v5.1.7 (Drummond et al., 2010). Barcode fragment alignments were assembled using an interface available with MAFFT v.7 (Katoh & Standley, 2013) within Geneious Pro, with a final edition by eye in the same platform. For the barcode sequences, we checked the nucleotide alignment and for the presence of pseudogenes in Geneious Pro, using the translated amino acid sequences based on the invertebrate mitochondrial genetic code. The best partitioning scheme and substitution model for each DNA partition was chosen under the Bayesian Information Criterion (BIC; Schwarz, 1978) using the 'greedy' search strategy in Partition Finder v.1.1.1 (Lanfear, Calcott, Ho, & Guindon, 2012; Lanfear, Calcott, Kainer, Mayer, & Stamatakis, 2014). The barcode fragment dataset was partitioned into first-, second- and third-codon positions with the appropriate nucleotide substitution model implemented for each codon position (TrN+I for the first [Tamura & Nei, 1993], F81 for the second [Felsenstein, 1981] and TrN+G for the third codon position [Tamura & Nei, 1993]). Sequences from two additional species, *P. meridionalis* and *Ergasilus* sp., were used as outgroup taxa.

The phylogenetic reconstruction was carried out using Bayesian Inference (BI) through MrBayes v.3.2.1 (Ronquist et al., 2012) and BEAST v1.8.0 (Drummond, Suchard, Xie, & Rambaut, 2012). The phylogenetic trees were reconstructed using two parallel analyses of Metropolis-Coupled Markov Chain Monte Carlo (MCMC) for 20×10^6 generations each, to estimate the posterior probability (PP) distribution. Topologies were sampled every 1000 generations. Once the average standard deviation of split frequencies was determined, it was less than 0.01, as suggested by MrBayes 3.2. For both MrBayes and BEAST analyses (Appendix S2 and Appendix S3, see supplemental material online), two separate runs were carried out and the last 10,000 trees from each run combined after establishing in Tracer v.1.5. that the runs had stabilized at similar likelihood values (correct 'mixing' of chains). The robustness of the clades was assessed using Bayesian PP, where PP > 0.95 was considered strongly supported. A majority consensus tree with branch lengths was reconstructed for each run after discarding the first 15,000 sampled trees, in both analyses. The Bayesian phylogenetic reconstructions were run through the CIPRES Science Gateway V.3.3 (Miller, Pfeiffer, & Schwartz, 2010).

A general mixed Yule-coalescent (GMYC) model (Pons et al., 2006), implement in R v.3.0.2. (R core team, 2013) package 'splits' v.1.9-19 (Ezard, Fujisawa, & Barraclough, 2009), was used for estimating species boundaries directly based on the phylogenetic tree topologies. Analyses were conducted with the phylogenetic topology obtained by MrBayes and BEAST, respectively, each one converted to an ultrametric tree using r8s v.1.7.1 (Sanderson, 2003). In addition, a Bayesian implementation of the GMYC model ('bGMYC' package v.1.0.2 for R, Ried & Cartens, 2012) was applied to a random sample of 100 of the last 500 trees from the two BEAST runs, setting the MCMC simulation at 50,000 generations with a

burn-in at 40,000 sampling every 100th generation. The default priors for the Yule and coalescent rate change parameters were used. A list of delimited GMYC species (described in the file's output as Maximum Likelihood entities) was compiled from the graphical output of the GMYC analysis in R. Additionally, the proportion (*p*) of absolute nucleotide sites (*p-distance*) (Nei & Kumar, 2000) was obtained to compare the genetic distance among and between lineages, with and without outgroups. The *p* value matrix was obtained using MEGA v.6.0 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013), with variance estimation, with the bootstrap method (500 replicates) and with a nucleotide substitution (transition + transversions) uniform rate.

Results

Bayesian morphometrics

In Fig. 2, the graphical representation of the measurements and the ratio values between the structures of *P.* cf. *fistula* on the different host species can be seen. The overlap in the range of the confidence limits between the measurements and relations prevents the definition of groups based on morphometric measurements (e.g., comparing the relation of the fourth segment length versus width and cephalothorax length versus neck length from copepods).

Molecular phylogenetic analysis

A total of 36 barcoding section sequences were obtained from individuals assigned to *P.* cf. *fistula* (25 specimens, ingroup), *M. antofagastensis* (four specimen, ingroup), *T.* cf. *tortuosus* (four specimens, ingroup), *P. meridionalis* (one specimen, outgroup), and *Ergasilus* sp. (two specimens, outgroup). In total, for the *P.* cf. *fistula*, we obtained sequences of barcoding region from Antofagasta Bay that were associated with two Haemulidae, one Kyphosidae, one Atherinopsidae, one Mugilidae, one Pinguipedidae, one Pomacentridae, one Pomadasidae, and one Sciaenidae species, respectively (Table 1). The barcoding dataset of *P.* cf. *fistula* consisted of 678 bp, except for five individuals that had 656 bp (three from *O. regia*, one from *G. laevifrons*, and one from *H. macrophthalmos*), two individuals that had 635 bp (one from *I. conceptionis* and one from *C. crusma*) and three individuals with 629 bp (from *M. cephalus*). *Metapeniculus antofagastensis* and *T.* cf. *tortuosus* also had 678 bp, with an exception of only one of these last species, with a 571 bp (from *S. oculatus*); and the *P. meridionalis* sequence obtained had 630 bp. For outgroups, barcoding sequences obtained in this study of *Ergasilus* sp. also had 678 bp, and the *P. meridionalis* 633 bp. The level of variation in the single nucleotide polymorphisms (SNPs) for each partition (first, second, and third codon positions) was 190/224/77 conserved, 36/2/149 variable, 32/0/146 parsimony informative and 4/2/3 singleton sites, respectively. In the partition of the protein-coding gene, the third codon position was the most variable, followed by the first and then the second position.

Bayesian phylogenetic relationships were inferred from 36 individuals of parasite copepods to assess species limits using GMYC analyses. The results of these analyses are presented in Table 2. The number of *species* − independent evolutionary lineages − detected by the GMYC analyses with the barcode dataset was 4 (Fig. 3). The genetic distance values among *P.* cf. *fistula* was 0.95%, and when compared with the outgroups, it was 17.86% with *M. antofagastensis*, 18.16% with *T.* cf. *tortuosus* (Table 3).

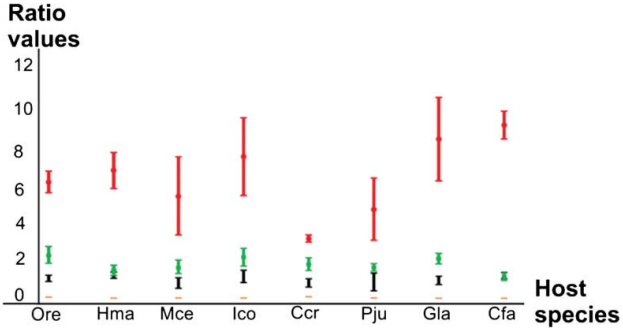

Fig. 2. Morphometric analyses with Bayesian statistics (mean and the credibility limit to the 95% significance level). Green lines = Mean and credibility limits of cephalothorax length *versus* neck length. Black lines = Mean and credibility limits fourth segment length *versus* fourth segment width. Orange lines = Mean and credibility limits fourth segment length *versus* trunk length. Red lines = Mean and credibility limits trunk length *versus* trunk width. Ore = *Odontesthes regia*, Hma = *Hemilutjanus macrophthalmos*, Mce = *Mugil cephalus*, Ico = *Isacia conceptionis*, Ccr = *Chromis crusma*, Pju = *Prolatilus jugularis*, Gla = *Girella laevifrons*, Cfa = *Cheilotrema fasciatum*.

