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Sea lice (Siphonostomatoida: Caligidae) diversity on littoral fishes from the south-eastern Pacific coast determined from morphology and molecular analysis, with description of a new species (*Lepeophtheirus confusum*)

M. Teresa González [a,*], Raúl Castro [b], Gabriela Muñoz [c], Zambra López [a]

[a] Instituto de Ciencias Naturales Alexander von Humboldt, Facultad de Ciencias del Mar y Recursos Biológicos, Universidad de Antofagasta, Antofagasta, Chile
[b] Depto. Ciencias Acuáticas y Ambientales, Facultad de Ciencias del Mar y Recursos Biológicos, Universidad de Antofagasta, Antofagasta, Chile
[c] Facultad de Ciencias del Mar y de Recursos Naturales, Universidad de Valparaíso, P.O. 5080, Viña del Mar, Chile

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ABSTRACT

Parasitic copepods of the family Caligidae are common on marine fish worldwide, and some species are responsible for disease outbreaks in aquaculture. Ten species of *Lepeophtheirus* have thus far been described in the south-eastern Pacific coast. Seven species have been recorded from littoral fish species. However, the latitudinal distribution and host ranges of these *Lepeophtheirus* spp. are not known. We evaluated, using morphology and molecular analyses, the taxonomic diversity, geographical distributions and host range of known *Lepeophtheirus* species. Seventeen fish species were examined for copepods. The collected parasites were identified according to their morphology and genetic sequences, based on rDNA 28S and COI genes. The recognition of *Lepeophtheirus chilensis* and *L. mugiloides* was indeed difficult due to the high morphological similarities between them. However, their taxonomic statuses were supported by the COI gene and ABGD analysis, with 6% of genetic distance. Moreover, a new species with a genetic distance of 19–22% with respect to known species was detected and described herein as *L. confusum*. This new species can be distinguished from other *Lepeophtheirus* spp. by a combination of characters (maxillary tine length and width; furca shape; the fifth leg position, shape and armature; maxillule tine length and thickness; and maxilliped armature on the myxal area). *Lepeophtheirus chilensis*, *L. mugiloides* and *L. frecuens* co-occurred on several littoral fish species, showing an extensive latitudinal distribution, whereas *L. confusum* was found only on *Eleginops maclovinus* from southern latitudes.

1. Introduction

Parasitic copepods of the genera *Caligus* and *Lepeophtheirus* (family Caligidae), known as sea lice, include a large number of species with a wide range of fish hosts [1]. Several of these parasites are responsible for disease outbreaks in marine farms, particularly in intensive salmonid aquaculture [2–7].

Along the south-eastern Pacific coast (SEP), caligid copepods are common on several marine fish species; 21 caligid species have been recorded, and 10 of them belong to the genus *Lepeophtheirus* [8]. Of note, three *Lepeophtheirus* species have been identified in specific localities off the coast of Chile: *L. selkirki* Atria, 1969 [9] and *L. interitus* Wilson, 1921 [10] were described on fish from the Juan Fernandez Archipelago, and *L. nordmanni* Milne-Edwards, 1840 was described on one oceanic fish species. Therefore, only seven species have been recorded on littoral fish species. Of these, three species parasitize specific fish hosts: *L. edwardsi* Wilson, 1905 infests fish of the family Paralichthydae, *L. yanezi* Stuardo and Fagetti, 1961 infests fish of the family Ophidiidae, and *L. dissimulatus* Wilson, 1905 has only been recorded on the wrasse *Semicossyphus darwinii* (Labridae). The remaining four *Lepeophtheirus* species have been found on several littoral fish species: *L. zbigniewi* Castro and Baeza, 1981, infesting mostly fish of Labrisomidae, but also some subtidal fish species distributed from 24°S to 32°S; *L. frecuens* Castro and Baeza, 1984 has only been reported on several subtidal fish (Serranidae, Oplegnathidae and Cheilodactylidae) from the lower latitudes (at approximately 24–26°S) [11]; *L. mugiloides* Villalba and Durán, 1986 has been recorded on two subtidal fish species [12–14] (*Pinguipes chilensis* Valenciennes, 1833 and *Eleginops maclovinus* Valenciennes, 1830) from 30°S to 44°S; and *L. chilensis* Wilson, 1905 has been reported on the demersal fish *Sebastes oculatus* Valenciennes, 1833 from 24°S to 52°S [15].

* Corresponding author at: Instituto de Ciencias Naturales Alexander von Humboldt, Facultad de Ciencias del Mar y Recursos Biológicos, Universidad de Antofagasta, Angamos 601, Antofagasta, Chile.
E-mail address: teresa.gonzalez@uantof.cl (M.T. González).

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The *Lepeophtheirus* species from the SEP are not differentiated by any single striking characteristic; instead, several features must be used in their identification [16]. In particular, specimens of *L. chilensis* and *L. mugiloides* are morphologically similar, and their combinations of structure differences are not sufficiently clear or precise to allow correct identification, making specimen identification confusing and producing doubts about the taxonomic status of these species. In these cases, molecular markers may help to establish the status of the taxonomic group or species, especially when morphology is not clear enough to make distinctions among them (e.g., [17,18–21]). The mitochondrial cytochrome *c* oxidase subunit I (COI) gene has been used successfully to identify parasitic copepod species (e.g., [19,20,22]).

The host-parasite relationship can be dramatically affected by human activities, including aquaculture, which can modify the transmission of parasites among hosts [4] and alter the geographic ranges of the parasite [23]. Therefore, precise knowledge of the taxonomy, geographic distribution and host ranges of parasite species is crucial to understanding the potential threats of emerging diseases [24]. In this study, we evaluated the taxonomic diversity, geographical distribution and host range of known *Lepeophtheirus* spp. infesting littoral fish species along the SEP. The use of molecular analyses, together with comparative morphological analysis, has allowed us to corroborate the taxonomic status of *L. chilensis* and *L. mugiloides* and to detect a new species, which is described and illustrated herein as *Lepeophtheirus confusum*.

2. Materials and methods

2.1. Copepod collection and morphology

From March 2011 to September 2014, seventeen fish species ($n = 330$) belonging to 12 fish families: *Sebastes oculatus*, *Pinguipes chilensis*, *Prolatilus jugularis*, *Cheilodactylus variegatus* (Valenciennes, 1833), *Paralabrax humeralis* (Cuvier and Valenciennes, 1828), *Hemilutjanus macrophthalmos*, *Acanthistius pictus*, *Anisotremus scapularis* (Tschudi, 1846), *Labrisomus philippi* (Steindachner, 1866), *Scartichthys viridis*, *Auchenionchus microcirrhis* (Valenciennes, 1836), *Girella laevifrons* (Tschudi, 1846), *Graus nigra* (Philippi, 1887), *Eleginops maclovinus*, *Semicossyphus darwini* (Jenyns, 1842), *Paralichthys adspersus* (Steindachner 1867) and *Genypterus chilensis* (Guichenot 1848) were captured from different latitudes along the Chilean coast (Fig. 1) and were examined for *Lepeophtheirus* species (Table 1). The recovered copepods were identified using specialised literature [11,14,16,25,26].

2.2. Molecular analysis

The egg sacs and complete bodies of *Lepeophtheirus* were isolated and placed into 1.5 ml Eppendorf tubes with one individual per tube. DNA extraction was performed following a modified protocol based on Miller et al. [27] and involved treatment with sodium dodecyl sulfate, digestion with Proteinase K, NaCl protein precipitation, and subsequent ethanol precipitation of the DNA [27].

Polymerase chain reaction (PCR) was performed to amplify the LSU D1-D2 region of the nuclear 28S gene and the Cytochrome c oxidase subunit I (COI) gene using primers described by Song et al. [28] for 28S, and Folmer [29], and Oines and Heuch [30] for COI. Each PCR included 0.025 U *Taq* polymerase, 1× buffer, 0.2 mM deoxynucleotide triphosphate (dNTP), 4 mM $MgCl_2$, 0.4 P/μl of each primer, 3.5–7 μl concentrated DNA, and 2.1 μl BSA (Biolabs) (10 mg/ml) and was brought to a final volume of 35 μl with water. The optimal cycling conditions for the 28S gene were an initial denaturing step at 94 °C (5 min), followed by 35 cycles at 94 °C (30 s), 54 °C (30 s), and 72 °C (1 min), and a final extension step at 72 °C (5 min). For the COI gene, the optimal cycling conditions were 95 °C (5 min), followed by 40 cycles at 95 °C (45 s), 50 °C (45 s) and 72 °C (1 min), and a final extension step at 72 °C (10 min). The PCR products were visualised on a 1.5% agarose gel and purified using an E.Z.N.A. commercial kit (Omega Bio-Tek). The PCR products were individually sequenced by Macrogen Inc., Seoul, Korea. (http://www.macrogen.com), using an ABI 3730XL automated capillary electrophoresis sequencer. The sequences were edited using ProSeq v 3.0 beta [31] and were aligned using Clustal W2 software [32].

