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Molecular and morphological analyses reveal that the pathogen *Benedenia seriolae* (Monogenea: Capsalidae) is a complex species: Implications for yellowtail *Seriola* spp. aquaculture

F.A. Sepúlveda [a,b,*,1], M.T. González [b,1]

[a] Programa de Doctorado en Ciencias Aplicadas, Mención Sistemas Marinos Costeros, Universidad de Antofagasta, P.O. Box 170, Antofagasta, Chile
[b] Laboratorio de Ecología Parasitaria y Epidemiología Marina LEPyEM, Instituto de Ciencias Naturales Alexander von Humboldt, Facultad de Ciencias del Mar y Recursos Biológicos, Universidad de Antofagasta, Antofagasta, Chile

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ABSTRACT

Monogeneans such as *Benedenia seriolae* are important pathogens of *Seriola* spp. that are farmed around the world. In northern Chile, cultures of *Seriola lalandi* are being developed, and *B. seriolae* has been recorded in farmed fish and in natural populations. In this study, specimens identified as *B. seriolae* collected from farmed *S. quinqueradiata* in Japan were compared with specimens of *B. seriolae* collected from natural populations of *S. lalandi* from the northern Chilean coast, and morphometrical and molecular analyses were used to evaluate whether these samples corresponded to the same genetic group. Additionally, our sequences were compared with published sequences of *Benedenia* spp. from Australia. The morphometrical characteristics of the specimens from Chile and Japan were evaluated using principal component analysis (PCA) and discriminant analysis (DA). For molecular analyses, regions within the 28S ribosomal DNA large subunit (28S rDNA) and mitochondrial gene cytochrome *c* oxidase 1 (COI) were used. Specimens of *S. lalandi* from Chile did not exhibit evident morphological (except by a slight difference in penis shape) and morphometrical differences compared with *S. quinqueradiata* from Japan. For 28S rDNA, the genetic distance between *B. seriolae* from Chile and Japan was 1.5%. For COI, the genetic distance between *B. seriolae* from Chile and Japan was 14% and 15.2% for *B. seriolae* from Chile and Australia. Similarly, the genetic distance between *B. seriolae* from Japan and Australia was 14.4%. Phylogenetic trees showed three clades: one group from Chile, one from Japan and one from Australia. Thus, our results indicate *B. seriolae* is a complex species, with three morphologically similar species, each one restricted to a distinct geographic area. Given the differences in biological aspects such as fecundity and development times between species, an effective management strategy to prevent these monogenean epizootics must begin with accurate species identification, which should be considered previous to chemical treatments. Consequently, more biological information must be obtained for *B. seriolae* from the Chilean coast to understand its developmental timeline and infestation dynamics to control and manage this parasite in farmed yellowtail.

1. Introduction

Monogenean parasites have been recognised as serious pathogens of finfish in sea cage aquaculture (Grau et al., 2003; Ogawa and Yokoyama, 1998; Tubbs et al., 2005). Infections with the monogenean *Benedenia seriolae* are a particularly persistent problem and a major barrier to efficient production and industry growth. This pathogen is a well-known parasite found on the epidermis of host yellowtail *Seriola quinqueradiata* and *S. dumerili* cultured in Japan (Ogawa and Yokoyama, 1998) and also on kingfish *S. lalandi* in Australia (Ernst et al., 2002), New Zealand (Sharp et al., 2003), México (Avilés and Castelló, 2004) and Chile (Whittington et al., 2001a).

B. seriolae attach to the host via a pair of anterior pads and an opisthaptor; the hamulis and accessory sclerites pierce the epidermis and penetrate the dermis of the host (Buchmann and Bresciani, 2006). The presence of large numbers of *B. seriolae* causes considerable irritation to fish and results in the fish 'rubbing' themselves along the bottoms and sides of tanks and cages. They cause external injuries to the skin that often lead to secondary infections by opportunistic pathogens such as bacteria and/or fungi (Buchmann and Bresciani, 2006). The total yearly yellowtail production in Japan is approximately 150,000 tonnes with an annual market value of >US$1 billion, but management of *B. seriolae* may increase production costs by >20% (Rohde, 2005).

* Corresponding author. Tel.: +56 55 637631; fax: +56 55 637804.
E-mail address: fabiola.sepulveda@uantof.cl (F.A. Sepúlveda).
[1] Present address: Angamos 601, Universidad de Antofagasta, Antofagasta, Chile.

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Yamaguti (1934) described *B. seriolae* (as *Epibdella seriolae* Yamaguti, 1934) from fish *S. aureovittata* Temminck & Schlegel (Carangidae) in the Inland Sea of Japan. Currently, *S. aureovittata* is considered to be synonymous with *S. lalandi* Valenciennes, a carangid with a broad distribution throughout tropical and warm-temperate seas of the southern hemisphere and northern Pacific (Smith-Vaniz et al., 1990). However, based on molecular markers, the existence of at least three distinct populations of *S. lalandi* in the western Pacific coast has been demonstrated (Miller et al., 2011; Nugroho et al., 2001). Therefore, taking into account the fact that monogeneans are a highly specific group among parasites (Whittington et al., 2000), it is doubtful that *B. seriolae* can be present with such an extensive geographic range and be found on different hosts.

