Molecular and morphological evidence indicates that *Pseudorhabdosynochus lantauensis* (Monogenea: Diplectanidae) represents two species

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SUMMARY

Sequences of the first internal transcribed spacer (ITS-1) and the D1-D3 domains of the large subunit (LSU) of the ribosomal DNA (rDNA) were determined for multiple specimens of 4 operational taxonomic units (OTUs) of the monogenean, *Pseudorhabdosynochus lantauensis*. OTUs were defined based on their collecting localities, host and/or morphological characteristics. All *P. lantauensis* specimens of one group (OTUs 1 and 3) differed in their sequences of the ITS-1 and partial LSU rDNA when compared with specimens of a second group (OTUs 2 and 4) by 12% and 2%, respectively. Results of the phylogenetic analyses of the LSU rDNA sequence data showed total (100%) bootstrap support for the separation of *P. lantauensis* into 2 distinct clades. At least 11 of the 18 nucleotide differences in the LSU sequence between the two *P. lantauensis* clades were derived (i.e. autapomorphic) characters when the morphologically distinct species, *P. epinepheli* and *P. coioidesis*, were used as outgroups. Furthermore, there were several autapomorphic character states for each *P. lantauensis* clade. This provides sufficient evidence to reject the null hypothesis that *P. lantauensis* represents a single species. Morphological and morphometric differences between these two clades provided additional strong support for the separation of *P. lantauensis* into two species. These two parasite species were found to co-exist on one of the two species of serranid fish (i.e. *Epinephelus coioides*) examined in the South China Sea (Guangdong Province, China).

Key words: *Pseudorhabdosynochus lantauensis*, first internal transcribed spacer, large subunit rDNA, Monogenea, *Epinephelus*, species delineation.

INTRODUCTION

Species of *Pseudorhabdosynochus* (Diplectanidae) occur on the gills of a variety of serranid fish, including those of *Epinephelus* (see Bu *et al*. 1999; Santos, Buchmann & Gibson, 2000). These fish are an important human food source and are cultured intensively in floating net cages in several countries, including China. However, infection by large numbers of *Pseudorhabdosynochus* in captive fish populations produces significant pathological effects and substantial economic losses (e.g., Leong, 1997; Vidal-Martínez & Mendoza-Franco, 1998). Given that serranid fish may be parasitized by several species of diplectanid (e.g., *P. lantauensis*, *P. coioidesis* and *Diplectanum grouperi* in *E. coioides* and *E. aerolatus*; see Bu *et al*. 1999), it is important that these parasites be identified in order to study their transmission patterns, epidemiology, and for the implementation of strategies to control the diseases they cause.

Pseudorhabdosynochus is characterized by the presence of a sclerotized, compartmental, bulb-shaped male copulatory organ (MCO). Identification of species within this genus is based on the size and shape of the MCO, vagina, squamodisc, ventral and dorsal bars, ventral and dorsal hamuli and the marginal hooklets, and on the number of rows of elements in the squamodisc (Beverley-Burton & Suriano, 1981; Kritsky & Beverley-Burton, 1986; Bu *et al*. 1999; Santos *et al*. 2000). However, there are sometimes difficulties in distinguishing among species with confidence because of the considerable within-species variation for some morphological characters (Santos *et al*. 2000). For example, the number of rows of elements in the squamodisc varies from 14–16 in *P. amplidiscatus* and *P. capurroi*, 14–17 in *P. epinepheli* and 15–16 in *P. sulamericanus* (see Santos *et al*. 2000). There are also reports of differences in the morphological measurements and/or descriptions of some species collected from different localities or hosts. For instance, Bu *et al*. (1999) noted

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Table 1. The number, host species and collection localities in Guangdong Province (China) for *Pseudorhabdosynochus* specimens used in the molecular and morphological analyses

Parasite species (OTU)	No. of specimens: Measured	No. of specimens: Sequenced	Host spp. (no. examined)*	Locality
P. lantauensis (1)	6	4	*E. coioides* (1)	Dayawan
(2)	5	4	*E. coioides* (1)	Dayawan
(3)	6	4	*E. brunneus* (5)	Huidong
(4)	5	4	*E. coioides* (4)	Huidong
*P. epinepheli**	0	4	*E. brunneus* (5)	Huidong
*P. coioidesis**	0	4	*E. coioides* (1)	Dayawan