Table 2. Number of species recovered and outputs obtained from the GMYC analyses performed from MrBayes and BEAST software using a DNA barcoding fragment.

	T	NC (ci)	NS (ci)	L0	LGMYC	LR
MrBayes Multiple	Na	4 (4-4)	7 (5-7)	174.49	181.92	14.88**
MrBayes Single	−0.05	4 (4-5)	5 (5-6)	174.49	181.43	13.88**
BEAST Multiple	Na	5 (4-6)	6 (5-18)	217.29	221.13	7.68*
BEAST Single	−0.01	6 (3-7)	7 (4-14)	217.29	220.48	6.39*

T., threshold genetic distance from the branch tips where the coalescent-speciation transition occurred; NC (ci), number of clusters (GMYC species with more than one individual) (ci, confidence intervals of GMYC species); NS (ci), number of GMYC species discriminated; L0, likelihood of null model; LGMYC, likelihood of GMYC model; LR, likelihood ratio with significance indicated by an asterisk (*$P < 0.05$; **$P < 0.01$).

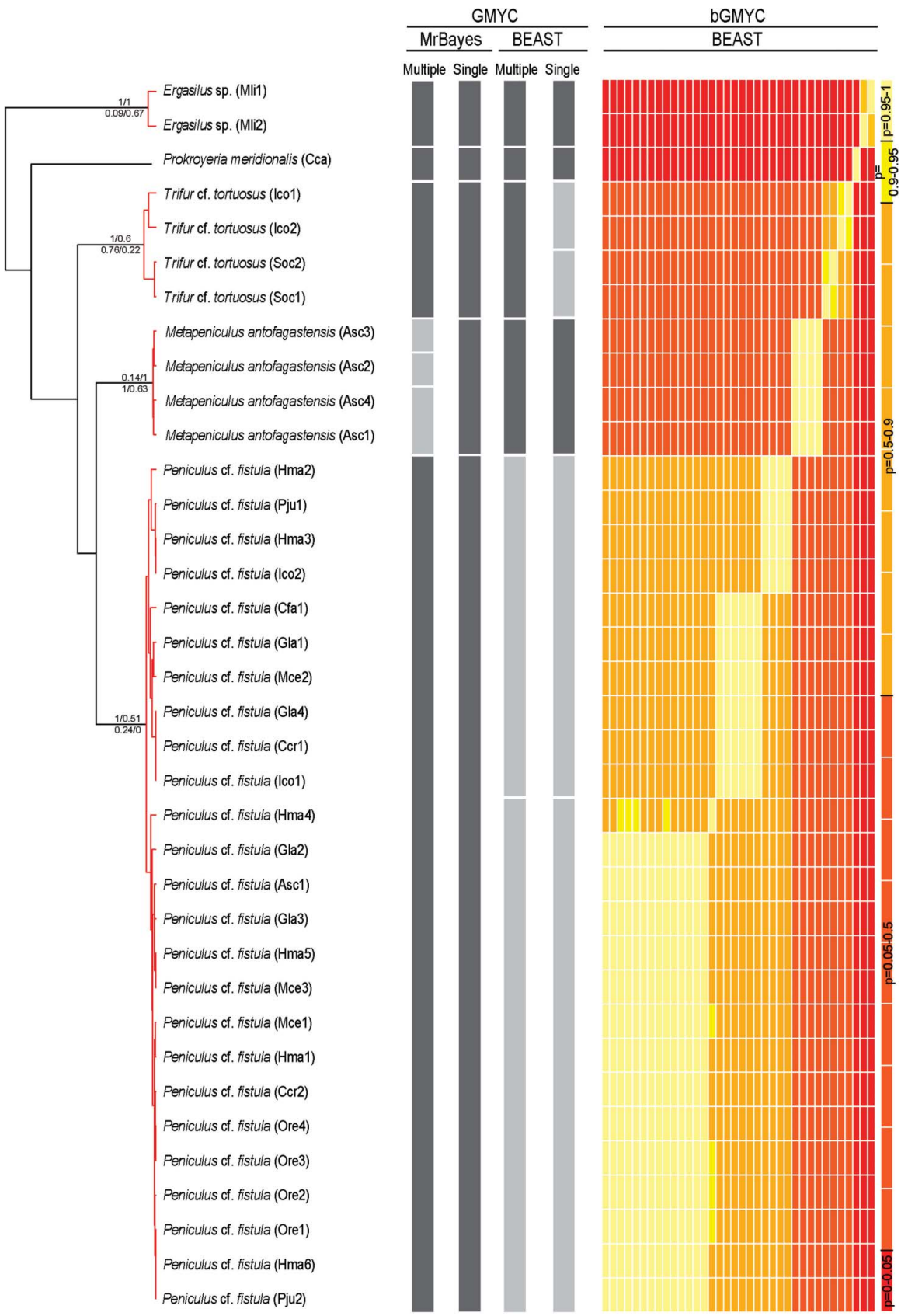

Fig. 3. Results of species delimitation analyses. Bayesian ultrametric tree inferred from the DNA barcoding dataset and subjected to GMYC analyses. Names of terminal taxa include in parentheses a code referring to the host species (three letters), and numbers indicating the isolate (for more information see Table 1). Values of probability posterior above/below branches represent Bayesian posterior probability 0.95. Light grey columns reveal differences in species delimitation in the GMYC analyses. Colour heat map represents the probability of species delimitation detected by bGMYC.

Table 3. Distance matrix of uncorrected p-distances among and within species derived from DNA barcoding analyses by the general mixed Yule-coalescence models.

	Ergasilus sp.	*P. meridionalis*	*T.* cf. *tortuosus*	*M. antofagastensis*	*P.* cf. *fistula*	Intraspecific
Ergasilus sp.						1.47
Prokroyeria meridionalis	29.86					-
Trifur cf. *tortuosus*	27.24	22.82				2.25
Metapeniculus antofagastensis	28.83	23.82	19.78			0.44
Peniculus cf. *fistula*	27.20	23.37	18.16	17.86		0.95

Genera diagnosis

In order to compile the information generated in this study, plus previously published (Boxshall, 1986; Castro-Romero & Baeza-Kuroki, 1985, 1986, 1988, 1989a, b; Castro-Romero, 2014; Delamare-Debouttevile & Nunes, 1951; Etchegoin, Lafranchi, & Timi, 2009; Kabata, 1979; Moon & Choi, 2014; Venmathi Maran et al., 2012; Wilson, 1917), for *Peniculus*, *Metapeniculus*, and *Trifur* an actualized diagnosis is presented.

Peniculus von Nordmann, 1832
(amended from Wilson, 1917; Kabata, 1979)

Description. Cephalothorax elongated, not bearing holdfast. Buccal area short, in some extending like a proboscis. Buccal tube characterized by a labrum composite (bearing two pair of lateral slits), labium armed with two pairs of scale-like plates. Intrabuccal stylet recurved at its base with simple setae. Intrabuccal armature with a central plate, lateral expansion simple. Mandible a dentate blade with a variable number of teeth (7−10). Maxillule biramous, inner lobe a papilla with two long setae, outer lobe a short papilla with a short seta. Maxilla uniramous, located near the buccal tube, close to the maxillule, lacertus armed with a spiniform process (in some with an annexed short spiniform process, or a medial distal margin cuticularized), lacertus with two flaps on inner surface, claw armed with row of fine setae. Neck, variable length, not reinforced with cuticular skeleton. Fourth segment fused or separated to trunk. Shape of the fourth segment subcircular or elongated. Trunk, variable wide. Abdomen not developed or more developed, in some bifid. Caudal ramus with six setae.