The presence of cryptic species, phenotypic plasticity and genetic variability among the characteristics used for species identification can lead to incorrect identifications. Therefore, in the last decades the use of molecular markers has been considered a powerful tool in taxonomy, systematics and phylogeny, allowing researchers to assess whether morphological variations correspond to different species or only represent differential phenotypic expression of the same species due to environmental variations (Hebert et al., 2004; Radulovici et al., 2010; Whittington, 2004). Using a region within the 28S ribosomal DNA large subunit (28S rDNA LSU), Whittington et al. (2001b) compared specimens of *B. seriolae* collected from *S. quinqueradiata* and *S. dumerili* in Japan and recorded 100% genetic similarity; they therefore concluded that both host fish species are parasitized by *B. seriolae*, suggesting it to be highly probable that *B. seriolae* is capable of infecting any *Seriola* spp. due to the cosmopolitan character of the genus *Seriola*.

In this study, morphometrical analysis and molecular markers (28S rDNA LSU region and cytochrome *c* oxidase subunit 1 (COI)) were used to compare specimens identified as *B. seriolae* collected from farmed *S. quinqueradiata* from Japan with specimens of *B. seriolae* collected from natural populations of *S. lalandi* from the northern Chilean coast to evaluate whether both samples corresponded to the same genetic group. Additionally, we compared our sequences with published sequences of *Benedenia* spp. from Australia.

2. Materials and methods

2.1. Sampling collection

Monogenean parasites were carefully removed from the skin of freshly killed *S. lalandi* specimens captured on the northern coast of Chile during the summer of 2012. Dr. Shirakashi provided samples of *B. seriolae* from *S. quinqueradiata* from Shirahama, Japan. All specimens were identified as *B. seriolae* according to Kearn (1992).

2.2. Morphology and morphometrical analysis

Five specimens from Chile and five from Japan, which were previously stored in ethanol, were stained with iron haematoxylin using routine parasitological staining techniques. Then, specimens were examined and compared based on morphological and morphometric characteristics following criteria provided by Kearn (1992) and Whittington et al. (2001a). The specimens were examined by microscopy, and all measurements were taken with the software programme Micrometrics 5.0, which was connected to an Olympus camera.

Morphometric analyses included 10 specimens measured in this study, and measurements of 14 specimens from Australia, Chile and Japan published by Whittington et al. (2001a; Table 7 of this paper). The source and number of data by host and geographical area are given in Table 1. Kruskal–Wallis tests were used to compare total body length (TL) and haptor length (HL) of *B. seriolae* specimens of *S. lalandi* from the different geographical area. Then, principal component analysis (PCA) and discriminant analysis (DA) were used for proportional morphometric measurements (Quinn and Keough, 2002). For PCA analysis, we used body proportion measurements divided by total body length (TL, included haptor) or by haptor length (HL). The proportion measurements were as follows: maximum body width/TL, haptor length/TL, haptor width/TL, accessory sclerite length/HL, anterior hamuli length/HL, posterior hamuli length/HL, anterior attachment organ length/TL, posterior attachment organ width/TL, pharynx length/TL and pharynx width/TL. For PCA analysis, the samples of *B. seriolae* were grouped by geographical area and host species: seven samples from Australia (all of *S. lalandi*); nine samples from Japan (three of *S. lalandi* and six of *S. quinqueradiata*) and eight samples from Chile (all of *S. lalandi*) (Table 1). Subsequently, the first five principal components (90% of variance) were used to perform DA (Quinn and Keough, 2002). These analyses were performed with the software programme Statistic 7.0.

Table 1
Number of data *B. seriolae* (N) used for PCA and DA analyses, showing geographical area, host and source information.

Geographic area	Host	N	Source
Japan (Ja1)	*S. quinqueradiata* (Sq)	5	This study
Japan (Ja2)	*S. quinqueradiata*(Sq)	1	GCK, Whittington et al. (2001a)
Chile (Ch1)	*S. lalandi* (Sl)	5	This study
Chile (Ch2)	*S. lalandi* (Sl)	3	Whittington et al. (2001a)
Japan (Ja3)	*S. lalandi* (Sl)	3	Yamaguti (1934) in Whittington et al. (2001a)
Australia (Au)	*S. lalandi* (Sl)	7	3 QM, Whittington et al. (2001a); 1 KR, Whittington et al. (2001a); 3 GCK, Whittington et al. (2001a).

GCK: samples from personal collection of Dr Graham Kearn; QM: Queensland Museum; KR: personal collection of Professor Klaus Rohde.

2.3. DNA extraction and amplification

Parasites were preserved in 70% ethanol and placed individually into 1.5 ml Eppendorf tubes for DNA extraction. DNA was extracted with the salting out method (Laitinen et al., 1994).

For molecular analyses, regions within the 28S rDNA LSU and mitochondrial gene cytochrome *c* oxidase 1 (COI) were used. The 28S rDNA LSU region is more conservative, and it is used for phylogenetic analyses, but in this study it was used to compare our sequences with published sequences available in GenBank. COI mtDNA is more variable and is commonly used to evaluate specific identifications (Hebert et al., 2004).