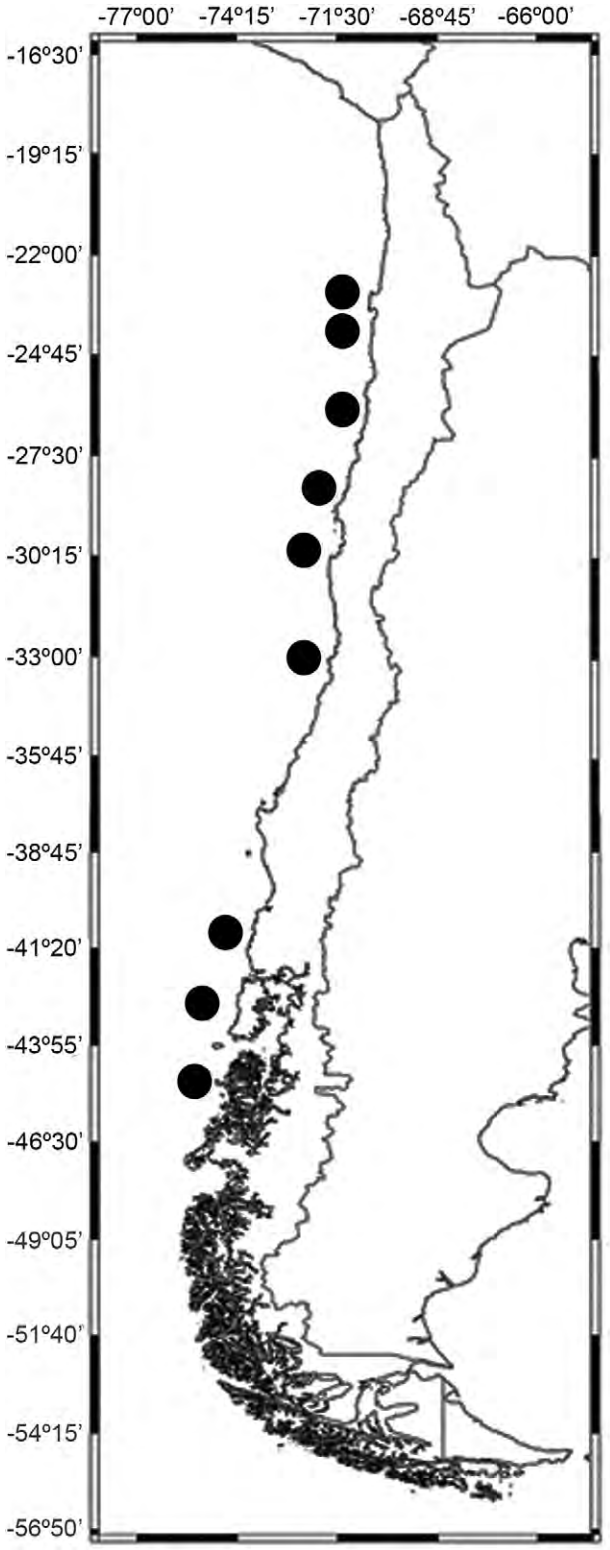

Fig. 1. Sampling areas along the Chilean coast, showing the latitudes (black circles) from where fish species were captured.

Table 1
Species of *Lepeophtheirus* collected from fish host species sampled at different latitudes along the Chilean coast. Host family and host distributional ranges are shown according to the information available in www.fishbase.org. This list is organized alphabetically by parasite species names, and number of examined fish by host species is given in parenthesis (n=).

Parasite species	Host species (n=)	Host family	Host distribution	Sampled latitudes
L. confusum	*Eleginops maclovinus* (15)	Eleginopsidae	35°S–52°S	39°S–44°S
L. chilensis	*Hemilutjanus macrophthalmos* (20)	Serranidae	2°N–40°S	24°S
L. chilensis, *L. frecuens*,	*Acanthistius pictus* (20)	Serranidae	20°N–30°S	23°S–24°S
L. chilensis, *L. frecuens*	*Cheilodactylus variegatus* (20)	Cheilodactilydae	20°S–38°S	23°S–24°S
L. chilensis, *L. frecuens*	*Prolatilus jugularis* (20)	Pinguipidae	11°S–44°S	23°S
L. chilensis, *L. mugilodes*	*Paralabrax humeralis* (20)	Serranidae	30°N–30°S	23°S–24°S
L. chilensis, *L. frecuens*, *L. mugilodes*	*Pinguipes chilensis* (30)	Pinguipidae	20°S–52°S	23°S–24°S–30°S–44°S
L. chilensis, *L. frecuens*, *L. mugilodes*	*Sebastes oculatus* (20)	Scorpaenidae	12°S–52°S	23°S–24°S
L. dissimulatus	*Semicossyphus darwini* (10)	Labridae	5°S–24°S	24°S
L. edwardsii	*Paralichthys adspersus* (10)	Paralichthyidae	2°N–46°S	24°S
L. frecuens	*Anisotremus scapularis* (20)	Haemulidae	1°N–24°S	23°S–24°S
L. frecuens	*Girella laevifrons* (25)	Kyphosidae	12°S–36°S	24°S
L. mugiloides	*Graus nigra* (10)	Kyphosidae	20°S–38°S	30°S, 32°S
L. yanezi	*Genypterus chilensis* (10)	*Ophidiidae*	6°S–54°S	23°S–24°S, 30°S
L. zbigniewi	*Auchenionchus microcirrhis* (25)	Labrisomidae	12°S–35°S	24°S–32°S
L. zbigniewi	*Scartichthys viridis* (30)	Blenniidae	12°S–36°S	24°S, 32°S
L. zbigniewi, *L. frecuens*, *L. mugilodes*	*Labrisomus philliphi* (25)	Labrisomidae	12°S–30°S	24°S

The 28S and COI data sets were analysed using Maximum Likelihood (ML), Neighbour-Joining (NJ) and Bayesian Inference (BI) methods. ML and NJ analyses were performed using Mega v6 software [33], and BI was performed using the software package MrBayes [34]. For the NJ analysis, the K2P evolution model was used, and for the ML and BI analyses, the GTR + G and GTR + G + I models, respectively, were used for the 28S and COI. The nodes support was statistically evaluated in ML and NJ by bootstrap analysis of 1000 samples [35]. Models for both genes were chosen according to the Akaike Information Criterion (AIC), implemented in Mega 6. To estimate BI inference, posterior probabilities were estimated over 5,000,000 generations via one run of three simultaneous Markov Chain Monte Carlo chains with every 1000th tree saved. The first 50,000 generations (10% burn-in) were discarded as suggested by Felsenstein [36], and the consensus trees were built with 1000 trees. *Caligus cheilodactylus* was used as an outgroup species.

The individuals were classified into groups according to species, and the distances were computed with the average number of mutations (substitutions). The divergence between groups was determined by applying the K2P and number of mutations in base pairs (bp).

The COI gene was used to aid in determining the number of species; specifically, we used the approximation of delineation of species boundaries in the automatic barcode gap discovery method (ABGD) [37,38]. This method delivers species circumscriptions based on patterns of pairwise genetic distances (ABGD), providing estimates of a maximum limit for intraspecific genetic divergence, and using this limit to group sequences belonging to the same species (with lesser divergences) from sequences belonging to different species (with greater divergences) [37,38].

2.3. Description of *L. confusum* n. sp.

The specimens used for the morphological and molecular analyses were taken from *Eleginops maclovinus* that had been collected from a site in San Ignacio, Puerto Montt in the South of Chile (42°S) and fixed in absolute ethanol. Drawings were made with the aid of a camera lucida attached to a light microscope (CH2 Olympus). Twenty six specimens were dissected, and the appendages were mounted in lactic acid when necessary to observe details. Measurements are expressed in micrometres (μm), based on ten females and six males, and the mean and the range (maximum and minimum) were recorded. Terminology follows that of Kabata [39,40] and Dojiri and Ho [41]. The leg armature's formula using Roman and Arabic numerals indicate spines and setae, respectively.