Metapeniculus Castro-Romero & Baeza-Kuroki, 1985
(amended from Castro-Romero & Baeza-Kuroki, 1985)

Description. Cephalothorax suborbicular, anterolateral angles projected into short lobes capable of expanding its cephalothorax, varying its shape (when changing its site of attachment). Buccal area, prolonged into a proboscis, highly muscular. Labrum simple, short. Labium armed with a pair of scale-like plates. Intrabuccal stylet short with a short seta. Intrabuccal armature with central plates plane and the lateral projection convoluted. Mandible apparently a stylet not denticulated. Maxillule biramous, with inner lobe, outer lobe. Maxilla uniramous, located at base of cephalothorax, at a distance from the buccal tube and the maxillule, lacertus armed with two spiniform processes, brachium without armature, claw with row of fine setae, the distal longer than the others. Neck long, with cuticular skeleton. Fourth thoracic segment fused to trunk. Three pairs of legs. Abdomen developed. Caudal rami armed with six setae. Egg sac straight.

Trifur Wilson, 1917
(amended from Wilson, 1917)

Description. Cephalothorax suborbicular distally, with a central part in which is located the buccal area, dorsally bearing the antennule and antenna. Armed with three holdfasts, of variable length and width, one oriented on each side and one posteriorly. Antennule apparently three segmented, provided with a long asthete 60−87% of the antennule length. Provided with bifid setae, two distally and another on basal segment, and the presence of plumose seta on basal segment and distal segment. Antenna bisegmented, chelated, with variable size of the first segment related to the second one. Buccal area, short, not forming proboscis, typical buccal tube, with three rings. Dorsally covered by the labrum, the latter simple, with short latero-distal projection. Labium armed with row of scale-like plates, some with serrated margin. Intrabuccal stylet short, simple base and distal short, simple seta. Mandible apparently divided into two parts, distal part bearing apically a dentate blade, with teeth on ventral margin, denticulation in some is difficult to define, with variation on presence of some denticle on distal margin in addition to the ventral teeth. Maxillule biramous, inner lobe a long papilla surmounted by two long setae, outer lobe a short papilla, external to the inner lobe, armed with a setae. Presence of a lobe of different developments, suborbicular to more elongated, ventrally to both rami. Maxilla, uniramous, lacertus strong, longer than brachium and claw together, with two strong spiniform processes. Brachium, shorter than lacertus, armed with a flap. Claw slender, armed with two rows of fine setae. Neck long, narrow, not bearing annexe structure. Bearing the legs, located at some distance each to the other, except the second pair which are very close to the first one. Legs: four

pairs, first and second biramous, and third and fourth pair uniramous, rami usually broken. Trunk sigmoid, the genitals are with a short projection. Abdomen long, widening and blunt distally. Caudal ramus not distinguishable. Eggs sacs coiled in those specimens located also inside the branchial chamber or in the palate of its host, or elongated in the case of those living attached externally to the host.

Species diagnosis

Peniculus* cf. *fistula von Nordmann, 1832
(Fig. 4–28)

VOUCHER SPECIMENS: No. 15113–15120, Museo Nacional de Historia Natural de Santiago de Chile (MNHNCL), Chile.

LOCALITY: Antofagasta Bay (South Pacific coast of Chile).

HOSTS: *O. regia*, *H. macrophthalmos*, *M. cephalus*, *I. conceptionis*, *C. crusma*, *P. jugularis*, *G. laevifrons*, *A. scapularis*, and *C. fasciatum*.

SITE INFECTION: All fins rays attached to rays.

MATERIAL EXAMINED: Seven specimens from *C. crusma*, eight from *G. laevifrons*, six from *H. macrophthalmos*, eight from *I. conceptionis*, six from *M. cephalus*, 23 from *O. regia*, nine from *P. jugularis*, one from *A. scapularis*, and one from *C. fasciatum*.

MEASUREMENTS: see Table 4.

Description

Female. Cephalothorax elongated (Fig. 4–9 and 25). The fourth segment variable in shape (sub-orbicular in specimens from *C. crusma*), and more elongated in specimens from *H. macrophthalmos* and *O. regia*. This segment is armed with three short spines (visible by transparency) (Fig. 22) in specimens from *O. regia*, and in specimens from *P. jugularis*, with two spines on the anterior part of the trunk (Fig. 23).

Table 4. Morphological measurements (in μm) of *Peniculus* cf. *fistula* associated with nine marine fish species. The codes of three letters are referring to the host species and correspond with those mentioned in Table 1.

Host	Total length	Cephalothorax length/neck length. Mean (Min.–Max.)	Trunk length/trunk wide. Mean (Min.–Max.)	Fourth segment length/ trunk length. Mean (Min.–Max.)	Fourth segment length/ fourth segment wide. Mean (Min.–Max.)
Ore	3.91 (3.19–4.62)	59 (54–63)	21 (19–24)	14 (7–20)	100 (67–158)
Hma	6.45 (5.79–7.10)	75 (69–81)	17 (14–19)	7 (6–8)	111 (90–123)
Mce	5.16 (3.55–6.76)	66 (56–75)	22 (15–29)	8 (4–11)	78 (54–107)
Ico	6.06 (4.80–7.32)	51 (41–60)	15 (11–20)	7 (5–9)	107 (87–131)
Ccr	2.96 (2.50–3.42)	60 (50–71)	35 (33–37)	12 (9–16)	79 (50–107)
Pju	4.72 (2.75–6.07)	66 (62–70)	24 (16–32)	9 (4–13)	83 (55–108)
Gla	6.76 (4.22–9.54)	54 (49–58)	14 (9–18)	6 (5–7)	94 (70–120)
Asc	2.55	56	17	15	77
Cfa	8.47 (8.06–8.74)	97 (91–100)	12 (11–13)	6 (6–7)	90 (79–112)

Abdomen (Fig. 4–9)**.** Development variable is not pronounced in some or well developed but blunt in others. Caudal rami (Fig. 24), armed with six setae (three long on distal margin, two of median size on surface near distal margin, and a short setae on distal outer margin).

Buccal area. Generally short (retractable into the cephalothoracic cavity, Fig. 25) or in others, more developed,

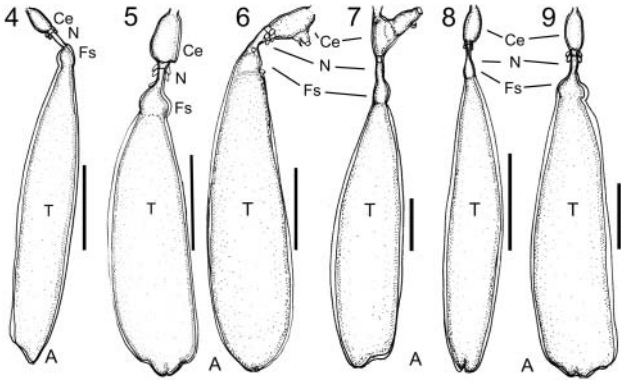

Fig. 4–9. 4. Polymorphism drawings of *Peniculus* cf. *fistula.* Specimen from *Hemilutjanus macrophthalmos.* **5.** Specimen from *Chromis crusma.* **6.** Specimen from *Girella laevifrons.* **7.** Specimen from *Odonthestes regia.* **8.** Specimen from *Mugil cephalus.* **9.** Specimens from *Prolatilus jugularis* (A = abdomen). Ce = Cephalothorax; N = Neck; Fs = Fourth segment. All scale bars: 1,000 μm.