28S LSU rDNA was amplified by polymerase chain reaction (PCR) with the forward primer C1 (5′ ACC CGCTGA ATT TAA GCA T 3′) and the reverse primer D2 (5′ TGG TCC GTGTTT CAA GAC 3′) (Chisholm et al., 2001); COI mtDNA was amplified using the forward primer JB3 (5′-TTTTTTGGGCATCCTGAGGTTTAT-3′) and reverse primer COX1 (5′AATCATGATGCAAAAGGTA-3′) as described by Leung et al. (2009).

Each PCR reaction had a final volume of 35 μl including: 5 standard units of *Taq* polymerase, 3.5 μl 10× PCR buffer, 5.6 μl $MgCl_2$ (25 mM), 1.75 μl BSA (10 mg/ml) (only for COI PCR), 0.7 μl of deoxynucleotide triphosphate (dNTP) (10 mM), 10 pM of each primer and 3.5 μl template DNA. A Boeco Ecogermany M-240R Thermal Cycler (Boeckel, Hamburg, Germany) was used with a cycling profile as follows: 30 temperature cycles programmed on a slow temperature ramp rate. Cycle 1 was 95 °C for 3 min, 45 °C for 2 min and 72 °C for 90 s. This was followed by four cycles of 95 °C for 45 s, 50 °C for 45 s and 72 °C for 90 s, then a further 25 cycles of 95 °C for 20 s, 52 °C for 20 s and 72 °C for 90 s. The mix was held at 72 °C for 5 min to complete extension and then dropped to 4 °C. For COI PCR, there was an initial denaturation step at 95 °C (2 min) followed by 40 cycles of 95 °C (30 s), 48 °C (40 s), and 72 °C (1 min) with a final extension step at 72 °C (10 min).

Double-stranded PCR products were cleaned using an E.Z.N.A TM Cycle-Pure Kit (Omega Bio-Tek, Inc., Atlanta, Georgia, USA), and both

DNA strands were directly sequenced (Macrogen, Seoul, Korea; http://www.macrogen.com).

2.4. Genetic analysis

Sequences were edited using ProSeq v 2.9 beta (Filatov, 2002) and aligned with Clustal X (Larkin et al., 2007). The sequences were compared using the Mega v.5.0 genetic analysis programme (Tamura et al., 2011) and PAUP* 4.0b10 (Swofford, 1998). The sequence of the monogenean *Neobenedenia* sp. was used as outgroup for gen COI mtDNA, and the sequence of *Capsala martinieri* was used for 28S LSU rDNA (see Table 2).

For 28S LSU rDNA, trees were generated using maximum parsimony, maximum likelihood (in PAUP*) and distance matrix analyses (in Mega v.5.0). For the distance analyses, the Kimura 2-parameter (K2P) was used to calculate a matrix. Maximum parsimony analyses were run using a heuristic search strategy. For all inferred parsimony and distance trees, branch support was tested using bootstrap analysis with 1000 replicates (Chisholm et al., 2001; Olson and Littlewood, 2002). For COI mtDNA, genetic distances were calculated using the K2P model, and a similitude tree was generated by neighbour joining phylogeny tested by bootstrap (1000 replicates) (Ferri et al., 2009). These analyses were performed in Mega v.5.0 with consideration to the following parameters: gaps/missing data, pairwise deletion; codon positions, 1st + 2nd + 3rd + noncoding; substitution model, K2P; substitutions to include, transitions and transversions; pattern among lineages, same (homogeneous); rates among sites, uniform rates.

All sequences were deposited in GenBank (see Table 2 for accession numbers).

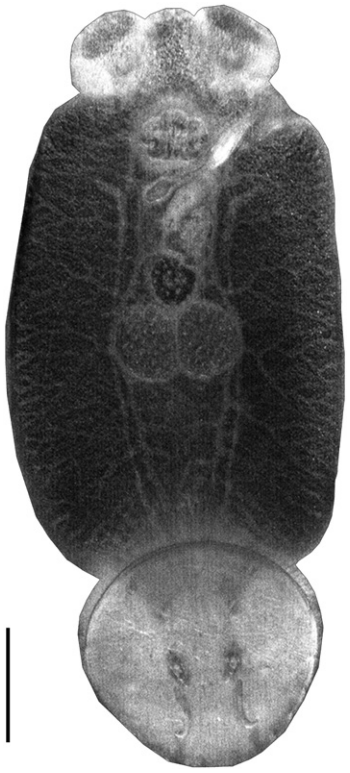

Fig. 1. *Benedenia seriolae* from *S. lalandi*, Chile stained with haematoxylin. Bar = 1.25 mm.

3. Results

3.1. Morphology and morphometry

Diagnosis based on live organisms and stained specimens showed typical characteristics of *Benedenia seriolae*. Principal characters such as reproductive system and attachment structures were the same as described by Hoshina (1968) and Kearn (1992) for *B. seriolae* (Fig. 1). The only slight difference was observed in the structure copulatory organ. Observed specimens *B. seriolae* of *S. quinqueradiata* showed blunt tip cirrus, whereas those of *B. seriolae* of *S. lalandi* presented a lanceolated cirrus (Fig. 2).