3. Results

3.1. Molecular analyses

Fifty-six sequences for 28S gene and 56 sequences for COI gene were obtained (Supplementary Table 1). The sequences of *Lepeophtheirus* spp. were submitted to GenBank under accession numbers: JX896325, JX896344, JX896347, JX896350, JX896376, JX896362-JX896375, KU317511-KU317548 for 28 S and KU317549 - KU317605 for COI). The total length of the analysed 28S rDNA sequences was 715 bp, and for the COI sequences, 688 bp.

Based on the 28S rDNA gene, the analysis showed seven groups, and in one of the groups, two species (*L. mugiloides* and *L. chilensis*) were grouped together. *Lepeophtheirus dissimulatus* showed the highest genetic distance (14.5% and 16.4%) from the other species, and it was the most distant species (Fig. 2), followed by *L. edwardsii*, which showed a genetic distance of 3.6%–6.3%, and *L. yanezi*, which showed a genetic distance of 4.6–5.1% with respect to the other species. *Lepeophtheirus zbigniewi*, *L. frecuens*, *L. chilensis* and the new species *L. confusum* showed genetic distances among themselves varying between 1.4 and 1.9, whereas *L. mugiloides* showed only a 0.1% genetic distance from *L. chilensis* (Table 2).

The phylogenetic relations among the *Lepeophtheirus* species based on 28S gene sequences showed that *L. edwardsii* is one of the ancestral species of the *Lepeophtheirus* species from the Chilean coast with a bootstrap support of 100% (ML) in this clade and an a posteriori probability of 1.0 (BI) (Fig. 2). In general, the bootstraps and a posteriori probability were high in the base of tree and in the terminal branches of *L. dissimulatus*, *L. edwardsii*, *L. yanezi* and *L. zbigniewi*. The others groups had low statistical support and resolution.

Based on the COI gene, the analysis showed seven groups, but in this gene *L. zbigniewi* was not included because the PCR was negative. The topology with the COI gene was different in comparison with the 28S gene. *Lepeophtheirus frecuens* showed the highest genetic distance with the COI gene (17.5% and 25.5%) from the other species, and it was the most distant of the species (Fig. 3). In general, the bootstraps and a posteriori probability were low and absent in the base of the tree, but were high in the terminal branches, clearly supporting each group, and the genetic distances among these groups were high (Table 3). In addition, with the high bootstrap (100%) and a posteriori probability (1.0), this analysis showed a close relation between *L. mugiloides* and *L. chilensis*, species that were not separated with the 28S gene. These species had a genetic distance of 6.3%. The new species (*L. confusum*) showed a genetic distance ranging from 19.7 (*L. frecuens*) to 22.7% (*L. chilensis*) with respect to the other species (Table 3).

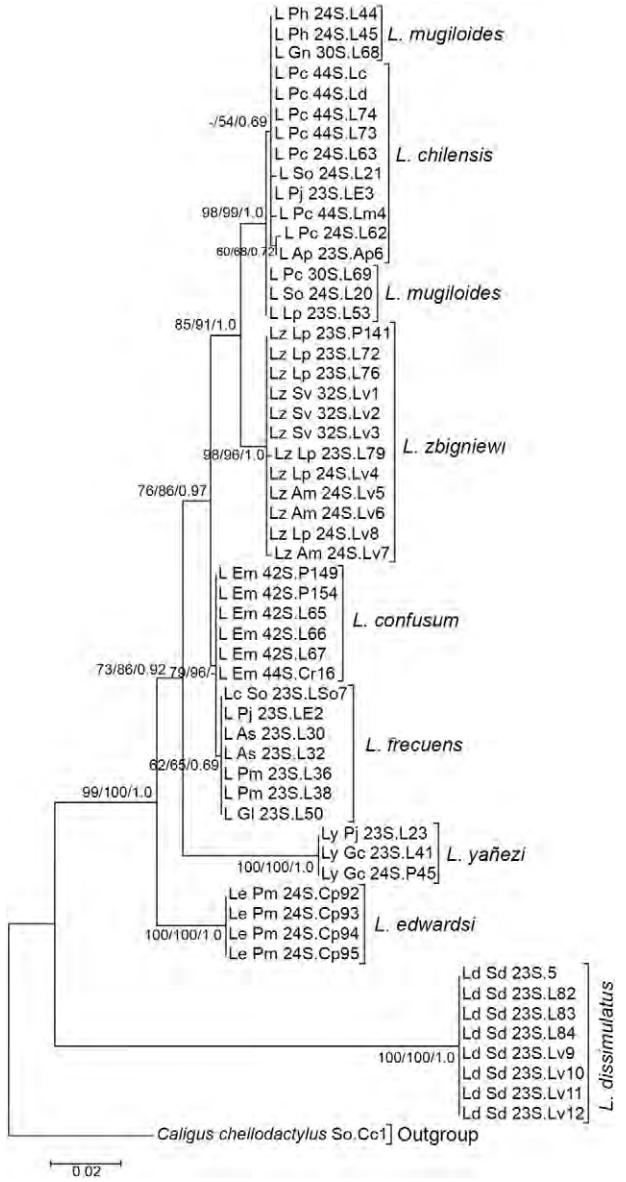

Fig. 2. Phylogenetic tree obtained with maximum likelihood (ML), using sequences of 28S gene belonging to 7 species of *Lepeophtheirus* infesting littoral fish species from southeastern Pacific coast and the new species *Lepeophtheirus confusum*. The values on nodes from left to right corresponding to the bootstrap values obtained for ML/NJ and a posteriori probability BI. The fish sampled were: *Acanthistius pictus* (Ap), *Anisotremus scapularis* (As), *Scartichthys viridis* (Sv), *Eleginops maclovinus* (Em), *Genypterus chilensis* (Gc), *Girella laevifrons* (Gl), *Graus nigra* (Gn), *Auchenionchus microcirrhis* (Am), *Labrisomus philippii* (Lp), *Paralabrax humeralis* (Ph), *Pinguipes chilensis* (Pc), *Prolatilus jugularis* (Pj), *Paralichthys microps* (Pm), *Semicossyphus darwini* (Sd) and *Sebastes oculatus* (So).

The ABGD analysis showed a tri-modal pairwise genetic distance (K2P) distribution with a gap located between 3 and 4% of the genetic distance and a second clear and wide barcode gap located between 7 and 17% of the genetic distance (Fig. 4a). Furthermore, the method detected seven stable candidate species with estimated prior maximum divergences of intraspecific diversity (P) as large as 3.8–4% (Fig. 4b) (one-tail 95% confidence interval). That is, the ABGD analysis distinguished 7 different species (*L. mugiloides*, *L. chilensis*, *L. confusum*, *L. frecuens*, *L. yañezi*, *L. edwardsi*, *L. dissimulatus*) and divergences (genetic distances) higher than 4% are useful to separate these different species. Notably, this result was consistent for the seven species used with this gene. The mean intraspecific variability varied among 0.09 and 1.02: *L. edwardsii* showed the lowest and *L. chilensis* the highest variability with a range of variation between 0 and 1.9% (0–13 bp). The intraspecific variations ranged between 0 and 1.2% in *L. frecuens*, 0.1 and 0.9% in *L. mugiloides*, and 0 and 1.0% in *L. confusum*. The other species showed minor intraspecific variations.

3.2. Morphology of copepods

Lepeophtheirus dissimulatus, *L. edwardsii*, *L. yanezi* and *L. zbigniewi* were clearly distinguished by morphological traits according to the original descriptions [16,26]. The first three species were recorded on specific fish species collected from 24°–26°S, whereas *L. zbigniewi* was recorded on 2 fish species of the family Labrisomidae, collected from 24°–26°S, and on one fish of the family Blenniidae, collected from 32°S (Table 1).

Lepeophtheirus frecuens, *L. chilensis* and *L. mugiloides* were found co-occurring on fish species belonging to different families across an extensive latitudinal range (24°S–45°S) (Table 1). *Lepeophtheirus frecuens* was differentiated by the lengths of the first maxilla segments and abdomen, which were the most distinctive of their observed features. Only a few specimens were recognised as *L. chilensis* (Table 1); the two-segmented abdomen of this copepod was observed as a light line between the abdominal segments, but this feature was not clearly distinguishing, as was determined in the original description [26]. Indeed, *L. chilensis* and *L. mugiloides* were very difficult to distinguish morphologically.