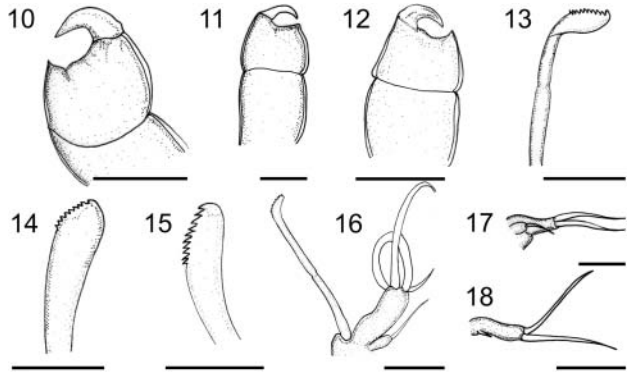

Fig. 10–18. 10. Appendages drawings of *Peniculus* cf. *fistula.* Antenna from *Hemilutjanus macrophthalmus*. **11.** Antenna from *Chromis crusma*. **12.** Antenna from *Prolatilus jugularis.* **13.** Mandible from *H. macrophthalmos*. **14.** Mandible (distal part) from *Mugil cephalus.* **15.** Mandible, distal end, from *Odonthestes regia.* **16.** Maxillule from *O. regia* entire and maxillule. **17.** Maxillule from *H. macrophthalmos.* **18.** Maxillula from *Girella laevifrons.* Scale bars: Fig. 14: 10 μm; Figs. 10, 13, and 17: 25 μm; Figs. 11–12, 15–16 and 18: 50 μm.

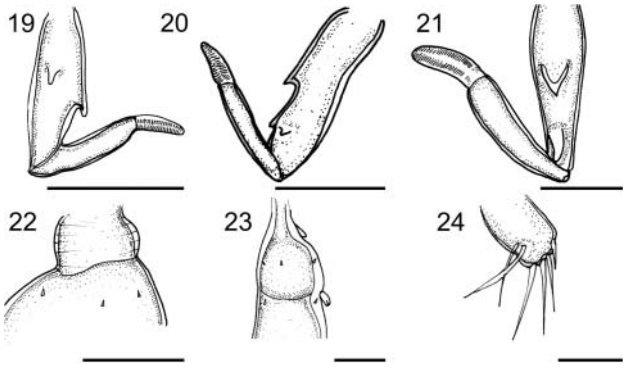

Fig. 19–24. 19. Maxilla from *Hemilutjanus macrophthalmos.* **20**. Maxilla from *Odonthestes regia.* **21.** Maxilla from *Prolatilus jugularis.* **22.** Fourth segment of *Peniculus* cf. *fistula* from *O. regia*. **23.** Fourth segment of *P.*cf. *fistula* from *P. jugularis*. **24.** Caudal rami of *P.*cf. *fistula*. Scale bars: Fig. 19–21: 50 μm; Fig. 22: 100 μm; Fig. 23: 200 μm; Fig. 24: 25 μm.

extended like a proboscis, like in specimens from O. *regia* (Fig. 26) not well muscularized.

Appendages. Antennula not detected. Second antenna (Fig. 10–12) usually covered by chitinous capsules, basal segment subrectangular, as wide as the second segment. The latter with straight margin in specimens from *P. jugularis* (Fig. 12) or convex margin in specimens from *H. macrophthalmos* (Fig. 10) the distal margin of this segment pronounced into a spiniform process, which receives the claw distal end. The medial distal margin with a soft sinuosity. Claw strong, wide, only in specimens from *C. crusma* appear more narrow (Fig. 11).

Mandible (Fig. 13–16)**.** With ten teeth in specimens from *H. macrophthalmos* (Fig. 13) and *O. regia* (Fig. 15), nine teeth in specimens from *M. cephalus* (Fig. 14), and only seven teeth observed in specimens of *A. scapularis* (Fig. 27). Maxillule (Figs 17 and 18) biramous; inner lobe with a long papilla bearing two setae, as long as the base, outer lobe with a short papilla bearing a single setae. In specimens from *H. macrophthalmos* the outer lobe is short (Fig. 17) and very reduced in *P.* cf. *fistula* from *G. laevifrons* (Fig. 18). Specimens from *A. scapularis* (Fig. 28) show the outer lobe with a papillae sub-circular.

Maxilla (Fig. 19–21)**.** With lacertus strong armed with only one spiniform process. Brachium with two flaps on inner surface, claw usually with two rows of fine setules.

Legs. Four pairs, usually broken in adult specimens.

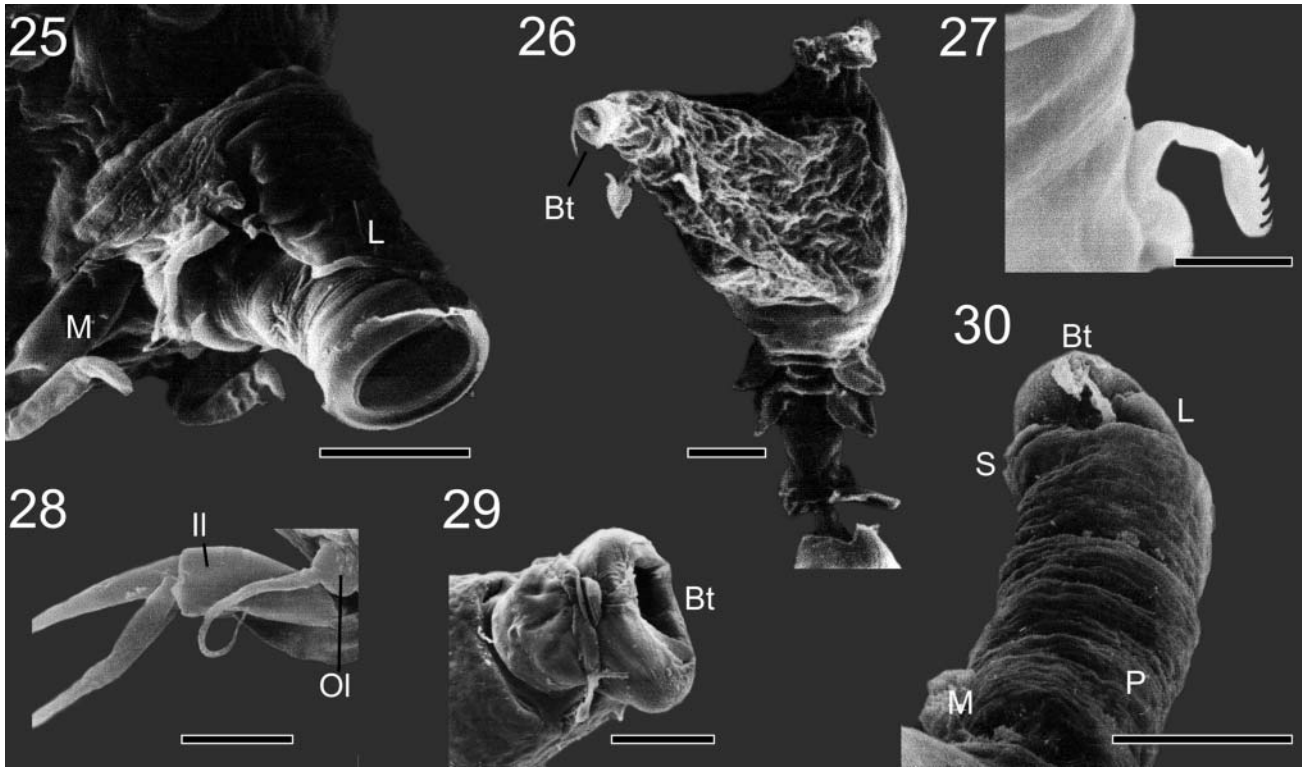

Fig. 25–30. 25. *Peniculus* cf. *fistula* buccal area front lateral view. **26.** Cephalothorax lateral view showing expansion-like proboscis in *P.* cf. *fistula* from *Odonthestes regia.* **27.** Maxillule of *P.* cf. *fistula* from *Anisotremis scapularis*. **28.** Maxillule of *P.* cf. *fistula* from *A. scapularis* in lateral view (Il = Inner lobe, Ol = Outer lobe). **29.** *Metapeniculus antofagastensis* showing the labrum and buccal tube. **30.** Proboscis of *M. antofagastensis.* Bt = Buccal tube, L = Labrum, M = Maxilla; .S = Scales on labium. Scale bars: Fig. 25: 50 μm; Fig. 26: 100 μm; Fig. 27–29: 10 μm.