Table 2
Parasite species, host species, geographic origin and GenBank accession number for the studied specimens.

Parasite species	Host species	Origin	GenBank accession no.	Gen
Benedenia seriolae	*Seriola lalandi*	Antofagasta, Chile	KC633872	COI, this study
B. seriolae	*S. lalandi*	Antofagasta, Chile	KC633873	COI, this study
B. seriolae	*S. lalandi*	Antofagasta, Chile	KC633874	COI, this study
B. seriolae	*S. lalandi*	Antofagasta, Chile	KC633875	COI, this study
B. seriolae	*S. lalandi*	Antofagasta, Chile	KC633876	COI, this study
B. seriolae	*S. lalandi*	Antofagasta, Chile	KC633877	COI, this study
B. seriolae	*S. lalandi*	Antofagasta, Chile	KC633878	COI, this study
B. seriolae	*S. lalandi*	Antofagasta, Chile	KC768334	28S, this study
B. seriolae	*S. lalandi*	Antofagasta, Chile	KC768335	28S, this study
B. seriolae	*S. lalandi*	Antofagasta, Chile	KC768336	28S, this study
B. seriolae	*S. lalandi*	Antofagasta, Chile	KC768337	28S, this study
B. seriolae	*S. lalandi*	Antofagasta, Chile	KC768338	28S, this study
B. seriolae	*S. lalandi*	Antofagasta, Chile	KC768339	28S, this study
B. seriolae	*S. lalandi*	Antofagasta, Chile	KC768340	28S, this study
B. seriolae	*S. quinqueradiata*	Shirahama, Japan	KC633879	COI, this study
B. seriolae	*S. quinqueradiata*	Shirahama, Japan	KC633880	COI, this study
B. seriolae	*S. quinqueradiata*	Shirahama, Japan	KC633881	COI, this study
B. seriolae	*S. quinqueradiata*	Shirahama, Japan	KC768341	28S, this study
B. seriolae	*S. quinqueradiata*	Shirahama, Japan	KC768342	28S, this study
B. seriolae	*S. quinqueradiata*	Shirahama, Japan	KC768343	28S, this study
B. seriolae	*S. hippos*	Australia	NC014291	mDNA, Perkins et al. (2010)
B. seriolae	*S. hippos*	Australia	HM222526	mDNA, Perkins et al. (2010)
B. seriolae	*S. quinqueradiata*	Japan	AY033941	28S, Whittington et al. (2001b)
B. rohdei	*Lutjanus carponotatus*	Australia	AY033940	28S, Whittington et al. (2001b)
B. lutjani	*L. carponotatus*	Australia	AY033939	28S, Whittington et al. (2001b)
B. lutjani	*L. carponotatus*	Australia	AF026106	28S, Mollaret et al. (1997)
Neobenedenia sp.	*Cheilodactylus variegatus*	Antofagasta, Chile	JQ782846	COI, This study
Capsala martinieri	*Mola mola*	United Kingdom	AF382053	Olson and Littlewood (2002)

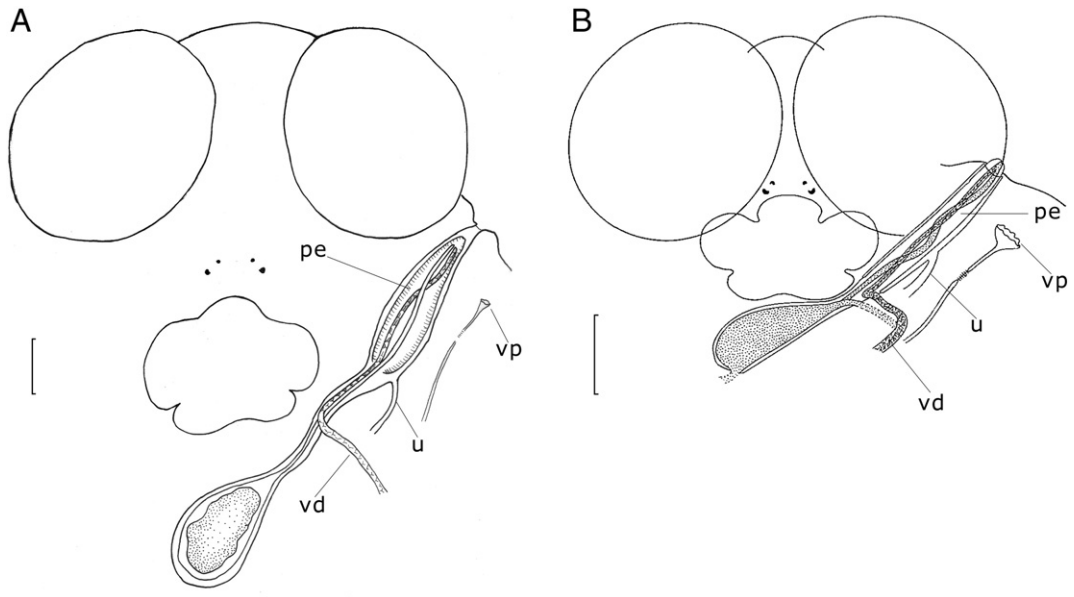

Fig. 2. Anterior ends of *B. seriolae* from (A) *S. lalandi*, Chile and (B) from *S. quinqueradiata*, Japan. (pe) penis, (u) uterus; (vd) vas deferens, (vp) vaginal pore. Bar = 0.25 mm.