* Specimens of *P. coioidesis* were collected from the same *E. coioides* individual as specimens of *P. lantauensis* OTU1 and OTU2, whereas specimens of *P. epinepheli* were collected from the same *E. brunneus* individuals as specimens of *P. lantauensis* OTU3.

differences in the shape of the dorsal bar and vagina of *P. lantauensis* from *E. coioides* (Malaysia and Indonesia) and *E. aerolatus* (Hong Kong) when compared to the original descriptions of Beverley-Burton & Suriano (1981) for *P. lantauensis* from *E. brunneus* and *E. fario* (Hong Kong). These differences were considered by Bu *et al.* (1999) to reflect geographical variation within the species or a consequence of comparing fixed with fresh specimens. Another possible explanation for these morphological differences is that *P. lantauensis* may represent two or more cryptic (i.e. genetically distinct but morphological similar) species.

DNA sequencing provides a valuable approach for the genetic characterization of parasite species (e.g. McManus & Bowles, 1996; Littlewood, Rohde & Clough, 1998) and for the detection of cryptic species (e.g. Andrews *et al.* 1998; Desdevises *et al.* 2000). Recent studies have demonstrated that sequences of the first internal transcribed spacer (ITS-1) and the partial large subunit (LSU) of the ribosomal DNA (rDNA) provide reliable genetic markers for the identification of monogenean species and for examining their phylogenetic relationships (e.g., Cunningham, 1997; Mollaret *et al.* 1997; Desdevises *et al.* 2000; Mollaret, Lim & Justine, 2000; Bentz *et al.* 2001; Chisholm *et al.* 2001; Whittington *et al.* 2004). In the present study, we compared the sequences of the ITS-1 and partial LSU rDNA, and the morphology of *P. lantauensis* from *E. coioides* and *E. brunneus* collected near Dayawan and Huidong (Guangdong Province, China) to test the null hypothesis that *P. lantauensis* from different hosts and sampling localities represents a single species.

MATERIALS AND METHODS

Parasites and morphological identification

Monogeneans were removed from the gills of 10 freshly killed fish. Some parasites were fixed in Bleasure's glue (Acacia gum 17·25%, glycerin 13·79%, chloral hydrate 34·48%, distilled water 34·48%) and their sclerotized parts examined using a dissecting microscope. All specimens (including those subsequently used for the DNA analyses) were identified morphologically to species level based on the existing keys and species descriptions (see Beverley-Burton & Suriano, 1981; Bu *et al.* 1999; Zhang, Yang & Liu, 2001). Measurements (in μm) of 22 specimens of *P. lantauensis* collected from 5 individuals of *E. coioides* and 5 individuals of *E. brunneus* from two locations in Guangdong, China (Table 1) were carried out as described previously (Bu *et al.* 1999). For the DNA analyses, 16 specimens (Psl 1-16) of *P. lantauensis* were divided into 4 operational taxonomic units (OTUs) based on host species, collecting localities (Dayawan or Huidong) and the size and shape of the vagina (e.g. presence/absence of a chelate diverticulum on the vagina) (Table 1). Specimens designated as OTU 1 and OTU 2 were collected from the same host individual (*E. coioides*) and locality (Dayawan), but differed in their morphology.

DNA extraction, PCR and DNA sequencing

Genomic DNA was isolated from 24 individual specimens of *Pseudorhabdosynochus* (i.e. 4 *P. coioidesis*, 4 *P. epinepheli*, and 4 of each *P. lantauensis* OTU; Table 1). Each specimen was placed in 20 μl of lysis solution (proteinase K 20 μg/ml, Tween-20 0·45%, NP-40 0·45%, EDTA 1 mM, Tris-HCl 10 mM), and incubated at 65 °C for 1 h, followed by incubation at 95 °C for 15 min to inactivate the proteinase. This lysate (6 μl) was used as template in PCR reactions to amplify two DNA regions, the ITS-1 and the D1-D3 domains of the LSU rDNA. The ITS-1 was amplified using primers IT1 (forward; 5′-GTCGTAACAAGGTTTCCGTAGG-3′) and IT2 (reverse; 5′-GCTGCACTCTTCATCGACGCRCG-3′) (Ding *et al.* 2003), whereas the D1-D3 domains of the LSU rDNA were amplified using primers C1 (forward; 5′-ACCCGCTGAATTTAAGCAT-3′) and D2 (reverse; 5′-TGGTCCGTGTTTCAAGAC-3′) (Li, Liao &