Remarks. The specimens examined cannot be considered conspecifics with the majority of the eight species known in the genus; from *Peniculus calamus* Nordmann, 1864 and *Peniculus minuticaude* Shiino, 1956 can be differentiated because the abdomen have a conformation bi-lobed and are short in these two species while not developed or blunt in the present specimens. *Peniculus truncatus* Shiino, 1956 can be discarded because they bear a straight abdomen, not developed. While a different shape in the present specimens, also the fourth segment is fused to the trunk in *P. truncatus*, but not so in the present reported specimens from *P. ostraciontis*.

From *Peniculus clavatus* Muller, 1779 and *Peniculus elongatus* Boxshall, 1986 both sharing the presence of a proboscis, can be considered not conspecific with the specimens from Chilean fishes because of the cephalothorax shape and the trunk distal end. Here care must be taken in the proboscis, which is present in *P. clavatus* and *P. elongatus*, but only in *P.* cf. *fistula* from *O. regia*. Is the length of proboscis a valid difference? It is worth mentioning that the proboscis in *Peniculus* sp. is highly contractile, and can be partially occulted in the cephalothoracic cavity, as has been shown previously for *P. fistula* (Moon & Choi, 2014).

Peniculus quadratus Moon & Choi, 2014 recently described, is differentiated from all the *Peniculus* species known (and from the present specimens) by the presence of subquadrangular cephalothorax, but its site of attachment on the fish palate could have produced such deformation of the shape (as demonstrated for *Metapeniculus* sp., which suffered great changes in the cephalothorax morphology when attached to other sites).

The other species reported for *Peniculus* sp., but were considered as species, are not valid: *Peniculus communis* Leigh-Sharpe, 1934 and *Peniculus elegans* Leigh-Sharpe, 1934 cannot be considered conspecific with the present examined specimens, because *P. communis* is a species with rectangular cephalothorax, and cuticular skeleton in the neck, not present in any species of *Peniculus*, but present in both *Metapeniculus* and the recently raised *Propeniculus* (Castro-Romero, 2014). *Peniculus communis* could belong to *Propeniculus*, but its morphology, buccal area, labrum type, and armature on labium should be investigated. *Peniculus elegans* shows a quadrangular cephalothorax and fourth segment fused with the trunk, on the other hand, the cephalothorax is elongated and the fourth segment separated in the present specimens. Consequently to the above expressed, the authors decided to let the specimens here studied as *P.* cf. *fistula*, by some similarities with the type species, e.g., range of morphological variation (neck, cephalothorax, fourth segment), and considering the morphotypes (Delamare-Debouttevile & Nunes, 1951). However, it is necessary that future research based on molecular data corroborate, or not, the genetic divergence of species from European (Mediterranean) waters, and other localities in which it has been reported.

Peniculus cf. fistula: intraspecific morphological variation

The *P.* cf. *fistula* specimens parasitic on the fishes from Antofagasta show morphological variations. The variations include: the cephalothorax length/neck length ratio (varied from 41 to 100%), the fourth segment shape (subcircular or more elongated, separated by a constriction from the trunk or in others continue to the trunk without such separation), the trunk length/trunk width ratio (varies from 9 to 37%), abdomen (with few or more development), the total length range (from a minimum length of 2.284 to a maximum length of 9.537), mandible distal blade (usually with nine teeth, or sometimes a major number appears (10), or in some specimens a minor number (as detected in specimens from *A. scapularis* with only seven teeth), maxilla in some specimens with the typical armature, but in others appears a chitinous flange, distal on the lacertus, at point of joint with the brachium). These morphological variations are found between *P. fistula* specimens from different hosts too (Delamare-Deboutteville & Nunes, 1951). Finally, the minimum mean size length observed was 2.960 (2.497−3.423) in *C. crusma* and the maximum length observed was for a specimen parasitic on *G. laevifrons* 6.760 (4.220−9.540), the size of all other specimens measured fell between those values.

Metapeniculus antofagastensis Castro-Romero & Baeza-Kuroki, 1985
(Fig. 29−30)
VOUCHER SPECIMENS: No. 210487−210488, United States National Museum (USNM), USA.
LOCALITY: Antofagasta Bay (South Pacific coast of Chile).
HOST: *Anisotremus scapularis*
SITE INFECTION: Buried into the musculature near base of caudal fins, pectoral, or ventral fins.
MATERIAL EXAMINED: Eight female specimens parasitic on *A. scapularis*.

Remarks. The species described by Castro-Romero and Baeza-Kuroki (1985), bear the cephalothorax suborbicular, with short lateral projection at both sides of the antenna position, the proboscis is highly muscular, and buccal tube displaced by the proboscis formation, and the maxilla remain near the cephalothorax on ventral surface, far from the buccal tube and maxillule. The labrum is a short, simple, convex plate and the labium is armed with a pair of scale-like plates. The long neck is armed with cuticular skeleton (see figures 8, 4e, 5, 6e, 6f, 9, 13, 14, 15 and 16 from Castro-Romero & Baeza-Kuroki, 1985).

Metapeniculus antofagastensis was documented with a big variation of the cephalothorax shape (Castro-Romero & Baeza-Kuroki, 1988) from suborbicular to subquadrangular, when attached in site not usually showing other morphological differences.

Trifur* cf. *tortuosus Wilson, 1917
(Figs 31–48)

VOUCHER SPECIMENS: Nos 15121–15122, MNHNCL.
LOCALITY: Antofagasta Bay (South Pacific Coast of Chile).
HOSTS: *Sebastes oculatus* and *Isacia conceptionis.*
SITE OF INFECTION: Branchial arcs and branchial chamber.
MATERIAL EXAMINED: Four adult females from *S. oculatus* and three adult females from *I. conceptionis*.
MEASUREMENTS. See Table 5.

Female from *S. oculatus*. Cephalothorax (Fig. 31–33), short, globose in dorsal view, dorsally bearing the antennules and antenna just on middle length, in some specimens in the base of them a lobular esclerite. The cephalothorax with three well-developed holdfasts (Fig. 32), one on the left side, another in the right side and the last posterior, the three oriented downwards.

Neck long, narrow. Trunk sigmoidal (Fig. 31), with fleshy lobules at each side, near the position of the genital opening. Abdomen (Fig. 33) almost cylindrical, oriented posteriorly. Caudal rami not detected.

Appendages. Antennule (Fig. 35), three segmented, first and second segment each armed with simple setae, distal segment with simple setae and two bifid setae plus a long aesthete, reaching 87% of the antennule length (measured from premetamorphosing stages from Castro-Romero & Baeza-Kuroki, 1986).

Antenna, basal segment strong, subquadrangular, a little longer than the second one, second segment subquadrangular, with distal inner margin shaping into a big projection which receives the claw distal end, claw narrow and slightly curved (Fig. 35).

Mandible (Fig. 36)**.** Bisegmented, distal blade with well-defined dentition, bearing nine teeth (all in ventral margin).

Maxillule (Fig. 36)**.** Biramous, inner lobe with papillae armed with two long setae, outer lobe with a short papillae and one setae. Intrabuccal stilet (Fig. 38) slender, with slightly curved base, distal seta short about a third of the base length.