Body measurements of the examined *B. seriolae* specimens of *S. lalandi* and *S. quinqueradiata* in this study are shown in Table 3. *B. seriolae* specimens of *S. lalandi* from Chile (including data of Whittington et al., 2001a) were longer than those specimens of *S. lalandi* from Australia and Japan ($H_{2,18} = 6.2$; $P = 0.048$), but when the analysis was performed, excluding specimens measured by Whittington et al. (2001a), the significant differences disappeared ($H_{2,15} = 2.6$; $P = 0.27$). On the other hand, haptor length did not vary significantly among *B. seriolae* specimens from the three geographical area ($H_{2,18} = 4.4$; $P = 0.11$) (Fig. 3). Similarly, PCA and DA did not show significant differences in body morphometrical proportional measurements between *B. seriolae* specimens of *S. lalandi* and those of *S. quinqueradiata* ($P > 0.05$), but the morphometry of the three *B. seriolae* specimens of *S. lalandi* from Japan were significantly different from the other specimens ($P < 0.05$) (Fig. 4).

Table 3
Comparative measurements (μm) of *B. seriolae* specimens of *S. lalandi* from Chile and *S. quinqueradiata* from Japan. Range (average ± standard deviation), and "n" number of examined specimens in this study.

Measurements (μm)	*S. lalandi* (n = 5) Chile	*S. quinqueradiata* (n = 5) Japan
Total length	5525.5–11209.5 (7818.1 ± 2349.4)	6313.7–8618.9 (7337.8 ± 834.2)
Maximum width	2553.1–5045.1 (3662.8 ± 1056.0)	3466.3–5010.3 (4077.7 ± 597.3)
Haptor length	1537.2–3289.3 (2216.1 ± 676.2)	1976–2065.5 (2136.3 ± 271.3)
Haptor width	1676.61–3420.5 (2348.6 ± 703.1)	1895.4–2852.4 (2347.0 ± 449.2)
Accessory sclerite length	307.3–620.0 (429.9 ± 129.5)	243.4–646.6 (438.7 ± 130.3)
Anterior hamulus length	375.8–699.3 (530.4 ± 135.8)	251.3–556.2 (439.9 ± 108.6)
Posterior hamulus length	83.1–118.4 (103.8 ± 15.5)	88.2–119.1 (103.6 ± 11.7)
Anterior attachment organ length	634.2–1229.8 (939.5 ± 236.1)	617.8–1367.2 (968.3 ± 246.2)
Anterior attachment organ width	621.7–1035.6 (803.4 ± 177.4)	514.7–1139.9 (784.1 ± 213.5)
Pharynx length	343.1–703.5 (538.6 ± 171.8)	350.3–847.8 (602.6 ± 186.0)
Pharynx width	412.3–878.0 (651.2 ± 191.8)	396.4–1054.5 (727.7 ± 239.7)

3.2. Molecular analyses

For the 28S rDNA LSU region, 7 sequences were obtained for *B. seriolae* from Chile and were compared with 4 sequences for *B. seriolae* from Japan (3 sequences from this study and one obtained from GenBank, Table 2). All sequences were 774 bp. A total of 11 polymorphic sites were identified from both parasite species. The genetic distance was 1.5% between studied specimens from Chile and Japan and >32% between specimens of *B. seriolae* from Chile and *B. lutjani* and *B. rohdei* parasitizing *Lutjanus carponotatus* (more than 100 polymorphic sites) (Table 4). The phylogenetic tree showed two clades for *B. seriolae* supported by 100% bootstrap values; one group included only specimens from Chile (99% bootstrap), and the other group included only specimens from Japan (100% bootstrap) (Fig. 5).

For COI mtDNA, 7 sequences for *B. seriolae* from Chile were compared with 3 sequences for *B. seriolae* from Japan (sequenced in this study) and 2 for *B. seriolae* from Australia (obtained from GenBank, see Table 2). All sequences were 584 bp. The genetic distance between *B. seriolae* from Chile and *B. seriolae* from Japan was 13.8 to 14.2%

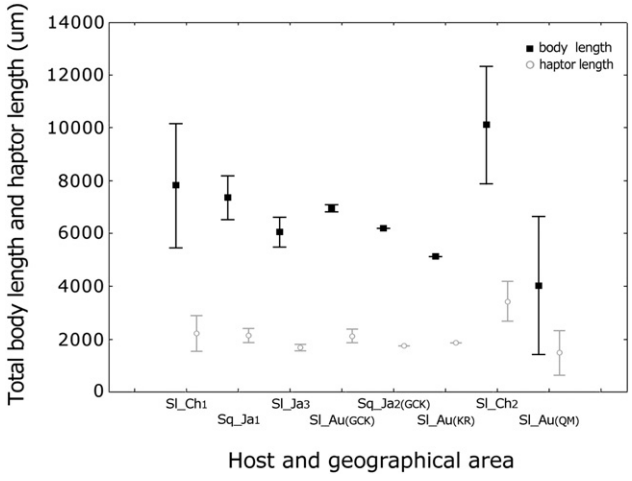

Fig. 3. Total body length and haptor length (μm) of *B. seriolae* specimens from different host species and geographical areas. Each bar show maximum, mean and minimum size of specimens. Symbols are explained in Table 1.