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Yang, 2000). PCR reactions (50 μl) were performed in 1·5 mM $MgCl_2$; PCR buffer (100 mM Tris-HCl, 500 mM KCl, 0·8% NP-40, pH 8·8) (TakaRa™); 200 μM of each dNTP; 0·8 μM of each PCR primer set and 2·5 units of Ex *Taq* polymerase (TakaRa™) in a thermocycler (MJ Research) using the following conditions: an initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min (denaturation); 56 °C for 1 min (annealing) and 72 °C for 1 min (extension), followed by a final extension at 72 °C for 5 min. Control samples with host (fish) DNA or without genomic DNA (no-DNA controls) were included in each PCR run, but in each case, no amplicons were detected. Aliquots (5 μl) of amplicons were detected in 1% agarose gels, stained with ethidium bromide, and photographed using trans-illumination. The remaining 45 μl of each amplicon was purified over a spin column (UltraPure™ PCR purification Kit, SBS) and subjected to automated DNA sequencing (ABI 373 DNA Sequencer, Shanghai United Gene Inc.) using the same primers (individually) as used for PCR.

Phylogenetic analyses

Sequences were aligned using the computer program ClustalX (Thompson *et al*. 1997). Pairwise comparisons were made of the sequence differences (D) among *P. lantauensis* OTUs and between species using the formula $D=1\text{-}(M/L)$, where M is the number of alignment positions at which the two taxa have a base in common, and L is the total number of alignment positions over which the two taxa are compared. Phenograms were constructed using the Unweighted Pair Group Method using Arithmetic averages (UPGMA; Sneath & Sokal, 1973). For the LSU rDNA sequence data, phylogenetic trees were constructed using a maximum parsimony (MP) analysis of the program PAUP* v.4b10 (Swofford, 1999). *P. epinepheli* (Pse 1-4) and *P. coioidesis* (Psc 1-4) were used as outgroups for these analyses. Characters were weighted equally and treated as unordered. Alignment gaps were treated as a fifth character state. A heuristic search with TBR-branch swapping was used to infer the shortest trees. A bootstrap analysis (using 1000 replicates) was conducted using heuristic searches and TBR branch-swapping with the MulTrees option, in order to determine the relative support for clades of the consensus tree.

RESULTS

DNA analyses

Sequences of the partial LSU and complete ITS-1 rDNA were obtained for 4 specimens representing each of the 4 OTUs of *P. lantauensis* and 4 specimens of *P. epinepheli* and *P. coioidesis*. Sequences have been deposited in the EMBL, GenBank™ and DDBJ databases (Accession numbers AY553622-AY553624 and AY596179-AY596183). The lengths of the ITS-1 and the D1-D3 domains of the LSU for all parasite specimens ranged 467–534 bp and 884–896 bp, respectively. The ITS-1 and partial LSU sequences were aligned over a consensus length of 566 bp and 897 bp, respectively (sequence alignments available from corresponding author upon request). No sequence variation was detected in the ITS-1 and LSU rDNA among individuals of a *P. lantauensis* OTU, nor among individuals of *P. epinepheli* or *P. coioidesis*. The 4 *P. lantauensis* OTU1 specimens had identical ITS-1 and LSU sequences as the 4 *P. lantauensis* OTU3 specimens. Similarly, specimens of *P. lantauensis* OTU2 had identical ITS-1 and LSU sequences when compared to specimens of *P. lantauensis* OTU4. However, sequence differences were detected between these two *P. lantauensis* groups (i.e. OTUs 1 and 3 *vs*. OTUs 2 and 4). There were 62 (12%) nucleotide differences in ITS-1 sequence between the two groups, representing 46 substitutional changes (25 transitions, 21 transversions) and 16 indels (i.e. insertion-deletions). This difference was less than that (25%) detected between specimens of the 2 morphologically distinct species *P. epinepheli* and *P. coioidesis*. The phenogram depicting the genetic differences in ITS-1 sequence among specimens of *Pseudorhabdosynochus* revealed that *P. lantauensis* OTUs 1 and 3 were genetically more similar to *P. lantauensis* OTUs 2 and 4 than to either *P. epinepheli* or *P. coioidesis* (Fig. 1A). The topology of the phenogram derived from the LSU rDNA data topology (Fig. 1B) was similar to that for the ITS-1. There were, however, relatively fewer nucleotide differences in LSU sequence (2%) between *P. lantauensis* OTUs 1 and 3 and OTUs 2 and 4. These differences in LSU sequence corresponded to 17 substitutional changes (11 transitions, 6 transversions) and 1 indel.