3.3. Description of the new species

Material examined: 20 females and six males collected on the surface of *Eleginops maclovinus* captured from San Ignacio and Puerto Montt, in the South of Chile (40°S–42°S). Material deposited in the Museo Nacional de Historia Natural, Santiago, Chile.

Holotype, one female, number: MNHNCL COP-15110
Paratypes, three females, numbers: MNHNCL COP-15112
GenBank accession number for COI: KU317590-KU317593
Prevalence of infection: 70%

Table 2
Mean distance genetic of the 28S rRNA gene between groups. Upper half shows the number of mutations between paired comparisons and lower half shows the percentage difference between the paired comparisons.

	Mean distance between groups	1	2	3	4	5	6	7	8	9
1	*Lepeophtheirus chilensis* (n = 10)		0.8	11.9	12.9	34.3	92.2	9.8	32.9	67.7
2	*Lepeophtheirus mugiloides* (n = 6)	0.1		11.5	12.5	33.5	91.5	9.3	32.5	67.5
3	*Lepeophtheirus frecuens* (n = 7)	1.7	1.7		1	32	90	10.8	24	61
4	*Lepeophtheirus confusum* (n = 6)	1.9	1.8	0.1		31	90	9.8	23	61
5	*Lepeophtheirus yanezi* (n = 3)	5.1	5.0	4.7	4.6		100	32.8	41	77
6	*Lepeophtheirus dissimulatus* (n = 8)	15.0	14.9	14.5	14.6	16.4		91.0	93	98
7	*Lepeophtheirus zbigniewi* (n = 12)	1.4	1.3	1.6	1.4	4.9	14.8		30	64.8
8	*Lepeophtheirus edwardsi* (n = 4)	4.9	4.9	3.6	3.4	6.3	15.3	4.5		64
9	Outgroup (n = 1)	10.4	10.4	9.3	9.3	12.0	16.0	10.0	10.0	

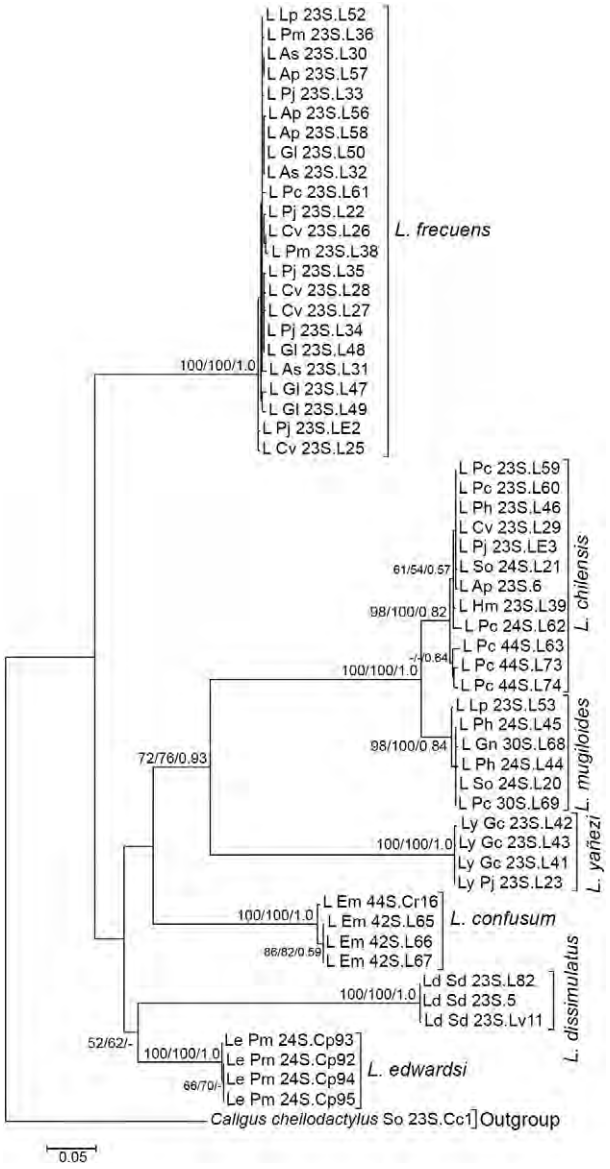

Fig. 3. Phylogenetic tree obtained with maximum likelihood (ML), using sequences of COI gene belonging to 6 known species from southeastern Pacific coast, and the new species *Lepeophtheirus confusum*. The values on nodes from left to right corresponding to the bootstrap values obtained for ML/NJ and a posteriori probability BI. The fish sampled were: *Acanthistius pictus* (Ap), *Anisotremus scapularis* (As), *Cheilodactylus variegatus* (Cv), *Eleginops maclovinus* (Em), *Genypterus chilensis* (Gc), *Girella laevifrons* (Gl), *Graus nigra* (Gn), *Hemilutjanus macrophthalmos* (Hm), *Labrisomus philippii* (Lp), *Paralabrax humeralis* (Ph), *Pinguipes chilensis* (Pc), *Prolatilus jugularis* (Pj), *Paralichthys microps* (Pm), *Semicossyphus darwini* (Sd) and *Sebastes oculatus* (So).

Measurements based on 10 ovigerous females (in micrometres - μm). Cephalothorax subcircular (Fig. 5a), longer than wide (width 83% of length), 3555 (3141–3999) long, 3159 (3026–3333) wide. Fourth thoracic segment wider than long, 525 (462–615) long, 1039 (974–1103) wide. Genital complex oblong, anterior part narrower than posterior width, with slightly pronounced posterior margin at both angles; 2059 (1846–2385) long, 1448 (1226–1795) wide. Egg sac 5538 (3359–7256) long, 412 (462–385) wide. Total body length, excluding distal setae, 7058 (6482–8024).

Antennule (Fig. 5b): two segments, proximal segment armed with 21 plumose setae and one naked seta on anterior margin and two short posterior distal processes, distal segment armed with 14 setae and one aesthetasc.

Antenna (Fig. 5c): three segments, proximal segment (coxa) is the shortest, with a short robust pointed process posteriorly, second segment (basis) longer, with a cuticular adhesion pad, oval, near distal margin. Third segment claw-like with a medial seta and other short seta more distally. Post-antennal process unbranched, with broad base, slightly curved.

Mandible blade, long and curved distally (Fig. 5e), bearing 12 teeth distally and medially. Maxillule with two tines of approximately equal length (Fig. 5e), the inner tines narrower than the other; anterior papilla bearing three setae, one long and the other of minor size and of sub-equal length. Maxilla with basal segment (syncoxa or lacertus) unarmed, medial segment (basis or brachium) longer than the first, on the outer medial margin, bearing a long flabellum (setose) (Fig. 6a, b). Distally bearing two unequal elements (calamus and canna Fig. 6b), calamus longer than canna, calamus with a serrated membrane, ventrally, and a row of fine setules located laterally on basal part. Canna with a serrated membrane.

Maxilliped (Fig. 5f) with strong and unarmed corpus protopod (or basal segment). Shaft shorter than claw, unarmed. Distal claw strongly curved, armed with a short seta near ventral margin. Sternal furca (Fig. 6c) box sub-rectangular, tines flat, blunt, slightly divergent, with hyaline margin sculptured.

First leg (Fig. 6d, e): protopod with one outer seta and other inner plumose seta (on surface) near ventral margin. Endopod: vestigial with a short setiform process. Exopod: first segment with a short seta on distal outer margin, second segment shorter than the first, armed with 3 long plumose setae on inner margin, and distal armature comprising four elements (Fig. 6e): spine 1 simple, second and third spines with accessory seta distally, the second the longest, fourth plumose seta as long as second spine.

Second leg (Figs. 6f, 7a) bi-ramous, both rami trisegmented, basis with one small seta on distal anterior margin and narrow hyaline membrane on outer margin and other wider on inner margin. Long plumose setae. Intercoxal plates with two groups of setules on each side. Exopod basal segment, longer than the others two armed with spine on outer distal margin, also a long and wide hyaline on outer margin. Second segment with a spine on distal margin and long plumose setae distally on inner margin. Third segment with 3 spines on outer margin. The first two with hyaline membrane on both sides, the third one with hyaline

Table 3
Mean distance genetic of the COI gene between groups. Upper half shows the number of mutations between paired comparisons and lower half shows the percentage difference between the paired comparisons.