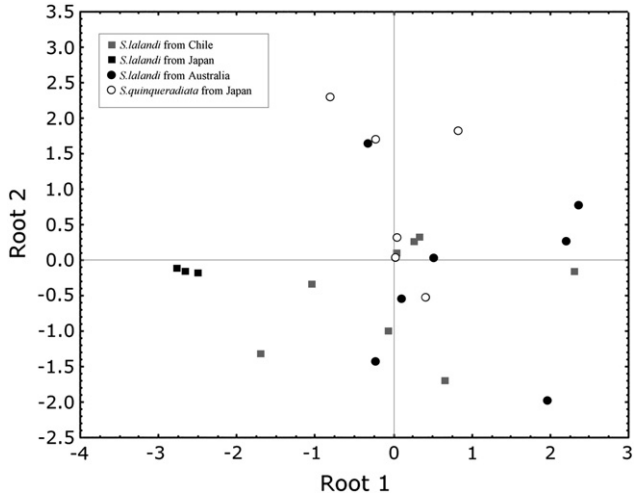

Fig. 4. Results of multivariate analyses, based on proportional measurements of *B. seriolae* from different host species and geographical areas. Grey square = *S. lalandi* from Chile; Black square = *S. lalandi* from Japan; White circle = *S. quinqueradiata* from Japan; Black circle = *S. lalandi* from Australia.

Table 4
Pairwise sequence divergences for 28S rDNA LSU region among species of *Benedenia* spp. The Kimura 2 parameters distance was calculated and it is showed as percentage with bp-pairwise differences between parentheses.

	No. of sequences	*B. seriolae* (Chile)	*B. seriolae* (Japan)	*B. rohdei* (Australia)	*B. lutjani* (Australia)
B. seriolae (Chile)	7	–			
B. seriolae (Japan)	4	1,5 (11)	–		
B. rohdei (Australia)	1	32.7 (183)	34 (190)	–	
B. lutjani (Australia)	2	32.9 (184)	34.3 (191)	2.9 (19)	–

(73 to 75 bp polymorphic sites) and 14.2 to 15.2% (76 to 80 bp polymorphic sites) between *B. seriolae* from Chile and those from Australia. Similarly, the genetic distance between *B. seriolae* from Japan and Australia was 14.2 to 14.4% (75 to 76 bp of 584 bp) (Table 5). The genetic intraspecific variability ranged between 0.3 and 1.4% (1–8 bp) for *B. seriolae* from Chile and 0.3% for *B. seriolae* from Japan. The phylogenetic tree showed three clades: one group from Chile, one from Japan and one from Australia, and each group supported by 100% bootstrap values (Fig. 6).

4. Discussion

Monogeneans such as *B. seriolae* are important pathogens of *Seriola* spp. farmed around the word (Grau et al., 2003; Ogawa and Yokoyama, 1998; Tubbs et al., 2005 Whittington et al., 2001a), and chemical treatments such as freshwater baths and hydrogen peroxide are used to control this disease in farmed fish (Chambers and Ernst, 2005). Currently, in northern Chile, cultures of *S. lalandi* are being developed, and *B. seriolae* has been recorded in farmed fish as well as in natural populations (prevalence: 16%, authors' unpublished data). Until the mid-1990s, reports of *B. seriolae* were restricted to Japan. Whittington (1996) then reported on this capsalid derived from the skin of *S. lalandi* from New South Wales, Australia, and Whittington and Horton (1996) identified this parasitic species among *Seriola* spp. off the Chilean coast. Since then, this parasite has been recorded in *S. quinqueradiata* from Japan (Whittington et al., 2001b), *S. lalandi* from Australia (Lackenby et al., 2007) and New Zealand (Sharp et al., 2003) and *S. hippos* from Australia (Perkins et al., 2010). Consequently, given the broad range of congeneric hosts and extensive geographic range of *B. seriolae*, Whittington et al. (2001a) suggested that *B. seriolae* is a cosmopolitan species. In this study, however, there was evidence that *B. seriolae* present in *S. lalandi* populations from the Chilean coast and *B. seriolae* present in Australia and Japan are genetically different, despite similar morphological characteristics (Kearn, 1992; Whittington et al., 2001a).