A maximum parsimony analysis of the LSU sequence data yielded a single most parsimonious tree (Fig. 2) with a length of 123, a consistency index (excluding uninformative characters) of 0·87 and a retention index of 0·96. There was total statistical support (bootstrap values 100%) for the separation of *P. lantauensis* OTUs 1 and 3 and *P. lantauensis* OTUs 2 and 4 into 2 distinct clades. When *P. epinepheli* was used as the outgroup in the cladistic analysis, 17 of the 18 nucleotide differences between the two *P. lantauensis* clades were considered as derived (i.e. autapomorphic) characters (Fig. 2A), 11 for OTUs 1 and 3 (positions 193, 410, 494, 545, 546, 727, 804, 820, 872 and 829) and 6 for OTUs 2 and 4 (positions 619, 669, 772, 777, 808 and 837) (Fig. 2A). When *P. coioidesis* was used as the outgroup, 16 of the 18 nucleotide differences were considered as derived characters (Fig. 2B), 8 for OTUs 1

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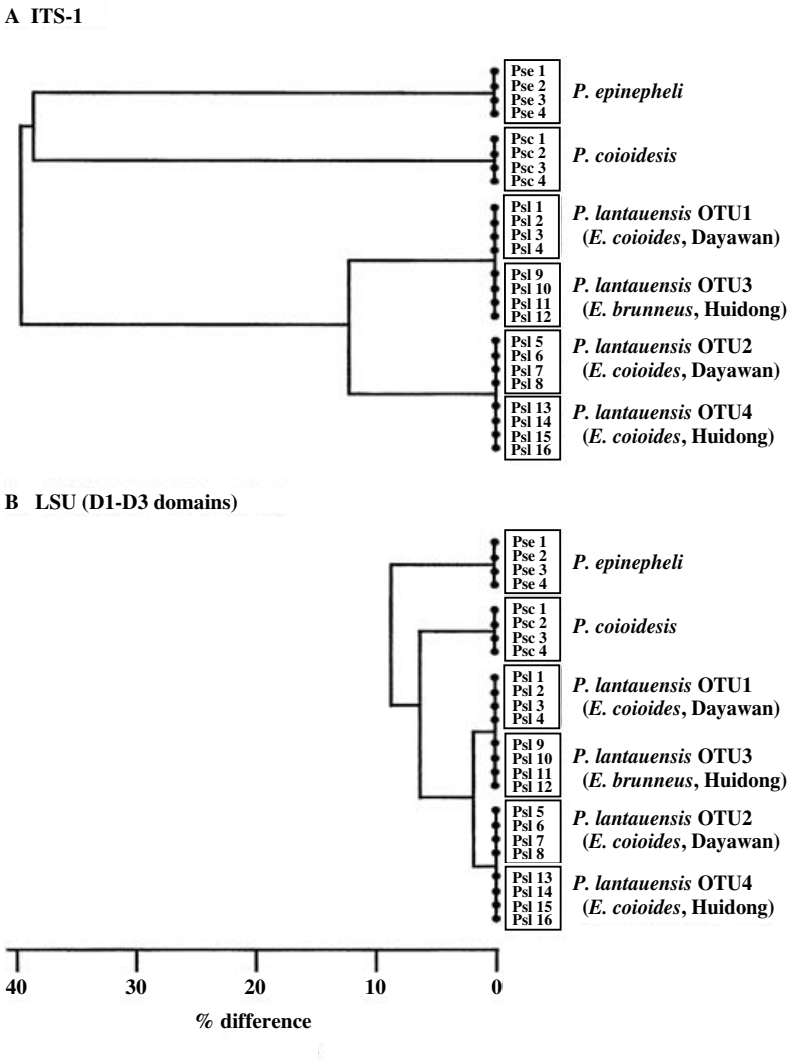

Fig. 1. Phenograms depicting the average percentage differences in sequences for the (A) ITS-1 and (B) LSU rDNA (D1-D3 domains) among *Pseudorhabdosynochus lantauensis* OTUs, *P. epinepheli* and *P. coioidesis*.

and 3 (positions 410, 494, 544, 669, 772, 804, 820 and 872) and 8 for OTUs 2 and 4 (positions 193, 546, 619, 727, 777, 808, 829 and 837) (Fig. 2B).