	Mean distance between groups	1	2	3	4	5	6	7	8
1	*Lepeophtheirus frecuens* (n = 23)		119.5	122.5	98.4	116.6	110.7	88.9	105.9
2	*Lepeophtheirus chilensis* (*n* = 12)	24.7		34.5	111.2	112.8	130.2	105	124.5
3	*Lepeophtheirus mugiloides* (n = 6)	25.5	6.3		108.3	111.4	126.8	106.3	132.3
4	*Lepeophtheirus confusum* (n = 4)	19.7	22.7	22.0		110.3	109.3	85.8	109.3
5	*Lepeophtheirus yanezi* (n = 4)	23.9	23.1	22.8	22.4		128.6	104.5	119.3
6	*Lepeophtheirus dissimulatus* (n = 3)	22.6	27.5	26.6	22.2	27.0		98.08	118
7	*Lepeophtheirus edwardsi* (*n* = 4)	17.5	21.3	21.6	16.7	21.1	19.6		96.3
8	Outgroup (n = 1)	21.2	25.9	27.9	22.1	24.5	24.5	19.0	

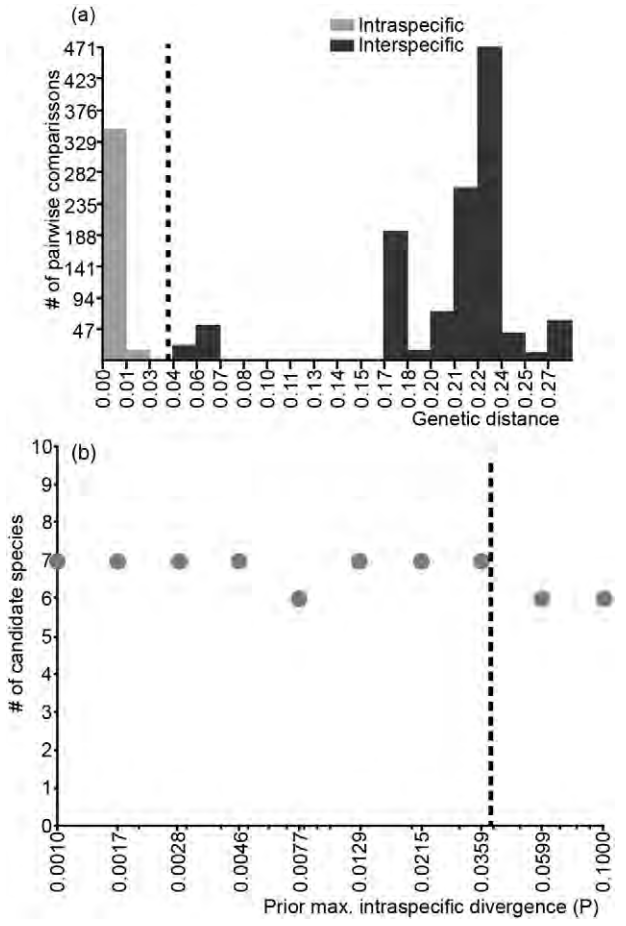

Fig. 4. Distribution of pairwise distances for the COI gene and automatic barcode gap discovery (ABGD). a) Frequency distribution of K2P distances between haplotype pairs for the COI gene. b) ABGD results showing the number of groups obtained for a range of prior maximum divergences of intraspecific diversity. Dashed lines (a and b) indicate the upper bound of estimated maximum limits for intraspecific genetic divergences that resulted in seven stable candidate species.

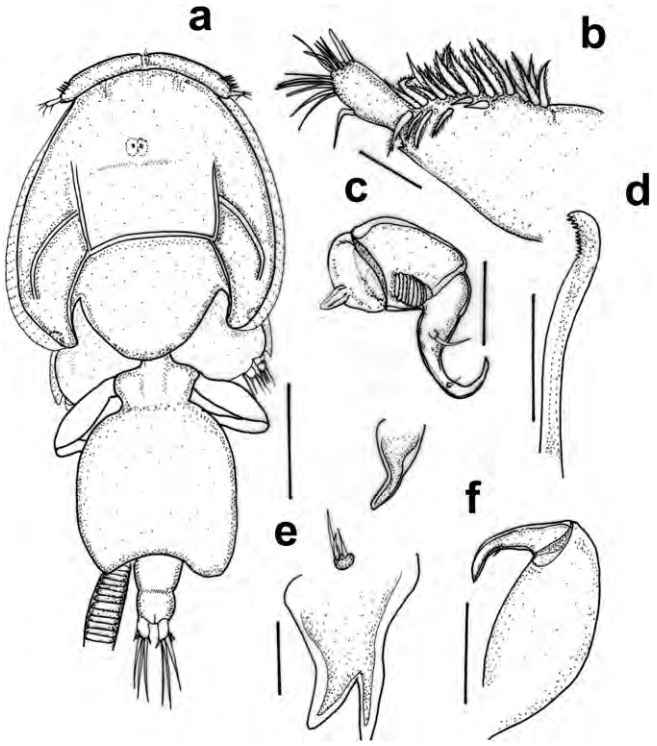

Fig. 5. Female morphology of *L. confusum* n. sp. a) Female entire dorsal, b) antennule, c) antenna entire, d) mandible, e) maxillule, f) maxilliped. Scales: a = 1000 μm, b = 100 μm, c = 20 μm, d = 100 μm, e = 100 μm, f = 200 μm.

membrane on outer margin and setules on inner margin, also 5 long plumose setae distally. Endopod basal segment as long as the second one. The first armed with only long plumose seta on inner distal border. Second segment with two long plumose setae on inner margin. Third segment smaller than others, with 6 plumose setae.

Third leg bi-ramous (Fig. 7b–d). Protopod (developed as an apron) with naked small seta on outer margin, and short plumose seta on inner margin, also bearing hyaline membrane (velum) between the rami. Exopod tri-segmented, endopod bisegmented. First exopod segment short, with long plumose seta on inner distal border and well developed spiniform process, with hyaline margin distally. Spine straight with narrow hyaline margin. Second segment longer than the first and third, respectively, with short spine on outer distal margin, and inner distal plumose seta, both margins of the segment with setules. Third segment bearing 3 short spines and 4 long plumose setae and setules on outer margin. First endopod segment with long plumose seta on inner distal margin and setules on outer margin. Second segment with 5 long and others short plumose setae plus setules on outer margin. Fourth leg (Fig. 7e). Protopod strong, armed with one short seta near disto-external margin, and two short setules near outer margin. Exopod tri-segmented, each one of the segments armed with a fringe on margin (not drawn). First segment with a short spine, at base with a short pecten, second segment longer than the others, bearing distally a plumose spine, of median size, armed with a pecten at its base. Distal segment armed with three distal spines, the innermost the longest, the other decreasing in length, the outer the shortest of all, each spine with a basal pectin (Fig. 7f). Spines with fine and narrow fringe at both margins. Fifth leg (Fig. 8a,b) located on disto-posterior surface of genital complex, slightly above the position of the egg sac attachment. Suborbicular (Fig. 8b), armed with three distal setae, the central the shortest, also at least seven spinules on the margin of the leg, and a papilla with one setae on disto-lateral margin of the genital complex, ventrally.

Legs armature.

	Coxa	Basi	Exopod	Endopod
L1	0-0	1-1	I-0; III;1–3	Vestigial
L2	0-1	1-0	I-1; I-1;III-5	0–1; 0–2; 6
L3	0-1	1-0	I-1; I-1; 4-3	0–1; 6
L4	0-0	1-0	I-0; I-0; III	Absent

Abdomen indistinctly bi-segmented (Fig. 8c), 690 (615–769) long, 485 (436–564) wide. Subrectangular, first segment as long as wide, longer than the second. Abdomen length only 34% of genital complex length. Caudal ramus (Fig.8c) subrectangular, armed with a median size seta, plus other shorter on outer margin and a short seta on disto-inner margin, and other three long plumose setae on distal border, the central setae the longest , the other two of approximately same size.