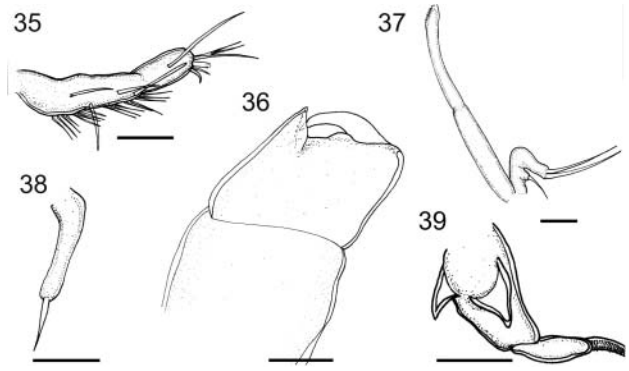

Fig. 35–39. 35. Antennule of *Trifur* cf. *tortuosus* from *Sebastes oculatus.* **36.** Antenna of *T.* cf. *tortuosus* from *S. oculatus.* **37.** Mandible and maxillule of *T.* cf. *tortuosus* from *S. oculatus.* **38.** Intrabuccal stylet of *T.* cf. *tortuosus* from *S. oculatus.***39.** Maxilla of *T.* cf. *tortuosus* from *S. oculatus.* Scale bars: Fig. 35–36, and 39: 50 μm; Fig. 37: 30 μm; Fig. 38: 15 μm.

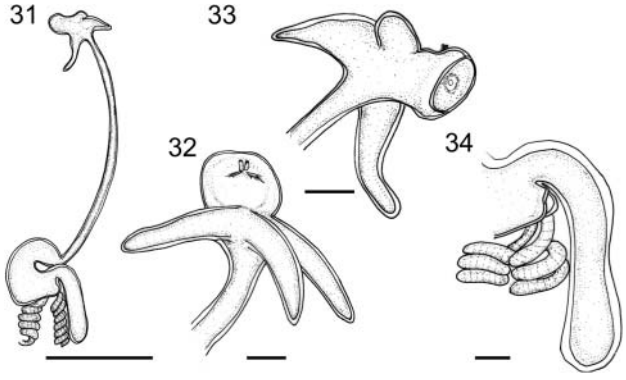

Fig. 31–34. 31. Female entire lateral view of *Trifur* cf. *tortuosus* from *Sebastes oculatus*. **32.** Cephalothorax dorsal view of *T.* cf. *tortuosus* from *S. oculatus*. **33.** Cephalothorax lateral view of *T.*cf. *tortuosus* from *S. oculatus* (other specimen). **34.** Abdomen lateral view of *T.*cf. *tortuosus* from *S. oculatus*. Scale bars: Fig. 31: 5000 μm; Fig. 32–34: 500 μm.

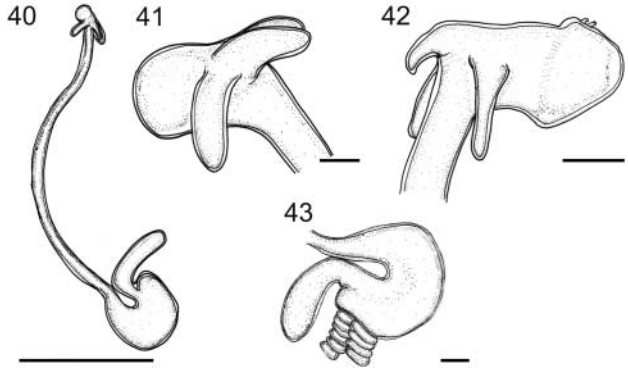

Fig. 40–43. 40. Female entire lateral view of *Trifur* cf. *tortuosus* from *Isacia conceptionis*. **41**. Cephalothorax lateral, left view of *T.* cf. *tortuosus* from *I. conceptionis*. **42.** Cephalothorax lateral, right view of *T.* cf. *tortuosus* from *I. conceptionis* (other specimen). **43.** Abdomen lateral right view of *T.* cf. *tortuosus* from *I. conceptionis*. Scale bars: Fig. 40: 5000 μm; Fig. 41–42: 500 μm; Fig. 43: 1000 μm.

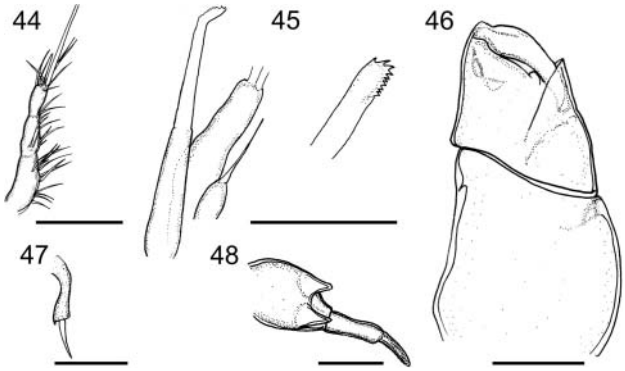

Fig. 44–48. 44. Antennule of *Trifur* cf. *tortuosus* from *Isacia conceptionis*. **45.** Mandible and maxillule, and detail of mandible distal part of *T.* cf. *tortuosus* from *I. conceptionis*. **46.** Antenna of *T.* cf. *tortuosus* from *I. conceptionis*. **47.** Intrabuccal stylet of *T.* cf. *tortuosus* from *I. conceptionis*. **48.** Maxilla of *T.* cf. *tortuosus* from *I. conceptionis*. Scale bars: Fig. 44: 100 μm; Figs 45–48: 50 μm; Fig. 47: 15 μm.

Maxilla (Fig. 39)**.** Lacertus with two long, strong, uniciform processes, brachium with unarmed, distal segment as a claw with two rows of fine setules. Legs: four pairs, the first positioned close to the end of cephalothorax, the second just at the anterior part of the neck, and the third separated, the fourth at a major distance.

Legs. Armature not recognizable, broken.

Female from *I. conceptionis*. Cephalothorax (Fig. 40–42), small, suborbicular in lateral view. Holdfast a little longer than cephalothorax. The lateral right, a little longer than the other holdfasts (Fig. 41), in some specimens one of the holdfasts (the left) is longer than the others, or in other specimens, the lateral holdfast pointed ventrally, elongated and a short, undeveloped right holdfast (Fig. 42).

Neck. Long, slightly curved medially, narrower at the end at the joint with the trunk. Trunk, suborbicular (Fig. 40) posterior margin slightly straight, genital margin less pronounced.

Abdomen (Fig. 43) with distal part separated from trunk and oriented upwards. Caudal rami not detectable.

Appendages. Antennule (Fig. 44), apparently three segments, first segment armed with 11 simple setae, second segment bearing eight simple setae, distal segment with seven setae (at least one of them bifid, and a long aesthete, reaching 66% of the antenna length).

Antenna (Fig. 46)**.** Bisegmented, chelated, basal segment subrectangular, longer than the second segment, with developed projection on distal inner margin in order to receive the claw, bearing a short seta. Intrabuccal stylet (Fig. 47) narrow base slightly curved proximally, bearing short setae (about a half of the base length).

Mandible (Fig. 45)**.** Distal blade with well-defined dentition, bearing 10 teeth (seven on the ventral margin and three on distal margin).

Maxillule (Fig. 45)**.** Biramous; inner lobe with papillae bearing two setae (broken), the outer lobe bearing a short papilla with setae longer than its base.

Maxilla (Fig. 47)**.** Lacertus strong, with two stout spiniform processes, as long as the brachium and claw together, brachium slender, not armature detected. Claw almost straight with two rows of fine setae.

Table 5. Morphological measurements (in μm) of *Trifur* cf. *tortuosus* associated with *Sebastes oculatus* (Soc) and *Isacia conceptionis* (Ico).