Morphological analyses

Table 2 shows the morphological measurements obtained for 22 specimens of *P. lantauensis*. Data for specimens representing OTUs 1 and 3 were combined, as were those for OTUs 2 and 4, based on the results of the molecular analyses and because there was no differences in measurements within each of these two groups. However, there were differences between the two groups (i.e. OTUs 1 and 3 *vs*. OTUs 2 and 4) in the distal length of the MCO and in the length of the vagina (Table 2). These structures were shorter in *P. lantauensis* OTUs 2 and 4. Also, *P. lantauensis* OTUs 2 and 4 had a larger body size (mean length × width: 611 × 184 μm *vs*. 533 × 164 μm), a greater ratio of the proximal to distal length in the MCO (1·9 : 1–3·7 : 1 *vs*. 0·8 : 1–1·4 : 1), a longer ventral bar and a larger squamodisc (mean length × width: 44 × 74 μm *vs*. 37 × 48 μm) than did *P. lantauensis* OTUs 1 and 3 (Table 2). Furthermore, the two *P. lantauensis* groups could be readily distinguished based on the shapes of the vagina (e.g. presence/absence of a chelate diverticulum on the vagina) and the dorsal bar (Fig. 3). All specimens of OTUs 1 and 3 had an overlapping dorsal bar and a longer, tubular vagina, whereas all specimens of OTUs 2 and 4 had a shorter, broader and more globular vagina (Fig. 3).

DISCUSSION

Sixteen specimens of *P. lantauensis* were divided into 4 OTUs based on host species, collecting localities (Dayawan or Huidong) and the shape and size of the vagina. OTUs 1 and 3 had 46 (12%) fixed differences in ITS-1 sequence when compared to OTUs 2 and 4. Similarly, there was 2% fixed sequence difference between two groups of *P. lantauensis* (i.e. OTUs

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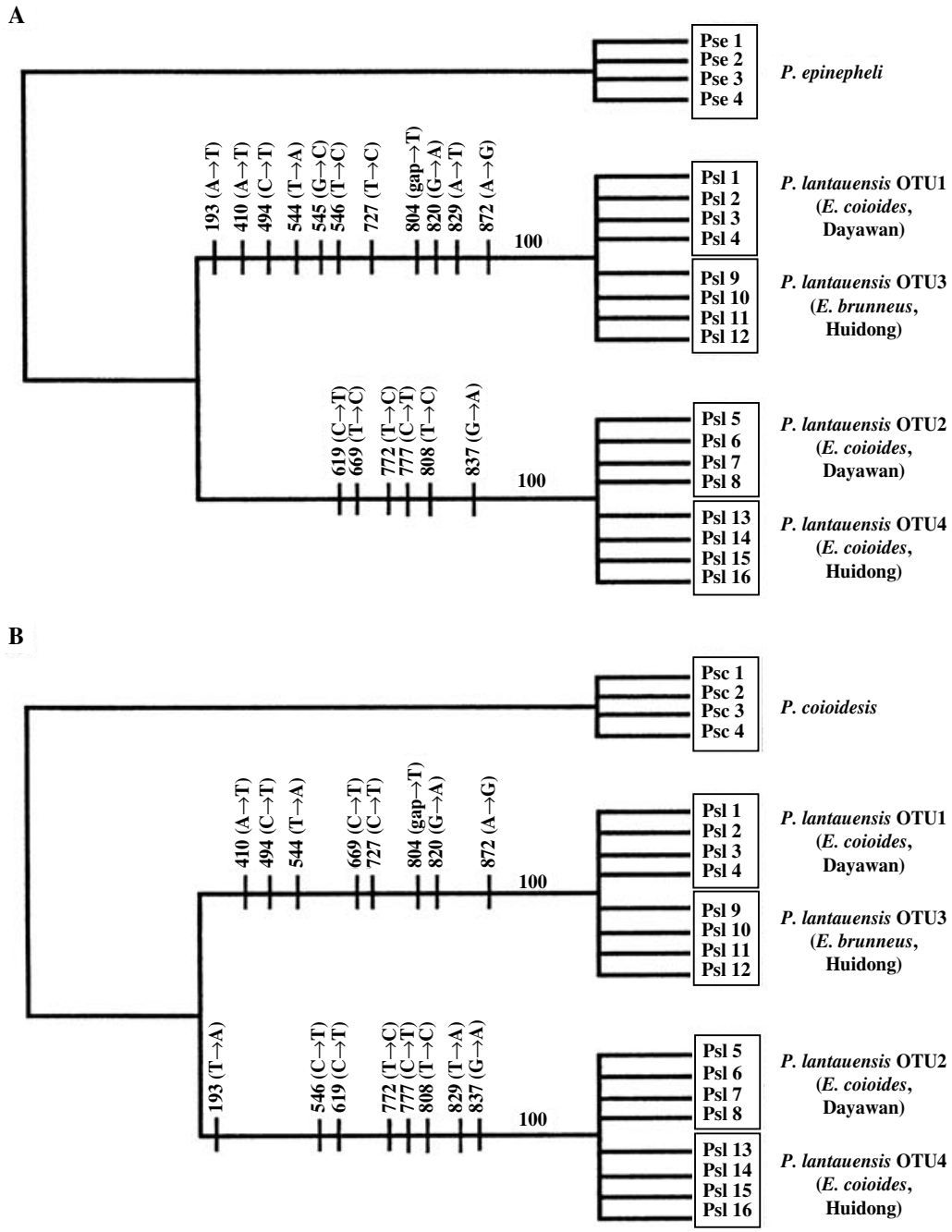