Male: Measurements based on 6 specimens (in micrometres - μm): Cephalothorax suborbicular, slightly longer 2282 (2128–2359) than wide 2051 (1923–2154). (Fig. 8d). Cephalothorax longer than the fourth segment plus the genital complex. Fourth segment 305 (244–376) long, 577 (508–629) wide. Genital complex slightly longer 702 (660–741) than wide 587 (548–660) (Fig. 9a), bearing a lateral lobule armed with four plumose setae, increasing its length from the anterior to the most posterior part, representing the fifth leg. Abdomen 344 (305–396) long, 290 (264–305) wide. Caudal ramus 195 (162–213) long, 134 (122–152) wide. Sixth leg just on disto-lateral margin, armed with three short setae. Total body length, excluding distal setae, 3994 (3549–5049).

Antenna three segmented (Fig. 9b), robust, basal segment the shortest of all, bearing a cuticular pad on the lateral surface. Second

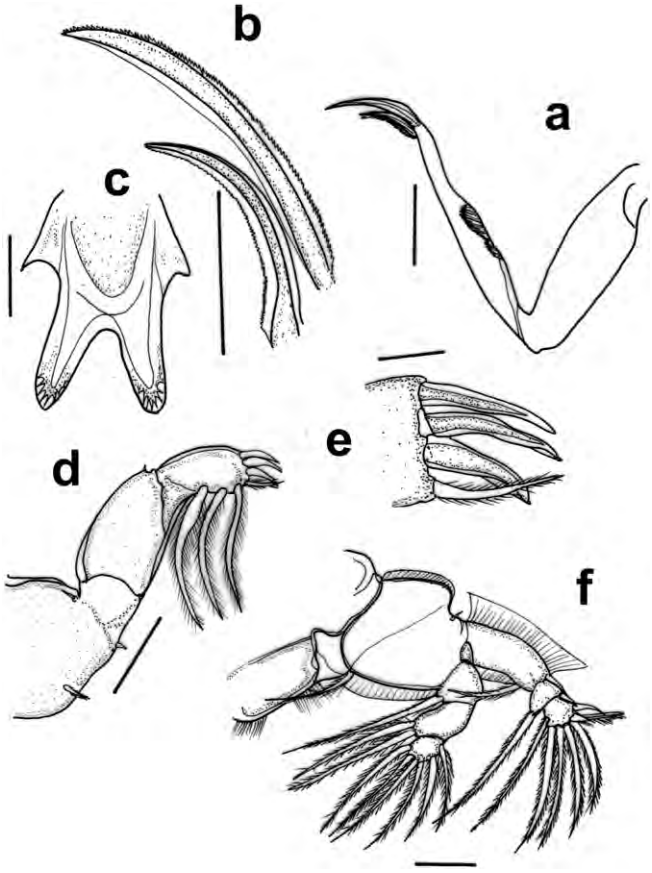

Fig. 6. Female morphology of *L. confusum* n. sp. a) Maxilla entire, b) detail distal end, c) furca sternal, d) first leg entire, e) first leg, detail of distal armature, f) second leg, entire. Scales. a = 200 μm, c = 100 μm, d = 200 μm, e = 50 μm, f = 250 μm.

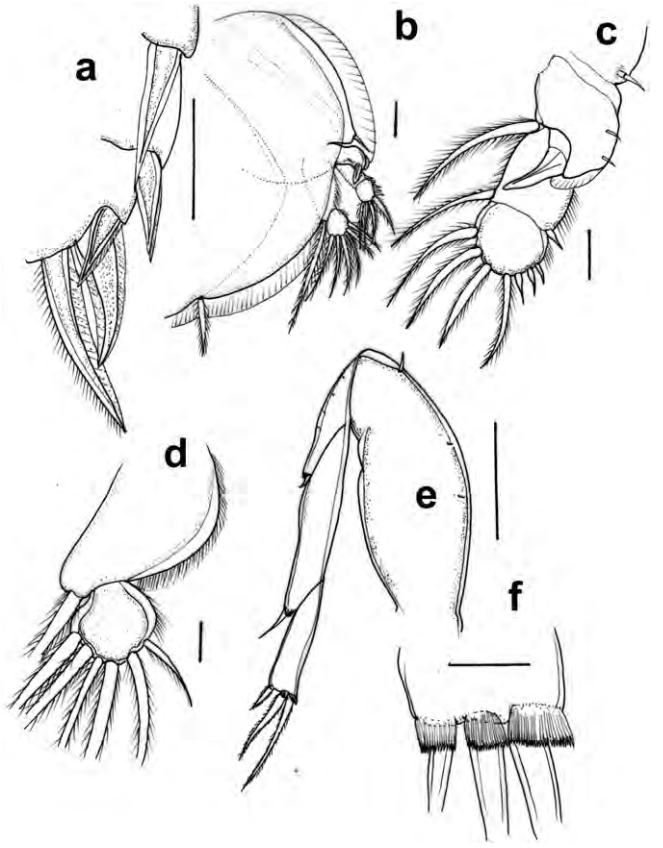

Fig. 7. Female morphology of *L. confusum* n. sp. a) Leg 2 detail, distal armature exopod, b) leg 3 entire, c) L3 exopod, d) L3 endopod, e) fourth leg, entire, f) detail of distal armature (pecten). Scales: a = 100 μm, b = 200 μm, c = 50 μm, d) 50 um, e) 75 um, f = 11 μm.

segment with as cuticular pad, wide, about a half of the segment length, and other two short pads near the distal margin of the segment, located transversally. Distal segment, a claw, curved distally, armed with three spiniform process, on its ventral margin and other on the surface near the dorsal margin. From the other side the claw, three processes on the lateral surface, the distal apparently three lobed proximally, the medial the shortest, also tri-lobed on its distal margin, the more basal process approximately triangular. Also a seta located on the lateral surface. Maxillule (Fig. 9c) with three tines, one strong, and the other two more narrow. The annexed cuticular pad elongated, wide posteriorly. Maxilliped (Fig. 9d,e), corpus narrow, myxal area with a short lobular projection (Fig. 9e), this armed with two short spines. Distal claw strong, slightly curved, bearing a seta just at the mid-length. Sternal furca (Fig. 9 f), box subrectangular, tines slightly divergent, blunt.

3.3.1. Remarks

Two *Lepeophtheirus* species (*L. chilensis* and *L. mugiloides*) are morphologically similar with *L. confusum* n. sp., which has probably contributed to erroneous records for hosts and localities. *Lepeophtheirus chilensis* parasitize *Sebastes oculatus* and has also been reported from several other hosts along the SEP [11,42]. *Lepeophtheirus mugiloides* parasitizes *Pinguipes chilensis* (= *Mugiloides chilensis*) and has also been reported on *Eleginops maclovinus* [43,44]. New observations and comparisons from the present study leave no doubt on the identity of the specimens on *E. maclovinus*, although *L. confusum* specimens do share the general outline of the cephalothorax and genital complex with *L. mugiloides* and *L. chilensis*.

Lepeophtheirus confusum n. sp. can be differentiated from *L. chilensis* because the female antenna medial segment bears a pad that is long and complex, whereas this pad is simple and short in *L. chilensis*. The maxillule bears tines that are slightly curved, whereas in *L. chilensis*, both are straight. Furthermore, in *L. confusum*, the sternal furca exhibits tines that are blunt and wide, whereas these tines are acuminated in *L. chilensis*. The basal segment of the exopod on the second leg bears a short spine that does not reach the base of the next segment, whereas in *L. chilensis*, that spine is longer, extending to the base of the next segment. The medial segment of the endopod of the fourth leg has a seta that extends only to the mid-length of the distal segment in *L. confusum*, whereas in *L. chilensis*, this seta extends to near the end of the distal segment. The fifth leg is suborbicular and oriented posteriorly, with the medial seta approximately the same size as the other external seta in *L. confusum*. In contrast, the legs are more lobular and oriented laterally, and the medial seta is half of the length of the other external seta in *L. chilensis*.

The male of *L. confusum* n. sp. presents other differences in comparison with *L. chilensis*. The medial tines are shorter than the other tines, whereas in *L. chilensis*, these times are of similar size. The pad located posterior to the maxillule is narrowed anteriorly and is slightly shorter than the maxillule length. In contrast, in *L. confusum* n sp., this pad is of the same width for all its length and half of the maxillule length. The antennal claw bears a simple basal process, whereas this process is bifid in *L. chilensis*. The medial segment of the antenna has two small pads near the disto-ventral surface in *L. confusum*, whereas in *L. chilensis*, only one small pad is present. The maxilliped corpus bears a lobular projection on the myxal area, and this projection is equipped with two short spiniform processes in *L. confusum*; by contrast, in *L. chilensis,* the myxal area bears only a short, narrow spiniform process, unarmed at that position.