		Soc	Ico
Cephalothorax	Length. Mean (Min.–Max.)	1.60 (0.87–2.82)	1.40 (1.18–1.62)
	Width. Mean (Min.–Max.)	1.77 (1.28–2.46)	0.90 (0.85–0.95)
Right holdfast	Length. Mean (Min.–Max.)	0.95 (0.77–1.08)	0.91 (0.80–1.03)
	Width. Mean (Min.–Max.)	0.65 (0.44–0.97)	0.36 (0.33–0.39)
Left holdfast	Length. Mean (Min.–Max.)	2.21 (1.74–3.08)	0.87 (0.82–0.92)
	Width. Mean (Min.–Max.)	1.38 (0.31–3.84)	0.36 (0.33–0.39)
Posterior holdfast	Length. Mean (Min.–Max.)	2 (1.28–3.08)	1.09 (1.15–1.03)
	Width. Mean (Min.–Max.)	0.69 (0.46–1.03)	0.37 (0.36–0.38)
Neck	Length. Mean (Min.–Max.)	9.42 (6.33–12.10)	12.63 (11.5–13.77)
	Width. Mean (Min.–Max.)	1.47 (0.385–2.64)	0.40 (0.31–0.49)
Trunk	Length. Mean (Min.–Max.)	2.69 (0.46–5.38)	2.43 (2.30–2.56)
	Width. Mean (Min.–Max.)	1.78 (0.77–3.59)	1.60 (1.54–1.67)
Abdomen	Length. Mean (Min.–Max.)	3.09 (2.10–3.72)	3.2
	Width. Mean (Min.–Max.)	1 (0.51–1.8)	0.88 (0.87–0.90)

Legs. Four pairs, the first close to the end of cephalothorax, the second just at the anterior part of the neck, and the third separated from the fourth by a major distance. Legs; armature unidentifiable, broken.

Remarks. *Trifur* cf. *tortuosus* specimens parasitic on *I. conceptionis* shows the holdfast reduced in length or with variation in wide, while of normal elongation in specimens from *Merluccius gayi* Guichenot, 1848 and *S. oculatus*. *Trifur* cf. *tortuosus*, show the antennule with three segments in those from *M. gayi* (see figures 5a, 5b and 6 from Castro-Romero & Baeza-Kuroki, 1989a), *S. oculatus* and *I. conceptionis* (from Antofagasta Northern Chile). But differing from those specimens described by Etchegoin et al. (2009) bearing two segmented antennules. Also it is necessary to define the exact chaethotaxy of the antennule, types of setae (nude or plumose, simple or bifid), and its position (this can be tested specifically from premetamorphosing specimens, considering that in the adult it can be lost). The aesthete length reaches 87% of the antennule length (from premetamorphosing stages; Castro-Romero & Baeza-Kuroki, 1986), 66% in specimens of *T.* cf. *tortuosus* from *S. oculatus*, while 82% in specimens from *I. conceptionis*, if compared with the 64.4% in specimens from *Pseudopercis semifasciata* Cuvier, 1829 from Etchegoin et al. (2009). These characters must be tested in other specimens, for possible variation or for detection, especially the antennule segmentation number (which could also show variation due to treatment for SEM).

The mandible, usually with a well-defined dentition, nine in specimens from *S. oculatus*, 10 in specimens from *I. conceptionis*, differing in the position of the teeth in the latter, while eight in specimens from Argentina (Etchegoin et al. 2009) parasitic on *Pseudopercis semifasciata* Cuvier, 1829, but care must be taken when observing the dentition under optical microscopy, especially by the terminal blade orientation, sometimes twisted, which can obscure some teeth.

Discussion

The high morphological polymorphisms detected for the taxa analysed in this study show the need to apply integrative taxonomy approaches (combined molecular and morphological data) to correctly delimit the species. Based on morphometric analyses we do not find significant statistical values that permit delimitation of species of *Peniculus* by association to morphological characters, e.g., the ratio cephalothorax length/neck length, fourth segment length/fourth segment wide, fourth segment length/trunk length, trunk length/width for the *P.* cf. *fistula* specimens from different hosts (Fig. 2). In this case, a particular diversity of forms of copepod parasites of marine fishes was attempted with DNA barcoding, analysed with independent methods without *a priori* defined groups, e.g., Bayesian phylogenetic analyses (BI) with four contrasting runs of GMYC plus bGMYC. The results obtained by the five analyses are relatively congruent, and show few differences in particular clades (subdivided or merged) (Fig. 3). We decided to delimit each terminal group (species), based on values of probability support nodes (> 0.95). In this context, we chose to follow a conservative approach by considerate as single species to independently evolving lineages with significant statistical values. Short branches for all terminals is a strong indicator of insufficient time to accumulate polymorphisms and to complete the speciation process (Cornils & Held, 2014; Martínez-Aquino et al. 2013). Therefore, in this study we show that the pennellids (genera and species) can show high morphological polymorphism with very low genetic differentiation, as here was discovered for *Peniculus*, not permitting its separation in taxonomic unities. On the other hand, both *Trifur* and *Metapeniculus* show low genetic divergence, among the studied specimens not enough for separation into species. In the next section, we briefly discuss the intraspecific morphological variation based on results obtained for each taxonomic group.

Peniculus cf. *fistula*

Based on the results of the molecular phylogenies using barcoding DNA, morphometric analyses, plus a strict morphological comparison with all other *Penniculus* species, we decided on the taxonomic status of the present specimens as *P.* cf. *fistula* until a molecular analysis of the type species can be done for testing the phylogenetic relationships between each of them from diverse host and localities and finally states its real identities with the present specimens. In future analyses, morphological characters can be used like the antenna medial margin of the second segment bearing plate with denticles variable in number, as probed here for the specimens from *M. cephalus*, differing in the number of denticles with those of *P. minuticaude* (also see Venmathi Maran et al., 2012). In this context, other aspects that must be tested are the antennules chaetotaxia, for premetamorphosing specimens, under high magnification (SEM), and also the presence of spinulation on the abdomen distal surface and margin. On the other hand, including specimens of *P. fistula* recorded in other marine systems around the world (e.g., Öktener, 2008), and associated to marine fishes with wide distribution (e.g., *M. cephalus*), permitted establishing whether *P. fistula* is a species with wide distribution or is a complex putative species (for more details referent to distributional area of *P. fistula* and theirs hosts, see Appendix 1, see supplemental material online).

The genetic distance among specimens of *P*. cf. *fistula* was low (0.95%); however, a high morphological variation was observed associated to hosts (although these are not necessarily observed in the ultrametric tree; Fig. 3). In general, the morphological variation among *P.* cf. *fistula* is associated with distinct hosts, e.g., the fourth segment shape, suborbicular, separated by a constriction from the trunk, in those from *C. crusma* and those from *G. laevifrons*, slightly separated in those from *I. conceptionis* and *P. jugularis* and on the other hand more elongated and not well separated from the trunk in those on *H. macrophthalmos* and *O. regia.* Some specimens bear the presence of short spines on the fourth segment (in those from *O. regia*), or others annexed in the trunk anterior part (in those of *P. jugularis*), which are not visible or not detectable in specimens from the other hosts studied. The abdomen showing variation, in some less developed (in specimens from *G. laevifrons* and *M. cephalus*), and in others more developed (in specimens from *C. crusma*, *H. macrophthalmos*, and *P. jugularis*). Here also it was observed that the second maxilla is armed with one or two spiniform processes as usual or, instead, a spiniform process and a strong chitinous border, vertical, distally on the lacertus as in *P. jugularis*.

It is important to mention the polymorphisms in some morphological aspect, especially for the cephalothorax shape, which can be modified if the specimens are attached to a distinct site (that is not the fins' rays), such could be the case of *P. quadratus* which live attached to the palate and could be inducing the extension of the cephalothoracic surface by the need to have a better attachment. Another character worth mentioning is the extension of the buccal area, similar to a proboscis (non-muscularized in *Peniculus* but highly muscularized in *Metapeniculus*), such as was verified in specimens from *O. regia*, but other specimens without such extension, that is probably because the buccal area can be easily contracted into a cephalic cavity or expanded outside this. This situation was also noted by Delamare-Deboutteville and Nunes (1951), detecting variation in specimens on a same host. A vision of the entire genus is desirable considering the molecular analysis in order to elucidate its real composition.