Numerous genetic studies have revealed complexes of cryptic species in many parasite taxa (Poulin and Morand, 2004). In these cases, morphological analyses have suggested the presence of one parasite species infecting several hosts; however, the genetic data revealed different parasite species showing high host specificity (Poulin and Keeney, 2008). The morphology of *B. seriolae*, particularly the reproductive organs, was previously reviewed by Kearn (1992). This author described in vivo specimens with genital pores that opened dorsally, and penis shaped like blunt tip but in other studies examining fixed specimens this characteristic has not been clear. In our fixed samples it was not possible either to determine whether the vaginal

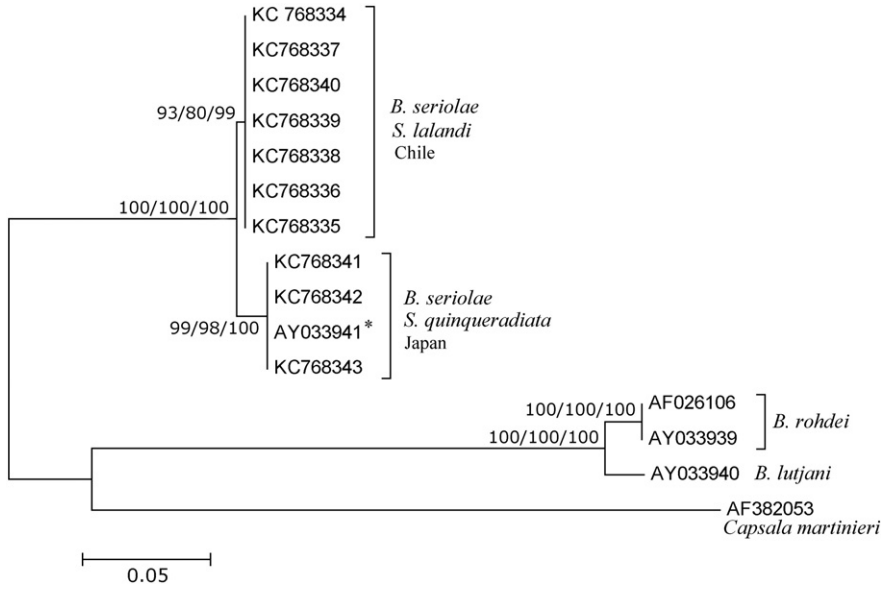

Fig. 5. Phylogenetic tree based on 28S rDNA LSU region of *B. seriolae* from Chile and Japan, inferred by maximum parsimony, maximum likelihood and maximum likelihood with Kimura 2 parameter model, supported with 1000 bootstrap and *Capsala martinieri* as outgroup. Numbers along branches indicate the percentage of support values resulting from different analyses in the order: MP/ML/ML K2P. *Sequence of *B. seriolae* from Japan taken from GenBank.

Table 5
Pairwise sequence divergences for the COI gene of mtDNA among species of genus *Benedenia*. The Kimura 2 parameter distance was calculated and it is shown in percentage (below the diagonal). The raw number of bp-pairwise differences is also shown (above the diagonal). 1–7, *B. seriolae* from Chile; 8–10, *B. seriolae* from Japan; 11–12, *B. seriolae* from Australia.

	1	2	3	4	5	6	7	8	9	10	11	12
1. KC633872		6	3	4	4	4	4	73	73	73	76	76
2. KC633873	1		7	8	8	8	8	73	73	73	80	80
3. KC633874	0.5	1.2		1	1	1	1	74	74	74	79	79
4. KC633875	0.7	1.4	0.2		2	2	2	73	73	73	78	78
5. KC633876	0.7	1.4	0.2	0.3		2	2	75	75	75	80	80
6. KC633877	0.7	1.4	0.2	0.3	0.3		0	73	73	73	80	80
7. KC633878	0.7	1.4	0.2	0.3	0.3	0		73	73	73	80	80
8. KC633879	13.8	13.8	14	13.8	14.2	13.8	13.8		0	2	75	75
9. KC633880	13.8	13.8	14	13.8	14.2	13.8	13.8	0		2	75	75
10. KC633881	13.8	13.6	14	13.8	14.2	13.8	13.8	0.3	0.3		76	76
11. NC014291	14.4	15.2	15	14.8	15.2	15.2	15.2	14.2	14.2	14.4		0
12. HM222526	14.4	15.2	15	14.8	15.2	15.2	15.2	14.2	14.2	14.4	0	

pore is open by ventral or dorsal. According to the key by Whittington et al. (2001a) for identification of *Benedenia* species, *B. seriolae* can present penis with blunt or tapering tip. However, we observed that *B. seriolae* specimens of *S. lalandi* from Chile showed all a lanceolated shape penis and those specimens of *S. quinqueradiata* from Japan showed blunt tip shape penis as described by Kearn (1992) for *B. seriolae* specimens of *S. quinqueradiata* from Japan.

On the other hand, Whittington et al. (2001a) identified *B. seriolae* of *Seriola* spp. from Chile based on morphological characteristics of the genus *Benedenia* but found important differences in body sizes between the specimens from Chile compared with those from Japan and Australia and they reported that the Chilean specimens were considerably larger. In our study, the *B. seriolae* specimens from *S. lalandi* were not significantly larger than those specimens from *S. quinqueradiata* or those of *S. lalandi* from other geographical area (See Fig. 3). Additionally, morphometric analyses based on body proportions, which included specimens examined by Whittington et al. (2001a), did not show significant differences in body morphometry of *B. seriolae* from different host species and geographical areas (nevertheless, our analyses were performed with a small sample size). A potential explanation for the morphological similitude of *B. seriolae* could be that this parasite species is undergoing allopatric speciation due to geographical isolation or as consequence of the cospeciation of this parasite with its hosts, which present allopatric distributions (Desdevises et al., 2002).