Fig. 2. Strict consensus trees, constructed using maximum parsimony and depicting the relationships of *Pseudorhabdosynochus lantauensis* OTUs 1–4, and the outgroups *P. epinepheli* (A) and *P. coioidesis* (B) based on sequence for the D1-D3 domains of the LSU rDNA. The derived character states (numbers correspond to sequence alignment positions) separating the two groups of *P. lantauensis* are shown on each branch. Numerals above the branches represent bootstrap values (%).

1 and 3 *vs*. 2 and 4). Although the magnitudes of difference in ITS-1 and LSU sequence among the two groups of *P. lantauensis* OTUs was considerably lower than that between the two morphologically distinct species, *P. epinepheli* or *P. coioidesis* (25% and 9%, respectively), they were within the range of sequence differences detected among closely related species of monogeneans (e.g. Cable *et al*. 1999; Bentz *et al*. 2001; Cunningham *et al*. 2001; Matejusová *et al*. 2001; Huyse & Volckaert, 2002; Whittington *et al*. 2004) and other platyhelminthes (e.g. Jousson, Bartoli & Pawlowski, 2000). However, it has been argued (e.g. Nadler *et al*. 2000) that the phylogenetic species concept should be used for species delineation rather than a comparative (i.e. yardstick) approach based on the magnitude of sequence differences.

Cladistic analyses were conducted on the D1-D3 domains of the LSU rDNA sequence data using *P. epinepheli* or *P. coioidesis* as outgroup to define

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Table 2. Comparative measurements (μm) of *Pseudorhabdosynochus lantauensis* specimens used in present study

(L, length; W, width.)

	P. lantauensis OTUs 1 and 3	*P. lantauensis* OTUs 2 and 4
Total body (L × W)	533 (444–586) × 168 (128–202)	611 (532–781) × 184 (166–230)
Haptor (L × W)	61 (50–82) × 207 (177–244)	73 (63–79) × 228 (204–251)
Copulatory organ proximal (L)	53 (49–59)	63 (58–66)
Copulatory organ distal (L)	48 (41–58)	26 (18–31)
Vagina (L)	49 (41–60)	31 (28–33)
Pharynx (L × W)	40 (35–47) × 50(45–56)	43 (36–58) × 51 (39–63)
Ventral hamuli		
Inner (L)	30 (27–33)	30 (21–35)
Outer (L)	42 (39–45)	48 (33–52)
Dorsal hamuli		
Inner (L)	21 (20–23)	27 (19–22)
Outer (L)	38 (36–41)	41 (38–45)
Ventral bar (L)	102 (92–111)	125 (113–137)
Dorsal bar (L)	79 (71–88)	73 (65–79)
Marginal hooklet (L)	10 (9–11)	10 (9–12)
Squamodisc (L × W)	37 (32–50) × 48 (41–57)	44 (31–56) × 74 (64–83)
Squamodisc rows	11	11 (10–12)
No. of specimens measured	12	10
Host	*E. brunneus*, *E. coioides*	*E. coioides*
Locality	South China Sea, Guangdong	South China Sea, Guangdong