Lepeophtheirus confusum n. sp. and *L. mugiloides* differ notoriously in the cephalothorax and genital complex to body ratios: the genital

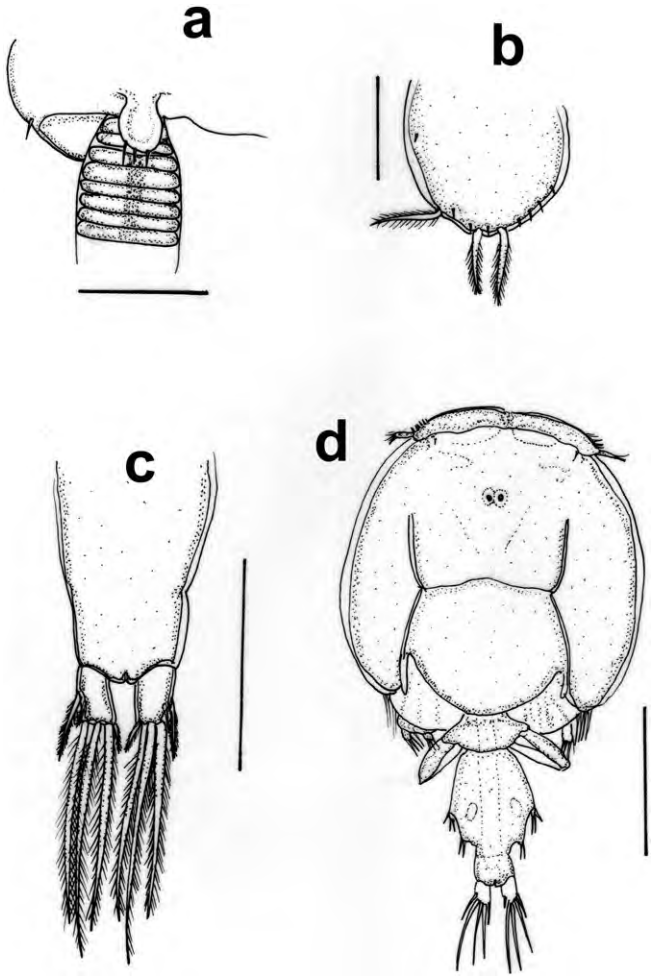

Fig. 8. Female and male morphologies of *L. confusum* n. sp. a) Fifth leg position on trunk ventrally, b) fifth leg entire, c) abdomen and caudal ramus, d) male entire dorsal view. Scales: a = 300 μm, b = 100 μm, c = 500 μm, d = 1000 μm.

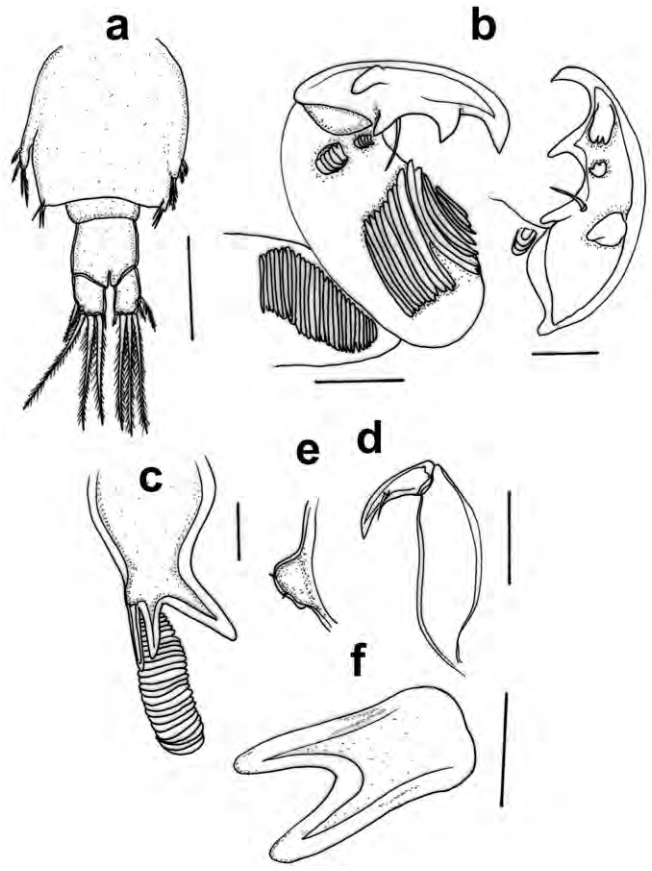

Fig. 9. Male morphology of *Lepeophtheirus confusum* n. sp. a) Genital complex, abdomen, b) antenna entire, and antenna claw detail other view, c) maxillule, d) maxilliped entire, e) detail of armature of myxal area, f) Furca sternal. Scales: a = 500 μm, b = 100 μm, c = 50 μm, d = 200 μm, f = 100 μm.

complex is approximately 58% of the cephalothorax length in *L. confusum* n. sp., whereas it reaches 25% of the cephalothorax length in *L. mugiloides*. Also, some morphological differences are apparent in the body. The female antenna bears an elongated and narrow claw in *L. confusum* n. sp., whereas in *L. mugiloides*, this antennal claw is wider. The maxillule bears a tine that is wider than the other, whereas both tines are of approximately equal length in *L. mugiloides*. The sternal furca bears tines that are distally blunter and more separated at the base in contrast to the tines that are more acuminated and more separated at the base in *L. mugiloides*. The maxilliped corpus of *L. confusum* is more robust, whereas in *L. mugiloides*, it is narrower. The leg armature on the outer margin of the exopod differs in the size of each of the spines, with the spines being shorter in *L. confusum*. The basal spine of the third leg in *L. confusum* n. sp. shows a short margin on the distal process, which is not present in *L. mugiloides*. The fifth leg is notoriously suborbicular and oriented posteriorly in *L. confusum* n. sp., whereas it is more lobular and oriented laterally in *L. mugiloides.*

The males of both species show other differences. The maxillule bears a medial tine shorter than the others in *L. confusum* n. sp., whereas in *L. mugiloides,* the outer tine is shorter than the other. The antenna shows differences in the distal claw; it bears a simple basal process in *L. confusum* n. sp., whereas it is bifid in *L. mugiloides.* The pads on the medial segment are also different between *L. confusum* n. sp. and *L. mugiloides,* especially due to the presence of two short pads on the distal ventral surface of the second segment in the former, whereas it bears only one pad on that location in *L. mugiloides*. Finally, differences occur on the maxilliped, where a projection on the myxal area is armed with two small spiniform processes on the surface in *L. confusum* n. sp. and is unarmed in *L. mugiloides.*

A comparison of the species of the genus studied herein shows that the other four species (*L. oblitus* Kabata, 1973, *L. parvus* Wilson, 1908, *L. pravipes*, and *L. scuitiger* Shiino, 1956) are morphologically more similar with the new species, but they differ in some characters. *Lepeophtheirus oblitus* can be distinguished from *L. confusum* n. sp. by exhibiting a female furca with tines widely separated because the tines are more closely spaced in *L. confusum* n. sp. In addition, seta 4 of the first leg distal armature is longer than all other setae, whereas in the new species, this seta is approximately the same length as the others. The male of *L. oblitus* differs from the present species because the antennal claw has only one medial process, whereas in the new species, two processes are present along with others that are located more basally. The maxilliped bears a patch of denticles in *L. oblitus*, whereas in the present species, only a lobular projection armed with two small spiniform processes is present.

Lepeophtheirus parvus Wilson, 1908 can be differentiated from the present specimens by the sternal furca of the female, which bears narrow and acuminated tines in *L. parvus*, whereas these tine are more blunt and strong in *L. confusum* n. sp. In *L. parvus,* on the fourth leg, the second spine on the distal segment is longer than the others, whereas in *L. confusum* n. sp., the first spine is the longest. Also the basal spine on the third leg shows a difference in the shape of the distal spiniform process. The second leg shows differences in the armature of the basal segment of the exopod, with the spine longest in *L. parvus* and shortest in *L. confusum* n. sp.