It is of interest that parasitic copepods of the genus *Peniculus* are attached to the fins' rays and present adaptive structures with morphological variation, e.g., length of the neck, fourth segment shape, its grade of separation from the trunk, and variation on the trunk wide/trunk length ratio. The principal morphological variation detected in this study of *P.* cf. *fistula* was in the neck length and the fourth segment shape. In this context, it is possible to infer that the association of *P.* cf. *fistula* on different host species from the coast of Antofagasta can explain the high morphological variation, because of a high environmental variation exposed by each host species, e.g., sea wave force in coastal waters, influencing the fish behaviour.

Metapeniculus antofagastensis

This species has previously been reported to suffer polymorphism, especially on the cephalothorax shape, expanding, sometimes, in big lobules, or adopting a subquadrate shape when the copepod attaches to a different site on the host, especially when attaching to a lateral musculature, separated from the fins' base (Castro-Romero & Baeza-Kuroki, 1988). The genetic variability could be compared with congeneric species *M. haemulonis* Alexander, 1983, associated in *Haemulon staindachneri* Jordan & Gilbert, 1882 from Brazilian waters, in order to test the status of both species.

Trifur cf. *tortuosus*

The analysis of the type species of *T. tortuosus* from *Salilota australis* Günther, 1878 from southern Chile with molecular markers (e.g., barcoding genes) is desirable in order to clarify (or test) if the specimens from Antofagasta are really the same species. Eventually, even Etchegoin et al. (2009) includes all the *Trifur* species as synonym of *T. tortuosus.* Furthermore, the morphological variation of *T.* cf. *tortuosus* does not give us clear character allowing the distinction among the specimens of *Trifur* here studied and with those previously known. The high variability of the cephalothoracic holdfast length and width as seen also by Etchegoin et al. (2009) and in the present observations, is not suitable to state differences among species (as seen too for *Lernaeenicus longiventris* Wilson, 1917 by Castro-Romero & Joyeux, pers. obs.). Some characters that need revision in all the specimens of *Trifur*, for an accurate comparison, are the antennules, which have three segments in the specimens from *M. gayi* (Castro-Romero & Baeza-Kuroki, 1989a), and in *S. oculatus* and *I. conceptionis*, while the description by Etchegoin et al. (2009) has two segmented or not well defined segmentation in specimens from *P. semifasciata*; and the chaetotaxia must be verified in each of the specimens for the possible variation in the setae number on each segment and the type of setae (simple or plumose), the number of bifid setae on the segments and distally, and finally the aesthete length. The mandible with normal dentition in specimens from *S. oculatus*, that has nine teeth on the ventral margin (as usual), contrary to the specimens from *I. conceptionis* in which the mandible shows three teeth on the distal margin and the other, a series of seven in the ventral margin (as detected from a young premetamorphosing female), while in specimens from *P. semifasciata* bear only eight teeth all on the ventral margin (Etchegoin et al. 2009). When observed on light microscopy care must be taken due to the fragile blade, which can be twisted, sometimes not allowing a good observation of the teeth number.

Taxonomic implications

DNA barcoding, used to delimit species and infer phylogenetic relationships between congeneric species, shows great potential for integrative taxonomy approaches due to recent improvements in statistical methods (Fujita, Leaché, Burbrink, McGuire, & Moritz, 2012; Jones et al., 2011; Puillandre, Lambert, Brouillet, & Achaz, 2012). In the case of marine copepods, the integration of barcoding phylogenies plus morphological evidence has been implemented in other studies (e.g., Blanco-Bercial, Cornils, Copley, & Bucklin, 2014; Cornils & Held, 2014). Based on averaged values of the genetic distances estimated using DNA barcoding for other crustacean groups (e.g., congeneric species of Decapoda, Cladocera, and Amphipoda), an average value of 17.16% was obtained (Costa et al., 2007). In particular groups of copepods (e.g., congeneric calanoid copepod species), these distance values can oscillate from 9 to 24% (Bucklin, Frost, Bradford-Grieve, Allen, & Copley, 2003). Recently in pennellid copepods, Muñoz et al. (2015) reported distances with an average of 20.3% between two species of Pennellidae (Pennellidae 'morphotypes', *Trifur* sp. 1 and *Trifur* sp. 2), and reported distances ranging from 11.9% to 30.1% between *Trifur* genus and pennellids taxa. These results are similar with the values that we reported in the present study from *Trifur* cf. *tortuosus*, *M. antofagastensis*, and *P.* cf. *fistula* (Table 2). On the other hand, Muñoz et al. (2015) also reported genetic distances of 3.3% and 0.9% between *Trifur* taxa and pennellids taxa; however, it is possible that these values are due to a misidentification. Future studies including more taxa of pennellids and more rigorous species delimitation can corroborate this finding. In any case, our study demonstrated the usefulness of DNA barcoding for species delimitation in parasitic copepods of the family Pennellidae. In the phylogenetic context, our results are not consistent with the phylogenetic analysis based on morphology for Pennellidae realized due to Boxshall (1986). This author mentioned that *Trifur* belongs to the 'Lernaeocera-group' in the tree is higher than *Peniculus* and justify their 'basal' phylogenetic position based on the sigmoid body (trunk shape). In our phylogenetic analysis, *Peniculus* is the sister group of *Metapeniculus* (described posteriorly to the Boxshall phylogenetic tree for Pennellidae) and *Trifur* is the basal group (plesiomorphic). This new position of the genera can be ratified when a complete analysis of all pennellids genera can be done based on molecular and morphological new antecedents (such as buccal area type, proboscis like extension, armature on labium, and type of labrum). Therefore, here is presented an amended diagnosis of the three genera (see below).

Conclusions

The specimens of *Peniculus*, *Metapeniculus*, and *Trifur* studied have high morphological variation, especially on the cephalothorax and annexed structures, such as the case of the holdfast (when present) with reduced size. This pattern of morphological variation was previously reported in other pennellids taxa, e.g., *Pennella* by Hogans (1987), *Metapeniculus* by Castro-Romero and Baeza-Kuroki (1988), and *Trifur* by Etchegoing et al. (2009). Furthermore, variation on the fourth segment for the *Peniculus* by Delamare-Debouteville and Nunes (1951) was also reported. Other pennellids probably also suffer such polymorphism of the holdfast, like in *L. longiventris* (Joyeux, pers. obs.). If it is not well known, this variability confuses the identification or the description of some species inside the genera of the family, due to the site of attachment on the host, or because of the age of collected parasite (maturity). Therefore, in order to establish the real morphological variability and genetic divergence through a range of distribution of their hosts, especially when the copepods parasites live buried in the host musculature with its cephalothorax, future systematic studies of the family Penellidae (genera and species) must be performed. In this context, beneficial approaches should combine integrative taxonomy with coevolutionary studies in order to detect hosts' specific morphological adaptations (e.g. Fontaneto, Flot, & Tang, 2015; Martínez-Aquino, 2016).

Acknowledgements

We thank Leo Campos (Postgraduate student of the Universidad de Antofagasta) for his help during fieldwork, and Z. Kabata (Canada) and A. Otkener (Turkey) for providing specimens of *Peniculus fistula* used for comparison. We also thank Dra. Fadia Sara Ceccarelli who reviewed the first draft of this manuscript, made very useful comments, suggestions and discussions of the phylogenetic analyses that we present in this contribution. We thank Sabrina Martorelli for her constructive criticism. We thank Paul Jones (English teacher) for editing the English text.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by a postdoctoral grant from CONACyT, Mexico (No. 238789) to A. M-A.

Supplemental data

Supplemental data for this article can be accessed at http://dx.doi.org/10.1080/14772000.2016.1158213.

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Associate Editor: Polly Hayes