autapomorphic (i.e. derived) character states within different lineages of *P. lantauensis*. The LSU was chosen for these analyses rather than the ITS-1 because of the greater confidence in comparing homologous characters (i.e. based on a more reliable sequence alignment). The results of the cladistic analyses showed that there was total statistical support (bootstrap values of 100%) for the separation of *P. lantauensis* into 2 distinct clades (i.e. OTUs 1 and 3 *vs*. OTUs 2 and 4). Furthermore, 17 of the 18 fixed nucleotide differences between these two groups were autapomorphic characters (11 for OTUs 1 and 3 and 6 for OTUs 2 and 4) when *P. epinepheli* was used as the group. There were 16 autapomorphic characters (8 for each *P. lantauensis* clade) when *P. coioidesis* was defined as the outgroup. The presence of autapomorphic character states for each of the two *P. lantauensis* clades provides strong evidence to reject the null hypothesis that *P. lantauensis* represents a single species.

Further evidence that *P. lantauensis* represents more than one species is provided by morphological and morphometric data. The two species could be readily distinguished from each other based on the difference in shape and size of terminal genitalia. *P. lantauensis* OTUs 1 and 3 possessed a differently shaped dorsal bar and a longer, tubular vagina, whereas *P. lantauensis* OTUs 2 and 4 had a shorter, broader and more globular vagina. The ratio of the proximal to distal length of the MCO was always higher in *P. lantauensis* OTUs 2 and 4 (1·9 : 1–3·7 : 1) than in *P. lantauensis* OTUs 1 and 3 (0·8 : 1–1·4 : 1), and the squamodisc of the former was wider than that of the latter. Previous studies have demonstrated that the characteristics of the MCO and vagina are of significance in delineating species within *Pseudorhabdosynochus* (see Beverley-Burton & Suriano, 1981; Kritsky & Beverley-Burton, 1986; Santos *et al*. 2000). Although the morphology of the haptor (including the hamuli, bar and hooklet) has also been considered important for distinguishing species of monogeneans (Yamaguti, 1963), there was overlap in the ranges of the length measurements for the haptor between *P. lantauensis* OTUs 1 and 3 and 2 and 4. This demonstrates that these structures are not useful diagnostic characters for this species complex. However, the shape of dorsal bar is one important morphological character for the delineation of the two *P. lantauensis* species (Bu *et al*. 1999).

The morphological features of *P. lantauensis* OTUs 1 and 3 were the same as those in the description of *P. lantauensis* from *E. brunneus* and *E. farrio* from Hong Kong (see Beverley-Burton & Suriano, 1981), whereas *P. lantauensis* OTUs 2 and 4 corresponds to the morphological description of this species from *E. coioides* collected in waters of Malaysia and Indonesia (see Bu *et al*. 1999). The morphometric measurements of *P. lantauensis* OTUs 2 and 4 are also in agreement with those of Bu *et al*. (1999). In contrast, specimens of *P. lantauensis* OTUs 1 and 3 were significantly larger than those recorded by Beverley-Burton & Suriano (1981). This may reflect methodological differences (i.e. measurements on fixed *vs*. fresh material) or the possibility of the presence of a third species within the *P. lantauensis* complex. Further study is therefore needed