Lepeophtheirus pravipes Wilson, 1912 exhibits a reduced abdomen compared with *L. confusum* n. sp. In addition, the male bears a distal

antennal claw with 4 processes located distally and one basally, whereas *L. confusum* n. sp. bears two processes separated on the margin of the claw and other more basal. In *L. pravipes*, the maxillule bears a tine shorter than other, whereas the tine is almost equal in *L. confusum* n. sp. The shape of the fifth leg is subtriangular and oriented laterally in *L. pravipes*, whereas in the new species, it is subcircular and posteriorly located.

Lepeophtheirus scutiger differs from *L. confusum* n. sp. in a combination of characters such as the maxilla and sternal furca, which bear tines widely separated in *L. scutiger*, whereas the tines are more closely placed in *L. confusum* n. sp. The fifth leg is subtriangular and laterally oriented in *L. scutiger*, whereas it is sub-circular and posteriorly oriented, in *L. confusum* n. sp. The abdomen is not segmented in *L. scutiger*, whereas the abdomen is bi-segmented in the new species.

In addition to morphological differences, the results of the molecular analysis based on mtDNA (COI gene) shows a genetic distance of 22.7% between *L. confusum* n. sp. and *L. chilensis* and 22% between *L. confusum* n. sp. and *L. mugiloides* (Table 2). Therefore, this analysis corroborates the existence of a new species of *Lepeophtheirus* parasitizing on *Eleginops maclovinus.*

Etymology: The specific name "*confusum*" comes from the Latin word "*confuses*" and refers to the difficulty in identifying the species of *Lepeophtheirus* on *E. maclovinus*, which has been confused several times with *L. mugiloides.*

This study corroborates the usefulness of female characters, such as the maxilla tine length and width; sternal furca shape and tine width; and the fifth leg position, shape and armature. These characters are used herein in addition to male characters, such as the number of claw processes and shape of the antenna, pad on the antennal segments, maxillule tine lengths and thickness, maxilliped armature on the myxal area, and the sternal furca shape, which sometimes show only minor differences among congeners. These differences are corroborated herein by the molecular analysis.

4. Discussion

In the south-eastern Pacific coast (SEP), caligid copepods are relatively common on marine fish, but most studies have reported *Lepeophtheirus* species only at the genus level [8] because the morphological identification is sometimes unclear. In fact, during this study, we discovered that the morphological descriptions used to distinguish *L. frecuens*, *L. chilensis* and *L. mugiloides* were sometimes difficult to apply because the differences described for these species were subtle. According to Castro and Baeza [11], *L. frecuens* can be distinguished from *L. chilensis* by its short abdomen, a first maxilla medial tine shorter than the lateral tine and a series of appendage differences, particularly in the second antenna in males. According to Villalba and Durán [14], *L. mugiloides* can be distinguished from other species by the first maxilla and an elongated and simple abdomen (approximately 1/3 the length of the genital segment). In this study, we based our morphological analysis on original descriptions or on re-descriptions, when available, of *Lepeophtheirus* species of Chile. However, we observed similarities of these characteristics in some specimens of different species. *Lepeophtheirus chilensis*, for example, is supposedly distinguished by the two-segmented abdomen, which was not clearly observed in some specimens of this study. In addition, the original description of *L. chilensis* and the additional description given by Stuardo and Fagetti [25] did not refer to several structures that may be important for the identification of the species [26].

The difficulty of among the morphological distinctions ascribed to *Lepeophtheirus* species has occurred because some authors have assumed that a fish species harbour one *Lepeophtheirus* species. However, in this study, we observed that *Lepeophtheirus* species are not host-specific. For example, *L. chilensis*, *L. mugiloides* and *L. frecuens* co-occur on *S. oculatus, P. chilensis*, and other littoral fish species [11]. Likewise, *L. mugiloides* possibly may co-occur with *L. confusum* on *Eleginops maclovinus* and *Pinguipes chilensis*, because the type host for each of these two *Lepeophtheirus* species have overlapping geographical range along the Chilean coast. Moreover, the distribution of *Lepeophtheirus* species along the Chilean coast is wider that it was known before this study. Phylogenetic analyses use different genetic markers to analyze the common ancestry of the species. The mutational rate of 28S gene is lower than COI gen. For this reason, the phylogeny of *Lepeophtheirus* with 28S gene was better supported (bootstrap) at the basal branches. On the other hand, COI gene present higher mutational rates; and therefore, the terminal branches were better supported by COI gene. Thus, the morphological distinction of *Lepeophtheirus* and the distributions of the species have been corroborated by DNA barcoding, which also demonstrated the suitability of the COI gene for identifying *Lepeophtheirus* species and had been applied successfully in the identification of other species previously [22,45]. For example, Morales-Serna et al. [22], using DNA barcoding, reported a pairwise genetic divergence between sister species of *Caligus* higher 4% (4.7 to 8%).

Host switching has been documented in several caligid copepods because they have a direct life cycle, several species are cosmopolitan, and some are generalists [18]. In addition, some fish host species have a high capacity for distribution and dispersal, which would mean that caligids could possibly extend their distribution and parasitize new host species as a result of chance or a random genetic change in the parasite or host populations [46]; furthermore, caligids can parasitize exotic species [4]. Therefore, it is possible that host switching has occurred along the Chilean coast.

The geographic distributions of the ectoparasites are dependent on the host fish distribution [47,48], predominantly due to the low migration capacity and the short duration of the copepod larval stages. Although most parasitic copepods are obligate and permanently infest their hosts, caligid copepods are mobile throughout the adult stage. However, they cannot move great distances and normally stay near fish in order to feed [18]. On the other hand, the planktonic larval nauplius stage of caligids can live in the water column for up to 48 h, and the copepodids (infective stage) can live up to 10 days, until they reach an appropriate fish host [49]. Thus, the larval stages cannot actively disperse in the water, and the extensive latitudinal distribution of the *Lepeophtheirus* species in the Chilean coast is likely determined by the fish host distributions: *L. chilensis*, *L. frecuens* and *L. mugiloides* are extensively distributed along the Chilean coast (18°S-45°S) according to host distributions, whereas the new species *L. confusum* n. sp. was found parasitizing the fish species *E. maclovinus*, which is distributed from 35°S to 52°S.

Lepeophtheirus zbigniewi, *L. yanezi*, *L. edwardsii* and *L. dissimulatus* were satisfactorily identified by morphological distinctions, which were confirmed by molecular analysis. *Lepeophtheirus zbigniewi* has been identified on intertidal and subtidal fish species, including fish of the family Labrisomidae, such as *Auchenionchus microcirrhis* (collected from 24°S) [11] and *Labrisomus phillipi* (collected from 24°S to 27°S), and fish of the Blenniidae family, including *Scartichthys viridis* (collected from 32°S). Additionally, undetermined *Lepeophtheirus* species have been recorded on intertidal fish from the south-central region of Chile [8]. Therefore, the latitudinal distribution and host range of *L. zbigniewi* is possibly wider than previously documented. *Lepeophtheirus dissimulatus* was reported for the first time along the Chilean coast by Castro and Baeza [16] on *Semicossyphus darwini*. Previously, *L. dissimulatus* had been found on fish from the Northern Hemisphere [26], mainly on *Acanthurus* species from Hawaii [50] and *Merluccius productus* from the Japanese coast [51]. *Lepeophtheirus dissimulatus* is not likely to be distributed throughout two hemispheres, particularly because the fish host species are not present in the Southern Hemisphere. Thus, even though the *Lepeophtheirus* specimens examined in this study were identified as *L. dissimulatus,* genetic analysis of the specimens from the original type host are important to confirm the presence of this copepod species along the South American coasts.

A variety of human activities, through ongoing climate change, biotic invasion and habitat modification, are dramatically modifying the biology of hosts and their parasites, which may subsequently become displaced within and outside their natural geographic ranges [23]. Therefore, the precise identifications of parasites such as sea lice are necessary to prevent epidemic outbreaks such as those observed in certain parasitic copepods on Chilean fish farms; these outbreaks have modified the parasite distributions and infectivity in new environments and hosts [4].

In summary, this study confirmed the presence of known species of *Lepeophtheirus* and a new species (*L. confusum*) infesting littoral fish species from the Chilean coast, demonstrating the importance of combining morphological and molecular markers (such as the COI gene) to identify and recognise caligid species and suggesting, moreover, that the richness of *Lepeophtheirus* species along the Chilean coast is probably greater than actually known, which is probably due to the reduced morphological differences among the species and because *Lepeophtheirus* species co-occur on the same hosts.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.parint.2016.08.006.

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