Molecular markers as 28S rDNA LSU region is useful to evaluate phylogenetic relationships (Olson and Littlewood, 2002), but also it has been used as tool to identify species. Using the 28S rDNA LSU region, Whittington et al. (2001b) compared 820 bp sequences of specimens of *B. seriolae* collected from *S. quinqueradiata* and *S. dumerili* and recorded a value of 100% similitude between them, concluding that all specimens corresponded to the same species. These authors also examined the genetic distances between species of *Benedenia* parasitising the same host species, *L. carponotatus*, from Australia, and they found that *B. lutjani* differed from *B. rohdei* by 2.68% (22 bp of 820 bp). These species exhibited, in turn, a genetic distance of 27% when compared with *S. quinqueradiata* from Japan. In other study, Whittington et al. (2004) found that *B. lutjani* and *B. rohdei* had a genetic distance of 3.17% (22 bp of 693 bp). Similarly, Wang et al. (2004) found a genetic distance of 2.08% between both species of *Benedenia* (sequences of approximately 350 bp). In this study, the genetic distance between *B. seriolae* from Chile and *B. seriolae* of *S. quinqueradiata* from Japan was only 1.5% (11 bp of 774 bp); however, the phylogenetic tree clearly showed two clades, suggesting the existence of two genetically different groups of *B. seriolae*.

On the other hand, DNA barcoding with COI mtDNA is a molecular marker validated to differentiate species (Hebert et al., 2003a, 2003b; Radulovici et al., 2010), but among monogenean capsalids there have not been any studies of COI mtDNA. Hebert et al. (2003a) analysed

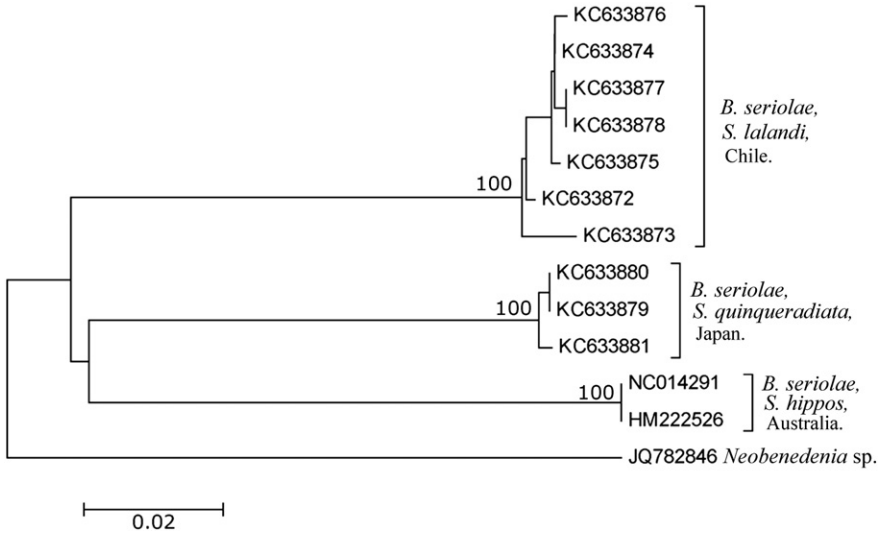

Fig. 6. Similitude tree based on COI mtDNA of *B. seriolae* from Chile, Australia and Japan, inferred by neighbour joining and Kimura 2 parameter model, supported with 1000 bootstrap and *Neobenedenia* sp. like outgroup.

sequences of the COI gene of different congeneric species pairs from several phyla and suggested that an average genetic distance of 11.3% is indicative of different species. In this study, a genetic distance higher than 13% (73–75 bp of 584 bp) was recorded between *B. seriolae* from *S. lalandi* from Chile and *B. seriolae* of *S. quinqueradiata* from Japan, and a percentage higher than 14% was found between *B. seriolae* of *S. hippos* from Australia and *S. lalandi* from Chile. In turn, *B. seriolae* of *S. quinqueradiata* from Japan was different from *B. seriolae* of *S. hippos* from Australia by 14%. Thus, our results indicate that there are three morphologically similar species of *B. seriolae*, each restricted to a distinct geographic area. Therefore, *B. seriolae* should be considered a complex species, which has also been demonstrated for *Neobenedenia melleni* by Whittington et al. (2004).

Effective management strategies to prevent monogenean epizootics need to incorporate accurate knowledge of the entire parasitic life cycle and the influence of environmental parameters (Ernst et al., 2005; Tubbs et al., 2005). In addition, chemical treatments must be applied carefully because these treatments may have undesirable effects if they are replicated with identical doses in geographic regions that have different physical and chemical characteristics or if these are applied to a misidentified species (e.g., a cryptic species). Therefore, more biological information must be obtained for *B. seriolae* from the Chilean coast to understand its developmental timeline and infestation dynamics to control and manage this parasite in cultures in northern Chile.

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