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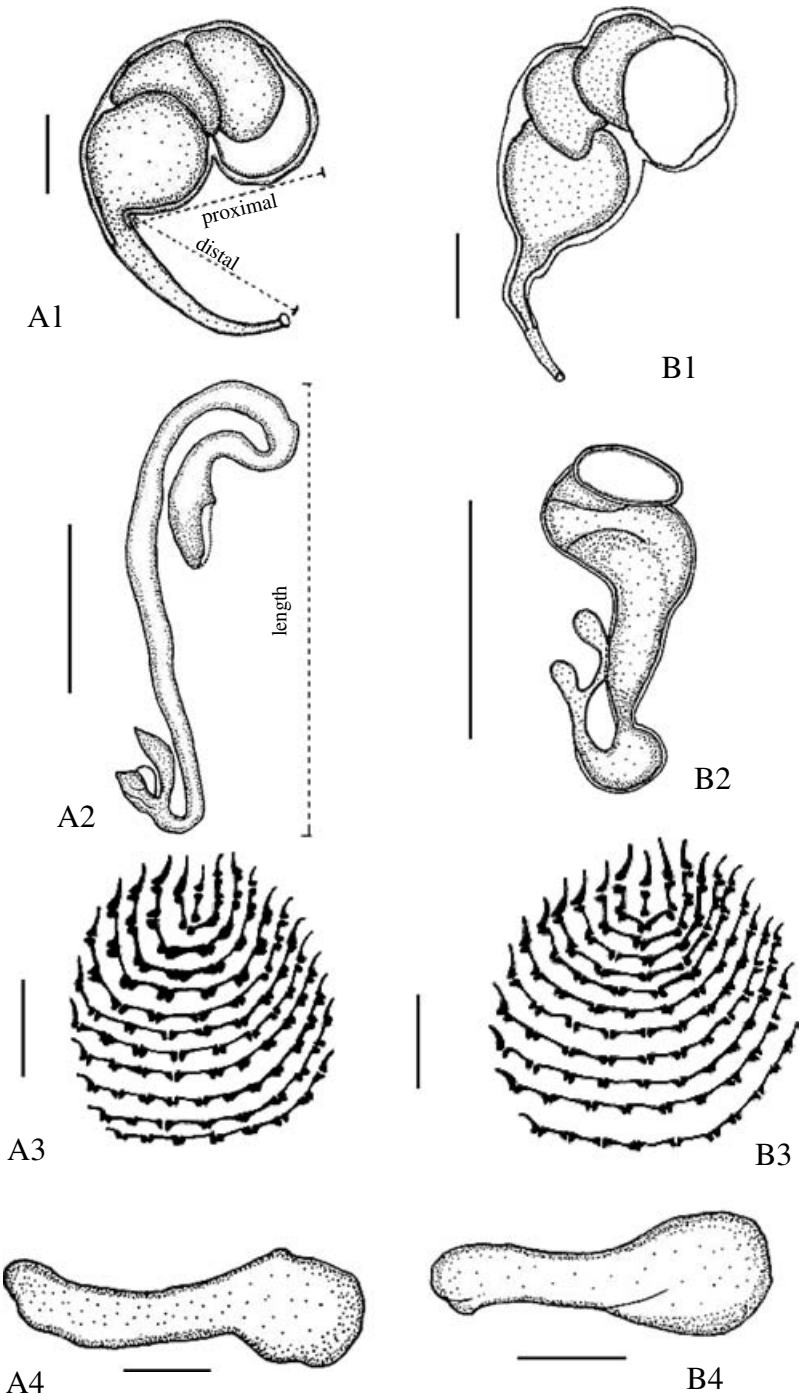

Fig. 3. Key morphological features of the two clades (A, OTUs 1 and 3; B, OTUs 2 and 4) of *Pseudorhabdosynochus lantauensis*. A1 and B1, male copulatory organs; A2 and B2, vagina; A3 and B3, squamodisc; A4 and B4, dorsal bar. Scale bars: 20 μm.

to elucidate the number of species within the *P. lantauensis* complex. This can be achieved by comparing, in a cladistic manner, the LSU and ITS-1 rDNA sequences for a large number of *P. lantauensis* from all geographical localities and species of hosts.

In conclusion, the combined molecular and morphological data obtained during the present study provide strong evidence to support the hypothesis that *P. lantauensis* from serranid fish collected in the South China Sea within Guangdong Province (China) represents at least 2 species. Both species were collected in sympatry from fish near Huidong. *P. lantauensis* OTUs 2 and 4 was only found in *E. coioides* (about 2000 individuals in 4 hosts), while *P. lantauensis* OTUs 1 and 3 was only detected in *E. brunneus* (about 1000 individuals in 5 hosts). In contrast, both parasite species (i.e. 42 and 27 individuals of *P. lantauensis* OTUs 1 and 2, respectively) were found in one *E. coioides* collected

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near Dayawan (i.e., located 60 km from Huidong). Detection of the presence of at least 2 co-existing species within what is currently considered as *P. lantauensis* has major implications for studying the transmission patterns and ecology of this socio-economically important group of parasites.

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